Journal of Nepal Biotechnology Association, 4 (1), 2023: 27-36 ISSN: 1728-1628

Research Article

In vitro Propagation and Assessment of Genetic Homogeneity using RAPD and ISSR Markers in *Tinospora cordifolia* (Wild.) Hook. F. & Thoms, An Important Medicinal Plant of Nepal

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ARTICLE INFO

Submission: 10/12/2022
Acceptance: 01/01/2023
Published: 05/03/2023

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Abstract

The Menispermaceae family includes the widely glabrous, succulent, climbing shrub *Tinospora cordifolia* (Gurjo), which has been found to have a variety of pharmacological and ethnomedicinal characteristics. *T. cordifolia* is also one of the most commercially exploited plants in pharmaceuticals. The nodal segment explants were initially cultured on Murashige and Skoog (MS) medium supplemented with various concentrations of 6-benzyl amino purine (BAP), and/or Kinetin (KIN) among which MS +BAP (2.0 mg/l) induced shoot initiation after 7 days of post-inoculation. The nodal segments were then excised and treated with various concentrations of BAP, BAP with KIN, and coconut water for the proliferation among which 5 mg/l induced significant nodal segment proliferation (9.0 nodal segments/per explant) and shoot length (8.0 cm). The efficacy of coconut water in increasing the nodal segment proliferation of *T. cordifolia* was tested and the shoot proliferation increased significantly at 5% and 10% of coconut water, however, the maximum response of shoot number (23.0), shoot length (12 cm) was in the MS medium supplementation with BAP (5mg/l) and 10% coconut water. The genetic fidelity of these plants was also confirmed by random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) markers in wild and in vitro cultures. This protocol is an efficient way for the in vitro mass propagation of true-to-type plantlets of *T. cordifolia* which provides a basis for germplasm conservation and sustainable utilization.

Keywords: Coconut water, Genetic fidelity, ISSR, Plant tissue culture, RAPD

Introduction

Tinospora cordifolia (Willd.) Hook. f. & Thoms is an extensively spreading, glabrous, succulent, woody climbing shrub belonging to the family Menispermaceae. It is distributed throughout the tropical region of Nepal, India, Sri Lanka and China ascending to an altitude of 1,200 m asl. It thrives in the tropical region in forests and other habitats (DPR, 2007). The genus *Tinospora* has around thirty-two different species distributed in tropical Africa, Madagascar, Asia to Australia, and the Pacific Island (Forman, 1981; Mabberley, 2005). In Nepal, only two species of *Tinospora* have been

reported are *Tinospora cordifolia* (Willd.) Hook. f. & Thoms. and *Tinospora sinensis* (Lour.) Merr. (Checklist of flowering plants of Nepal, 2000). It is known as Gurjo in Nepali. *T. cordifolia* is a rather large, widely disseminating, glabrous, dioecious, perennial deciduous climber that may be found growing on a variety of hedges and trees up to an altitude of 1000 m. It is often found growing in dry deciduous woods in tropical and subtropical countries. The plant has been demonstrated to possess multiple ethnomedicinal, pharmacological, and medicinal activities but systematically updated information is lacking on the therapeutic effectiveness of *T. cordifolia*. *T. cordifolia* is one of the most commercially exploited plants in pharmaceuticals. In the Indian system of medicine (ISM), there is an estimated 10,000 tonnes of annual demand for this species, which is used to prepare crude herbal medications (Singh, 2004). This plant has medicinal use primarily because it contains a variety of bioactive substances, including glucosides and alkaloids like berberine (Singh et al., 2003). Especially in Ayurveda, stems, roots, leaves, and starch derived from the stems and roots are used for therapeutic purposes (Singh et al., 2003). The root is a powerful emetic and is used for visceral obstructions. Its water extract is used in leprosy (Nayampalli et al., 1982). The root also exhibits an antidiabetic effect (Gupta et al., 1967). The extracts of stems, leaves, barks and roots show strong antioxidant activities (Stanley et al., 1999). Decoction of the leaves is used to treat gout, while the bitter substance in the stem is used to treat debility, dyspepsia, fever, and urinary illness (Singh et al., 2003). The leaves, bark, and roots of this plant are primarily responsible for its pharmaceutical significance because they contain a variety of bioactive substances, including alkaloids, glycosides, lactones, saponins, tannins, steroids, polysaccharides, and aliphatic compounds with various medicinal uses. Intense interest in the plant has been sparked by the identification of its active ingredients and their biological significance in the prevention of disease (DPR, 2007). A wide number of chemical compounds like aporphine, alkaloids, diterpenes, berberine, palmatine, tembertarine, magniflorine, choline and tinosporin have been isolated from this plant (Forman et al., 1981). It has been demonstrated that an extract from the stems of *T. cordifolia* increases the immunological protection response to a commercially available vaccine for infectious bursal illness in chicks (Sachan et al., 2019). The surface glycoprotein, receptor binding domain, RNA-dependent RNA polymerase, and major protease of the SARS-CoV-2 virus had strong binding affinities for berberine, isocolumbin, magnoflorine, and tinocordiside in in vitro investigations (Sagar & Kumar, 2020).

