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Regeneration and Genetic Fidelity Analysis of *Chlorophytum borivilianum* Using Flower Stalk as Explant Source

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Abstract

Chlorophytum borivilianum is a critically endangered plant well known for its medicinal properties for diabetes mellitus, diarrhea, arthritis, sterility, and erectile dysfunction, etc. Due to low viability and long dormancy of seeds, *in vitro* regeneration is required for large scale cultivation of this plant. In the present study, direct plant regeneration was optimized using flower stalk as explant. Nodal segments of flower stalk were sterilized and kept for direct regeneration on different combinations of BAP and KIN supplemented media. The highest, 15.27 ± 1.14 number of shoots were produced on medium containing BAP (2 mg/L) per nodal segment. The multiple shoot clumps regenerated from flower stalk were separated carefully and kept on rooting media. A maximum of 16.87 ± 1.53 roots per plant was observed in MS media having 0.5 mg/L of NAA. The rooted plantlets were shifted into the pot containing soilrite for hardening and acclimatization. The genetic stability of hardened plants was confirmed by start codon targeted, and inter simple sequence repeats molecular markers. All the 18 randomly selected plantlets showed similar genetic homogeneity to the mother plant. It is the first report on *in vitro* regeneration along with the genetic fidelity analysis of the regenerated plantlets from flower Stalk of *C. borivilianum*. As the standardized method of regeneration and mass multiplication is quite efficient and genetically stable, the protocol will be useful for the large-scale production of *C. borivilianum* to meet the market demand.

Keywords

Chlorophytum borivilianum, *In Vitro* Regeneration, SCoT, ISSR, Genetic Fidelity

1. Introduction

Chlorophytum borivilianum is one of the most valuable medicinal plants of *Li-*

liaceae family. Pharmaceutically, it is a very important plant because of its tuberous roots which contains steroidal saponins, alkaloids, steroids, flavonoids, triterpenoids, tannins, and phenolic acids [1] [2]. The medicinal properties of this plant are well known for curing weaknesses, physical illnesses, diabetes mellitus, diarrhea, dysuria, arthritis, sterility, and erectile dysfunction [2] [3]. Dried roots are most commonly used in traditional medicine due to their aphrodisiac and natural tonic properties [4]. Traditionally, it is grown using seeds and tubers. However, vegetative propagation via traditional methods is not sufficient for large scale cultivation [5]. The long dormancy period of *C. borivilianum* seeds, poor seed germination, and low seed viability (11% - 24%) limits its natural regeneration methods [6]. Due to these is limitations, *in vitro* culture based methods are in demand and have been successfully used for micropropagation of different species of Chlorophytum [7] [8]. Tissue culture which includes callus induction, micro-propagation and *in vitro* tuberization has been reported in different species of Chlorophytum such as *C. comosum* [9], *C. borivilianum* [10], *C. amanienese* [11] and *C. arundianceum* [8]. *In vitro* micropropagation depends on the type of explants and growth regulators used in the medium. The clonal propagation of *C. borivilianum* through multiple shoot induction has been reported using shoot buds, stem discs and, from callus and hypocotyls of geminated seedling using 6-benzylaminopurine (BAP), Kinetin (KIN) and Thidiazuron. As per the previous reports, BAP and KIN (0.5 to 15 mg/L) individually and in combinations with auxins (2,4-D, IAA and IBA) are efficient in shoot induction, proliferation and multiplication. [6] [7] [12] [13] [14] [15]. But the major concern for *in vitro* regenerated plants is the incidence of somaclonal variations in the form of DNA polymorphism, chromosomal aberrations and sequence changes as the culture techniques, composition of nutrient media, amount and, combinations of growth hormones and growth conditions can cause somaclonal variations in micro propagated plants [16].

Differentiated plant organs (leaves, stems and roots) have the tendency to produce more variations than explants like axillary shoot buds or inflorescences having meristematic tissue [17]. Also, young tissues are less prone to genetic variations as compare to the older tissue [18]. Studies suggest that direct regeneration of plantlets from the explants like apical shoot buds and inflorescence is least prone to genetic modifications [19]. However, a little possibility of occurrence of somaclonal variations still exists as genetic variations do occur in undifferentiated cells, isolated protoplasts, calli, tissues and morphological traits of *in vitro* raised plants [16] [20] [21]. Genomic fingerprinting can differentiate between individuals, species and populations and is useful for the detection of the homogeneity of the regenerated plants. Therefore, genome based approaches, which include sequence based and direct polymorphism methods, have been developed for the authentication of medicinal plants [22]. The DNA based markers such as SCoT (start codon targeted) and ISSR (inter simple sequence repeats) have been successfully utilized to investigate genetic stability or variability in the tissue culture raised plants in several plant species [14] [23] [24]

[25].

