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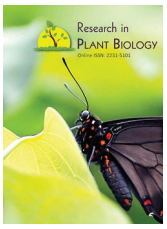
Research Article



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Micropropagation, genetic fidelity assessment and phytochemical studies of *Clerodendrum thomsoniae* Balf. f. with special reference to its anti-stress properties

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ABSTRACT

Clerodendrum thomsoniae commonly known as bleeding heart vine or bag flower. In this study, *in-vitro* callus regeneration of *C. thomsoniae* through nodal culture has been tried. Murashige and Skoog's medium (MS) with BAP was found suitable for shoot and root development. To detect clonal fidelity in *C. thomsoniae*, RAPD and ISSR markers were used. Ten RAPD decamers produced 65 amplicons, while ten ISSR primers generated 75 bands in both *in-vitro* plantlets and mother plants. The amplified products of parent plants and the regenerated plants were found to be monomorphic in RAPD and ISSR analyses. A number of compounds with potential therapeutic and biological activity had been detected with the help of GC-MS analysis. Among this the compounds namely 3-Hydroxybutyric acid, Azelaic acid, Linoleic acid, Oleic acid, Squalene, Stigmasterol are the important compounds mainly responsible for anti-cancer, anti-inflammatory, antiasthma, antimicrobial, antistress activity and so on. Taking into account of all the GC-MS and *in-silico* molecular docking data, it can be concluded that some of these compounds may be potential to future drug industry.

KEYWORDS: *Clerodendrum thomsoniae*, RAPD, ISSR, nodal stem segment, genetic fidelity, GC-MS

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INTRODUCTION

India has a long tradition of growing ornamental flowers that have recently been exploited both in domestic and international market [1]. However, due to the never ending search of the customers for new flowering plants lead the floriculture industries to explore the new species. Bleeding heart vine (*Clerodendrum thomsoniae*) belonging to the family Lamiaceae, is a potent ornamental plant recently exploited by the floriculture industries [2]. Floriculture industry mainly depends on two important components, firstly, trade of flowering inflorescences and type of foliage, and secondly, supply of nursery stocks such as tubers, seeds, bulbs, cutting-raised plants and tissue culture raised plantlets [3].Propagation through conventional techniques have some limitations and cannot fulfill the high demand of ornamental plants, therefore, there is in need of urgent attention for *in-vitro* multiplication [4].

Clerodendrum thomsoniae is a well known cultivated ornamental plant with beautiful flowers and has been extensively used in the horticulture as pot plants for its attractive flowers. The species is relatively new to the floriculture industry because unavailability and lesser number of seed production. *C. thomsoniae* is a rambling, twining, vine like shrub native to tropical West Africa. Bleeding-heart vine or Bag-flower is the common name of *Clerodendrum thomsoniae* [5,4].

Apart from having immense floriculture and horticulture importance, this plant has been utilized in traditional medicine. The other members of the genus *Clerodendrum* like *C. indicum*, *C. viscosum*, *C. serratum*, *C. colebrookianum* etc. are medicinal and used as folk medicine [6-9]. The leaves and flowers of *C. thomsoniae* are the main sources of several medicinally important phytocompounds. These phytocompounds protect human body from oxidative stress by its own competent defense mechanism and curing diversified diseases like bruises, cuts, skin

rashes and sores etc. [10]. Oxidative stress induced by reactive oxygen and nitrogen species (ROS and RNS) is a major causative agent in the induction of several brain disorders including neurogenerative diseases [11] and depression [12].

Apart from some studies on propagation, plant growth retardants etc., no attempt so far has been made to regenerate *C. thomsoniae* through *in-vitro* methods. However, proper molecular characterization and profiling of phytochemical constituents of *C. thomsoniae* still remains largely obscure. So, initiatives were taken to explore molecular biology and medicinal property detection through GC-MS. Keeping all these lacunae in mind, the aim of the present study is to standardize the working protocol of regeneration of *C. thomsoniae* by way of regeneration through callus culture, detection of somaclonal variations, if any and medicinal characterization of both the mother plant and tissue culture raised plantlets. Within the same scope we will try to evaluate whether the bioactive compounds present in *C. thomsoniae* could be responsible for reducing depression with an *in-silico* molecular docking approach.