There is always a chance of variation happening within the in vitro-raised plants for their genetic homogeneity whenever a plant tissue culture technology is utilized for plant multiplication. Observations of somaclonal differences in in vitro-raised plants are frequent. Because of their excellent reproducibility, reliability, simplicity, and cost-effectiveness, RAPD and ISSR markers have the most widespread uses among the many markers and are more frequently utilized by researchers (Sultana et al., 2022). Molecular markers are frequently employed to examine the variation within the germplasm to determine the genetic similarity between the in vitro-grown plantlets and the mother plant (Mittal & Sharma, 2017). Simple sequence repeats (SSR), inter simple sequence repeats (ISSR), and random amplified polymorphic DNA (RAPD) have historically been utilized extensively in the assessment of clonal fidelity in a variety of plant species (Bhattacharya et al., 2015; Oliya et al., 2021; Pandey et al., 2020). Also, it is important to use many molecular markers to assess the clonal fidelity of plants grown in tissue culture (Palombi & Damiano, 2002). Because *T. cordifolia* contains a lot of phenolics, the advancement of plant tissue culture technology holds tremendous promise for the quick reproduction of plant germplasm. For the short- to medium-term conservation of significant plant species, it is a potent instrument. The return of plants into their original habitats and large-scale plant propagation are made possible through tissue culture technologies (Leander & Rosen, 1988; Lernda & Svensson, 2000). The present investigation was carried out to produce a reproducible protocol for in vitro propagation and assessment of genetic homogeneity in *T. cordifolia*.

Materials and Methods

Plant material and surface sterilization

Fresh *T. cordifolia* plants were procured from Dabur Nepal, Kavre, and Banepa and planted in the garden of the Tribhuvan University, Central Department of Botany. Excised nodal segment explants were

sterilized with a few drops of liquid detergent Tween 20 (Himedia, India) and then washed in sterile water for 30 minutes to eliminate all soil and debris. To get rid of any remaining $HgCl_2$ residues, the explants were treated with 0.1% $HgCl_2$ for five minutes. They were then rinsed five times with sterile water.

Inoculation of explant, shoot proliferation, elongation and root formation

Nodal segments explants were inoculated on MS basal medium supplemented with different concentrations and combinations of PGRs, which were chosen based on the primary response of the cultures which were BAP (0.5-2 mg/l), Kinetin (KIN) (0.5-2 mg/l), and combination of BAP (0.5 mg/l) + KIN (0.5 mg/l), BAP (1.0 mg/l) + KIN (0.5 mg/l), BAP (2 mg/l) + KIN (1.5 mg/l), BAP (2 mg/l) + KIN (2 mg/l), MS+ BAP (5 mg/l) and MS+BAP (5 mg/l)+ coconut water (5, 10%). After the proliferation of nodal segments, the shoots were inoculated in MS+IBA (0.5-2 mg/l) for rooting. All cultures were incubated under a 16/8-hour photoperiod using white fluorescent tubes (Phillips, India) at 25 ± 2°C. After 4 weeks of inoculation, the frequency and number of shoots developed were counted. Visual observations were used to record morphological modifications.

Genetic stability of in vitro-developed plants compared with mother plants

Selection of samples for DNA isolation: Young and healthy leaves harvested from the wild (mother plant collected from natural habitat) and five explants, each set derived from in vitro culture nodal segments grown on MS+BAP (5 mg/l) + 10% coconut water were used as explants for DNA extraction.

