

# Efficient In Vitro Regeneration of *Gladiolus grandiflorus* L. Through Direct Organogenesis and Somatic Embryogenesis

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**Abstract.** *Gladiolus grandiflorus* is an economically important ornamental geophyte with a low multiplication rate and high susceptibility of corms to fungal infection under field conditions, thus limiting the availability of healthy planting material. Therefore, the development of an efficient in vitro regeneration system is essential for rapid clonal propagation and disease-free plant production. This study established an efficient regeneration system using corm explants. Direct regeneration was achieved, with the highest shoot induction frequency (83.33%) obtained on Murashige and Skoog (MS) medium fortified with 0.5 mg/L 6-benzylaminopurine (BAP) and 0.5 mg/L 1-naphthalene acetic acid (NAA), and the maximum number of shoots (4.03 shoots per explant) was recorded at 1.0 mg/L BAP. The first report of this cultivar included high-frequency direct somatic embryogenesis induced on MS medium containing 2 mg/L BAP and 2 mg/L NAA. The embryogenic origin of somatic embryos was confirmed by scanning electron microscopy and histological analyses. Plantlet conversion and rooting were observed on MS medium containing 2.0 mg/L BAP plus 1.0 mg/L NAA and 1.0 mg/L indole-3-butyric acid, respectively, with an ex vitro survival rate of 83%. Flow cytometry showed comparable 2C nuclear DNA contents (33.58–33.61 pg) among regenerated plantlets and the field-grown (mother plant), indicating ploidy stability. Start codon targeted marker revealed 95.13% genetic similarity with predominantly monomorphic banding patterns, confirming clonal fidelity. The established regeneration system provides an efficient approach for the production of disease-free and genetically stable *G. grandiflorus* plants.

## Introduction

Bulbous decorative plants are regarded as economically significant plant genetic resources in the floriculture industry (Jabbar et al. 2018). *Gladiolus* (*Gladiolus grandiflorus* L. ‘Pusa Kiran’), a member of the Iridaceae family, is a perennial bulbous plant of significant commercial value. The Latin term “*Gladius*” means “sword”; because of the sword-like morphology of its foliage, it is known as “Sword Lily” (Gautam et al. 2020). Well-known in the field of decorative gardening, it is also referred to as the “Queen of Bulbs” (Gangwar et al. 2023). It is cultivated in natural field settings in the summer in moderate to cool climatic zones

and in warm temperate zones during the cold season (Naithani et al. 2024). Its origin can be traced back to indigenous species found in the Mediterranean region as well as south and central Africa (Hassan et al. 2024). The United States (Florida and California), Holland, Italy, France, Poland, Bulgaria, Brazil, India, and Australia are the leading producer nations of *Gladiolus* (Vasanthakumar et al. 2015). *Gladiolus* is produced worldwide because of its eye-catching spikes with large appealing florets, striking coloration, varied sizes, and extended shelf life, which make it perfect as a cut flower (Belwal et al. 2024). In addition to its aesthetic value, the plant has therapeutic potential in the pharmaceutical industry. *Gladiolus* species

have been used to cure various ailments such as arthritis, rheumatism, epilepsy, headaches and fungal infections (Ngoupaye et al. 2013; Odhiambo et al. 2010). *Gladiolus dalenii* Van Geel has been proven effective for treating psychotic illnesses and depression (Ngoupaye et al. 2013).

The *Gladiolus* propagation method involves using seeds or corms and cormels. Although propagating plants using seeds is a successful approach, they could fail to develop a clonally uniform population. Conversely, multiplication of *G. grandiflorus* by corms and cormels is slow because the main corm produces only one corm per harvest cycle, along with two to three secondary cormels that require at least three seasons to produce a flowering spike. This suggests that the plant typically multiplies quite slowly, which affects its availability (Kumar et al. 2024). In addition, multiplication by corms and cormels is influenced by pathogenic infections caused by *Fusarium* (fungi) and viruses leading to substantial corm rotting, diminished plant growth, and reduced floral output (Naik et al. 2024). Plant tissue culture is a powerful tool for enhancing plant production in terms of both quality and yield. The regrowth of a wide range of horticultural crops for large-scale propagation has been aided by advances in the field of tissue culture (Kavinaya Shri et al. 2025). The in vitro regenerated plantlets rely on two basic approaches: somatic embryogenesis and organogenesis (Desai et al. 2022). Direct organogenesis is the best regeneration method for producing clonal plantlets with morphological and genotypic traits identical to those of the mother plant (Bekheet 2013). However, regeneration via somatic embryogenesis is regarded as an essential event for the mass multiplication and genetic manipulation of plantlets (Du et al. 2020). It provides an ideal in vitro model for investigating the developmental progression of embryos (Mujib et al. 2016). Although direct/indirect organogenesis (Deshmukh et al. 2021; Dutta and Gantait 2016; Remotti and Löffler 1995; Sheena and Sheela 2010) and indirect somatic embryogenesis (Mujib et al. 2016; Remotti 1995) have been extensively studied in *G. grandiflorus*, a limited embryogenic response from the explant surface under auxin treatment has been observed (Mujib et al. 2017). A consistent and replicable direct somatic embryogenesis protocol that achieves successful plant regeneration has not been documented.

Clonal regenerants frequently acquire genetic and epigenetic modifications called somaclonal variations as a result of the sustained application of diverse plant growth regulators (PGRs) and regularity and span of sub-cultures (Bansal et al. 2022). Changes caused by somaclonal variations are undesirable when consistent elite lines are produced on a large-scale. Therefore, it is extremely vital to establish methods of thoroughly evaluating the genetic fidelity of in vitro propagated plantlets (Das et al. 2025). DNA-based molecular markers such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplification of polymorphic DNA (RAPD), start

codon targeted (SCoT), and inter-simple sequence repeat (ISSR) markers are used to verify the genetic resemblance between the parent plant and in vitro propagated plants. The SCoT molecular marker has become increasingly significant in plant genome research because of its advanced, reliable, effective, replicable, and gene-targeted DNA marker technique (Rai 2023; Wasi et al. 2024). Recently, it has been successfully implemented to assess genetic homogeneity in numerous significant plant species such as *Cicer arietinum* L. (Sadhu et al. 2020), *Carthamus tinctorius* L. (Ejaz et al. 2022), *Tylophora indica* (Mamgain et al. 2022), *Dactylorhiza hatagirea* (Jaryal et al. 2025). Furthermore, flow cytometry is commonly used to determine the 2C DNA content in cultures grown in vitro because of its excellent precision and speed (Gomes et al. 2025). This technique was used to examine the genetic integrity of in vitro-grown plantlets from various plant species, including *Allium sativum* L. (Malik et al. 2020) and *Boswellia serrata* (Naikawadi et al. 2025).

The present study aimed to overcome the limitations associated with conventional propagation of *G. grandiflorus* by developing an efficient, reliable, and reproducible in vitro regeneration protocol through direct organogenesis and direct somatic embryogenesis using corm explants. The genetic stability and fidelity of the regenerated plants were confirmed using flow cytometry and SCoT marker techniques. To the best of our knowledge, this study provides the first comprehensive report of the optimization of the direct somatic embryogenesis protocol along with the use of SCoT markers for genetic fidelity assessment in *G. grandiflorus*.

