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ORIGINAL ARTICLE

Somatic embryogenesis and genetic homogeneity assessment of regenerated plants of *Crinum brachynema* (Amaryllidaceae): an endemic critically endangered medicinal plant

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Abstract

Crinum brachynema is a bulbous plant belonging to the family Amaryllidaceae which is restricted to Western India. Due to its high rarity and low distribution range, it has been classified as "critically endangered". Establishing an efficient and unprecedented somatic embryogenesis protocol is necessary for its conservation and large-scale propagation. In this study, regeneration was achieved through somatic embryogenesis using bulb explants on MS media supplemented with various concentrations of 2,4-D alone and in combination with N^6-benzyl-adenine. Different advanced phases with maturation of somatic embryo were obtained on MS medium with different ratios of picloram and thidiazuron (TDZ). The highest number of somatic embryos (50.33 ± 1.52) was obtained after eight weeks in the medium supplemented picloram (2.0 mg L^{-1}) in combination with TDZ (0.5 mg L^{-1}). MS medium with reduced concentration of salts in combination with GA_3 (1.0 mg L^{-1}) was used for somatic embryo germination. The maximum embryo germination frequency (82.22) was recorded on half-strength MS medium fortified with 1 mg L^{-1} GA_3. The genetic true-to-typeness of regenerated plants was confirmed by ISSR, SCoT and RAPD primers based molecular analyses. This confirmed their genetic homogeneity compared to the mother plant and it also demonstrated the reliability of our somatic embryogenesis system for *C. brachynema.* The protocol developed may facilitate efforts in reintroduction, restoration, and ex situ conservation of *C. brachynema* in its natural habitat and its potential commercial utilization.

Key message

We provided the first report on somatic embryogenesis system in *C. brachynema*. SEM indicated the morphogenesis and several molecular markers revealed genetic homogeneity of the regenerated plants.

Keywords Conservation · *Crinum Brachynema* · Critically endangered · Genetic fidelity · Molecular markers · Somatic embryo

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Abbreviations

ANOVA	Analysis of variance
BA	6-Benzyladenine
2,4-D	2,4-Dichlorophenoxyacetic acid
CTAB	Cetyl trimethyl ammonium bromide
DMRT	Duncan's multiple range test
dNTP	Deoxynucleotide triphosphate
GAL	Galanthamine
GA_3	Gibberellic acid
IUCN	International union for conservation and nature
ISSR	Inter simple sequence repeat
LED	Light-emitting diode
MS	Murashige and Skoog
PCR	Polymerase chain reaction

PGRs	Plant growth regulators
PPFD	Photosynthetic photon flux density
RAPD	Random amplified polymorphic DNA
SCoT	Start codon targeted polymorphism
SE	Somatic embryogenesis
SEM	Scanning electron microscope
TAE	Tris-acetate EDTA
TDZ	Thidiazuron

Introduction

The family Amaryllidaceae includes around 1600 species, distributed in 100 genera, which grow throughout the tropical and warm-temperate regions of the globe (Chahal et al. 2021; Kaur et al. 2022a). *Crinum* L. is one of the prominent genera of this family with more than 110 species distributed globally (Chahal et al. 2021). In India, a total of 13 *Crinum* species are found, of which 5 are endemic (Lekhak and Yadav 2011; Patel and Patel 2019). Indian *Crinum* species have been documented with several potential therapeutic applications such as antidiabetic, anti-inflammatory antiviral, antiaging, antioxidant, antimalarial, antidiarrheal analgesic, antitumour, antimicrobial and anti-Alzheimer due to the presence of valuable active alkaloids (Fennell and Van Staden 2001; Uddin et al. 2012; Refaat et al. 2013; Ghane et al. 2018; Sikder et al. 2021; Kaur et al. 2022b; Patel et al. 2022).

Crinum brachynema, locally known as "Karnaphul", is a critically endangered herb restricted to Northern region of Western ghats of India (Punekar 2001; Kaur et al. 2022a). Like other *Crinum* species, *C. brachynema* is known for its beautiful fragrant flowers and as a potential source of valuable alkaloids (viz. galanthamine and lycorine (Jagtap et al. 2014; Kaur et al. 2022b). This plant is well known for its biological activities as anti-Alzheimer and anticancer, due to the presence of alkaloids skeletons like galanthamine (GAL) and lycorine (Fig. 1E, F) and could be used commercially in pharmaceutical and perfume industries as well (Jagtap et al. 2014; Roy et al. 2018; Santos et al. 2020; Lekhak et al. 2022).

GAL is a reversible, selective isoquinoline alkaloid, which acts as a competitive inhibitor of acetylcholinesterase and modulator of acetylcholine receptors (Heinrich and Teoh 2004; Jagtap et al. 2014; Argade et al. 2022). It is also sold under the product names Nivalin® and Reminyl® as a potential drug for Alzheimer's disease (Heinrich 2010). *C. brachynema* is rarely cultivated and over the past few years wild populations of *C. brachynema* were over-exploited, threatening the very existence of this valuable endemic flora (Kaur et al. 2022a). Furthermore, limited availability, anthropogenic pressure, human activities, over-exploitation of bulb material and forest fires make them difficult to propagate in their natural habitat and it faces the threat of extinction. This plant has been also categorized as "critically endangered" by the International Union for Conservation of Nature (IUCN), under the red list measures (POWO 2019). Its extreme destruction and status underline the need to develop novel biotechnological strategies for in vitro regeneration and conservation, essential to develop rapid propagation and conservation systems as tools to save and expand the populations of *C. brachynema*.