Extraction of DNA by CTAB method: Genomic DNA of in vitro plant leaves and wild leaves was extracted by using the modified CTAB method (Doyle, 1990). Approximately 0.2 g of leaf samples were taken and ground to fine powder by using a motor and pestle in liquid nitrogen. About 750 µl of CTAB buffer (2% CTAB, 0.5M EDTA, 5M NaCl, 1M Tris-HCl, pH 8.0, 0.2% β-mercaptoethanol) was added to make the fine paste and transferred into a clean sterilized microcentrifuge tube. Samples were incubated at 65 °C for 45 minutes in a recirculating water bath. After incubation, samples were centrifuged at 10,000 rpm for 5 minutes to spin down cell debris. A clean, sterilized microcentrifuge tube was then used to transfer the supernatant, and an equal volume of chloroform and isoamyl alcohol (24:1) was added. This mixture was then gently inverted several times (5-8 minutes). The upper aqueous phase was transferred to the microcentrifuge tube after it was centrifuged once more for 10 minutes at 12,000 rpm. An approximately equal volume of ice-cold propanol was added to each sample. To precipitate the DNA, the tube was slowly inverted many times. The tube was kept at -20 °C for one hour. The pellet was then created by spinning DNA at 12,000 rpm for five minutes. After discarding the supernatant, 400 µl of ice-cold 70% ethanol were used to wash the particle. To remove the salt, it was centrifuged once more for 5 minutes at 10,000 rpm. The ethanol was then thrown away, and the pellet was allowed to dry for 30 minutes, during which ethanol was evaporated. It was then re-dissolved in TE buffer (40µl) and kept at 4°C. Using a UV-spectrophotometer and agarose gel electrophoresis at a concentration of 1%, the quality and amount of the isolated DNA were evaluated. At the end, DNA samples were stored at -20°C after the concentration of the extracted DNA was adjusted to 10–20 ng.

DNA amplification and RAPD, ISSR analysis

The chosen explant DNA was amplified using five 14-decamer ISSR primers and a set of ten 10-decamer RAPD primers. The RAPD and ISSR assays were performed using a polymerase chain reaction (PCR) reaction in a thermal cycler using a 15 µl reaction volume, nuclease-free water, 1µ l Taq polymerase (0.5 U µl), 0.5 l dNTPs (0.2 mM), 1 l primer, and template DNA (50 ng) (Biorad, USA). Cycling condition was also optimized for identifying the best cycling conditions. The amplification cycle for RAPD primers was initial denaturation at 92 ºC for 5 min, 92 ºC for 1 min, 45 cycles of 35-60 ºC for 1 min, 72 ºC for 1 min, 72 ºC for 5 min. The amplification cycle for ISSR markers was initial denaturation at 95 ºC, 94 ºC for 1 min, 35 cycles of 42-60 ºC, 72 ºC for 2 min and 72 ºC for 7 min.

Data analysis

Triplicates of each sample were used for analysis. The samples' triplicate analysis results were expressed as means (n = 3) with SD. With the use of the SPSS, all the data were then further examined

using a one-way analysis of variance (ANOVA) and Duncan's multiple range test ($p < 0.05$). $p < 0.05$ was regarded as indicative of significance, and the DMRT analysis was used to rate the response percentage. Manual scoring was done on the banding pattern in the genetic fidelity analysis using RAPD and ISSR markers.

Results and Discussion

Establishment of in vitro cultures from nodal segment explant

In the present study, the nodal segment explants were initially cultured on an MS medium supplemented with various concentrations of BAP, and/or KIN for axillary shoot initiation and proliferation (Table 1). This treatment was chosen based on the positive response of the nodal segment in different concentrations of plant growth hormones. Nodal segment explants cultivated on medium enriched with BAP (2.0 mg/l) (Figure 1A) produced shoot from the nodal explants after 7 days of inoculation, among the tested concentrations of BAP (Figure 1B).

