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Original Research Article

*Meta*Topolin-driven breakthrough in lemon beebalm (*Monarda citriodora*) regeneration: A molecular fidelity study for genome engineering applications

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Abstract

Plant cell and tissue regeneration has become the key platform for genome editing and integrated approaches. However, there is a lack of reliable and reproducible *in-vitro* conservation methods for several plant species. In addition, collecting plant material from natural habitats has decreased species numbers, prompting efforts to create growing methods that support conservation. Biotechnological approaches such as plant tissue culture have increasingly satisfied this growing demand. Thus, optimization of *in-vitro* regeneration system in *Monarda citriodora* Cerv. ex-Lag., was done using nodes as explants. The nodal explants were cultured on Murashige and Skoog's (MS) medium blended with different concentrations of 6-benzylaminopurine (BAP), kinetin (Kn), thidiazuron (TDZ), *meta*Topolin (*m*T) alone or in combination. The highest regeneration efficiency (90.31 ± 0.15%) with multiple shoots (13.22 ± 0.26) induction and shoot length (4.32 ± 0.06 cm) was achieved on shoot regeneration medium (SRM) containing MS salts fortified with 1-mg/L *m*T. Furthermore, we found that the combined application of 1-mg/L *m*T+ 0.5 mg/L α-naphthaleneacetic acid (NAA) resulted in an increased shoot number (25.30 ± 0.45) with a shoot length of 6.13 ± 0.02 cm. A higher rooting percentage (85.51 ± 0.42) was recorded in plants grown in half-strength MS media. The start codon targeted (SCoT) and inter simple sequence repeat (ISSR) markers revealed that the *in-vitro* regenerated and greenhouse-acclimatized plants showed a similar banding pattern to that of the *in-vivo* grown mother plant, thus confirming the absence of a polymorphic nature of regenerated *M. citriodora* plants. These results signify that *m*T enhances the regeneration and development rate of *M. citriodora* plants.

Keywords: Molecular markers, *Monarda citriodora*, MS basal, Nodal explants, Regeneration.

Introduction

Monarda citriodora Cerv. ex Lag. (Lamiaceae) is an annual aromatic herb known as lemon mint, horse mint, lemon bee balm, and lemon bergamot (Davidson, 2007; Mattarelli *et al.,* 2017). This temperate ornamental and medicinal plant is native to North America and has grown in some parts of Europe (the United Kingdom and Russia) (Collins *et al.,* 1994; Pivovarov & Dryagin, 1991). It has been introduced to India in the Shivalik hill regions (Himachal Pradesh, Jammu & Kashmir, and Uttarakhand states) and grown for essential oil production (Katoch *et al.,* 2017). Aerial parts (leaf and inflorescence) are the primary source of essential oil extraction. The plant with the inflorescence may yield approximately 3.34 to 3.83% of essential oil (Gontar *et al.,* 2022). Various researchers reported essential oil extraction and studied the constituents of the essential oil; it contains around 30 constituents, which include thymol, b-cymene, a-phellandrene, 1,8-cineole, p-cymene, carvacrol, 7-octen-4-ol and terpinen-4-ol (Dorman & Deans, 2004; Lu *et al.,* 2011). The concentration of constituents differs from geographical regions and the varieties of *M. citriodora,* but mostly thymol remains the main constituent (Pathania *et al.,* 2013). Monarda essential oils are widely used in pharmaceutical preparations, food products, cosmetics, agronomic, and veterinary industries. Each plant part of this species is important in various aspects. It is consumed as a flavoring or seasoning agent in different dishes and beverages (Grzeszczuk *et al.,* 2020). The citrusy fragrance of leaves is used as an efficient insect repellent (Meena *et al.,* 2017). Since historical times, native

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American tribes have used aerial parts to treat various problems associated with the respiratory, digestive, skin, and inflammatory systems. An infusion is used internally to treat colds, catarrh, headaches, sore throat, nausea, menstrual pain, sleep disorder, and whooping cough (Singh *et al.,* 2009). It also has antibacterial, antioxidant, antifungal, and anticancer properties (Katoch & Pull, 2017). According to the Business Research Insights report, the global thymol demand was USD 100.2 million in 2021 and is expected to increase to USD 141.58 million in 2031 (BRI, 2024).