Plant tissue culture techniques are being used to conserve rare, endangered, and threatened species all over the world. Somatic embryogenesis (SE) has several advantages for increasing the rate of propagation of genetically uniform phenotypes (Chahal et al. 2023; Uma et al. 2023). Plant cells and tissues undergo several genetic changes during in vitro regeneration, including DNA methylation and morpho-physiological changes, chromosomal restructuring and/or doubling (aneuploidy/polyploidy), collectively referred to

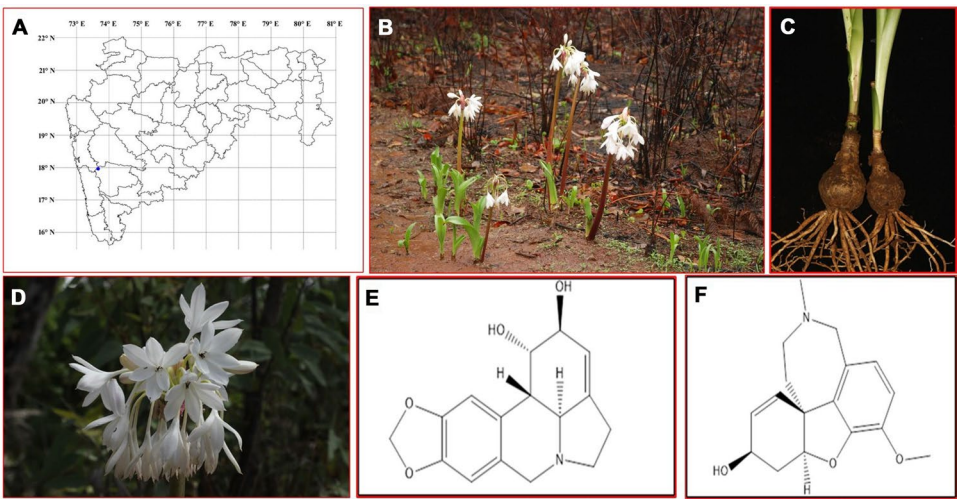

Fig. 1 **A** Schematic map of collection site of *Crinum brachynema*, **B** *C. brachynema* in natural habitat. **C** The freshly harvested and young bulbs of *C. brachynema*, **D** Flower of *C. brachynema*. **E** Chemical structure of lycorine, **F** Chemical structure of galanthamine

as "somaclonal variation" (Phillips et al. 1994; Bairu et al. 2011; Takagi 2011).

The somaclonal variations may arise due to several factors such as type of explant, exogenous plant growth regulators (PGRs) and excessive subculturing during in vitro propagation (Bhattacharyya et al. 2018; Chirumamilla et al. 2021). Different genetic markers including random amplified length polymorphism (RAPD) (Sebastiani and Ficcadenti 2016; Bajpai and Chaturvedi 2021), amplified fragment length polymorphism (AFLP) (Yin et al. 2013), direct amplification of minisatellite DNA (DAMD) (Parab et al. 2021; Longchar and Deb 2022), inter-simple sequence repeat (ISSR) (Jaiswal et al. 2021) and start codon targeted polymorphism (SCoT) (Thakur et al. 2016; Bhattacharyya et al. 2018) have been extensively used to discover the genetic variations of in vitro plant species. True-to type plants are vital to preserve the genetic resources of rare, endangered, and threatened species and are crucial for conservation practices and secondary metabolite production.

To date only one recent study (Kaur et al. 2022a) has reported on regeneration and conservation of *C. brachynema*. A regeneration of in vitro clones through somatic embryogenesis in a shorter period of time is advantageous for rapid propagation and production of bioactive compounds (Kumar et al. 2015, 2017; Lema-Rumińska et al. 2019; Marković et al. 2021; Mishra et al. 2022; Chahal et al. 2023). Therefore, we undertook this aim to establish an efficient protocol to produce true-to-type plantlets via somatic embryogenesis for the first time. The RAPD, ISSR, and SCoT markers were utilized to examine the true-to typeness of in vitro regenerated plants.

Materials and methods

Plant collection, and somatic embryogenesis

Pant material of *C. brachynema* were obtained from Satara district (17°56.270′N, 73°41.488′E), Maharashtra, India. (Fig. 1A). The species was identified by a botanist and a voucher was deposited at the herbarium of Shivaji University, Kolhapur, Maharashtra, India.

To obtain bulb explants, plant leaves were taken away, and the outer scale and root were harvested. The bulbs (5–7 cm) (Fig. 1C) were rinsed thoroughly with running tap water and surface sterilized by using our recently published protocol (Kaur et al. 2022a). The disinfected bulb explants were cut longitudinally to give twin scales with basal plate (2.0 cm) inside the laminar air flow under sterile conditions. Sterilized explants were placed in culture flasks containing MS (Murashige and Skoog 1962) media supplemented with sucrose (3%), agar powder (0.8%) and with the plant growth regulators (PGR) 2,4-D, BA, picloram and TDZ, added individually or in mixture for induction of somatic embryogenesis. Bavistin and agar powder, Picloram, TDZ, 2,4-dichlorphenoxyacetic acid (2,4-D), 6-benzyladenine (BA), vitamins, and myo-inositol were procured from Hi-Media®, Pvt. Ltd. India. The pH of the MS medium was adjusted to 5.8 before autoclaving for 20 min at at 121 °C and 1.06 kg cm^{-2} of pressure. The inoculated culture tubes were maintained and incubated at 25 ± 2 °C in a culture room with a 16/8 h photoperiod from 40 W fluorescent tubes (Philips, India) emitting a photon flux density of 50 µmol m^{-2} s^{-1}, with a relative humidity of 60% retained by using a humidifier. The medium without any PGR served as control. All cultures were kept under the same conditions as above. The percentage of embryogenic calli was calculated as the total number of embryogenic explants/total number of explants used ×100. Friable embryogenic calli were selected and transferred to MS medium fortified with 0.5–5.0 mg L^{-1} picloram and 0.5 mg L^{-1} TDZ. The mean number of somatic embryos was recorded after 8 weeks of culture.