## Materials and Methods

**Plant material collection and explant disinfection.** *Gladiolus grandiflorus* L. 'Pusa Kiran' corms were collected from the herbal garden (lat. 28° 30' 48.0" N, long. 77° 14' 51.4" E), Jamia Hamdard, New Delhi, India. This plant species was identified by Dr. Tapas Kumar Chowdhuri (Floriculture and Landscaping

Department, BCKV, West Bengal, India). For surface disinfection of corms, the outer skin of the corm explants was removed, and corms were immersed in a 20% Teepol (Qualigen, Mumbai, India) solution for 15 min. Thereafter, they were rinsed for 15 min under running water to eliminate the superficial pollutants. To complete surface disinfection, corms were immersed in 0.1% mercuric chloride solution for 3 min, followed by 70% ethanol for 2 min. Then, the explants were washed thrice with double-distilled sterile water to remove the disinfectants.

**Growth media and culture environment.** For in vitro cultures, full-strength Murashige and Skoog (MS) medium (Murashige and Skoog 1962) was prepared by combining macronutrients, micronutrients, iron, vitamins, glycine, and meso-inositol using their respective stock-based solutions along with 3% (w/v) sucrose. The PGRs were added to the medium in  $\mu\text{M}$  concentrations, and pH was adjusted to  $5.7 \pm 0.1$  using 1 N sodium hydroxide (NaOH) and/or 1 N hydrochloric acid (HCl). Subsequently, 0.8% (w/v) agar was added to the medium and heated to dissolve; thereafter, it was transferred to 25- × 150-mm test tubes (Borosil, Mumbai, India). The medium was autoclaved at 121°C for 20 min. Cultures were incubated at  $24 \pm 2^\circ\text{C}$  under white fluorescent tube lights (irradiance at  $50 \mu\text{mol}/\text{m}^2/\text{s}$  photosynthetic photon flux) during a 16-h/8-h photoperiod at 70% relative humidity.

**Direct organogenesis.** To induce shoot regeneration, the surface-disinfected corms were chopped into thin slices ( $1\text{-cm}^2$  explants) and one explant per tube containing MS medium supplemented with 6-benzylamino purine (BAP; 0.5, and 1.0 mg/L) was cultured alone or in combination with 1-naphthalene acetic acid (NAA; 0.5, and 1.0 mg/L) or 2,4-dichlorophenoxyacetic acid (2,4-D) at 0.5 mg/L alone. For culture maintenance, the explants were subcultured onto the same media every 3 weeks. The regeneration study included three replicates, with each replicate containing 24 explants. Following 6 weeks of culture, the percentage of explants that generated shoots and the average number of shoots per explant were recorded for 72 explants per treatment.

**Direct somatic embryogenesis.** To optimize the protocol for inducing somatic embryogenesis, the explants were inoculated onto MS medium supplemented with different concentrations of independent 2,4-D (0.5 or 1.0 mg/L) and BAP (0.5, 1.0, 2.0, or 4.0 mg/L) and NAA (0.5, 1.0, 2.0, or 4.0 mg/L) in combination. Additionally, NAA (1.0 mg/L) was further tested in combination with kinetin (1.0 mg/L). The cultures were maintained under

the laboratory conditions described previously, with routine passages every 3 weeks. After the induction and proliferation of somatic embryos, the culture was transferred into a germination medium supplemented with varying concentrations of BAP (1.0 or 2.0 mg/L) and NAA (0.5 or 1.0 mg/L). Each treatment was replicated thrice, and each replicate was represented by 24 test tubes. Following 6 weeks of culture, the embryogenesis percentage, number of embryos per explant, and regeneration of plantlets from somatic embryos were calculated.

**Scanning electron microscopy.** For the scanning electron microscopy (SEM) analysis, the embryo samples were initially kept in a Karnovsky fixative for 14 h at  $4^\circ\text{C}$ . After fixation, the samples were moved to a cold 0.1 M phosphate buffer. Then, a dehydration procedure was followed in a graded acetone series at concentrations of 30%, 50%, 70%, 90%, and 100% at 15-min intervals. Then, samples were subjected to critical point drying at 11,000 Psi. Next, they were carefully mounted on aluminum stubs and sputter-coated using 35-nm-thick gold film. Finally, samples were examined and photographed using a scanning electron microscope (Zeiss, Oberkochen, Germany) operating at a rising voltage of 20 kV.

**Histological observations.** To perform a histological examination of the embryo, embryo tissue was placed in solution comprising formalin, glacial acetic acid, and 70% ethanol (FAA; 1:1:18) for 24 h. Thereafter, the sample was sequentially diluted with ethanol to perform dehydration and subsequently fixed in a paraffin block. Then, the sample was processed into fine sections ( $8 \mu\text{m}$ ) using a rotary microtome (Spencer; American Optical Company, Buffalo, NY, USA) fitted with a steel blade. Following de-waxing, the sections were placed on fresh glass slides and dyed using 2% eosin and 5% hematoxylin. Ultimately, the dyed specimens were mounted on Canada balsam and viewed through a light microscope (Nikon Optiphot, Japan).

**Root induction in vitro.** To induce root growth, the regenerated plantlets (5–6 cm) were transferred to MS medium with varying doses of indole-3-butyric acid (IBA; 0.5–2.0 mg/L). The rooting study was conducted using three replicates, with each replicate consisting of 24 explants. The percentage of root development and number of roots following 3 and 6 weeks of culture were recorded for 72 explants per treatment. Subsequently, the well-rooted plantlets were moved to plastic pots filled with a 1:1 mixture of soil rite and soil for their acclimatization and survival in ex vitro conditions.

Table 1. List of primers and their attributes used for *Gladiolus grandiflorus* start codon targeted (SCoT) study.

| No. | Primer | Sequences (5' - 3') | %G/C |
|-----|--------|---------------------|------|
| 1   | SCoT1  | CAACAATGGCTACCACCA  | 50   |
| 2   | SCoT3  | CAACAATGGCTACCACCG  | 56   |
| 3   | SCoT5  | CAACAATGGCTACCACGA  | 50   |
| 4   | SCoT11 | AAGCAATGGCTACCACCA  | 50   |
| 5   | SCoT17 | ACCATGGCTACCACCGAG  | 67   |
| 6   | SCoT33 | CCATGGCTACCACCGAG   | 67   |

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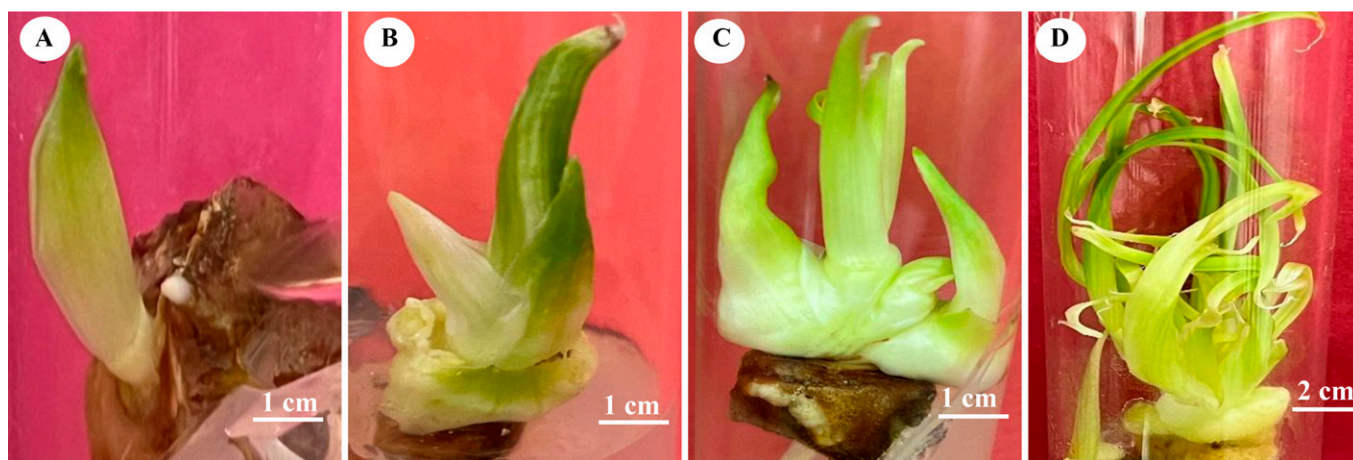


Fig. 1. In vitro shoot regeneration of *G. grandiflorus* through direct organogenesis from corm explants after six weeks of culture on MS medium supplemented with 0.5 mg/L BAP and 0.5 mg/L NAA. (A) Induction of shoot; (B, C) multiplication of shoots; and (D) regenerated plantlet.