The *in-vitro* regeneration system in *M. citriodora* has not yet been reported. It is one of the main hurdles for genetic improvement with biotechnological tools. Recently, in *M. citriodora,* transcriptome data unveiled the responsible genes for monoterpenes biosynthesis, a detailed study of trichome types, and significant trichome density variation at different developmental stages of leaf influencing the impacting concentration of monoterpenes (Sharma *et al*., 2023). This will help researchers enhance essential oil production with modern biotechnological tools in *M. citriodora.* Conventional breeding and cultivation systems have limitations concerning the time required to develop desired traits. The intervention of genetic and genome engineering-based trait improvement necessitates the availability of an efficient *in-vitro* regeneration system. Earlier *in-vitro* studies have been reported for closely related species of *M. fistulosa* using axillary buds (Calugaru-Spataru, 2023; Manushkina *et al.,* 2022), and cell cultures of *M. citriodora* have also been established (Bulya *et al.,* 2023).

Genetic variability is one of the main concerns in *in-vitro* regenerated plantlets because the chance of occurrence for somaclonal variations is very high (Martín & González-Benito, 2024). Therefore, tissue culture-raised plants need to be studied for their genetic fidelity. Different molecular markers such as inter simple sequence repeats (ISSR) and start codon targeted (SCoT) are highly efficient and used to evaluate the genetic variability of plantlets in diverse plant species (Bisht *et al.,* 2024; Igwe *et al.,* 2021). ISSR markers use non-coding DNA regions for genetic stability scrutiny (Zietkiewicz *et al.,* 1994). These amplicons are then compared with wild (control) for genetic stability assessment (AI *et al.,* 2023; Bisht *et al.,* 2024). Genetic fidelity assessment through SCoT polymorphism markers is a targeted fingerprinting technique. These markers are generated from conserved genome regions flanked by the start codon ATG. However, it is worth noting that SCoT markers are derived from transcribed regions and might be associated with gene functionality in some plant varieties (Al-Qurainy *et al.,* 2015). Compared with all other molecular markers, in most plant systems, SCoT primers are highly reproducible (Bisht *et al.,* 2024). Thus, an efficient shoot regeneration system has not been developed for the genus *M. citriodora*. This study aimed to develop an efficient and monomorphic *in-vitro* regeneration system in *M. citriodora* using nodal explants.

Materials and Methods

Establishment of in-vitro cultures

M. citriodora seeds were collected from the CSIR-Indian Institute of Integrative Medicine (CSIR-IIIM) experimental fields at Chatha village, Jammu, Jammu and Kashmir. Collected seeds were germinated in pots containing garden soil, coco peat, and vermiculite (1:1:1) and maintained under greenhouse conditions. In 2-month-old greenhouse-grown seedlings used as a source for healthy nodal segments ~ 0.5 to 1.0 cm to establish aseptic cultures.

Surface sterilization of explants

Excised nodal explants were kept under running tap water for 15 minutes. After that, a few drops of 0.5% Tween 20 were added and shaken thoroughly for 15 minutes, followed by three consecutive washes using autoclaved distilled water. Further, explants were submerged in 0.1% $HgCl_2$ for 2 minutes and subsequently washed with sterile distilled water 3 to 4 times under aseptic conditions (Singh *et al.,* 2017).

Nutrient media and culture conditions

For the inoculation of surface sterilized nodes, Murashige and Skoog (MS) basal (Murashige & Skoog, 1962) was prepared by supplementing with 3% sucrose, and pH was taken at 5.8 before adding 0.8% agar and sterilized at 121°C for 15 minutes at 1.05 kg/cm^2. All the cultures were incubated *in-vitro* under cool fluorescent tube lights of 30 µmol $m^{-2}s^{-1}$ light intensity for a 16-hour photoperiod and 60 to 70% relative humidity at 25 ± 1°C.

Shoot induction and multiplication

The nodal explants were collected from aseptically grown culture and inoculated vertically in the test tube (Borosil®, 150x 25 mm) containing MS medium enriched with various concentrations (0.5, 1.0, 1.5. 2.0 mg/L) of individual cytokinins like 6-benzylaminopurine (BAP), kinetin (Kn), thidiazuron (TDZ), *meta*Topolin (*m*T) (Table 1). The effective concentration of cytokinin for nodal regeneration was optimized and denoted as shoot regeneration medium (SRM), which was further used to study the impact of auxins. SRM with various concentration levels (0.1, 0.5, 1.0, 1.5, 2.0 mg/L) of individual auxins like α-naphthaleneacetic acid (NAA), and Indole-3-acetic acid (IAA) were employed to increase the shoot multiplication/induction percentage (Table 2), and MS medium without any plant growth regulators (PGR) was taken as control. This induction medium was denoted as shoot multiplication medium (SMM). After three weeks, percent shoot bud induction, proliferation, average number of shoots, and shoot length were recorded.