Scanning electron microscopy

During scanning electron microscope (SEM) analysis, different stages of somatic embryos were collected and fixed in 2.5% of glutaraldehyde solution and dehydrated with an ethanolic series (10, 30, 50, 70 and 100%). Dehydrated samples were dried in a critical point dryer and coated with gold in ion coater (D II-29030SCTR). The coated samples were visualized under SEM (FESEM, JSM-7610 F, Oxford Instruments X-Max N) operated at 15–25 kv and images were taken at different magnifications.

Somatic embryo germinations

To study the embryo germination into complete plantlets, modifications were made in MS medium by reducing the sucrose and salt (macro and micronutrients) concentrations. The cotyledonary somatic embryos were transferred to half and full-strength MS medium with 1.0 mg L^{-1} gibberellic acid (GA_3). The inoculated culture tubes were maintained and incubated under the same conditions as above. The embryo germination rate was determined after 8 weeks of time.

Clonal fidelity analysis

Genomic DNA extraction

The genomic DNA was extracted from 4 to 5 cm pieces of fresh leaves of randomly selected seven in vitro regenerated plantlets and from a mother plant to compare the genetic fidelity assessment. Briefly, 500 mg of leaf sample were acquired from plant genomic DNA using the

standard method of Doyle and Doyle (1987). CTAB buffer was prepared freshly with a composition of 2% N-cetyl-N,N,N-trimethylammonium bromide (CTAB; w/v), 0.2% β-mercaptomethanol (v/v), 1 M Tris-HCL, (pH 8.0), 20mM ethylene diamine tetra acetic acid (EDTA) and 5.0 M NaCl). The genomic DNA quality and quantity were obtained with a UV–VIS spectrophotometer and confirmed on 1.0% agarose gel electrophoresis.

Polymerase chain reaction

dNTPs were purchased from Takara, Japan, DNA ladder were obtained from Thermofischer, India, whereas primers were ordered from Operon Technologies, USA. The PCR reaction was done with eight primers of RAPD and ten primers of each ISSR and SCoT marker using 96 well plate in a Gradient Thermal Cycler (iGene Labserve or G-storm). The reaction mixture was containing 10× PCR buffer, Taq DNA polymerase (3u/µl), 10mM dNTPS (Takara, Japan) and 2 µL of SCoT, ISSR and RAPD primers (Operon Technologies, USA). The amplification reaction was carried out using a cycling program consisting of initial denaturation at 94 °C for 3 min followed by 32 cycles of each at 94 °C for 30s, extension at 72 °C for 2 min and final extension at 72 °C for 10 min. During PCR reaction analysis, annealing temperature was varied between 50 and 58 °C based on the Tm (melting temperature) of the primer. The amplified PCR products were separated using 2.5% 1× TAE agarose gel, and a 100 bp or 50 bp DNA ladder (Thermo Fisher Scientific, USA) were used to compare the fragmentation pattern of the bands. All the chemicals and reagents used during the study were of analytical grade.

Data analysis

Every experiment was conducted three times with three replicates. The data of embryogenesis (%) and number of somatic embryos in mean values were recorded. All experiments were evaluated by using analysis of variance (ANOVA). Duncan's multiple range test was used to analyze the significant difference among means with 95% confidence level ($P \leq 0.05$).

Results and discussion

Induction of somatic embryogenesis

Somatic embryogenesis is a very efficient tool for large-scale production of regenerated plantlets and has the potential to develop the conservation practices for valuable and endangered species. Categorized as "critically endangered" and an endemic species, the development of a long-term conservation strategy for *C. brachynema* needs urgent attention. In the present investigation, an efficient plant regeneration system via somatic embryogenesis was established (Figs. 2 and 3). A range of different concentrations of 2,4-D (1.0–10.0 mg L^{-1}) alone and in combination with BA (1.0 mg L^{-1}) were used for somatic embryogenesis induction using bulb explants. Further, combinations of Picloram (1.0–5.0 mg L^{-1}) and TDZ (0.5 mg L^{-1}) were examined to achieve the