**Flow cytometry.** Following the Galbraith protocol (Galbraith 2009), the 2C DNA contents of three randomly selected plants from each plant group, including field-grown, organogenic-derived, and somatic embryo-derived plants, were determined using flow cytometry. *Allium sativum* was used as a reference standard with a known 2C DNA content of 33.56 pg (Malik et al. 2020). Initially, Galbraith buffer (nuclei extraction buffer) was prepared using 45 mM MgCl<sub>2</sub>, 20 mM MOPS, 30 mM sodium citrate, and 0.1% (v/v) Triton X-100 and kept at 4°C after adjusting the pH to 7. Next, 100 mg of each sample leaf was weighed and finely chopped in 1.5 mL of ice-cold buffer placed in an ice-chilled petri dish. Then, the nuclei suspensions were filtered through nylon mesh with a pore size of 50 µm. Thereafter, 500 µL of PI RNase (Propidium iodide RNase; Sigma-Aldrich, St. Louis, MO, USA) was added to stain the samples, which were kept in the dark for 15 min and later incubated at 4°C for 1 h. Finally, the samples were examined using Diva 9.0.1 software with a BD FACSaria III flow cytometer. The data from each sample were collected and analyzed using the formula (Dolezel et al. 2007):

$$2C \text{ DNA content of the sample (pg)} = \frac{33.56 \times \text{Mean position of } \frac{G_0}{G_1} \text{ peak of sample}}{\text{Mean position of } \frac{G_0}{G_1} \text{ peak of standard}} \quad [1]$$

**SCoT marker analysis.** The SCoT analysis was performed for three randomly selected plant sets, with each set including a field-grown plant (mother plant), organogenic-derived plant, and somatic embryogenic-derived plant. The total genomic DNA of nine arbitrarily selected plants (three from each plant group) was extracted from 15 mg of lyophilized leaves using a modified CTAB-based procedure (Doyle 1990). The qualitative and quantitative evaluations of total genomic DNA were examined using electrophoresis on a 0.8% (w/v) agarose gel containing 0.5 µg/mL ethidium bromide in 1× TBE buffer (Sambrook et al. 1989). A

ultraviolet-Vis spectrophotometer (BL-295; Shimadzu, Kyoto, Japan) was used to measure the DNA concentration, and each aliquot

was diluted until the final concentration was 20 to 30 ng/µL. Afterward, the samples were stored at 4°C. Each plant set was analyzed

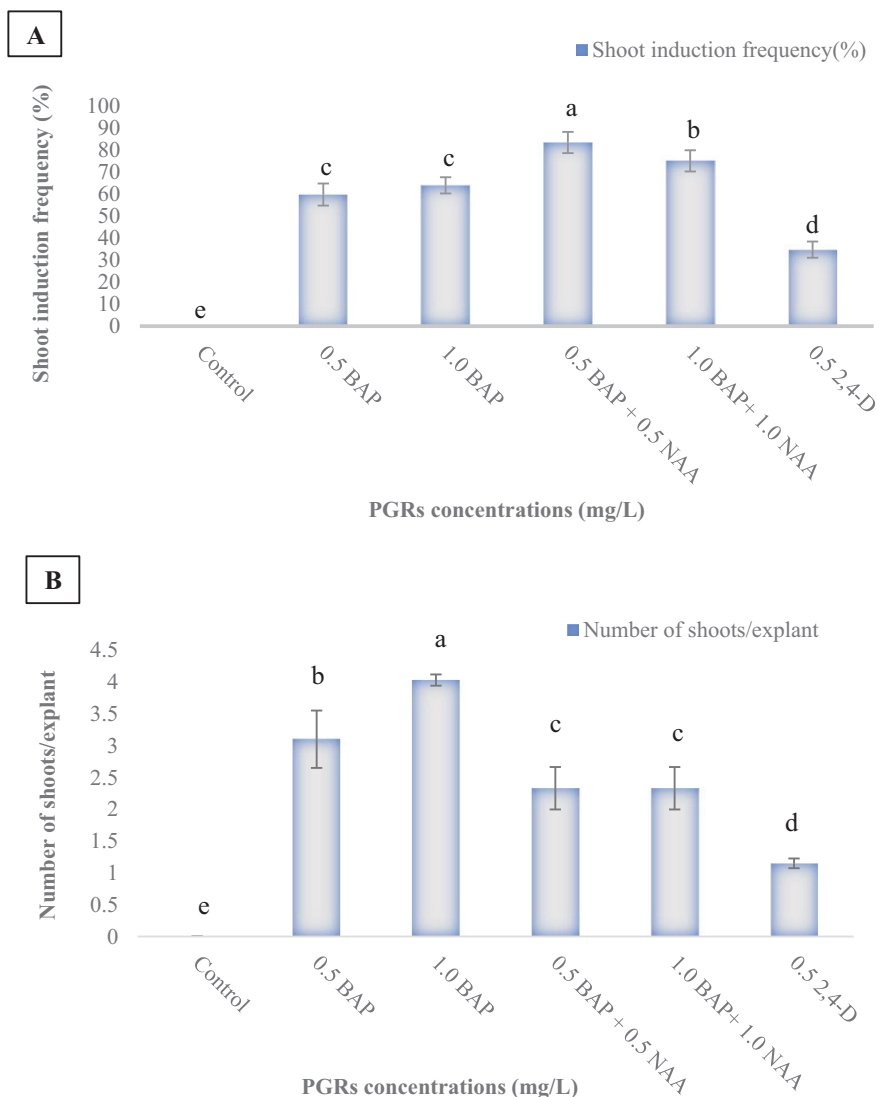


Fig. 2. Direct shoot regeneration in *G. grandiflorus* from corm explants. (A) Shoot induction (%) and (B) average shoot count per explant in the presence of cytokinin (BAP) and auxins (NAA and 2,4-D). The data were collected following six weeks of culture, and the values denote means ± standard error (n = 3). According to Duncan's multiple range test (DMRT), mean values with distinct letters differ significantly from one another at  $P \leq 0.05$ .

using six SCoT primers (Table 1) for genetic uniformity investigations, which yielded desired and repeatable amplified fragments that were then used for additional amplification processes. For the polymerase chain reaction (PCR) analysis, a 10- $\mu$ L reaction mixture containing 50 ng template DNA in 1 $\times$  reaction buffer, 1.5 mM MgCl<sub>2</sub>, 10 pmol random primers, and 1 U Taq DNA polymerase (Bio tools) was prepared. All PCR reactions were conducted using a Gene Amp PCR 9700 thermal cycler beginning with an initial denaturation step (5 min at 94°C). This was followed by 35 cycles of denaturation (30 s at 94°C), annealing (30 s at 50°C), and elongation (1 min at 72°C), with a final extension step (5 min at 72°C). Afterward, electrophoresis was used to resolve DNA amplicons in 1.5% (w/v) agarose gels. The gel documentation system (Azure Biosystem, Dublin, CA, USA) was used to display the bands and document gel images. A comparison with the 1-kb DNA ladder (Gene DireX, Inc., Taoyuan, Taiwan) was conducted to assess the size of the amplified fragments. The resultant bands were manually scored for presence (designated as “1”) and absence (designated as “0”). Using Jaccard’s similarity coefficient, the genetic similarity scores among sample groups were calculated. The similarity matrix was used to generate a dendrogram via the unweighted pair group method of arithmetic averages method (Sneath and Sokal 1973), which grouped the samples into related clusters. For statistical analyses, NTSYSpc version 2.21 (Applied Biostatistics, Setauket, NY, USA) was used (Rohlf 2000).