Micropropagation in *C. borivilianum* has been established using young shoot buds [12] [26], seeds [6], stem discs [27], immature floral buds [15], apical meristems in *C.comosum* [9] and leaf from *in vitro* grown plant from *C. borivilianum* seed [28] as an explants. However, disadvantages like frequent bacterial contamination with stem discs due to their direct contact with soil and poor viability of seeds [10] have been seen.

Present study was designed in accordance to the recent studies of *in vitro* regeneration and genetic fidelity analysis in different medicinal plants [29] [30] [31]. In our study, we have used flower stalk (nodal segment) as an explant for plant regeneration and multiple shoot induction. The genetic fidelity of regenerated plantlets was then checked using SCoT and ISSR markers. The main idea behind using nodal segments was the presence of active meristems which provide the best platform for multiple shoot induction and regeneration. Moreover, direct regeneration methods would help in maintaining genetic homozygosity. The objective of the study was achieved by healthy flower stalk selection, *in vitro* regeneration of the nodal segments to produce plantlets, greenhouse acclimatization and genetic fidelity analysis of the hardened plants using molecular markers.

2. Materials and Methods

2.1. Surface Sterilization and Explant Preparation

Plants of *C. borivilianum* were maintained in culture room of department of biotechnology at Panjab University, Chandigarh. Approximately, 2 - 3 Flower stalks each having 4 - 5 internodes were taken, washed thoroughly under running tap water. The surface sterilization was done with 0.1% mercuric chloride for 2 - 3 min and them washed with autoclaved distilled water for 4 - 5 times. Nodal segments of flower stalk were then cut into 3 - 4 cm pieces and kept on shooting media.

2.2. Media Preparation and Culture Conditions

Murashige and Skoog's (MS) basal medium [32] having calcium chloride, vitamins, sucrose and agar (Himedia, India) was used for *in vitro* experiments. MS media was dissolved in deionized water, growth regulators [Benzyl aminopurine (BAP) and Kinetin (KIN)] were added, and pH was adjusted to 5.8. All cultures were kept at 25˚C ± 2˚C under 16 h light and 8 h dark photoperiod having light intensity of 3000 lux.

2.3. Media Optimization for Direct Regeneration Using Flower Stalk

Direct regeneration of multiple shoots from nodal segment of flower stalk was optimized using different cytokinin (BAP/KIN) concentrations varied from 0 - 4 mg/L (**Table 1**). 5 - 6 Sterilized nodal explants were inoculated per Petri dish

having different hormone combinations. The cultures were kept in light as mentioned above and after every 14 - 15 days the fresh medium was used for the subculturing of explants. The number of regenerated shoots was counted after 6 - 8 weeks of initiation of cultures.

2.4. Root Media Optimization

Multiple shoots clumps originated from somatic embryos and nodal segment were separated for rooting. The individual shoots were kept onto different media. MS medium supplemented with root initiation medium (RIM) having different concentrations of NAA (0.25, 0.5 and 1 mg/L) and IAA (0.25, 0.5 and 1mg/L) were used for root induction. MS full strength and MS half strength media were used as controls to check the effect of growth regulator free media on root induction. The root number and length were observed after 6-8 weeks of initiation of cultures (**Table 2**).

2.5. Hardening and Acclimation

Each well rooted plantlet was taken out from a bottle or a test tube and washed carefully with tap water to remove agar from the roots. Plants were transferred to pots containing soilrite and covered with perforated polythene bags. These poly bag covered pots were shifted to Percival growth chamber (Percival, USA) for 2 weeks under controlled conditions. After 2 weeks poly bags were removed and again kept for one week in growth chamber. Finally, after one-week plants were exposed to the external environment.