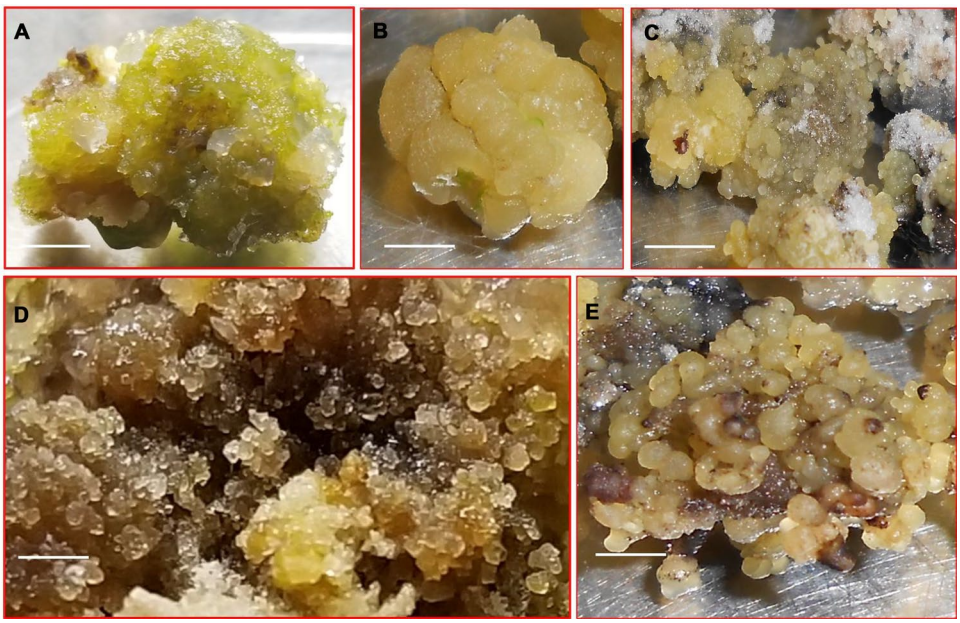

Fig. 2 Somatic embryogenesis showing embryogenic callus induction and formation of somatic embryos in *C. brachynema*. **A**, **B** Induction and proliferation of embryogenic callus. **C**, **D** Embryogenic callus with cluster of emerging globular somatic embryos. **E** Development and maturation of various somatic embryos *Scale Bar: A–E* = 5 mm

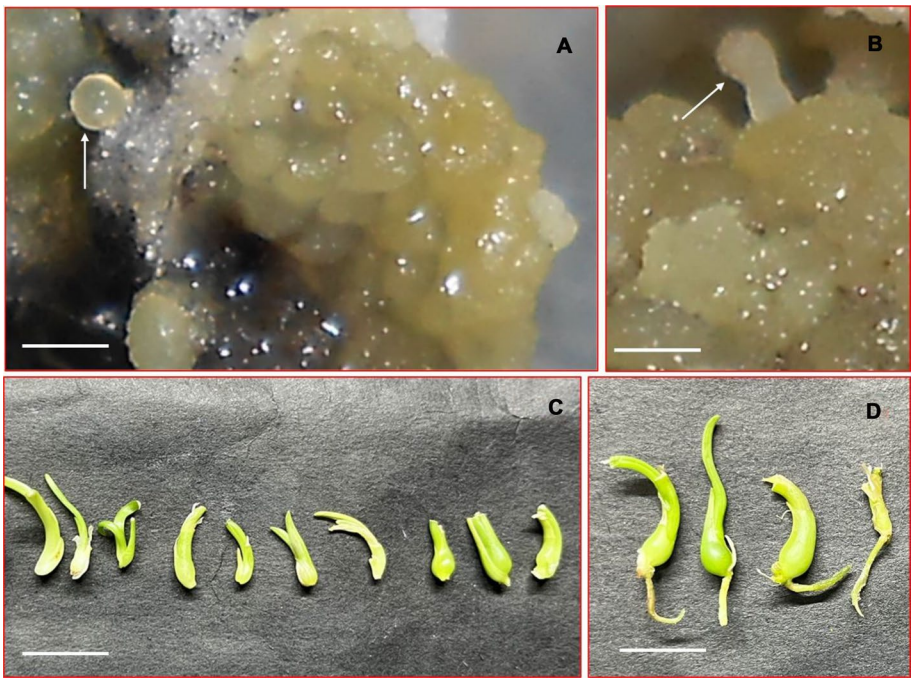

Fig. 3 **A** Translucent globular-shaped embryo (arrow indicating the globular embryo). **B** Initiation of cotyledonary-shaped somatic embryo globular-shaped embryo (arrow indicating the appearance of cotyledonary embryo). **C** Cotyledonary-shaped somatic embryos, **D** Cotyledonary embryos with radicle

maturation of somatic embryos. The somatic embryogenesis induction percentage and production of somatic embryos varied with the PGRs tested (Fig. 4). Alone, 2,4-D induced embryogenic calli and had a significant effect on the production of somatic embryos, in agreement with similar results for other Amaryllidaceae species (Mujib et al. 1996; Ptak et al. 2013; Priyadharshini et al. 2020; Chahal et al. 2023). The addition of BA with 2,4-D significantly increased the formation of somatic embryos. A combination of 8.0 mg L^{-1} 2,4-D and 1.0 mg L^{-1} BA influenced the induction of embryogenic calli (78.5%), and was coupled with a significant impact on the mean number of somatic embryos (35.44) achieved. Our results concur with reports on the embryogenic potential of other species such as *Allium schoenoprasum* L. (Zdravković-Korać et al. 2010), *Chrysanthemum* sp. (Naing et al. 2013), *Vitis vinifera* (Dai et al. 2015) d *malabaricum* (Chahal et al. 2023). Furthermore, a combination of picloram (2.0 mg L^{-1}) and TDZ (0.5 mg L^{-1}) significantly enhanced the mean number of somatic embryos (50.33) in *C. brachynema* (Fig. 4F). Although, 2,4-D is the most commonly used PGR during somatic embryogenesis (Jouini et al. 2023), the application of picloram for somatic embryo production has been well-documented in other species (Takamori et al. 2015; Campos-Boza et al. 2022; Ferreira et al. 2022a, b), including several other bulbous plant species such as *Lilium longiflorum* (Tribulato et al. 1997), *Leucojum aestivum* (Ptak et al. 2013), *Lachenalia virdiflora* (Kumar et al. 2016) *Crinum malabaricum* (Chahal et al. 202320232023).