**Experimental design and statistical analysis.** A completely randomized design was used for the in vitro study. All experiments were repeated thrice, with 72 explants per treatment. The data are presented as mean  $\pm$  standard error. Assumptions of normality and homogeneity of variances were evaluated, and data were transformed when required before the analysis of variance (ANOVA). A one-way ANOVA using SPSS (software version 15; IBM, Chicago, IL, USA) was performed during statistical analyses of organogenesis, embryogenesis, and rooting percentage experiments. A two-way repeated-measures ANOVA was conducted to analyze the effects of the IBA concentration and time (3 and 6 weeks) on the root count. When the ANOVA revealed significant differences, Duncan’s multiple range test (DMRT) was used to assess mean comparisons at  $P \leq 0.05$  (Duncan 1955). In the SCoT analysis, DNA amplification using each primer was conducted thrice to ensure reproducibility.

## Results

**Direct shoot induction response of *G. grandiflorus* from corm explants.** Direct shoot regeneration was observed on the explant surface, devoid of any intermediate callus growth phase, when MS medium was fortified with auxins and cytokinins, either alone or in combination (Fig. 1). Shoot initiation from the explant occurred following 2 weeks of culture. The highest percentage of shoot initiation

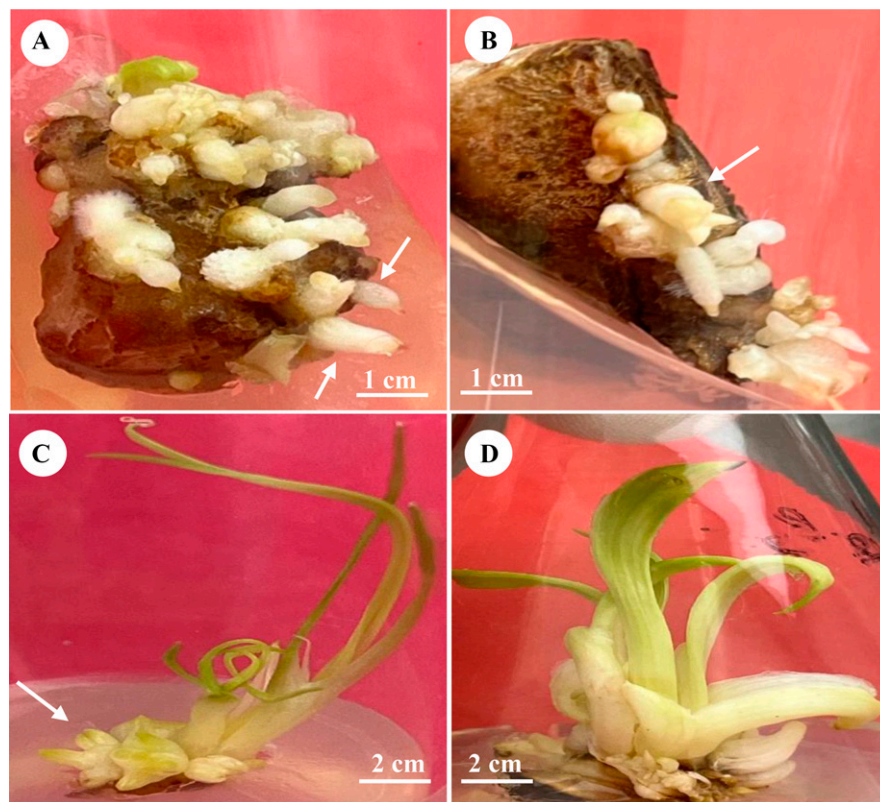


Fig. 3. Various stages of plant morphogenesis from somatic embryos to plantlets. (A, B) Close-up image of somatic embryos (arrow heads) growing from corm explant cultured onto MS medium supplemented with 2.0 mg/L BAP and 2.0 mg/L NAA; (C) germinating somatic embryos (arrow head) with different developmental stages into plantlets; and (D) conversion of somatic embryos into fully developed plantlet.

(83.33%) was obtained on medium containing 0.5 mg/L BAP and 0.5 mg/L NAA (Fig. 2). The BAP treatments at concentrations of 0.5 mg/L and 1.0 mg/L significantly increased the number of shoots per explant as compared with other treatments. The highest count (4.03 shoots per explant) was observed on medium supplemented with 1.0 mg/L BAP. However, when BAP was combined with NAA, the mean shoot count did not exhibit a significant increase, even at higher BAP concentrations. The medium fortified with 0.5 mg/L 2,4-D had the lowest shoot induction rate (34.72%) and lowest mean

shoot count per explant (1.15), thus demonstrating minimal significance as compared with other PGR concentrations. Medium devoid of PGRs showed no response, indicating that PGRs are mandatory for shoot regeneration of *G. grandiflorus*.

**Development of somatic embryos directly from corm explants.** Small, faint white protrusions began to appear directly on the surface of the corm explants following 2 weeks of culture on MS medium supplemented with different levels of auxins and cytokinins. These protrusions gradually progressed to the globular

Table 2. Effects of types and concentrations of plant growth regulators (PGRs) on direct somatic embryogenesis from *Gladiolus grandiflorus* corm explants.

| PGR treatments (mg/L) |     |       |     | Embryo induction (%)                       | Number of somatic embryos/explants        |
|-----------------------|-----|-------|-----|--------------------------------------------|-------------------------------------------|
| BAP                   | NAA | 2,4-D | Kn  |                                            |                                           |
| 0.0                   | 0.0 | 0.0   | 0.0 | 0.00 $\pm$ 0.00 <sup>c</sup>               | 0.00 $\pm$ 0.00 <sup>f</sup>              |
| 0.0                   | 0.0 | 0.5   | 0.0 | 8.33 $\pm$ 2.40 <sup>d</sup>               | 1.33 $\pm$ 0.33 <sup>e</sup>              |
| 0.0                   | 0.0 | 1.0   | 0.0 | 11.11 $\pm$ 3.67 <sup>d</sup>              | 1.50 $\pm$ 0.29 <sup>e</sup>              |
| 0.5                   | 0.5 | 0.0   | 0.0 | 25.69 $\pm$ 4.22 <sup>c</sup>              | 4.50 $\pm$ 0.29 <sup>d</sup>              |
| 1.0                   | 1.0 | 0.0   | 0.0 | 27.78 $\pm$ 7.35 <sup>c</sup>              | 5.20 $\pm$ 0.60 <sup>c</sup>              |
| 2.0                   | 2.0 | 0.0   | 0.0 | 45.83 <sup>i</sup> $\pm$ 1.20 <sup>a</sup> | 9.30 <sup>j</sup> $\pm$ 0.88 <sup>a</sup> |
| 4.0                   | 4.0 | 0.0   | 0.0 | 40.97 $\pm$ 4.55 <sup>b</sup>              | 7.00 $\pm$ 0.58 <sup>b</sup>              |
| 0.0                   | 1.0 | 0.0   | 1.0 | 0.00 $\pm$ 0.00 <sup>c</sup>               | 0.00 $\pm$ 0.00 <sup>f</sup>              |

<sup>i</sup>Indicates the greatest values in response to the optimal medium.