2.6. Genomic DNA Isolation and Polymerase Chain Reaction (PCR)

Total genomic DNA was isolated from 40 - 45 hardened plants and mother plants using DNeasy Plant Mini Kit (QIAGEN, Germany). The purity of DNA was checked on 0.8% agarose gel. The DNA was quantified using spectrophotometer (Bio-Rad, U.S.A.). Each DNA sample was diluted with sterile distilled water to make the concentration 100 ng/µl. A total of 8 SCoT and 4 ISSR primers were selected from previous genetic fidelity studies (**Table 3**) [32]. PCR was performed using Dream *Taq polymerase* enzyme (Thermo Fisher, U.S.A.) and 100 ng DNA as template. Each PCR reaction contains, 2.5 µl 10X buffer, 1 µl 10 mM dNTP, 10 pmol primer 1 U Dream taq enzyme, and nuclease free water in total volume up to 25 µl. The PCR reaction was carried out in a thermal cycler (Bio-Rad, U.S.A.). The amplification cycles conditions were as follow: initial denaturation: 94˚C for 3 minutes; 35 cycles of denaturation: 94˚C for 45 seconds; annealing: 42˚C - 45˚C for 1 minute; extension at 72˚C for 90 seconds and final extension at 72˚C for 10 minutes for total 35 number of cycles. PCR products were then separated onto 1% agarose gel using Good view staining dye (Br Biochem, India) in Tris acetic acid EDTA (TAE) buffer. The bands were visualized using gel documentation system (Bio-Rad, U.S.A.). Banding patterns were analyzed for polymorphism.

Table 1. Effect of Cytokinins (BAP and KIN) on shoot regeneration using flower stalk of *C. borivilianum*.

MEDIA	BAP (mg/L)	Kinetin (mg/L)	No. of shoot per nodal explant
Control (MS)	-	-	0.87 ± 0.12^{g}
SIM1	1	-	7.40 ± 1.04^{ef}
SIM2	2	-	15.27 ± 1.14^{a}
SIM3	3	-	11.13 ± 0.64^{c}
SIM4	4	-	9.13 ± 1.21^{d}
SIM5	-	0.5	6.20 ± 1.00^{f}
SIM6	-	1	12.60 ± 1.20^{b}
SIM7	-	2	9.13 ± 0.31^{d}
SIM8	-	3	8.60 ± 0.72^{de}

N = 3 with each replicate consists of 5 explants per treatment. Value in column represents mean ± Standard deviation. Mean with same letter with in column are not significantly different ($P \leq 0.05$). *N = 3 (Each replicate of regeneration studies consists minimum of 5 cultures).

Table 2. Effect of media and auxins (NAA/IAA) on root induction and proliferation of regenerated shoots of *C. borivilianum*.

MEDIA	NAA (mg /L)	IAA (mg /L)	Root number (mean ± SD)	Root length (mean ± SD)
Control 1	-	-	4.8 ± 0.60^{e}	4.28 ± 0.39^{e}
Control 2	-	-	6.8 ± 1.0^{e}	5.29 ± 0.22^{d}
RIM1	0.25	-	11.47 ± 0.95^{cd}	5.57 ± 0.08^{cd}
RIM2	0.5	-	16.87 ± 1.53^{ac}	6.55 ± 0.20^{a}
RIM3	1.0	-	11.20 ± 1.11^{b}	5.87 ± 0.29^{bc}
RIM4	-	0.25	12.13 ± 1.01^{bcd}	5.53 ± 0.28^{cd}
RIM5	-	0.5	14.27 ± 0.76^{b}	6.23 ± 0.08^{ab}
RIM6	-	1.0	10.60 ± 0.60^{d}	5.31 ± 0.06^{d}

Control 1 (full strength MS media) and Control 2 (half strength MS media). N = 3 with minimum of 5 explants per treatment. Value in column represents mean ± Standard deviation. Mean with same letter with in column are not significantly different ($P \leq 0.05$).

Table 3. List of Primers used and genetic homogeneity analysis of regenerated plantlets:

S. No.	Primer ID	Primer Sequence	Tm (˚C)	No. of amplified bands	Amplified product range (KB)
1	SCoT_1	CAACAATGGCTACCACCA	42	3	0.9 - 1.5
2	SCoT_2	CAACAATGGCTACCACCC	42	4	0.8 - 2.0
3	SCoT_3	CAACAATGGCTACCACCG	42	4	1.0 - 2.5
4	SCoT_4	CAACAATGGCTACCACCT	42	6	1.0 - 2.5
5	SCoT_5	ACGACATGGCGACCGCGA	45	6	0.6 - 2.2
6	SCoT_6	ACCATGGCTACCACCGCC	45	3	0.9 - 2.0
7	SCoT_7	CATGGCTACCACCGGCCC	45	5	0.6 - 2.4
8	SCoT_8	GCAACAATGGCTACCACC	45	2	1.1 - 1.3
9	ISSR_1	TCTCTCTCTCTCTCTCRT	45	4	0.9 - 2.0
10	ISSR_2	TCTCTCTCTCTCTCTCRG	45	5	0.4 - 2.4
11	ISSR_3	TGTGTGTGTGTGTGTGRA	45	3	1.0 - 1.6
12	ISSR_4	CACACACACACACACARC	50	4	0.4 - 2.0