The cytokinin TDZ has been widely used in somatic embryo production in different plant species (Baskaran et al. 2012, 2014; Dhekney et al. 2016; Ghahremani et al. 2021). The present investigation revealed that MS medium supplemented with a combination of picloram and TDZ is essential for maximum number of somatic embryo production in *C. brachynema*. A similar result was found for somatic embryo production in *Merwilla plumbea* (Baskaran et al. 2012), *Drimia robusta* (Baskaran et al. 2014), *Lachenalia virdiflora* (Kumar et al. 2016), *Cyrtanthus mackenii* (Kumari et al. 2017), *Stewartia sp*. (Gladfelter et al. 2021) *Crinum malabaricum* (Chahal et al. 2023).

SEM investigation of *C. brachynema* samples showed the development of all stages of somatic embryogenesis on the surface of explants, inlcuding globular-shaped somatic embryos (Fig. 5b–d), cotyledonary-shaped somatic embryos (Fig. 5e, f), as well as somatic embryos with well-developed cotyledon with a distinct shoot tip (Fig. 5g, h).

Embryo germination and plantlet development

Successful conversion of somatic embryos into well-developed plantlets is vital for effective plant regeneration. In this study, embryo germination was 51.11% on half-strength MS medium, whereas full-strength MS medium produced 40% germination. Interestingly, half-strength MS medium supplemented with 1 mg L^{-1} GA_3 gave a maximum frequency of somatic embryo germination (82.22%) with well-rooted plantlets (Fig. 6). The growth