Embryo induction frequency was determined by calculating the percent of explants that produced somatic embryos in relation to the total count of explants inoculated. Data were collected following 6 weeks of culture. Values are presented as the mean  $\pm$  standard error ( $n = 3$ ). According to Duncan’s multiple range test, mean values with distinct superscript letters across a column differ significantly from one another at  $P \leq 0.05$ .

BAP = 6-benzylaminopurine; Kn = kinetin; NAA = 1-naphthalene acetic acid.

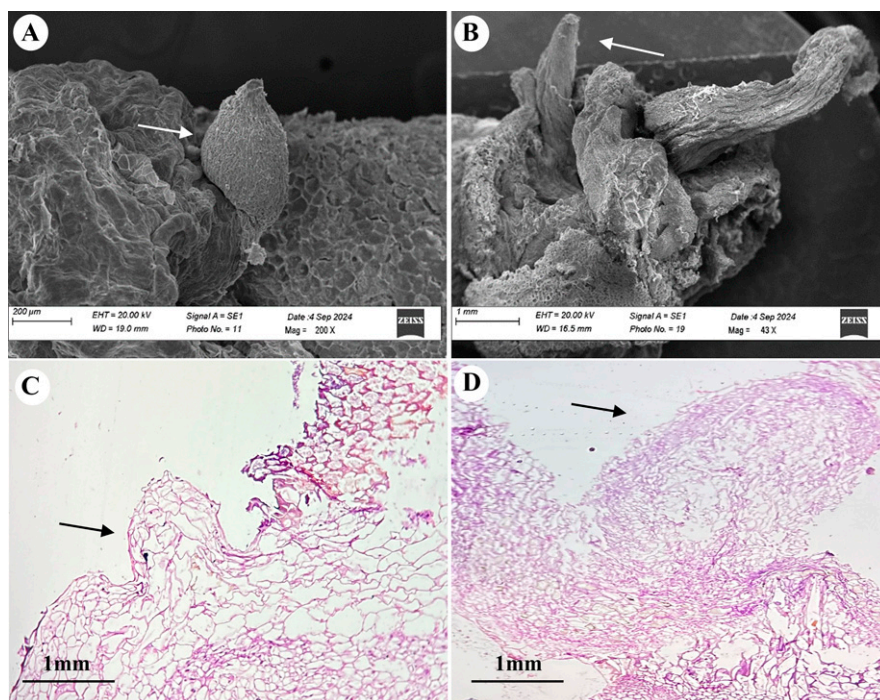


Fig. 4. Scanning electron microscopy and histological observations of *G. grandiflorus* somatic embryogenesis. (A, B) Electron micrographs of somatic embryos at early stages (arrow head) and (C, D) histological sections showing early embryonic structures (arrow head).

phase, marking the initiation of the somatic embryogenesis pathway. Following another 4 weeks of subculture on the same medium, the count of somatic embryos doubled, and asynchronous growth was simultaneously exhibited (Fig. 3A, 3B). The highest rate of somatic embryo induction ( $45.83 \pm 1.20$ ) and greatest mean count of embryos per explant ( $9.30 \pm 0.88$ ) were achieved with 2.0 mg/L BAP and 2.0 mg/L NAA following 6 weeks of explant culture (Table 2). Low dosages of these PGRs (0.5–1.0 mg/L BAP and

0.5–1.0 mg/L NAA) resulted in reduced embryogenesis and reduced embryo counts per explant, while 0.5 to 1.0 mg/L 2,4-D demonstrated a delayed response, with somatic embryo induction occurring following 4 weeks of culture. The high PGR dosage (i.e., 4.0 mg/L BAP and 4.0 mg/L NAA) also showed a good response, but the response was lower than that with the combination of 2.0 mg/L BAP and 2.0 mg/L NAA. Conversely, treatment comprising 1.0 mg/L NAA and 1.0 mg/L kinetin was ineffective for somatic embryo induction.

**Histology and scanning electron microscopy analysis.** Histological and SEM analyses (Fig. 4) were performed to determine the origin of the somatic embryos. The microphotographs resulting from the SEM analysis are presented in Fig. 4A and 4B. The presence of distinct pointed structures was observed, thereby confirming that the embryos originated directly from the corm explant surface.

Table 3. Conversion frequency of *Gladiolus grandiflorus* somatic embryos to plantlets following 6 weeks of culture on Murashige and Skoog (MS) medium fortified with different concentrations of 1-naphthalene acetic acid and 6-benzylaminopurine.

| MS + PGRs (mg/L) |     | Embryo conversion (%) |
|------------------|-----|-----------------------|
| BAP              | NAA |                       |
| 0.0              | 0.0 | $0.00 \pm 0.00^c$     |
| 1.0              | 0.5 | $23.41 \pm 2.60^d$    |
| 2.0              | 1.0 | $56.75^1 \pm 2.60^a$  |
| 1.0              | 0.0 | $46.43 \pm 2.08^b$    |
| 2.0              | 0.0 | $39.28 \pm 2.64^c$    |

<sup>1</sup>Indicates greatest values in response to the suitable media.

Embryo conversion frequency was calculated as the percent of somatic embryos converted into complete plantlets out of the total number of somatic embryos inoculated in each treatment. The data were collected following six weeks of culture and the values denote means  $\pm$  standard error ( $n = 3$ ). According to Duncan's multiple range test, mean values with distinct superscript letters across a column differ significantly from one another at  $P \leq 0.05$ . BAP = 6-benzylaminopurine; NAA = 1-naphthalene acetic acid; PGR = plant growth regulator.

The histological sections (Fig. 4C, 4D) depicted cellular division, which resulted in the creation of an embryonic mass that protruded from the peripheral surface of the corm explants.

**Somatic embryo conversion and successful plantlet regeneration.** Mature somatic embryos, when transferred to MS medium supplemented with 2.0 mg/L BAP and 1.0 mg/L NAA, provided the maximum conversion rate (56.75%) of somatic embryos to plantlets (Table 3). Successful conversion was also observed in BAP-alone medium; however, the conversion rate decreased with an increased BAP concentration and was lower than that with medium containing 2.0 mg/L BAP and 1.0 mg/L NAA, indicating that BAP alone was not optimal. In contrast, 1.0 mg/L BAP was effective for promoting shoot elongation and proliferation in the embryo-derived plantlets (Fig. 3D) following an efficient conversion process (Fig. 3C).

**Rooting and acclimation of plants.** Different concentrations of IBA (0.5–2.0 mg/L) significantly influenced the rooting percentage as well as the number of roots per explant (Table 4). Root initiation was observed following 1 week of culture, and the MS medium containing 1.0 mg/L IBA exhibited the highest root formation rate (79.17%), with an average count of 22 roots per plant following 6 weeks of culture. Both thick and thin roots were observed (Fig. 5A, 5B). According to the repeated measures ANOVA, the IBA concentration and culture time had significant effects on the number of roots per plantlet. After 3 weeks, only 1.0 mg/L IBA (9.33) produced a significantly higher count than that produced with 2.0 mg/L IBA (5.33); all other concentrations responded similarly ( $P > 0.05$ ). Compared with 0.5 mg/L IBA (17.33), 1.5 mg/L IBA (13.83), and 2.0 mg/L IBA (8.33), 1.0 mg/L IBA produced the maximum mean root count (22.00) after 6 weeks. Following the development of roots, the plants were transferred to plastic pots for acclimatization (Fig. 5C, 5D). The in vitro-propagated plants of *G. grandiflorus* were adapted to ex vitro conditions, with a high survival rate of 83%.