2.7. Statistical Analysis

All data presented were the mean values of at least 3 independent experiments with 3 replicates each. Analysis of variance (One way ANOVA) at $P \leq 0.05$ in SPSS version 17 (SPSS Inc., Chicago, USA), followed by Duncan's post hoc test to calculate the mean significant difference using SPSS software.

3. Results

3.1. Direct Regeneration from Nodal Segment of Flower Stalk

The flower stalk (Figure 1(a)) which contains 6 - 8 nodal regions was cut down into small pieces and used as an explant. Explant which did not have nodal region did not respond to shooting media (Figure 1(b)). Few explants were grown in MS medium without any growth regulator produced one shoot after 4 weeks (Figure 1(c)). The explants inoculated on SIM2 and SIM6 media showed small greenish callus like structure at nodal region after 13 - 15 days of culture initiation, which further turned into multiple shoot clumps (Figure 1(d)). During subculture cycle these multiple shoot clumps were trimmed off from the flower stalk segment and divided into 4 - 5 sections for proper growth and multiplication of shoots (Figure 1(e) and Figure 1(f)). These shoots clumps were allowed to grow for 8 - 10 weeks on shoot induction media under continuous subculturing (Figure 1(g)). The number of shoots per explant on different media was calculated after 6 weeks and is tabulated in Table 1. The SIM2 media showed best shoot regeneration of 15.27 ± 1.14 per explant as compared to SIM6 where 12.60 ± 1.20 were regenerated. Rest of the shooting media showed decrease in shoot number with increase or decrease of cytokinin concentration.

3.2. *In Vitro* Rooting and Acclimatization

Individual shoots separated from multiple shoots clump were separated carefully and kept on rooting medium (Figure 2(a)). After 6 weeks, roots formed from a single separated shoot of inoculation on different media were counted and is shown in Table 2. MS full strength media showed 4.8 ± 0.60 roots per shoot with a root length of 4.28 ± 0.39, whereas few separated plants did not respond to the media. However, half MS showed better response 6.8 ± 1.0 roots per explant with the root length of 5.29 ± 0.22. Addition of auxins such as IAA and NAA in MS medium enhanced the roots formation. RIM2 and RIM5 were found to be more efficient in inducing roots, producing 16.87 ± 1.53 and 12.13 ± 1.51 with a root length of 6.55 ± 0.20 and 5.53 ± 0.28, respectively (Table 2). Rooted plants were taken out from tube and washed with water to remove excess of agar without damaging roots (Figure 2(b)). Plantlets were then transferred into pots having soilrite and covered with transparent poly bags for 2 weeks acclimatization (Figure 2(c)). All acclimatized plants did not show any morphological variations and tubers were formed within 4 - 5 months (Figure 2(d) and Figure 2(e)).

Figure 1. (a) Flower stalk (explant) of *C. borivilianum*; (b) Inoculation on SIM2 media after sterilization; (c) Shoot proliferation on hormone free media (MS only); and (d) on SIM2 media; (e) Shoot regeneration after excising proliferating meristematic region into smaller segments on SIM2 media; (f) Shoot growth from one segment (after excising) of meristematic region on SIM2 media; (g) Multiple Shoot growth on SIM2 media.