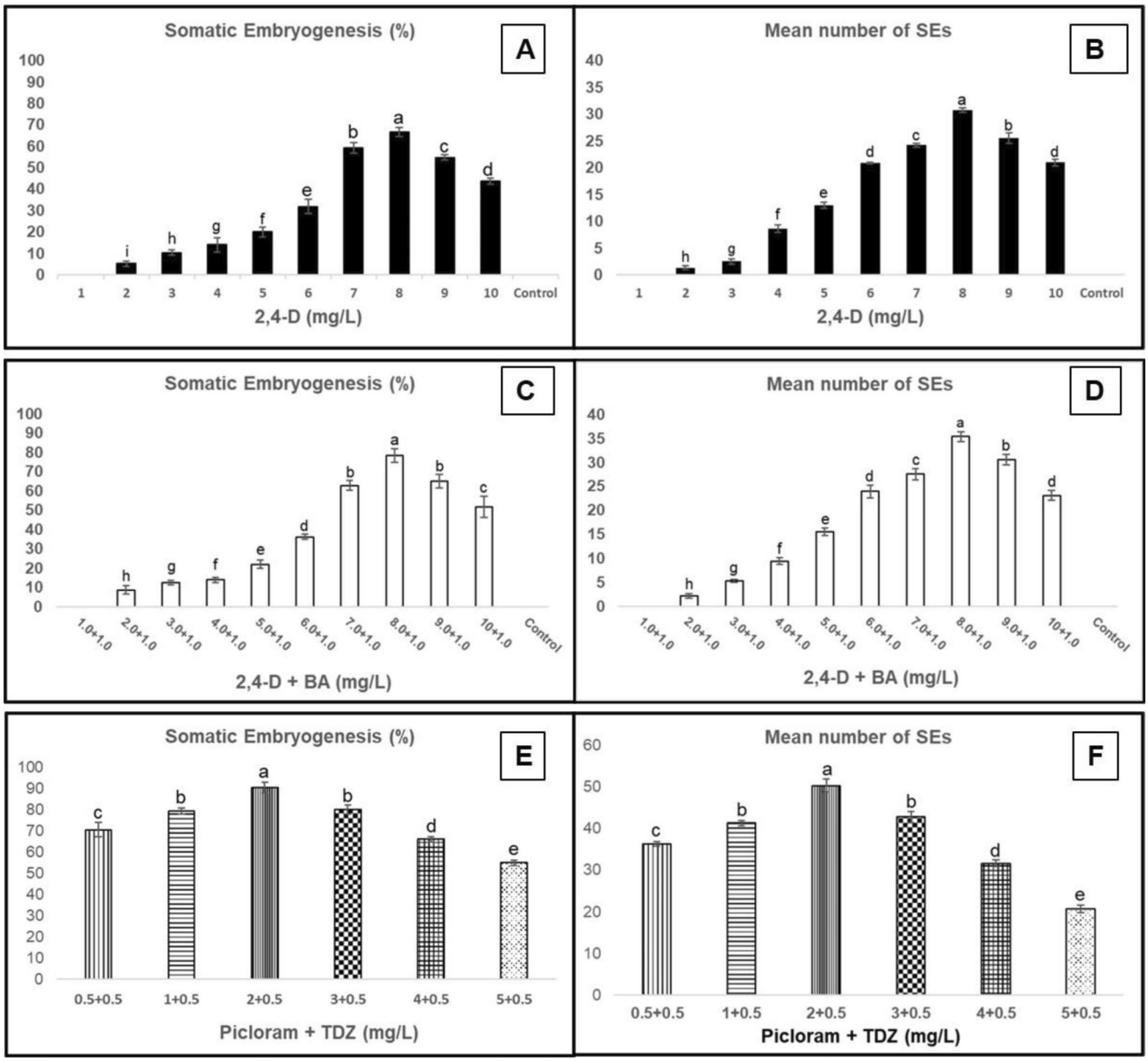

Fig. 4 **A** and **B** Effect of 2.4-D alone on somatic embryogenesis induction in *Crinum brachynema*. **C** and **D** Showing the effect of 2,4-D in combination with BA on somatic embryogenesis induction in *Crinum brachynema*. **D** and **E** The effect of picloram and thidiazuron on somatic embryo development in *Crinum brachynema*. Mean ± S.D followed by different letters indicate significant differences analysed by Duncan's multiple range test at a 95% confidence level ($P \leq 0.05$)

and expansion of the root and cotyledonary leaves were more pronounced in half-strength MS medium compared to the full-strength medium. Our results agree with similar findings on several other species (Paul et al. 2011; Raju et al. 2013; Baskaran and Van Staden 2014; Khan et al. 2015; Ferreira et al. 2022a; Lekshmi and Swapna 2022). Similarly, the supplementation of GA_3 and its beneficial effect on somatic embryo germination has been reported for several plant species (Cangahuala-Inocente et al. 2007; Siddiqui et al. 2011; Baskaran and Van Staden 2012; Raomai et al. 2014; Fang et al. 20222022).

Clonal fidelity assessment

The genetic stability studies help in the assessment of the true-to-typeness between the regenerated clones and mother plants (Hou et al. 2022; Chahal et al. 2023; Uma

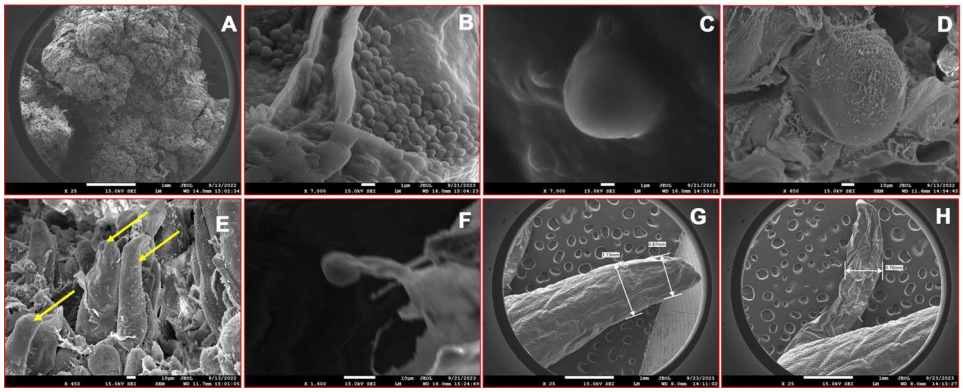

Fig. 5 Different stages of *C. brachynema* somatic embryos viewed under scanning electron microscope. **A** SEM micrograph showing the appearance of embryogenic calli. **B** Globular-shaped somatic embryos emerging from the surface of embryogenic calli. **C**, **D** Single globular-shaped somatic embryo. **E** Cluster of globular-shaped somatic embryos **E**, **F** SEM micrograph of developing cotyledonary-shaped somatic embryos. **G**, **H** Well-developed cotyledonary-shaped embryo with single shoot *Scale Bar:* $A = 1$ mm; $B–D = 1$ µm; E, $F = 10$ µm; G, $H = 1$ mm