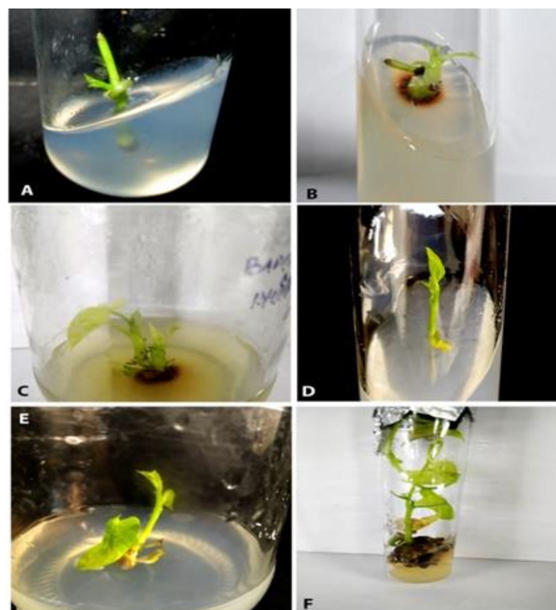

Figure 1: In vitro regeneration of *T. cordifolia* nodal explant. (A) inoculation in MS+BAP (2 mg/l); (B) after 7 days of inoculation in BAP (2mg/l); (C) after 25 days of inoculation in BAP (2 mg/l); (D) inoculation in MS+KIN (2 mg/l); (E) after 7 days of inoculation in MS+KIN (2 mg/l); (F) after 25 days of inoculation in MS+KIN (2 mg/l).

Nevertheless, it was found that when transferred to MS+KIN (2 mg/l), the proliferation rate was modest (Figure 1C), and only two shoots per explant were formed at the end of 30 days (Figure 1D). After 10 days, the proliferation rate significantly decreased, and even two subcultures onto a new medium spaced two weeks apart had little effect on the proliferation rate (i.e., number of shoots and roots). However, compared to different concentrations of BAP, MS+KIN (2 mg/l) was effective in producing shoots with more nodal segments (7.33±0.57) and (3.06±0.057 cm shoot length) (Figure 1F, Table 1).

Table 1: Effect of different concentrations and combinations of PGRs on the proliferation of nodal explant of *T. cordifolia*.

PGRs			Average shoot number	Average shoot length (cm)
BAP (mg/l)	Kinetin (mg/l)	Coconut water (%)		
0	0	0	0.66±0.57	0.23±0.057
1.5	-	-	2.66±0.57	0.6±0.10
2	-	-	1.66±0.57	1.16±0.15
0	1.5	-	4.33±0.57	2.06±0.15
0	2	-	7.33±0.57	3.06±0.057
0.5	0.5	-	2.3±0.57	2.13±0.15
1	0.5	-	3.66±0.57	3.23±0.15
2	1.5	-	2.33±1.15	2.10±0.17
2	2	-	5.66±1.15	3.040.057
5	0	-	9.33±0.57	8.63±0.55
5	-	5	13.33±1.52	9.43±0.38
5	-	10	20.33±0.57	12.10±0.10

Mass multiplication and rooting of the nodal segment

The nodal explants from in vitro generated shoots cultured in a medium containing BAP (2 mg/l) were inoculated into MS + BAP (5 mg/l) alone and supplemented with coconut water (5, 10%) in order to investigate the effectiveness of coconut water in boosting the shoot proliferation of *T. cordifolia*. The shoot proliferation increased significantly at 5 and 10% (Figure 2D), however, the maximum response was attained with the supplementation of 10% coconut water-containing medium producing more shoots (20.33 ±0.57) with 12.10± 0.10 cm (Figure 2H) as compared other concentration of coconut water. All the experiment was performed using control MS medium without plant growth regulators. Coconut water (10%) supplementation resulted in a maximum response about shoot numbers (20.0) and shoot length (12.0 cm). The

results indicated that medium supplemented with coconut water significantly increased the shoot induction of nodal explants (Figure 2H) as compared to the control MS basal medium (Table 1, Figure 2E, Figure 3). Similarly, rooting was also observed when the shoots grew in MS+BAP (5 mg/l)+10% CW containing medium was inoculated in MS+IBA (0.5 mg/l) (Figures 2F and 2G).