MATERIALS AND METHODS

Establishment of Aseptic Culture

The nodal segments of *C. thomsoniae* were collected from our laboratory garden for aseptic culture. The explants were washed in 1% tween 20 for 30 minutes and then rinsed several times with double distilled water (DDW). The explants were then surface sterilized with 0.1% $HgCl_2$ for 5 min and then rinsed several times with sterile DDW. Explants were finally treated with 70% alcohol for 45 seconds and washed several times with sterile DDW to remove the traces of alcohol. The explants were then trimmed to approximately 3 cm. in length and blot dried. All these operations were conducted under aseptic environment of laminar air flow cabinet.

Callus Culture and Plantlet Regeneration

The trimmed explants were aseptically inoculated in MS medium [13] and Woody Plant medium [14], containing various concentration of growth regulators (BAP and NAA) along with three different concentrations of sucrose (Table 1) and 0.8% agar. The pH of the media was adjusted to 5.6-5.8 before adding agar and autoclaved at 121°C for 20 min at 15 psi.

Different concentrations of cytokinins like BAP (6-benzyl amino purine) and auxins like NAA (1-napthaleneacetic acid) were used in this study for initiation of callus and shoot. Controlled cultures with no hormone were also prepared. Sub-culturing was done at 2 weeks interval in the same media having the same hormonal composition [15]. BAP was applied at the rate of 1, 2, 3 and 4 mg/l and NAA at the rate of 0.5mg/l to observe various stages of *in-vitro* callusing and subsequent shoot formation. The cultures were incubated under 2000-3000 lux light intensity for 16 h/day at 25° ± 2° C temperature. Root initiation and growth took place in the same media used for callus culture.

Table 1: Effect of sucrose on regeneration of shoot buds from callus of *C. thomsoniae*

BAP (mg/l)	NAA (mg/l)	Sucrose (%)	Mean no. of shoots/callus
1 mg/l	0.5 mg/l	1	2.76±0.39
		2	3.71±0.25
		3	6.5±0.5
2 mg/l	0.5 mg/l	1	6.98±0.21
		2	9.07±0.17
		3	13±1.5
3 mg/l	0.5 mg/l	1	7.54±0.24
		2	8.24±0.30
		3	9.5±0.5
4 mg/l	0.5 mg/l	1	1.31±0.21
		2	2.5±0.10
		3	4±1.0

Hardening and Transfer of Plants to Soil

After 4 weeks of *in-vitro* root development, the plantlets were carefully taken out of the culture bottle without causing much damage to the plant parts. The roots were washed gently under running tap water to completely remove the medium. Then the plantlets were transferred to a mixture of sandy soil and farm yard manure with a ratio of 1:1 (v/v). Hardening of plantlets was conducted for 30 days in green house condition and was finally transferred to the field. The survival percentage of the acclimatized plantlets was recorded.

Molecular Analysis

The genomic DNA of field grown plant and the *in-vitro* propagated plantlets was extracted using Genelute Plant Genomic DNA kit (Sigma, St. Louis, MI, USA, Cat# G2N-70). Based on the spectrophotometric analysis, the DNA was diluted to a concentration of 25 ng/μl. Initially, a total of 20 RAPD and 15 ISSR primers were screened for PCR amplification. Both RAPD and ISSR amplifications were performed using 25 μL of PCR mixture containing 12.5 μl PCR Master Mix 2X (Promega, Cat# M7122), 1.25 μl of primer (0.25 μm), 2 μl of template DNA (25 ng/μl) and Pyrogen-free water to a final volume of 25 μl. The PCR reactions were performed on an Applied Biosystems Thermocycler 2720. The amplification condition of RAPD was 94 °C for 4 min, followed by 40 cycles of amplification with 1 min denaturation at 94 °C, 1 min annealing at 37 °C, 2 min primer extension at 72 °C, and a final extension at 72 °C for 10 min. The conditions of the thermal cycle for ISSR amplifications were 94 °C for 5 min, followed by 35 cycles of amplification having 45 s for denaturation at 94 °C, 1 min annealing at 52 °C, 1 min primer extension at 72 °C, and a final extension at 72 °C for 7 min. The PCR products were electrophoresed on 1.5% (w/v) agarose gel with two DNA markers- λ DNA/EcoRI/HindIII double digest (Chromous Biotech, Cat# MAN 06) and a 100 base pair (bp) DNA ladder (Chromous Biotech, Cat# LAN 02), and were photographed with Gel Documentation system (UVi).