Table 1: Effect of cytokinins on shoot induction and multiplication of *M. citriodora* nodal explants

PGRs (mg/L)				*Percent shoot induction*	*Number of shoot/ explant*	*Shoot length (cm)*
Kin	*BAP*	*TDZ*	m*T*			
-	-	-	-	-	-	-
0.5	-	-	-	31.71 ± 0.96^{j}	1.07 ± 0.02^{k}	0.51 ± 0.01^{m}
1.0	-	-	-	34.44 ± 0.55^{i}	1.49 ± 0.02^{j}	0.95 ± 0.01^{k}
1.5	-	-	-	35.90 ± 0.45^{i}	1.76 ± 0.02^{ij}	1.05 ± 0.03^{k}
2.0	-	-	-	21.81 ± 0.90^{k}	1.51 ± 0.04^{j}	0.73 ± 0.02^{l}
-	0.5	-	-	70.47 ± 0.47^{d}	6.55 ± 0.07^{e}	2.00 ± 0.03^{g}
-	1.0	-	-	81.52 ± 0.28^{c}	8.14 ± 0.06^{d}	2.81 ± 0.08^{d}
-	1.5	-	-	65.55 ± 0.55^{e}	6.75 ± 0.05^{e}	2.13 ± 0.03^{f}
-	2.0	-	-	60.63 ± 0.63^{f}	5.71 ± 0.06^{f}	1.50 ± 0.03^{i}
-	-	0.5	-	55.56 ± 0.80^{g}	2.17 ± 0.03^{h}	1.24 ± 0.01^{j}
-	-	1.0	-	65.00 ± 0.00^{e}	3.05 ± 0.05^{g}	1.92 ± 0.02^{g}
-	-	1.5	-	55.71 ± 0.71^{g}	2.95 ± 0.05^{g}	1.75 ± 0.03^{h}
-	-	2.0	-	45.87 ± 0.87^{h}	1.86 ± 0.04^{hi}	1.32 ± 0.01^{j}
-	-	-	0.5	85.47 ± 0.23^{b}	8.77 ± 0.01^{c}	3.67 ± 0.04^{b}
-	-	-	1.0	90.31 ± 0.15^{a}	13.22 ± 0.26^{a}	4.32 ± 0.06^{a}
-	-	-	1.5	86.14 ± 0.21^{b}	9.69 ± 0.27^{b}	3.26 ± 0.03^{c}
-	-	-	2.0	80.63 ± 0.31^{c}	9.05 ± 0.1^{c}	2.63 ± 0.02^{e}

The data is represented as mean ± standard error. Means followed by the same letter are not significantly different ($p \leq 0.05$) as per Duncan's test. The highlighted text denotes the best response.

Table 2: Effect of auxins with optimized cytokinin on shoot induction and multiplication of *M. citriodora* nodal explants

S.No.	*Plant growth hormone (mg/L)*			*Percent shoot induction*	*Number of shoots/ explant*	*Average shoot length (cm)*
	m*T*	*NAA*	*IBA*			
1	1	0.1	-	90.31 ± 0.15^{b}	20.88 ± 0.20^{c}	5.75 ± 0.02^{bc}
2	1	0.5	-	95.07 ± 0.07^{a}	25.30 ± 0.45^{a}	6.13 ± 0.02^{a}
3	1	1.0	-	85.00 ± 0.00^{c}	18.26 ± 0.10^{e}	5.22 ± 0.15^{de}
4	1	1.5	-	80.31 ± 0.31^{d}	16.19 ± 0.16^{g}	5.32 ± 0.01^{d}
5	1	2.0	-	69.86 ± 0.93^{g}	14.19 ± 0.07^{i}	4.86 ± 0.03^{f}
6	1	-	0.1	86.14 ± 0.21^{c}	19.63 ± 0.23^{d}	5.61 ± 0.02^{c}
7	1	-	0.5	90.61 ± 0.14^{b}	21.96 ± 0.13^{b}	5.81 ± 0.04^{b}
8	1	-	1.0	76.15 ± 0.65^{e}	17.33 ± 0.10^{f}	5.10 ± 0.05^{e}
9	1	-	1.5	71.38 ± 0.78^{f}	15.33 ± 0.16^{h}	4.59 ± 0.01^{g}
10	1	-	2.0	67.16 ± 0.50^{h}	14.39 ± 0.17^{i}	4.22 ± 0.02^{h}

The data is represented as mean ± standard error. Means followed by the same letter are not significantly different (p≤0.05) as per Duncan's test. The highlighted text denotes the best response.