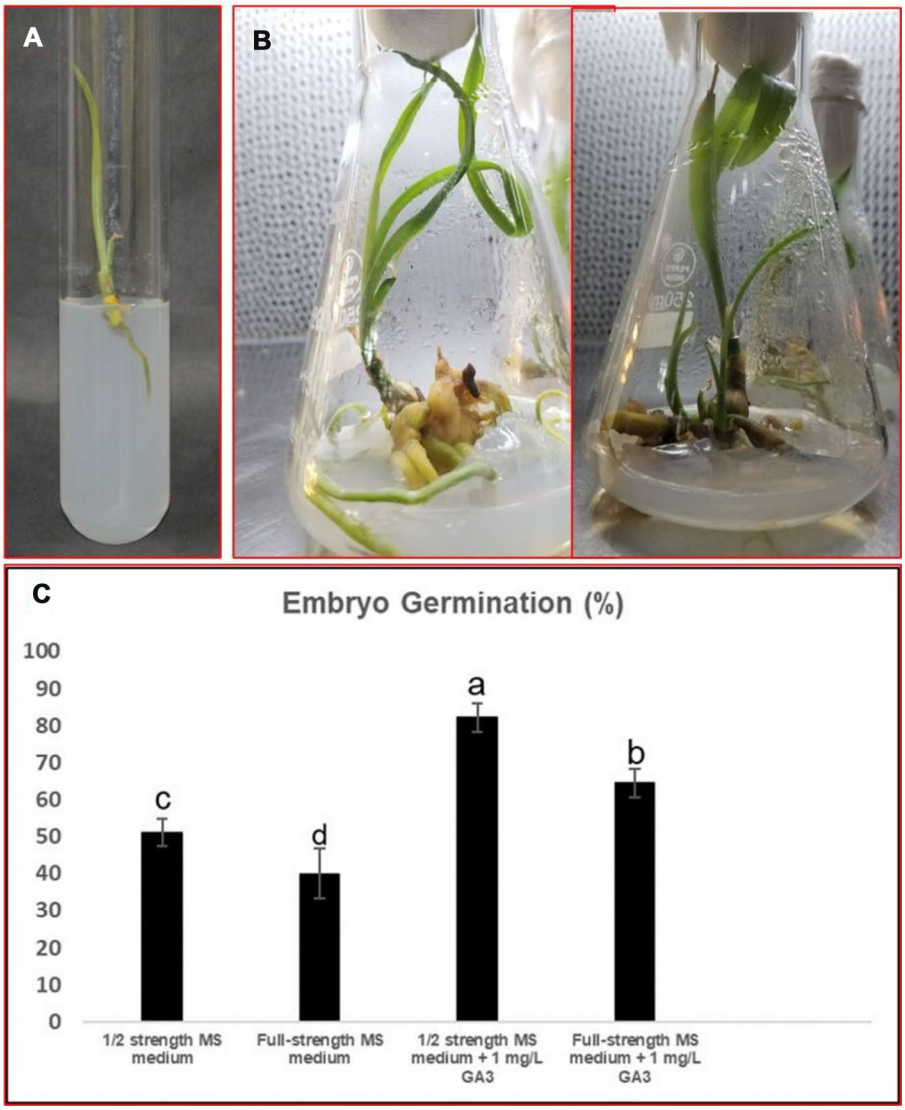

Fig. 6 *C. brachynema* somatic embryos germination and plantlet conversion. **A** Germinated cotyledonary-shaped embryos in germination medium. **B** Well-developed rooted plantlets of *(C) brachynema*. **C** The effect of MS medium (full strength and half strength + 1.0 mg L^{-1} GA_3) on the germination of somatic embryos of *C. brachynema*. All the experiments were repeated thrice with at least three replicates. The significant difference between mean values was obtained via Duncan's multiple range test at a 95% confidence level ($P \leq 0.05$)

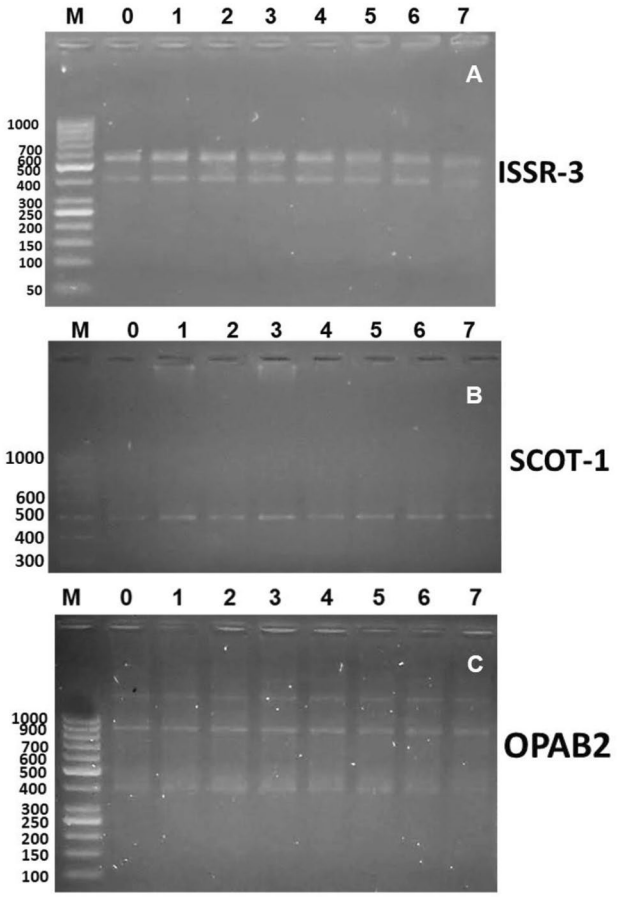

Fig. 7 Genetic stability assessment in somatic embryogenesis derived plantlets and mother plant of *C. brachynema*. **A** ISSR amplification profile with primer ISSR-3. **B** SCoT amplification profile with primer SCoT-1. **C** RAPD amplification profile with primers OPAB-2. Lane 1 represents the 100 bp DNA ladder (M) followed by mother plant (0) in lane 2. Lane 3–9 labelled 1–7 are the in vitro raised plant lines via somatic embryogenesis

et al. 2023). RAPD and ISSR markers are considered as advanced marker types, yet simple approaches to assess the clonal fidelity of regenerated plantlets (Bhardwaj et al. 2018; Sadhu et al. 2020; Rohela et al. 2020; Bajpai and Chaturvedi 2021; Mood et al. 2022). SCoT markers were proven to be more reliable and highly reproducible (Gorji et al. 2011) that can target the starting codon region of plant genes, and the same primer serves as forward and reverse (Collard and Mackill 2009; Elayaraja et al. 2019; Verma et al. 2022). Moreover, it is always beneficial to utilize more than one marker to assess the clonal fidelity of in vitro regenerated plants (Palombi and Damiano 2002; Rohela et al. 2020; Mishra et al. 2023). Therefore, we undertook to investigate the clonal fidelity assessment of somatic embryo-derived plantlets with the ex vitro mother plant using three (RAPD, ISSR and SCoT) different markers (Fig. 7).