GC-MS Analysis

The bio-active compounds of the mother and tissue cultured plants were identified by GC-MS analysis according to standard methods with slight modifications [16].

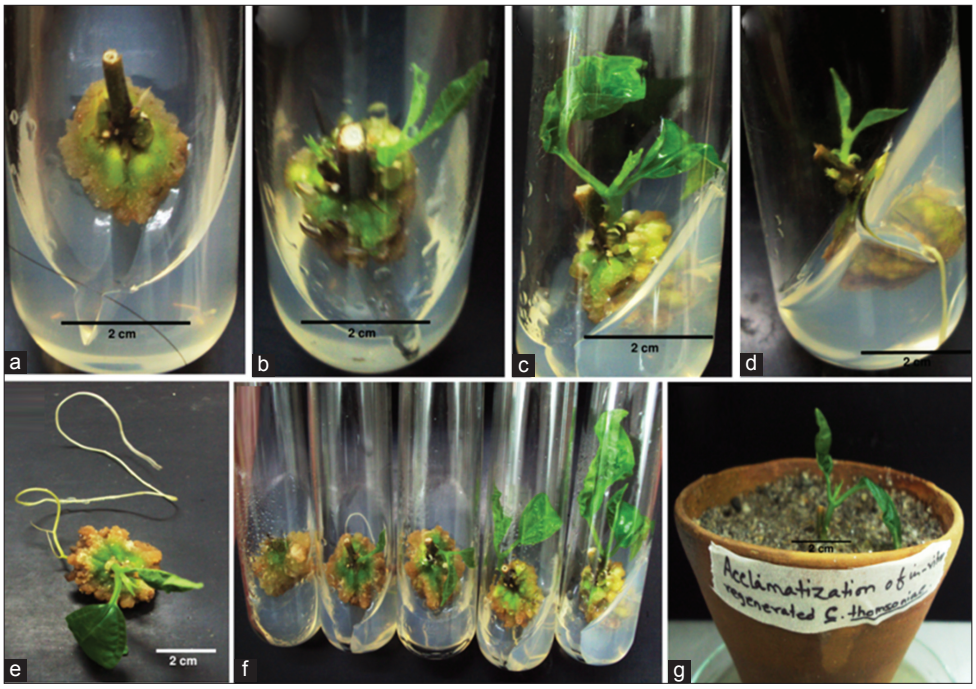

Figure 1: Stages of callus induction and regeneration. (a) Callus induction; (b) Formation of *in-vitro* shoot; (c) Development of leaves; (d) *In-vitro* root induction; (e) Plantlet with well developed roots; (f) Different stages of shoot induction; (g) Acclimatization of plant in clay pot containing mixture of soil and sand.

Table 2: Effect of different concentrations of BAP with the optimal concentrations of NAA on callus induction from nodal segments of *C. thomsoniae*

Medium	Plant growth regulators (mg/l)		Callus induction frequency (%)	Mean weight of the callus (gm)
	BAP	NAA		
MS	0	0	0	0.0
	1	0.5	25	0.11±0.015
	2	0.5	85	0.56±0.076
	3	0.5	40	0.3±0.02
	4	0.5	30	0.2±0.025

Molecular Docking

The most prominent and active compounds present in tissue cultured plants, detected through GC-MS analysis were subjected to molecular docking analysis against protein. The protein selected here is Brain-derived neurotrophic factor (BDNF) which is proved to play a role in depression [17]. The X-ray crystallographic structure of the protein available in the Protein Data Bank (http://www.rcsb.org) was used. Molecular docking was conducted using AutoDockVina [18].