Root induction

Fully developed *in-vitro* regenerated shootlets (2–3 cm) were excised individually and inoculated on different rooting media. MS full-strength and half-strength media with and without varying auxin concentrations (IAA, IBA) were used to study the impact of root induction. Data were observed as rooting percentage, mean number of roots, and root length after three weeks of inoculation.

Acclimatization and hardening

Healthy plantlets were separated from the half-strength rooting medium (HRM), and adherent agar was rinsed with sterile distilled water. Fully grown and washed

Table 3: Effect of different concentrations of IAA and IBA supplemented to full and half MS basal media on root induction of *M. citriodora in-vitro* regenerated shoots

S. No	*Growth regulators (mg/L)*	*Rooting percentage*	*Number of roots/shoots*	*Root length (cm)*
1	MS Basal without PGR	75.39 ± 0.39^{c}	6.14 ± 0.30^{b}	1.38 ± 0.04^{cd}
2	Half MS without PGR	85.51 ± 0.42^{a}	7.08 ± 0.46^{a}	1.84 ± 0.02^{a}
MS +PGR				
3	0.2 IAA	66.10 ± 0.55^{g}	3.85 ± 0.07^{fgh}	1.06 ± 0.05^{fghi}
4	0.4 IAA	69.39 ± 0.60^{f}	4.14 ± 0.18^{defg}	1.20 ± 0.03^{ef}
5	0.6 IAA	56.42 ± 0.71^{i}	3.50 ± 0.12^{hij}	0.99 ± 0.05^{ghi}
6	0.8 IAA	39.66 ± 0.82^{l}	3.12 ± 0.12^{ijk}	0.64 ± 0.02^{lmn}
7	1.0 IAA	31.20 ± 0.60^{m}	2.79 ± 0.04^{k}	0.50 ± 0.01^{n}
8	0.2 IBA	70.00 ± 0.00^{ef}	3.94 ± 0.02^{efgh}	1.12 ± 0.02^{fg}
9	0.4 IBA	72.28 ± 0.43^{d}	4.41 ± 0.23^{de}	1.29 ± 0.01^{de}
10	0.6 IBA	61.26 ± 0.63^{h}	3.89 ± 0.02^{efgh}	1.11 ± 0.2^{fgh}
11	0.8 IBA	46.02 ± 0.80^{k}	3.65 ± 0.08^{ghi}	0.64 ± 0.06^{lmn}
12	1.0 IBA	38.72 ± 0.63^{l}	2.99 ± 0.07^{jk}	0.62 ± 0.01^{mn}
Half MS+PGR				
13	0.2 IAA	71.38 ± 0.78^{de}	4.34 ± 0.13^{def}	0.94 ± 0.09^{hij}
14	0.4 IAA	76.15 ± 0.65^{c}	5.22 ± 0.28^{c}	1.46 ± 0.08^{bc}
15	0.6 IAA	70.47 ± 0.47^{def}	4.11 ± 0.08^{defg}	0.92 ± 0.06^{ijk}
16	0.8 IAA	65.55 ± 0.55^{g}	3.45 ± 0.13^{hij}	0.92 ± 0.06^{ijk}
17	1.0 IAA	50.79 ± 0.79^{i}	3.13 ± 0.03^{ijk}	0.69 ± 0.08^{lm}
18	0.2 IBA	75.39 ± 0.39^{c}	4.58 ± 0.02^{d}	1.19 ± 0.09^{ef}
19	0.4 IBA	80.31 ± 0.31^{b}	5.46 ± 0.10^{c}	1.56 ± 0.04^{b}
20	0.6 IBA	70.47 ± 0.47^{def}	4.25 ± 0.04^{def}	0.79 ± 0.04^{jkl}
21	0.8 IBA	56.42 ± 0.71^{i}	4.08 ± 0.10^{defg}	0.75 ± 0.05^{klm}
22	1.0 IBA	51.58 ± 0.79^{j}	3.19 ± 0.14^{ijk}	0.68 ± 0.03^{lm}

The data is represented as mean ± standard error. Means followed by the same letter are not significantly different ($p \leq 0.05$) as per Duncan's test. The highlighted text denotes the best response.

plantlets were shifted to paper cups (5 cm radius) containing a proportion of sterile cocopeat, garden soil, and vermiculite (1:2:1) and covered with transparent perforated polythene bags for maintaining proper air and humidity (Figure 2E). These cups were kept inside the growth room at 25 ± 2°C temperature. For up to two weeks, one-fourth of the inorganic solution of MS medium was added to the paper cups every alternate day. After two weeks, the cups were irrigated with water for eight weeks to acclimatize the condition. After eight weeks, plants were transferred into pots (10 cm radius) for optimum growth. Finally, after two months, the *in-vitro* hardened plants were transferred to a greenhouse for growth and development.