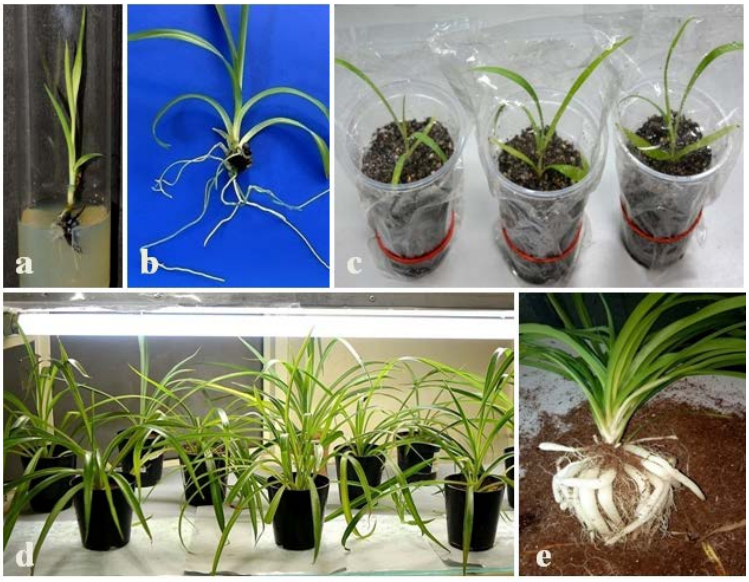

Figure 2. (a) Inoculation of individually separated shoot on root induction media; (b) Fully grown plantlet showing healthy shoot and roots after 6 weeks of inoculation on rooting media; (c) Acclimatized plantlets with no morphological changes after3weeks of hardening; (d) Hardened plants after 4 months on soilrite; (e) Fully grown healthy plant after 5 months.

3.3. SCoT and ISSR Analysis

Genetic homogeneity of 18 randomly selected plantlets was done using SCoT and ISSR primers. Each SCoT and ISSR primer showed the amplified bands within the range of 0.4 to 2.5 KB (Table 3). Eight SCoT primers generated total 33 reproducible PCR bands with an average of 4.08 bands per primer. In case of ISSR, a total of 16 bands were amplified using 4 primers with an average of 4 bands per primer. All the *in vitro* grown plantlets showed same banding pattern as identical to the mother plant (Figure 3).

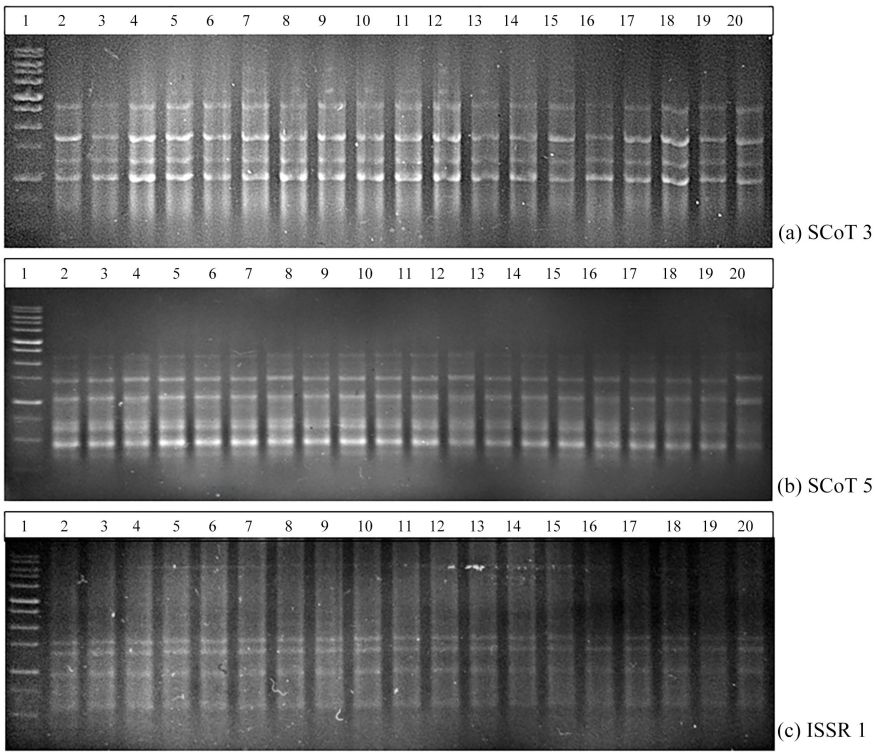

Figure 3. Showing DNA profiles of mother plant and 18 tissue culture raised plantlets of *C. borivilianum*; SCoT_3 (3a), SCoT_5 (3b), ISSR_1 (3c). Lane 1: 1 KB DNA ladder; Lane 2: DNA banding pattern of mother plant; L3 - L120 showing DNA banding pattern of direct regenerated plantlets.