Statistical Analysis

All the experiments were repeated thrice with minimum 10 explants each. The values were presented as a mean ± SE of three experiments. The data were analyzed by Student's unpaired *t* test and treatment mean values were compared at $P \leq 0.05–0.01$.

RESULTS AND DISCUSSION

Establishment of Aseptic Culture

Fungal and bacterial contamination was the main problem during the early stage of the culture initiation. To avoid this problem various surface sterilants (tween 20, 70 % ethanol, 0.1% mercuric chloride solution) were used Almost similar methods like 70 % ethanol and 0.1 % mercuric chloride ($HgCl_2$) have been used to disinfect the explants of *Clerodendrum inerme* [19]. Labolene (5%) in combination with 0.1% $HgCl_2$ has been used in the tissue culture study of *Clerodendrum serratum* by Sharma and his coworkers [20].

Callus Induction

In-vitro maintained nodal explants started swelling within 6–8 days and fully developed callus like structures were observed after 12-15 days of inoculation. All the calli were observed to

Table 3: Influence of different concentrations of plant growth regulators on regeneration of shoot buds and elongation of proliferated shoots from callus of *C. thomsoniae*

Medium	Plant growth regulators (mg/l)		Mean no. of shoots/ callus	Mean shoot length (cm)
	BAP	NAA		
MS	0	0	0.0	0.0
	1	0.5	6.5±0.5	1.33±0.3**
	2	0.5	13±1.5	3.5±0.5**
	3	0.5	9.5±0.5	2.3±0.15**
	4	0.5	4±1.0	1.6±0.07^{NS}

Data expressed as mean±S.D (n=3). **p<0.01; ^{NS}-Non significant when compared with mean no. of shoots/callus.

Table 4: Effect of different concentrations of BAP and NAA on rooting of *in-vitro* raised elongated roots in *C. thomsoniae*

Medium	Plant growth regulators (mg/l)		Mean no. of roots/ explant	Mean root length (cm)
	BAP	NAA		
MS	0	0	0.0	0.0
	1	0.5	1.33±0.35	7.8±0.76**
	2	0.5	3.5±0.5	21.3±1.52**
	3	0.5	1.9±0.13	13±1.0**
	4	0.5	1.7±0.05	10.5±0.5**

Data expressed as mean±S.D (n=3). **p<0.01 when compared with mean no. of roots/explants.

Table 5: PCR amplification using RAPD and ISSR primers

Primer ID	Primer sequence (5′-3′)	Total bands amplified	No. of monomorphic bands	Band size (bp)
OPA 01	CAGGCCCTTC	4	4	324-1310
OPA 02	TGCCGAGCTG	8	8	325-1646
OPA 03	AGTCAGCCAC	13	13	224-1434
OPA 07	GAAACGGGTG	3	3	220-1128
OPA 08	GTGACGTAGG	5	5	357-1453
OPA 09	GGGTAACGCC	4	4	286-1218
OPA 10	GTGATCGCAG	7	7	346-1605
OPA 11	CAATCGCCGT	5	5	383-1383
OPA 12	CAGCACCCAC	9	9	459-1544
OPA 18	AGGTGACCGT	7	7	363-1589
Total bands		65	65	
UBC 807	(AG) 8T	7	7	226-949
UBC 808	(AG) 8C	8	8	139-1340
UBC 810	(GA) 8T	12	12	225-935
UBC 811	(GA) 8C	6	6	185-847
UBC 813	(CT) 8T	3	3	250-975
UBC 815	(CT) 8G	4	4	425-1260
UBC 818	(CA) 8G	8	8	326-1030
UBC 822	(TC) 8A	8	8	288-1250
UBC 824	(TC) 8G	8	8	235-1225
UBC 825	(AC) 8T	11	11	323-1110
Total bands		75	75	