Genomic DNA extraction and clonal fidelity analysis

The fresh leaves (500 mg) were taken from 2-month-old fully grown mother plant (wild) and randomly selected *in-vitro* regenerated plantlets of 2nd, 7th, and 11th subcultured lines to isolate genomic DNA using the CTAB method (Doyle & Doyle, 1987). Quantification of DNA was done using NanoDrop^{TM}2000 (Thermofisher). The genetic uniformity of micropropagated plantlets was evaluated using 10 ISSR and 8 SCoT primers (Table 4). DNA sample concentration was diluted to 150 ng/µL with Mili Q water. The PCR reaction mixture (10 µL) was comprised of 1-µL of 150 ng/µL genomic DNA, 1-µL of 10 pmol each primer, 1-µL of 2.5 mM dNTPs, 0.2 µL of 5 U/µL Taq DNA polymerase, 1-µL 10X buffer and 10 µL of final volume was made up with sterile water. The reaction was performed in a thermocycler (ProFlex, Thermo Fisher) for 35 cycles (PCR setup: 94°C for 5 minutes, 94°C for 30 seconds, 50°C for 40 seconds, 72°C for 2 minutes 15 seconds). The PCR products were analyzed and documented using BIO-PRINT© (Vilber) gel documentation unit.

Statistical analysis

In-vitro shoot and root morphogenic data were documented after three weeks. All experiments were conducted thrice using a minimum of 20 explants per replica. One-way ANOVA and the difference between means were examined using Duncan's multiple range test ($p \leq 0.05$) in IBM SPSS version 23. Data were described in the form of mean ± standard error

Results

Shoot bud induction and multiplication

Aseptic nodal explants were inoculated on MS medium supplemented with various PGRs (Figure 1A). Shoot bud initiation was observed after 5 to 6 days of incubation in all tested cytokinins (Kn, BAP, TDZ, *m*T) at different concentrations (Figure 1B), but 1-mg/L *m*T (SRM) showed the highest shoot induction percentage of 90.31 ± 0.15. SRM induced maximum multiple shoots (13.33 ± 0.26) and shoot length (4.32 ± 0.06 cm) without intervening any callus (Figure 1C, D). Medium devoid of any PGR (control) failed to induce shoot buds (Table 1). Apart from *m*T, higher concentrations of TDZ and BAP also induced multiple shoots.

Optimized cytokinin interactive effect with multiple auxins

The effect of optimized *m*T (SRM) concentration and varying concentrations of auxins (IBA and NAA) was studied for inducing morphogenetic response in nodal explants (Table 2). SRM medium fortified with 0.5 mg/L NAA (SMM) induced the highest shoot regeneration efficiency of 95.07 ± 0.07%. The number of multiple shoots and shoot length on SMM medium increased to 25.30 ± 0.45 and 6.13 ± 0.02 cm, respectively (Table 2 and Figure 1E-G). SRM and higher concentrations of NAA, i.e., 1 to 2 mg/L, showed a significant decrease in regeneration efficiency and shoot multiplication number. SRM, combined with IBA, also produced an increased number of multiple shoots, but in comparison with SMM, the regeneration efficiency was significantly lesser (Table 2).

Rooting and hardening

Single excised shoots were shifted to MS containing half-

Table 4: ISSR and SCoT primers, their sequence, number of bands, and size range of amplified fragments of *M. citriodora*

S.No.	*Primer code*	*Primer sequence 5′-3′*	*Number of bands amplified*	*Length of amplified fragments (bp)*
1	ISSR 1	TCTCTCTCTCTCTCTCC	4	1100–2500
2	ISSR 2	ACTGCTAGAGAGAGAGAGAG	6	1600–4600
3	ISSR 3	AGAGAGAGAGAGAGAGC	6	800–4000
4	ISSR 4	AGAGAGAGAGAGAGAGG	3	1700–3200
5	ISSR 5	AGAGAGAGAGAGAGAGTC	8	1600–4600
6	ISSR 6	GCCGAGAGAGAGAGAGA	4	1500–3100
7	SCoT 1	ACGACATGGCGACCAACG	3	800–2700
8	SCoT 2	CAACAATGGCTACCACCC	2	700–2600
9	SCoT 3	ACCATGGCTACCACCGCC	9	400–3300
10	SCoT 4	GCAACAATGGCTACCACC	2	2800–5500