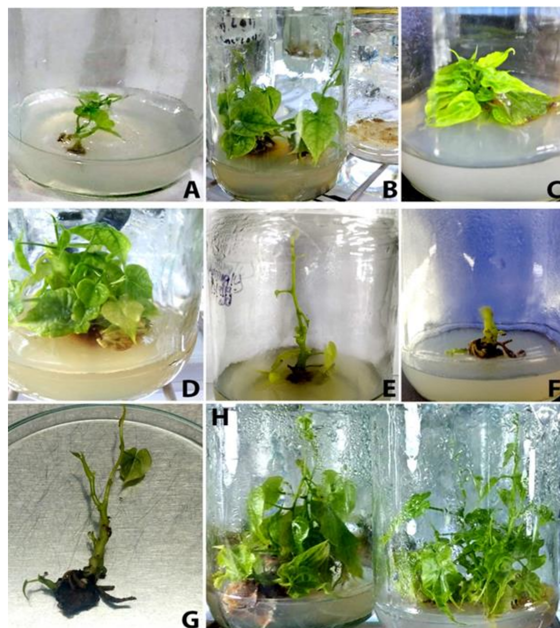

Figure 2: Mass multiplication of *T. cordifolia* nodal explant. (A) inoculated in MS+BAP (5 mg/l); (B) after 10 days of inoculation in BAP (5 mg/l); (C) after 25 days of inoculation in BAP (5 mg/l); (D) inoculated in MS+BAP (5 mg/l)+ 5% CW; (E) after 15 days of inoculation in MS basal medium; (F, G) rooting in MS+BAP (0.5 mg/l); (H) after 25 days of inoculation in MS+BAP (5mg/l)+ 10% CW.

Different protocols have been reported for the in vitro propagation of *T. cordifolia* (Sultana et al., 2013) reported the development of multiple shoots in high frequency was achieved in the nodal explant culture. They used MS basal medium throughout this investigation with different combinations and concentrations of BAP, Kinetin and Thidiazuron (TDZ). Within 30 days of inoculation, MS media with BAP (2 mg/l), Kinetin (4 mg/l), and TDZ (0.20 mg/l) generated a maximum of 10.29 shoots per explant, while half-strength MS medium supplemented with IBA (2 mg/l) produced roots. Earlier, a micropropagation protocol was developed using *T. cordifolia* collected from Northeast India (Handique et al., 2009). MS basal medium was used throughout this experiment with different combinations and concentrations of BAP, Kinetin and IAA. In this experiment, the nodal segments produced fewer shoots in MS+ BAP (2 mg/l) and KIN (2 mg/l) (Figure 1). Increasing the concentration of coconut water in MS+BAP (5 mg/l) containing medium supported the growth of the plant resulting in a higher number (20.33±0.57) of nodal segments from shoots with high shoot length (12.10± 0.10) (Table 1, Figure 2H). This is because the enhanced growth activity of coconut water can be attributed to its growth regulator content specifically cytokinin (Chugh et al., 2009), and this has been reported in *V. jatamansi* (Pandey et al., 2020). Similarly, high concentrations of IBA (1, 1.5 and 2 mg/l) did not produce many roots, as compared to IBA (0.5 mg/L) which produces prominent roots in *T. cordifolia* (Figure 2G). This is the first report of in vitro propagation of this species with the supplementation of coconut water in this species in Nepal.

RAPD and ISSR analysis

In the present study, ten RAPD primers (OPA O8, OPA 10, OPA 18, OPB 07, OPD 04, OPE 08, OPC 11, UBC 210, UBC 292 and UBC 465) gave similar and reproducible banding patterns between the in vitro raised plantlets and the mother plant (Table 2). The number of bands varied from 1 to 4 with a total number of 22 bands for RAPD primer with band size between 100-1200 bp (Figure 4). Similarly, out of the 10 primer sets of ISSR markers, 5 primers (HB8, HB9, HB10, HB11 and 17898B) gave reproducible bands patterns between the in vitro raised plantlets and the mother plant (Table 3). The number of bands varied from 1 to 3 with a total number of bands for ISSR primer with band size between 100-400 bp (Figure 5).

In the case of plants that are significant commercially, determining the genetic homogeneity between the mother plant and in vitro regenerated plants is crucial. There are numerous variables, including dietary content, stress, hormonal balance, and culture time, which are significant contributors to in vitro variability (Khan et al., 2011). It is crucial to determine if the in vitro regenerants are genetically identical to the mother plant or not, particularly if the research's goal is to commercialize the specific plant species for its medicinal value. ISSR and RAPD primers have been successfully used to demonstrate clonal stability and to detect possible genetic variations within the in vitro-