be initiated from the cutting edge of the explants (Fig. 1). The calli formed were fast growing, yellowish green and compact. Though in our tissue culture studies, MS and WPM were used for preliminary screening, but better responses were observed in cultures of MS media. So, for propagation of *C. thomsoniae* only MS was employed for further regeneration. This indicates that some of the essential component required by *Clerodendrum* for its regeneration is not available in WPM [21]. Similar observation on other species of *Clerodendrum* tissue culture is available. Callus was induced only when nodal explants were inoculated in MS medium supplemented with BAP and NAA. The callus induction frequency was found optimum in MS medium supplemented with 2 mg/l BAP and 0.5 mg/l NAA (85%), followed by 40% with 3 mg/l BAP and 0.5 mg/l NAA (Table 2). Further increase in the hormone concentration resulted in reduction of callus formation. In our present study, the highest callus weight (0.56±0.076gm) was noted with 2 mg/l BAP and 0.5 mg/l NAA followed by 3 mg/l BAP and 0.5 mg/l NAA (0.3±0.02gm) (Table 2).

Shoot Regeneration

The regenerative ability of the compact calli was studied by the application of two important plant hormones cytokinins and auxin. After 2 weeks of the culture, most of the calli turned green and emergence of shoot primordia took place (Fig. 1). The number and length of shoots varied with the concentration of phytohormones used. MS medium supplemented with 2 mg/l BAP in combination with 0.5 mg/l NAA produced the highest number of shoot buds (13±1.5) followed by 3 mg/l BAP in combination with 0.5 mg/l NAA (9.5±0.5) (Table 3). BAP is most commonly used cytokinin mainly due to twofold reasons, firstly, it is cheap and secondly, it can be autoclaved [22]. The highest shoot length (3.5±0.5 cm) was noted with 2 mg/l BAP and 0.5 mg/l NAA followed by 2.3±0.15 cm with 3 mg/l BAP in combination with 0.5 mg/l NAA. In the present workflow, BAP was found to be rather effective incase of shoot multiplication. These findings are in accordance with the earlier work on *in-vitro* propagation of other *Clerodendrum* species like *C. inerme* [19], *C. serratum* [20], *C. colebrookianum* [23], *C. inerme* [24] where BAP has been widely used and was found to be effective.

In our present experiment, it was noted that root development took place without changing the medium and hormones. In BAP assisted with MS, rooting was initiated little later 10 ± 3 days after shoot development. About 90 % of shoots rooted in MS medium supplemented with 2 mg/l BAP in combination with 0.5 mg/l NAA. The highest number of roots (3.5±0.5) and maximum length of roots (21.3±1.52 cm) were recorded with 2 mg/l BAP with 0.5 mg/l NAA followed by 3 mg/l, 4mg/l BAP (Table 4; Fig. 1).

Acclimatization of *In-vitro* Regenerated Plants

The *in-vitro* propagated plantlets were successfully transferred to clay pots containing sandy soil and farm yard manure in a ratio of 1:1 (v/v) exhibited 70 % survival rate and grew in the greenhouse (Fig. 1). After a month, these acclimatized plants were successfully transferred to the field.

Genetic Fidelity among *in-vitro* Raised Plantlets

Plantlets regenerated indirectly from the nodal explants via callus culture were used for the study of somaclonal variations. No differences were detected by RAPD and ISSR analysis in