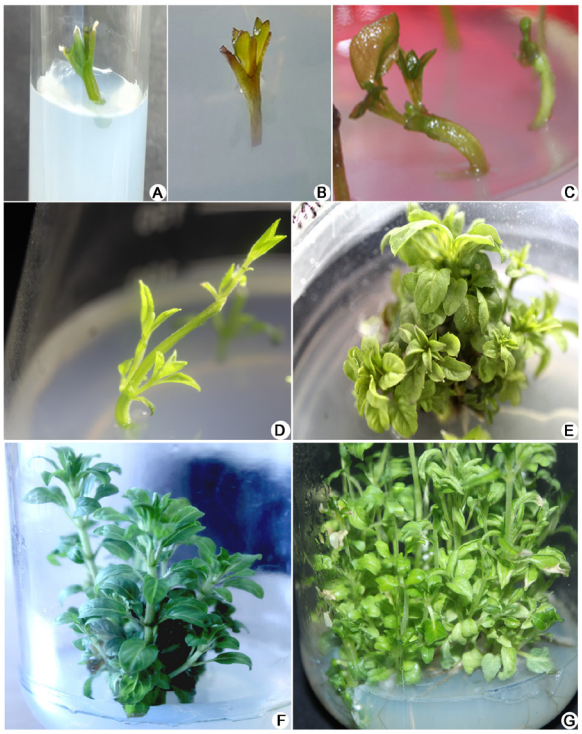

Figure 1: *meta*Topolin (*m*T) induced *in-vitro* regeneration of *M. citriodora* using the nodal explants. (A) Inoculated nodal explant on MS media supplied with different PGR (B) Axillary bud breaking from nodal explant on SRM (1 mg/L *m*T) (C,D) Shoot proliferation on SRM (E,F,G) Shoot multiplication and elongation on SMM (1-mg/L *m*T + 0.5 mg/L NAA)

strength and full-strength medium, along with different concentrations of IAA or IBA (Figure 2A). After 14 to 18 days of inoculation, initiation of roots was observed from the shoots (Figure 2E). The best rooting (85.51%) response was seen in half-strength MS medium without PGR (HRM) with 7.08 ± 0.46 roots per shoot and with a mean length of 1.84 ± 0.02 cm (Table 3) (Figure 2 B-D). All plantlets acclimatized well in greenhouse and field conditions with a 100 percent survival rate (Figure 2 F-H).

Evaluation of genetic stability using ISSR and SCoT primers

The genetic uniformity of *in-vitro* regenerated plants and control mother plants was confirmed by employing molecular markers such as ISSR and SCoT. Out of 10 ISSR and 8 SCoT primers tested, based on the compatibility, 6 ISSR and 4 SCoT primers were chosen for confirmation. 6 ISSR primers generated 31 reproducible bands ranging from 800 to 4600 bp (Figure 3A-F). At the same time, 4 SCoT markers produced 16 reproducible bands ranging from 400 to 5500 bp (Table 4) (Figure 4 A-D). The banding pattern confirmed the monomorphic nature of all regenerated plantlets compared to the control mother plant.

Figure 2: Root induction and acclimatization of *in-vitro* regenerated shoots of *M. citriodora.* (A) Inoculation of single excised shoots on HRM (B) Initiation of roots in HRM (C,D) Elongated roots in the same HRM (E) Maintaining humidity for acclimatization of *in-vitro* regenerated plants in the culture room (F,G) Acclimatized *in-vitro* regenerated plants in greenhouse condition