4. Discussion

Ease of explant availability, less contamination frequency and genetic stability in regenerants are the most important factors which were considered during explant selection. In previous reports, mostly shoot buds, and stem discs have been used as initial explants [26] [27]. The major limitations of these explants are sacrificing whole plant and higher contamination probability. In some other studies, immature inflorescence was used, however its availability is limited as compared to flower stalk which remains with the plant even after perishing of the inflorescence [8] [15]. Moreover, flower stalk is less delicate and easy to handle as compare to immature inflorescence and axillary buds during *in vitro* culturing. The plant hormones and their optimum concentration play a key role in decid-

ing the fate of plant tissues and also, maintaining the genetic fidelity. In earlier studies of direct shoot regeneration, young shoot buds [26], stem discs [27], immature inflorescence and floral buds [8] [15] were used as explant for micropropagation in *C.borivilianum*. For shoot regeneration, young shoot buds [26] were inoculated onto MS medium supplemented BAP and KIN. In another study, immature floral buds attached with inflorescence axis were used as explant for direct regeneration. MS medium supplemented with 2 mg/l kinetin and 0.1 mg/l 2, 4-D showed best response for multiple shoot induction [15].

In earlier study, Samantray *et al.* (2009) used middle segment located just behind the apex of immature inflorescence for shoot regeneration where BAP (3 mg/L) in combination with $AdSO_4$ and NAA produced 7.4 shoots per explant and no response was observed using KIN supplemented media [8]. In our study, using single nodal segment a maximum no. of 15.27 ± 1.14 and 12.60 ± 1.20 shoots were regenerated on SIM2 and SIM6 supplemented media, respectively. The no. of shoots was quite higher on both media as compare to earlier reports [8]. The protocol established by Sharma and Mohan (2006) using immature floral buds took approximately 50 weeks to field transfer of *in vitro* grown plants which is quite a long time as compare to our established protocol where 24 - 25 weeks were taken to produce hardened plants. MS media having 0.5 - 1 mg/L IAA/NAA/IBA showed better rooting frequency as compare to other growth regulators, which is in accordance with the previous reports of *C. borivilianum* [15] [26]. In our study, NAA (0.5 mg/L) showed best rooting response over IAA. Number of roots started to decrease with the increase in concentration.

To check the genetic fidelity of tissue culture raised plants various molecular markers are available. Nowadays, SCoT and ISSR markers are gaining much attention due to their better resolvability over other markers [14] [33] [34]. Moreover, use of more than one marker *i.e.*, ISSR and SCoT is advantageous to justify the results more evidently. The SCoT markers have start codon (ATG) in their sequence [35] whereas, ISSR marker are based on the non-coding regions of the DNA [33]. In recent studies, SCoT and ISSR markers have been frequently used for assessing the genetic stability of several medicinal plants such as *Morus alba*, *Pittosporum eriocarpum*, *Cleome gynandra* and *Albizia julibrissin* [14] [23] [33] [35] where the results were quite promising as compare to the other markers. A previous study showed polymorphic information content (PIC), of SCoT was more informative with better distinction power than the other markers. SCoT and ISSR markers dendrograms also showed a close relationship in bamboo genotypes [36]. In another study where SCoT, ISSR and directly amplified minisatellite DNA (DAMD) markers were studied together for fingerprinting of chickpea genotypes showed that SCoT and ISSR markers were quite informative as compare to other markers for the genetic assessment [37]. In case of *C. borivilianum*, there are very few reports on genetic assessment of *in vitro* grown plants. Arora *et al.* (2006) reported that long-term embryogenic cultures of *C. borivilianum* showed variation using RAPD markers [28]. In earlier study of direct rege-

neration in *C. borivilianum* using stem disc as an explant, Samantray and Maity, (2010) using RAPD markers showed monomorphism with the mother plant [38]. In our study using SCoT and ISSR markers where 18 randomly selected *in vitro* raised plants using flower stalk internodes showed similarity to the mother plant, hence maintaining the genetic homogeneity (**Table 3**).

The developed protocol using flower stalk internodes significantly reduced the risk of somaclonal variations as compare to other regeneration methods. This is the first report assessing genetic stability of regenerated plantlets using SCoT and ISSR markers in *C. borivilianum*. Thus, this method could be used for micro propagation for its efficient mass multiplication and conservation using flower stalk to maintain genetic stability of the *C. borivilianum*.

5. Conclusion

C. borivilianum is the endangered medicinal plant, whose mass propagation is in demand for agricultural and commercial purposes. We have optimized media for direct regeneration using flower stalk of the plant. For the first time, *in vitro* propagation through direct regeneration using nodal segments and their genetic fidelity analysis has been done successfully. The used markers and its amplified band pattern might be helpful to screen large populations to detect genetic variation in *C. borivilianum*.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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