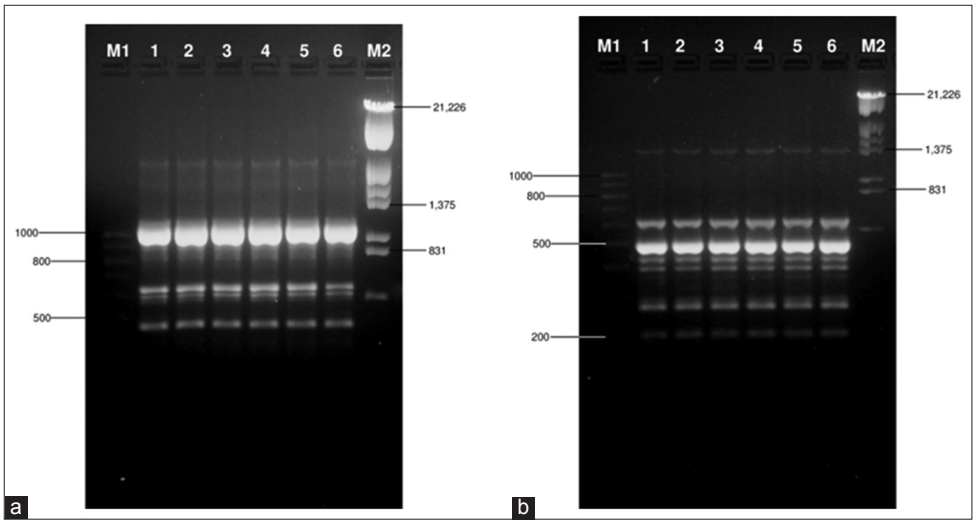

Figure 2: DNA fingerprinting pattern of *in-vitro* callus regenerated plantlets of *C. thomsoniae*. (a) using RAPD primer OPA 13 and (b)using ISSR primer UBC 808. Lane 2–6: micropropagated plantlets compared with mother plant (lane1); Lane M1: 100 bp molecular marker; Lane M2: λ DNA/EcoRI/HindIII double digest DNA ladder.

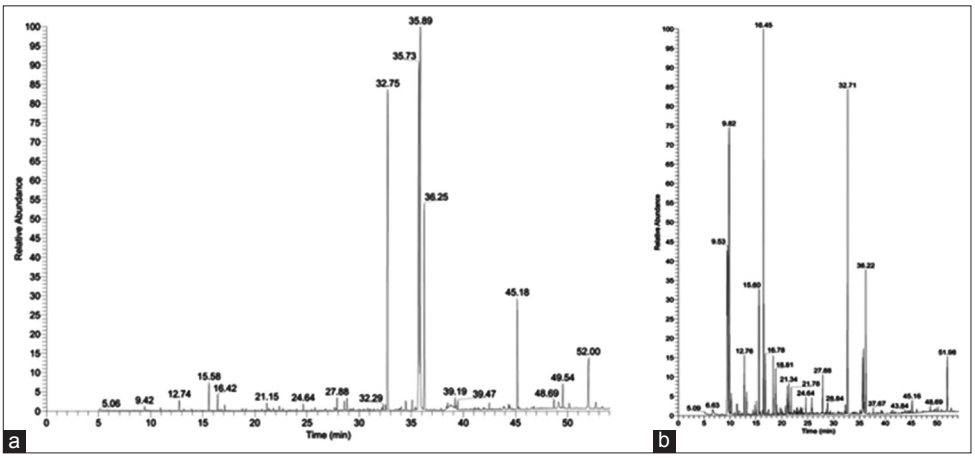

Figure 3: GC-MS fingerprinting of (a) mother plant and (b) tissue culture plant.

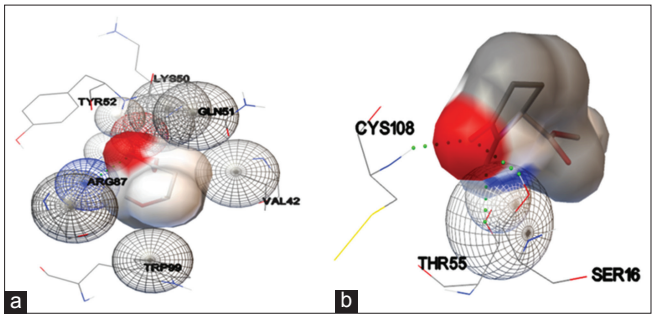

Figure 4: Molecular surface view of BDNF protein with (a) 3-Hydroxybutyric acid and (b) Pyroglutamic acid docked into its binding site.