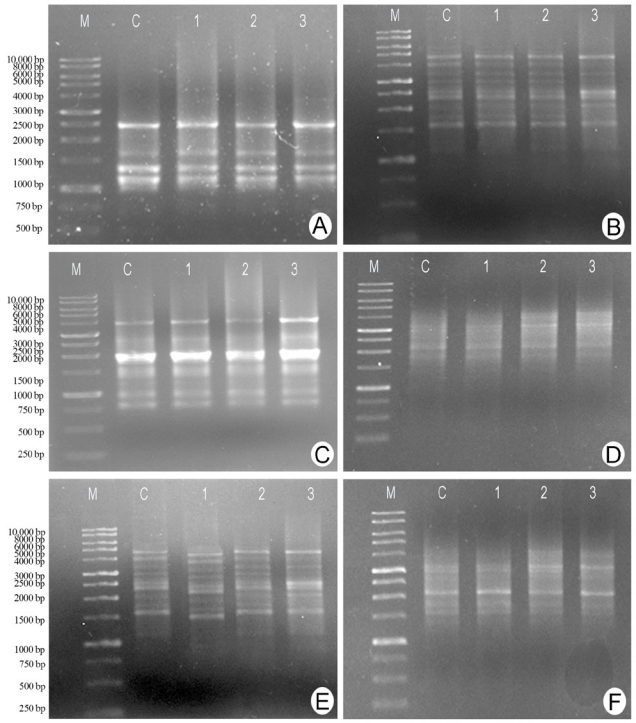

Figure 3: Generated amplicon patterns of the wild and regenerated plants of *M. citriodora* using ISSR primers. (*lane M*: 1- kb marker, *lane C:* mother plant, *lane 1-3: in-vitro* propagated hardened plant) (A) ISSR 1, (B) ISSR 2, (C) ISSR 3, (D) ISSR 4, (E) ISSR 5, (F) ISSR 6

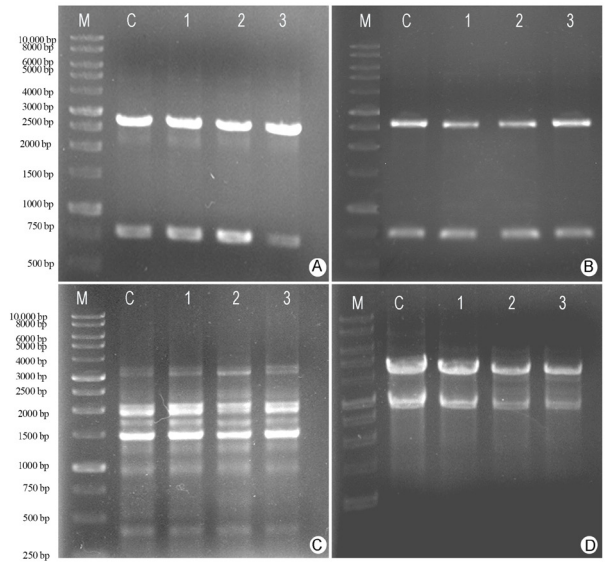

Figure 4: Genetic uniformity confirmation of control wild and regenerated plants of *M. citriodora* using SCoT primers (*lane M*: 1- kb marker, *lane C:* mother plant, *lane 1-3: in-vitro* propagated hardened plant) (A) SCoT 1, (B) SCoT 2, (C) SCoT 3, (D) SCoT 4

Discussion

The regular cytokinins, BAP, KIN, TDZ, *m*T are used for the plant *in-vitro* regeneration studies. Compared to all cytokinins, *m*T and TDZ are highly potent in induced regeneration in various plant systems (Fatima *et al.,* 2021; Murthy *et al.,* 1998). This study was based on observation of the effect of different cytokinins (BAP, KIN, TDZ, *m*T) on percent shoot induction and multiplication using the nodal explants of *M. citriodora*. This showed that the shoot induction occurred in all tested cytokinins, but on *m*T-containing medium, there was a remarkable increase in the percent induction, number of multiple shoots, and shoot length. *m*T, a natural, highly active aromatic cytokinin, was isolated from the source of poplar leaves and widely used in recalcitrant plant regeneration studies (Strnad *et al.,* 1997a). The prime metabolic product and storage form of *m*T is *O*-glucoside, which can easily and rapidly be translocated in plant tissues. This stable form is non-toxic and later converts into possible cytokinin bases required for plant cells (Werbrouck *et al.,* 1995). In different explants, the uninterrupted availability of cytokinins at the physiological level over an extended period will result in higher shoot induction percentage and elongation rates (Strnad *et al.,* 1997b). Similarly, *m*T enhanced the shoot induction percentage and multiplication in *S.cumin* (Naaz *et al.,* 2019) *A. polyphylla* (Bairu *et al.,* 2007). Compared to other cytokinins like TDZ, Kn, and BAP, *m*T induced a notably higher percentage of shoot elongation in *C. sativa* (Lata *et al.,* 2016) and *P. sidoides* (Moyo *et al.,* 2012). Besides this, *m*T showed anti-senescence activity (Işlek, 2021), reduced abnormalities, better acclimatization, rooting, and improved secondary metabolite production (Koç, 2021). One synthetic PGR (plant growth regulator), TDZ, effectively induces somatic embryogenesis, callus, organogenesis, and proliferation. In our experiment, TDZ induced hyperhydricity in the regenerated shoots. Similar results were observed in *C. wilsonii* (Sivanesan *et al.,* 2010), *A. unedo* (El-Mahrouk *et al.,* 2010), *P. lanceolata* (Kher *et al.,* 2014), *A. polyphylla* (Ivanova & Van Staden, 2010). In contrast, other researchers reported that TDZ induces healthy and significant shoot regeneration (Aggarwal *et al.,* 2012; Lata *et al.,* 2008).