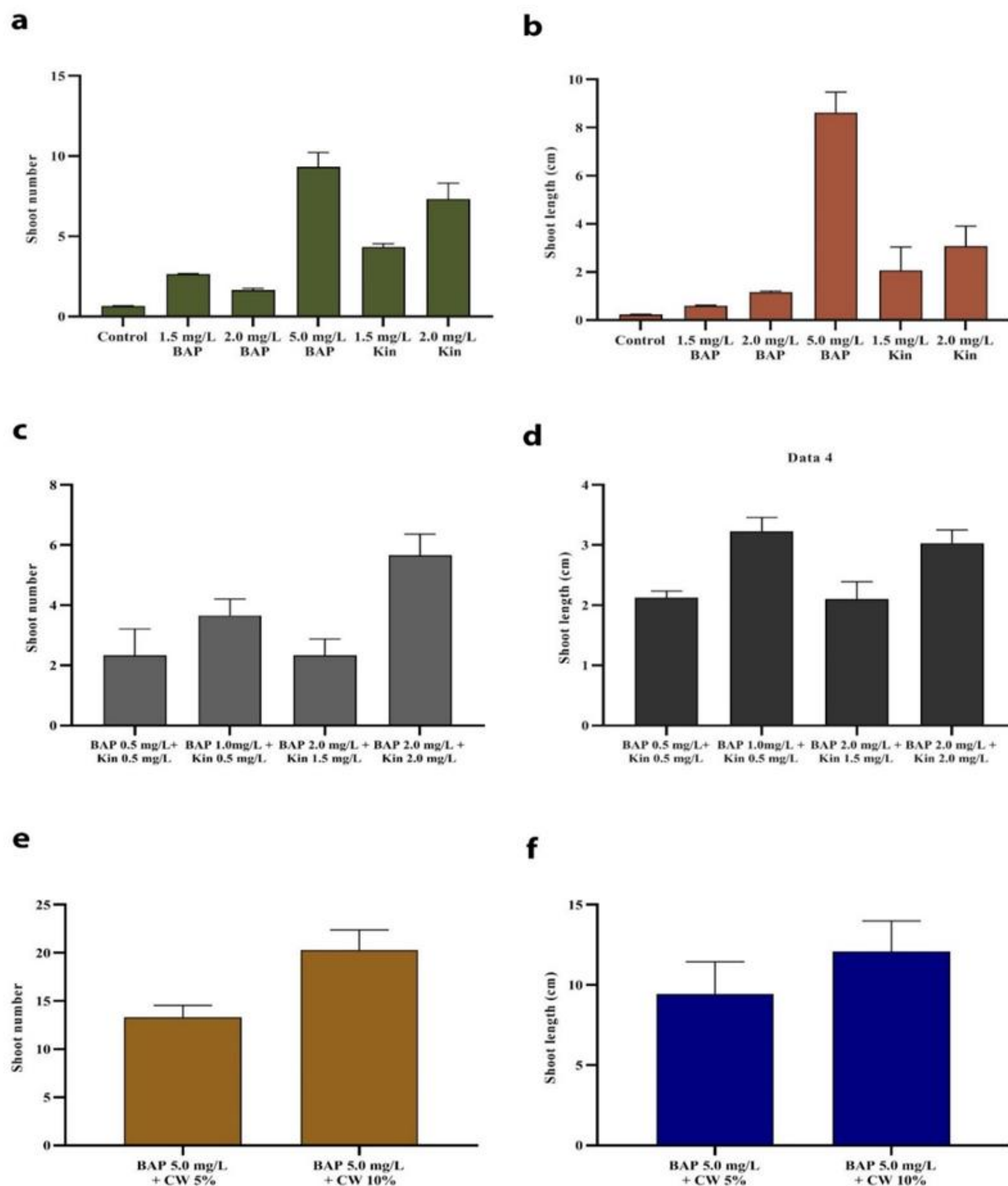

Figure 3: Effect of PGRs on shoot induction from nodal explants of *T. cordifolia*. (**a**) number of shoots per explant with different concentrations of BAP and Kin; (**b**) shoot length with different concentrations of BAP and Kin; (**c**) number of shoots per explant with different concentrations of BAP and Kin; (**d**) shoot length with different concentrations of BAP and Kin; (**e**) number of shoots per explant with different concentrations of BAP and coconut water; (**f**) shoot length with different concentrations of BAP and coconut water.

regenerated plantlets in several in vitro propagated medicinal and commercially important plant species like *Withania sominifera* (Nayak et al., 2012), *Alhagi maurorum* (Agarwal et al., 2015) and *Withania coagulans* Dunal (Tripathi et al., 2018). It is known that micropropagated tissues are easily exposed to somaclonal variations, especially during long-term cultures (Larkin & Scowcroft, 1981). In this regard, RAPD and ISSR markers are the favoured method used to determine the genetic stability of regenerated plants and wild plants of *T. cordifolia* (Mittal et al., 2017). The RAPD analysis revealed that the in vitro-derived plants of *T.*

cordifolia exhibited the same banding patterns as that of mother plants which produced 22 bands of approximately 100-1400 bp confirming that no genetic variation occurred in the DNA of in vitro regenerated plantlets (Figure 4, 5).