**Flow cytometry and SCoT analysis.** Flow cytometry histograms revealed that the mother plant and in vitro-derived plants had identical peak values (Fig. 6). The 2C nuclear DNA

Table 4. Rooting of in vitro-derived shoots of *Gladiolus grandiflorus* on Murashige and Skoog (MS) medium supplemented with varying concentrations of indole-3-butyric acid following 3 and 6 weeks of culture.

| IBA (mg/L) | Root count per plantlet |                      | Root formation (%)   |
|------------|-------------------------|----------------------|----------------------|
|            | 3 weeks                 | 6 weeks              |                      |
| 0.0        | $0.00 \pm 0.00^c$       | $0.00 \pm 0.00^c$    | $0.00 \pm 0.00^c$    |
| 0.5        | $7.60 \pm 0.33^{ab}$    | $17.33 \pm 1.33^b$   | $66.67 \pm 6.36^b$   |
| 1.0        | $9.33 \pm 0.88^{*a}$    | $22.00^1 \pm 1.73^a$ | $79.17^1 \pm 2.40^a$ |
| 1.5        | $6.95 \pm 0.71^{ab}$    | $13.83 \pm 1.36^c$   | $63.54 \pm 2.62^c$   |
| 2.0        | $5.33 \pm 0.33^b$       | $8.33 \pm 0.88^d$    | $59.72 \pm 2.78^d$   |

<sup>1</sup>Symbolizes greatest values in response to the suitable media.

Data were collected following 3 and 6 weeks of culture. Values are presented as the mean  $\pm$  standard error ( $n = 3$ ). According to Duncan's multiple range test, mean values with distinct superscript letters across a column differ significantly from one another at  $P \leq 0.05$ .

IBA = indole-3-butyric acid.

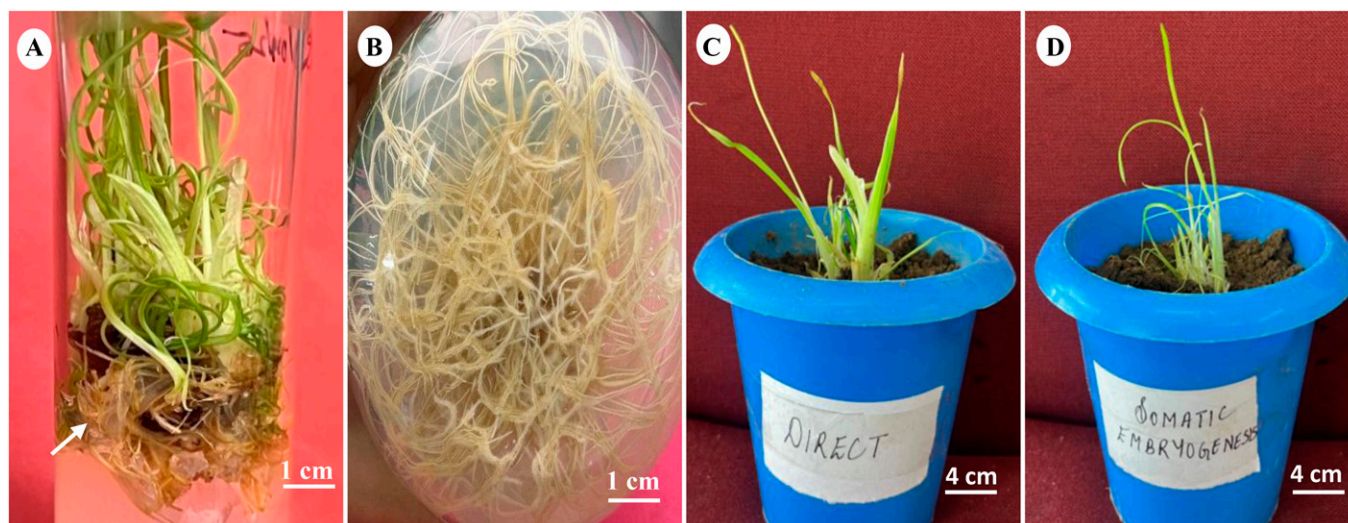


Fig. 5. In vitro rooting and acclimatization of *G. grandiflorus* plantlets. (A) Root induction (arrow head) onto MS medium supplemented with 1.0 mg/L IBA; (B) rooting observed following 6 weeks of culture; and (C, D) transfer of plantlets to plastic pots for acclimatization.

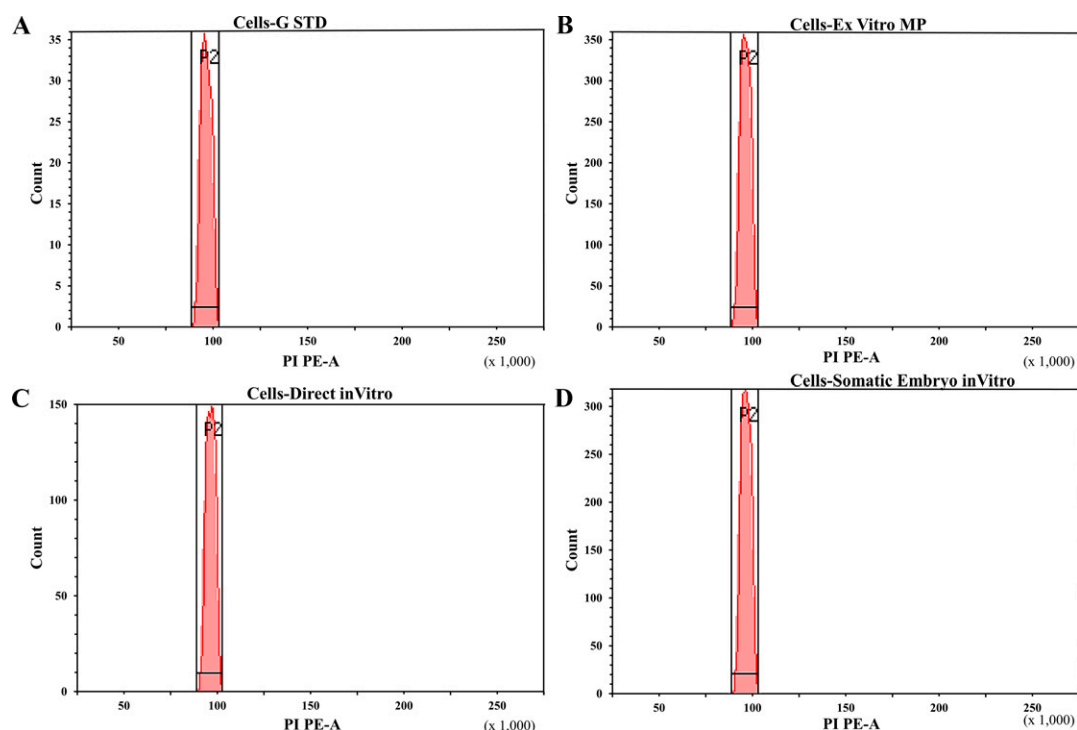


Fig. 6. Flow cytometry histogram profiles displaying peaks derived from nuclear suspensions labeled with pIRNase stain for the relative 2C DNA content extracted from leaf tissues of (A) field-grown *Allium sativum* (standard); (B) field-grown *G. grandiflorus* mother plant; (C) *G. grandiflorus* plant regenerated through direct organogenesis; and (D) *G. grandiflorus* plant regenerated through somatic embryogenesis.