Several researchers have reported the cytokinin-auxin synergistic effect initiated potential signaling molecules that regulate the meristematic activity, i.e., cell division and elongation required for the growth and development of a whole plant (Su *et al.,* 2011). It can be ascribed that critical balancing of exogenous and endogenous hormones is required to achieve the minimum threshold hormonal level for optimum shoot multiplication (Gupta *et al.,* 2020). In our present finding, *m*T combined with NAA induced the highest multiple shoots compared to *m*T alone. Similar results reported that *m*T in combination with auxin proved superior in producing foremost shoot regeneration frequencies in plant sps like *S. cumini* (Naaz *et al.,* 2019), *C. album* (Fajinmi *et al.,* 2014), *H. hystrix* (Amoo & Van Staden, 2012), and *P. marsupium* (Ahmad & Anis, 2019). In *O. gratissimum,* combining cytokinin and auxin does not synergize shoot regeneration (Saha *et al.,* 2012). MS medium (full and half strength) was found to be better than in combination with various auxins for stimulation of root initiation. The effectiveness of full and half-strength hormone-free MS medium has been well-documented in plants like *A. scaber* (Boo *et al.,* 2015), *J. curcas* (Sujatha & Mukta, 1996), and *V. vinifera* (Khan *et al.,* 2015). In our work, the maximum root induction efficiency was achieved on HRM. The efficacy of low-strength MS medium has been reported for higher rooting frequency (Sen *et al.,* 2023; Waseem *et al.,* 2009). HRM was found to be the most suitable rooting medium. Similar results were found in *Portulaca pilosa* (Chen *et al.,* 2020), *Portulaca* sps. (Bhuiyan & Adachi, 2002). The nitrogen ions concentration required for root formation is much lower than for shoot formation (Driver & Suttle, 1987). This study observed root induction on media augmented with IAA and IBA at different concentrations. However, neither significantly increased the induced root number compared to the hormone-free medium.

Genetic homogeneity is a prerequisite for developing true-to-type plantlets through *in-vitro* propagation for any plant of interest. Genetic uniformity is nonstable in different subcultured lines. Therefore, it is imperative to confirm genetic uniformity among different subculture lines. In various studies, a plethora of molecular markers such as SSR, ISSR, SCoT, RFLP, RAPD, and AFLP were

used for genetic fidelity testing (Mir *et al.,* 2021). Out of all, ISSR and SCoT are PCR- based methods with high reproducibility as well as cost-effective in confirming genetic fidelity (Rohela *et al*., 2019). In the case of other markers, some limitations, such as high cost for AFLP, low reproducibility of RAPD, and RFLP-length process that include involvement of restriction enzyme as well as blotting techniques, are required (Bednarek *et al.,* 2021). Our results of SCoT analysis were similar to previous reports of genetic fidelity of micropropagated plants *C. forskohlii* (Badhepuri *et al*., 2023), *F. stobilifera* (Sirikonda *et al*., 2023), *V. planifolia* (Rathore *et al*., 2013), and *A. vulgaris* (Thakur *et al*., 2016). Similarly, ISSR primer-based confirmation analysis of *in-vitro* raised plantlets was observed in *L. javanica* (Mood *et al*., 2022), *O. majorana* (Lata *et al*., 2009), *S. taccada* (Kumar *et al*., 2011), and *A. echioides* (Sharma *et al*., 2014). This molecular analysis confirms the genetic uniformity of micropropagated plants in various subcultured stages.

Conclusion

In *M. citriodora,* an efficient regeneration protocol was developed through nodal explants. *meta*Topolin has been found to be the most effective cytokinin to induce multiple shoots. The regenerated plant's genetic purity was confirmed using ISSR and SCoT molecular markers. This established regeneration protocol can be used for genetic transformation and genome editing studies for trait improvement.

Author's contribution

Y.S. and V.S. performed experiments and drafted the manuscript. S.S. maintained the cultures and contributed to data tabulation. M.B. reviewed the manuscript. P.M. provided the plant material and reviewed the manuscript. S.K. planned and supervised the whole experiment and reviewed the manuscript.

Conflict of Interest

All authors read and approved the manuscript and declare no conflict of interest.

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