A total of ten SCoT and ISSR primers and eight RAPD primers-based fingerprinting was performed by randomly selecting seven regenerants derived from somatic embryos in vitro compared with the *ex vitro* mother plant (Tables 1, 2 and 3). While validating the clonal fidelity, SCoT amplified primers showed good amplification with the generation of total 18 scorable DNA bands with amplified band sizes of 220 to 2000 bp (Table 1). The ISSR primers scored 22 bands with sizes of 250 to 950 bp (Table 2), while the RAPD primers produced 10 bands in a range of 400 to 1300 bp (Table 3).

The generated bands produced with SCoT, ISSR and RAPD were monomorphic and since true-to-type propagules were obtained via somatic embryogenesis no variation was detected (Fig. 7). SCoT, ISSR and RAPD markers assessments were also performed in several other species such as *Eclipta alba* (Singh et al. 2012), *Angelica glauca* (Rawat et al. 2018), *Artemisia vulgaris* (Jogam et al. 2020), *Iris* × *hollandica* (Verma et al. 2022), C. *malabaricum* (Chahal et al. 2023), Congenesis-Robusta (C×R) cultivar (Mishra et al. 2023). The current report revealed the application of more than one marker, as also confirmed by several researchers (Sadhu et al. 2020; Mood et al. 2022; Uma et al. 2023), which clearly shows that utilization of one single marker is

Table 1 Genetic homogeneity analysis of in vitro regenerated *C. brachynema* using SCoT markers

S. No.	Primer code	Primer sequence (5′–3′)	Number of scorable bands	Band length of amplicons
1.	SCoT-1	CAACAATGGCTACCACCA	1	500
2.	SCoT-2	CAACAATGGCTACCACCC	2	700, 300
3.	SCoT-3	CAACAATGGCTACCACCG	2	600, 220
4.	SCoT-4	CAACAATGGCTACCACCT	1	510
5.	SCoT-5	CAACAATGGCTACCACGA	2	700,400
6.	SCoT-6	CAACAATGGCTACCACGC	4	2000, 1100, 700, 400
7.	SCoT-7	CAACAATGGCTACCACGG	2	1100, 250
8.	SCoT-8	ACGACATGGCGACCAACG	1	250
9.	SCoT-9	ACCATGGCTACCACCGAC	1	2000
10.	SCoT-10	CCATGGCTACCACCGCAG	2	1000, 800

Table 2 Genetic homogeneity analysis of in vitro regenerated *C. brachynema* using ISSR markers

S. No.	Primer code	Primer sequence (5′–3′)	Number of scorable bands	Band length of amplicons
1.	ISSR-1	AGAGAGAGAGAGAGAGC	3	400, 300, 250
2.	ISSR-2	AGAGAGAGAGAGAGAGG	1	450
3.	ISSR-3	GAGAGAGAGAGAGAGAC	2	580, 420
4.	ISSR-4	TCTCTCTCTCTCTCTCC	4	700, 600, 500, 450
5.	ISSR-5	AGAGAGAGAGAGAGAGYT	2	700, 480
6.	ISSR-6	AGAGAGAGAGAGAGAGTC	3	950, 900, 600
7.	ISSR-7	CACACACACACACACARC	–	–
8.	ISSR-8	TGTGTGTGTGTGTGTGRA	1	500
9.	ISSR-9	ACACACACACACACACT	4	800, 600, 430, 300
10.	ISSR-10	CTCCTCCTCCTCCTCCTC	3	900, 650, 550

Table 3 Genetic homogeneity analysis of in vitro regenerated *C. brachynema* using RAPD markers

S. No.	Primer code	Primer sequence (5′–3′)	Number of scorable bands	Band length of amplicons
1.	OPAA-01	AGACGGCTCC	2	700, 450
2.	OPAA-02	GAGACCAGAC	2	750, 600
3.	OPAB-01	CCGTCGGTAG	3	900, 700, 400
4.	OPAB-02	GGAAACCCCT	3	1300, 900, 400
5.	OPAD-01	CAAAGGGCGG	–	–
6.	OPAD-02	CTGAACCGCT	–	–
7.	OPAH-01	TCCGCAACCA	–	–
8.	OPAH-02	CACTTCCGCT	–	–

not effective enough to reliably validate the genetic variations of in vitro regenerated plants.

Conclusions

This first report for the development of somatic embryogenesis system of *C. brachynema* offers an opportunity to ensure germplasm conservation, to control destruction threats, and it also delivers a potential system for the analysis of secondary metabolites. The induction and production of somatic embryos was significantly influenced by the combination of picloram (2.0 mg L^{-1}) and TDZ (0.5 mg L^{-1}). The SEM analyses affirm the developmental differentiation of somatic embryos. The banding pattern from SCoT, ISSR and RAPD markers analyses proved that the in vitro clones highly resembled to their mother plant which ruled out any somaclonal variation induced during somatic embryogenesis. The established model system enables the mass multiplication of genetically stable *C. brachynema* plants and could be employed as a potential source of active biomolecules in several ailments. In addition to the considerable work carried out, in-depth studies including genetic and metabolic engineering should also be performed in future to maximize the productivity of valuable alkaloids present in this species.

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Declarations

Conflict of interest Authors declare that there is no conflict of interest. Two of the authors are members of the editorial board, the editor-in-chief (S. Ochatt) and one of the associate editors (V. Kumar) and, as such, they have not been involved in any stage of the evaluation of the submission. This status had no bearing on the editorial consideration of the manuscript.

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