content values computed for the mother plant, somatic embryo-derived plants, and direct organogenesis-derived plants were 33.58 pg, 33.61 pg, and 33.58 pg, respectively (Table 5). These results demonstrated similarity in the genome size of the mother and in vitro-regenerated plantlets. For the SCoT marker analysis, genomic DNA was successfully isolated from the mother plant, somatic embryo-derived plants, and direct organogenesis-derived plants and quantified on 0.8% agarose gel, resulting in intact bands (Fig. 7A). For one representative plant set, six SCoT primers (SCoT\_1, SCoT\_3, SCoT\_5, SCoT\_11, SCoT\_17, SCoT\_33)

produced 65 monomorphic bands out of 68 total bands scored (250–2000 bp), ruling out the chance of somaclonal variation. Representative amplification profiles obtained with three of the six SCoT primers are shown in Fig. 7B–7D. For each primer, an average of 11 detectable bands were produced, with SCoT\_3 giving the highest number of monomorphic bands ( $n = 8$ ) (Table 6). These monomorphic SCoT profiles revealed the genetic integrity of the tissue culture-raised plantlets, with the mother plant depicting 95.13% genetic similarity according to Jaccard's similarity matrix (Fig. 8). The overall schematic and experimental layout adopted for

the clonal propagation and genetic fidelity analysis of *G. grandiflorus* are illustrated in Fig. 9.

## Discussion

Plant cells possess complete cellular potency, thus enabling the emergence of a young shoot by organogenesis and somatic embryogenesis (Zhang et al. 2021). Thus far, several regeneration pathways involving direct/indirect organogenesis and embryogenesis have been successfully established for several plant types, including *Gladiolus* species (Devi et al. 2019; Emek and Erdag 2007;

Table 5. Genome size (Mbp) and nuclear 2C DNA content (pg) assessments and comparisons with validated genetic uniformity of in vitro-grown *Gladiolus grandiflorus* plantlets.

| Plant material                   | Nuclear DNA content (pg)  |                           | 2C genome size (Mbp) <sup>ii</sup> |
|----------------------------------|---------------------------|---------------------------|------------------------------------|
|                                  | 2C DNA <sup>i</sup>       | 1C DNA <sup>i</sup>       |                                    |
| Mother plant                     | 33.58 ± 0.00 <sup>a</sup> | 16.79 ± 0.00 <sup>a</sup> | 32,841.24 <sup>a</sup>             |
| Direct in vitro-derived plantlet | 33.58 ± 0.02 <sup>a</sup> | 16.79 ± 0.01 <sup>a</sup> | 32,841.24 <sup>a</sup>             |
| Somatic embryo-derived plantlet  | 33.61 ± 0.03 <sup>a</sup> | 16.81 ± 0.02 <sup>a</sup> | 32,870.58 <sup>a</sup>             |

<sup>i</sup>Values are presented as the mean ± standard error (n = 3). According to Duncan's multiple range test, mean values with distinct superscript letters across a column differ significantly from one another at  $P \leq 0.05$ .

<sup>ii</sup>1 pg = 978 Mbp (Dolezel 2003).

Tripathi et al. 2017). An essential consideration for an efficient in vitro morphogenesis protocol is the type of explant because it plays a major role in establishing a regenerative pathway. As reported for *Gladiolus* and the majority of the Iridaceae species, corms are the significantly favored explants (Tan Nhut et al. 2004). In line with this, Aftab et al. (2008) suggested that cormel explants generated a higher shoot count in *G. hybridus* in contrast to meristem or leaf. Additionally, *G. dalenii* (Kabir et al. 2014) and *Gladiolus hybridus* Hort. (Naik et al. 2024) were subjected to a great in vitro development approach using corm slices as explants, further emphasizing the importance of corms as an optimal explant type.

In this study, direct shoot morphogenesis from the corm explant was observed without any intervening callus stage, and maximum shoot regeneration efficiency was noted in medium containing the PGR mixture with equivalent amounts of auxin (NAA) and cytokinin (BAP). According to Ngomuo et al. (2013), PGR combinations are extensively used in media because the growth characteristics of in vitro plant tissues can be effectively modified by adjusting and varying the quantities of auxins and cytokinins. It was previously noted in *Crocus sativus* L. that cytokinins and auxins in combination work effectively to encourage shoot development (Lagram et al. 2023). On the contrary, in *G. grandiflorus*, BAP-enriched medium exhibited a significant increase in the

mean shoot number per explant. A previous study of *Gladiolus grandiflorus* L. also demonstrated the effectiveness of the BAP dosage of 2.0 mg/L for encouraging shoot growth (Devi et al. 2019). A similar trend of enhanced shoot proliferation with BAP treatment was noted in other monocot members, including *Eleusine coracana* (L.) Gaertn. (Atul Babu et al., 2018) and *Cymbopogon citratus* (DC.) Stapf (Cárdenas-Aquino et al. 2023). Somatic embryogenesis is a sequential developmental pathway beginning with the emergence of proembryogenic structures that subsequently leads to the induction of somatic embryos, their morphological differentiation, and, eventually, complete transition to plantlets (Von Arnold et al. 2002). According to Zuta-Puscan et al. (2025), PGRs are crucial to the mechanism of somatic embryogenesis because they provide auxins and cytokinins as the key components involved in cell destiny specification. The present work demonstrated that the corm could effectively produce the highest count of somatic embryos directly on the corm explant surface in MS media fortified with 2.0 mg/L BAP and 2.0 mg/L NAA, but that BAP alone was inefficient for initiating embryogenesis. Additionally, the maximum shoot regeneration efficiency of *G. grandiflorus* was observed using MS media supplied