between the genetic material of mother plant and plantlets regenerated from nodal explants. A total of 45 primers (30 RAPD and 15 ISSR) were used to screen somaclonal variations, of which clear and scorable amplification products were produced by 10 each RAPD and ISSR primers. Distinct and scorable bands were obtained from the RAPD analysis (65) and ISSR analysis (75) (Table 5). In case of RAPD primers number of bands varied from 3-13 whereas in case of ISSR primers the number of bands ranged from 3-12. All the bands generated through both RAPD and ISSR analyses were found to be monomorphic i.e. bands generated were common in parental genotypes and the *in-vitro* raised plantlets. A representative of RAPD and ISSR profile is depicted in Fig. 2. Our fingerprint analyses confirm genetic stability and clonal fidelity of *C. thomsoniae*. Importance of RAPD and ISSR fingerprints to detect clonal fidelity of *in-vitro* raised plantlets has already been reported by Goyal et al. [25].

GC-MS Analysis

The present study was extended for the analysis pertaining to the identification of active compounds in mother plant and tissue culture plant using GC-MS method. A total number of nineteen (19) and twenty seven (27) phytocompounds have been identified in mother plant and tissue culture plant respectively (Table 6), which corresponds to Fig. 3. Ten

Table 6: List of phytocompounds identified in *C. thomsoniael*eaf extract (mother plant and tissue culture plant) by GC-MS analysis

Sl. No.	Compound name	Formula	Mol. Wt.†	Mother plant	Tissue culture plant
1.	Acetic acid	$C_2H_4O_2$	55	×	✓
2.	Propanoic acid	$C_3H_6O_2$	73	×	✓
3.	3-Hydroxy-2-butanone	$C_4H_8O_2$	87	×	✓
4.	Glyceric acid	$C_3H_6O_3$	89	✓	×
5.	3-Hydroxybutyric acid	$C_4H_8O_3$	103	×	✓
6.	Diethylene glycol	$C_4H_{10}O_3$	105	×	✓
7.	2-Furancarboxylic acid	$C_5H_4O_3$	111	×	✓
8.	2-Hexenoic acid	$C_6H_{10}O_2$	113	✓	✓
9.	Butanedioic acid	$C_4H_6O_4$	117	✓	✓
10	1,2,3-Benzenetriol (pyrogallol)	$C_6H_6O_3$	125	×	✓
11	Pyroglutamic acid	$C_5H_7NO_3$	127	×	✓
12.	Pentanedioic acid (Glutaric acid)	$C_5H_8O_4$	131	✓	✓
13.	Malic acid	$C_4H_6O_5$	133	✓	×
14.	1,2-Ethanediol, phenyl	$C_8H_{10}O_2$	137	×	✓
15.	Benzoic acid, 3-hydroxy (m-Salicylic acid)	$C_7H_6O_3$	137	×	✓
16.	4-Hydroxyphenylethanol (Tyrosol)	$C_8H_{10}O_2$	137	×	✓
17.	Adipic acid	$C_6H_{10}O_4$	145	×	✓
18.	Heptanedioic acid (Pimelic acid)	$C_7H_{12}O_4$	159	×	✓
19.	Octanedioic acid (Suberic acid)	$C_8H_{14}O_4$	173	×	✓
20.	1-Dodecanol	$C_{12}H_{26}O$	185	×	✓
21.	Azelaic acid	$C_9H_{16}O_4$	187	✓	✓
22.	Dodecanoic acid (lauricaicd)	$C_{12}H_{24}O_2$	200	✓	✓
23.	Ethyl tartrate	$C_8H_{14}O_6$	205	×	✓
24.	Tetradecanoic acid	$C_{14}H_{28}O_2$	227	✓	✓
25.	Hexadecanoic acid (Palmitic acid)	$C_{16}H_{32}O_2$	255	✓	✓
26.	Heptadecanoic acid	$C_{17}H_{34}O_2$	269	✓	×
27.	Linoleic acid	$C_{18}H_{32}O_2$	279	✓	✓
28.	Oleic acid	$C_{18}H_{34}O_2$	281	×	✓
29.	Stearic acid	$C_{18}H_{36}O_2$	283	✓	×
30.	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl	$C_{20}H_{40}O$	295	✓	×
31.	Eicosanoic acid	$C_{20}H_{40}O_2$	311	✓	×
32.	Palmitelaidic acid, trimethylsilyl ester	$C_{19}H_{38}O_2Si$	325	✓	×
33.	Heptacosane	$C_{27}H_{56}$	379	✓	×
34.	Squalene	$C_{30}H_{50}$	409	✓	✓
35.	Stigmasterol	$C_{29}H_{48}O$	412	✓	✓
36.	α-Tocopherol	$C_{29}H_{50}O_2$	429	✓	×