Table 2: RAPD primers with total number of bands amplified.

Primer	Sequence (5'-3')	Length (bp)	Annealing temp.	DNA band produced
OPA 08	GTGACGTAGG	10	44.2	2
OPA 10	GTGATCGCAG	10	44.2	1
OPA 18	AGGTGACCGT	10	44.2	2
OPE 08	TCACCACGGT	10	44.2	2
OPC 11	AAAGCTGCGG	10	44.2	2
UBC 292	TGCCGAGCTG	10	44.2	1
OPB 07	GGTGACGCAG	10	45.0	3
OPD 04	TCTGGTGAGG	10	46.0	4
UBC 210	CCGGGGTTTT	10	46.0	2
UBC 465	AGCTGAAGAG	10	44.2	3

Because of genetic variation, the resultant plant could not have the same qualities as the parent plant. Physiological/biochemical, morphological, and molecular methods can all be used to identify these somaclonal variants (Bairu et al., 2011). Morphological and biochemical approaches are inferior to molecular techniques among them. random amplified polymorphic DNA markers are frequently used in genetic variation studies in tissue-culture-derived plants because they require less DNA, are simpler to use, less expensive, more reliable, take less time, and don't require prior knowledge of the nucleotide sequence of the organism being studied (Devarumath et al., 2002). Various RAPD-based studies in *T. cordifolia* have been reported which were specific to wild populations only, however, the results of such studies could not be so reliable due to low reproducibility and low level of polymorphism of RAPD marker.

Table 3: ISSR primers with total number of bands amplified.

Primer	Sequence (5'-3')	Length (bp)	Annealing temp.	DNA band produced
HB 08	GAGAGAGAGAGAGG	14	52.3	1
HB 09	GTGTGTGTGTGTGG	14	52.3	3
HB 10	GAGAGAGAGAGACC	14	52.3	2
HB 11	GTGTGTGTGTGTCC	14	52.3	1
17898 B	CACACACACACAGT	14	52.3	2

Contrarily, the ISSR markers revealed higher heterozygosity and thus can be attributed to the detection of high polymorphism which makes them the most preferred DNA marker (Lade et al., 2020). This study reports confirmation of genetic homogeneity with RAPD and ISSR markers of the in vitro and mother plant.

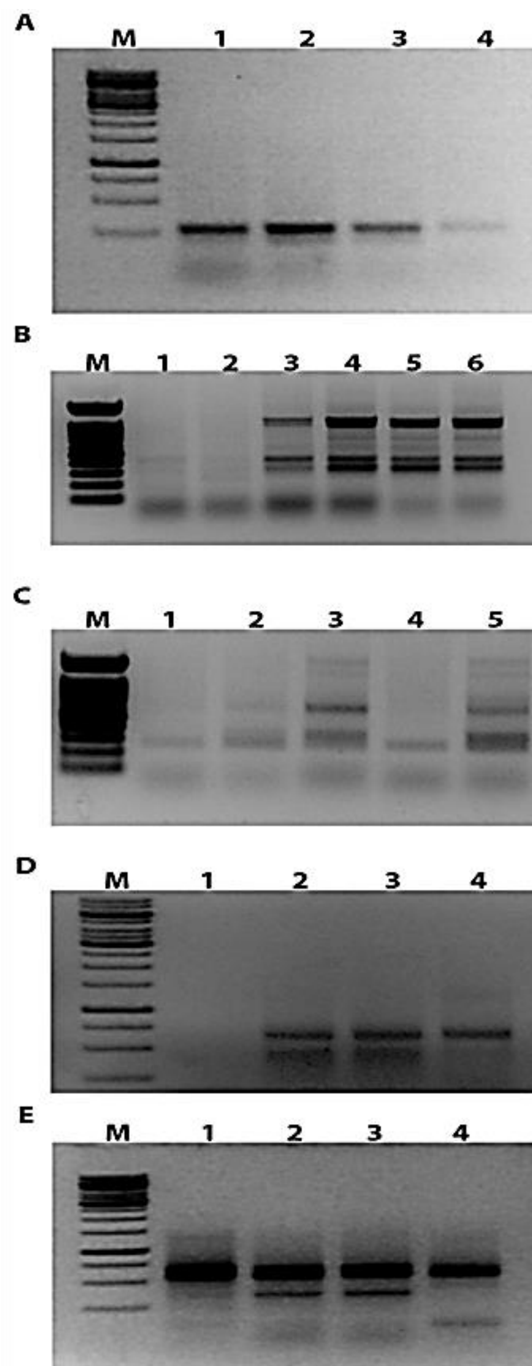

Figure 4: Representative images depicting the genetic fidelity analysis of in vitro and mother plant of *T. cordifolia* using RAPD markers depicting the molecular ladder of 100 bp -1.5 kb. (A) OPA 10 primer, 1 mother plant, 2 to 4 in vitro plants; (B) OPB 07 primer, 3 mother plant, 4 to 6 in vitro plants; (C) OPD 04 primer, 1 to 4 in vitro plants, 5 mother plant; (D) OPE 08 primer, 2 mother plant, 3 and 4 in vitro plants; (E) OPA 18 primer, 1 mother plant, 2 to 4 in vitro plants.

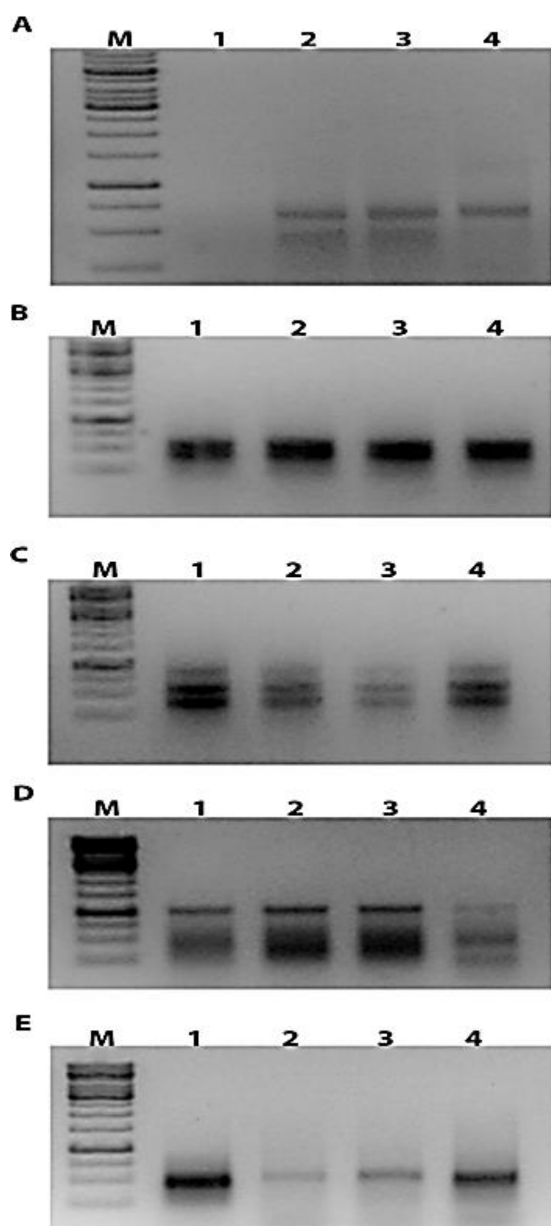

Figure 5: Representative images depicting the genetic fidelity analysis of in vitro and mother plant of *T. cordifolia* using ISSR marker depicting the molecular ladder of 100 bp -1.5 kb. (A) 17898 B primer, 1 to 3 in vitro plants, 4 in mother plant; (B) HB 08 primer, 1 to 3 in vitro plants, 4 in mother plant; (C) HB 09 primer, 1 to 3 in vitro plants, 4 mother plant; (D) HB 10 primer, 1-mother plant, 2-4 in vitro plants; (E) HB 11 primer, 1-mother plant, 2-4 in vitro plants.

Conclusion

An efficient *in vitro* propagation technique was developed in *T. cordifolia,* using nodal segment by using coconut water in the highly effective culture medium. The plants produce through nodal segment culture generated true-to-type plants. Also, the genetic fidelity was confirmed using RAPD and ISSR primers. The current strategy can be an alternative way for germplasm conservation and producing *T. cordifolia* plantlets in large quantities which is highly useful in pharmaceutical industries.

Acknowledgements

Authors acknowledge the University Grants Commission, Nepal for providing PhD research fellowship (Grant No. PhD/73-74/S&T-10) to Sushma Pandey.

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