compounds are common between mother plant and tissue culture plant, but interestingly seventeen (17) compounds were exclusively present in tissue culture plant of which a number of compounds are of potential therapeutic significance. Several phytochemicals belonging to long chain fatty moiety and their derivatives such as Hexadecanoic acid; Dodecanoic acid; Tetradecanoic acid; Heptanedioic acid; Octanedioic acid; Adipic acid etc has been identified in the samples. Interestingly, these long chain fatty acids play an important role in plant development [26]. linoleic acid (LA), oleic acid (OA), squalene and stigmasterol are the main bioactive compounds having different medicinal properties. LA is one of the essential fatty acids that human need in diet. Deficiency of LA may lead to growth retardation, infertility, skin and kidney degeneration and abrupt changes in fatty acid composition of lipids [27]. Besides, LA has been reported to suppress human tumor [28] and lung tissue cancer [29]. Another metabolite, OA has been reported to have potential protective effect against breast cancer and colon carcinomas in rats [30,31]. 3-Hydroxybutyric acid has anti-stress activity [32]. In addition, squalene and stigmasterol were reported as potent antioxidants as well as beneficial against several carcinogens [33,34]. Hence, it seems likely that the tissue culture plant contain plenty of medicinally important constituents which might be a good source of natural medicine.

Molecular Docking

The compounds detected by GC-MS for the mother plant and the tissue cultured progeny largely contain the same set of phytocompounds indicating that the basic chemical architecture of both is same. However, some of the compounds present in tissue cultured plant were missing in the mother plant (Table 6). Among them interestingly 3-Hydroxybutyric acid and Pyroglutamic acid are also present. They are both reported for anti-stress activity and their presence indicates the intelligent development scheme for the micropropagated progeny [35,32]. To further understand their chemical behavior from a different perspective we conducted *in-silico* molecular docking tests. Here the receptor protein used is Brain-derived neurotrophic factor (BDNF), which is reported to have an upper hand in depression and stress related lifestyle hazards [32]. The two compounds are seen to have good interactions with our BDNF receptor, 3-Hydroxybutyric acid has a binding affinity of -4.4 kcal/mol and Pyroglutamic acid has a binding affinity of -4.7 kcal/mol (Fig. 4). So, it can be inferred that the 3-Hydroxybutyric acid and Pyroglutamic acid present in the micropropagated plants can have a potential to bind with BDNF receptor. Hence, besides having all the therapeutic uses of the plant itself, the tissue cultured plants can possess an added anti-stress property in itself.

CONCLUSION

The present study is the first report of establishment of standard protocol for *in-vitro* regeneration of *C. thomsoniae*. The regenerated plantlets were healthy and RAPD and ISSR analysis showed that there was virtually no difference among the micropropagated plantlets and the mother plant of *C. thomsoniae* and thus can be concluded that the *in-vitro* regenerated plantlets were genetically stable and identical to their parental counterpart. Potential medicinal molecules derived during the regeneration process of *C. thomsoniae* can be a biotic, aseptic and low cost source of phytochemical for pharmaceutical industry.

AUTHOR CONTRIBUTIONS

PK, AKC, MB and TM carried out the research, collection and analyzing data and wrote the first draft of the manuscript. AKC carried out statistical and docking part of the manuscript. AS conceived of the study, and participated in its design and coordination helped to draft the manuscript. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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