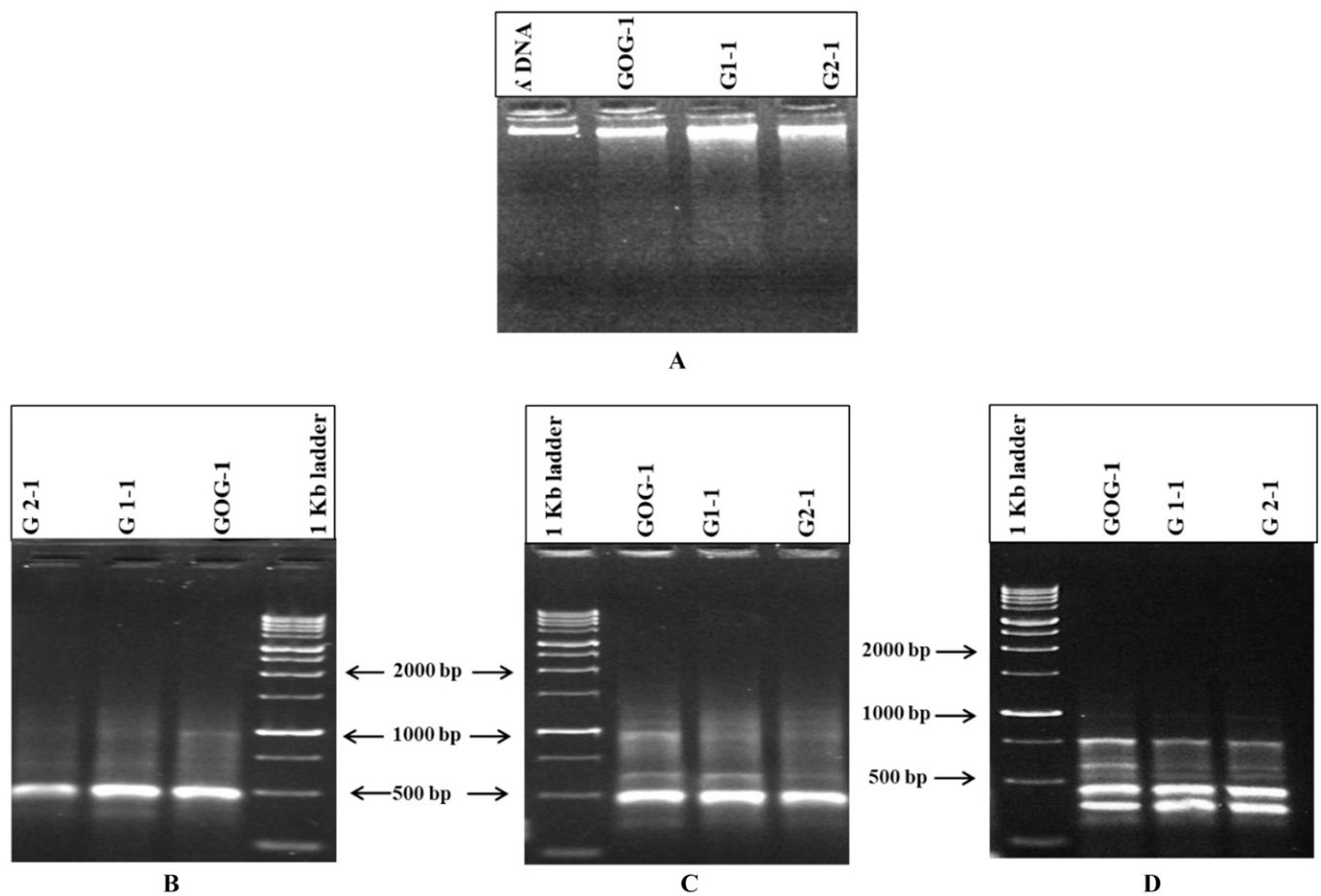


Fig. 7. (A) Genomic DNA quantification of *G. grandiflorus* on 0.8% agarose gel. GOG: mother plant (field grown); G1-1: in vitro plant regenerated by 0.5 mg/L BAP and 0.5 mg/L NAA induced direct organogenesis; G2-1: in vitro plant regenerated by 2.0 mg/L BAP and 2.0 mg/L NAA induced somatic embryogenesis; (B) representative SCoT profile of *G. grandiflorus* plants generated with the SCoT\_1 primer; (C) representative SCoT profile of *G. grandiflorus* plants generated with the SCoT\_11 primer; and (D) representative SCoT profile of *G. grandiflorus* plants generated with the SCoT\_33 primer.

Table 6. A record of primers detailing their melting temperature and counts of detectable and monomorphic bands generated by the SCoT analysis of *Gladiolus grandiflorus* plantlets grown in vitro.

| No.   | Primer  | Melting temperature | No. of detectable bands <sup>1</sup> |    | Total bands | Monomorphism % |
|-------|---------|---------------------|--------------------------------------|----|-------------|----------------|
|       |         |                     | G1                                   | G2 |             |                |
| 1     | SCoT 1  | 50 °C               | 2                                    | 2  | 4           | 100            |
| 2     | SCoT 3  | 50 °C               | 8                                    | 8  | 16          | 87.5           |
| 3     | SCoT 5  | 50 °C               | 6                                    | 6  | 12          | 83.33          |
| 4     | SCoT 11 | 50 °C               | 4                                    | 4  | 8           | 100            |
| 5     | SCoT 17 | 50 °C               | 7                                    | 7  | 14          | 100            |
| 6     | SCoT 33 | 50 °C               | 7                                    | 7  | 14          | 100            |
| Total |         |                     | 34                                   | 34 | 68          | 95.13          |

<sup>1</sup> G1 and G2 represent direct and somatic embryo-derived plantlets, respectively.

DNA band lengths were determined using the 1 kb Thermo Scientific GeneRuler DNA Ladder (Waltham, MA, USA).

SCoT = start codon targeted.

with 2.0 mg/L BAP and 1.0 mg/L NAA. In *Caladium × hortulanum* ‘Fancy’ (Syeed et al. 2022), the same combination was found to be effective for inducing direct somatic embryos. However, Mujib et al. (2017) observed very low-frequency direct somatic embryos in *Gladiolus* but did not provide information regarding embryo count, induction efficiency, and a detailed structural analysis. In addition, the maturation and regeneration efficiency of direct somatic embryos were not demonstrated. Most of the results provided depict embryogenic callus-generated somatic embryos. Therefore, the present study addressed this gap by establishing a high-frequency direct somatic embryogenesis protocol for *G. grandiflorus*.

Histological and SEM investigations confirmed the occurrence of somatic embryogenesis in *G. grandiflorus*, thus validating embryonic development and its progression. A histological investigation is a crucial method of researching the morphogenic development and tissue organization of somatic embryos (Qin et al. 2025). However, SEM investigations are commonly conducted to identify the origin of somatic embryos (Bansal et al. 2022). In this study, SEM micrographs validated the origin of somatic embryos and illustrated their initial

stage. The histological study revealed an early stage of somatic embryos arising from the surface of the corm explant. Similar SEM and histological investigations of somatic embryo progression in several geophytic plant species, such as *Crocus sativus* L. (Devi et al. 2014), *Iris sibirica* (Stanišić et al. 2015), and *Lachenalia viridiflora* (Kumar et al. 2016), have also been conducted.

One crucial stage that affects the overall effectiveness of the clonal propagation method is plantlet acclimation by promoting the formation of robust and strong roots. Auxins are crucial for the induction and development of roots, thus emphasizing their relevance in the accomplishment of in vitro plant development (Rezapour et al. 2025). The primary element that affects the rooting process is the auxin variant and dosage, which significantly affect the rooting efficiency of tissue culture-derived shoots (Okello et al. 2021). In this study, *G. grandiflorus* microshoots were rooted onto medium containing different concentrations of IBA, and 1.0 mg/L was the most effective concentration for healthy root formation. Auxins were previously used to induce roots in a number of plant species, such as *Iris sanguinea* (Wang et al. 2018) and *Gloriosa superba* L. (Mosoh et al. 2024).

Genetic mutations, epigenetic modifications, or chromosomal abnormalities may arise in tissue culture-derived plantlets because of harsh conditions in media, such as long-term exposure to varying PGR concentrations, and subculture durations (Delparte et al. 2014; Gawronski and Dyduch-Sieminska 2022). Therefore, it is imperative to evaluate the genetic homogeneity of the in vitro (direct and somatic embryo-derived) plantlets and compare them with the mother plant to ensure that the in vitro plants are devoid of somaclonal variations (Sarkar et al. 2026). Thus, to enable a detailed analysis of genetic integrity, our study used two techniques: flow cytometry and a PCR-based SCoT marker analysis. The most frequently encountered genomic alterations in laboratory-grown plantlets, such as changes in genome size and 2C DNA content, may be assessed and analyzed in relation to the mother plant using the flow cytometry approach. This allows ascertainment of whether the in vitro-grown plantlets exhibit any ploidy alteration (Bahmankar et al. 2024). The use of flow cytometry for assessing ploidy alterations and culture-derived genetic variations of in vitro-raised plantlets compared with field-grown plantlets has been validated among different plants, such as *Mentha arvensis* (Faisal et al. 2014) and *Uvaria hamiltonii* (Mohanty et al. 2025). In the present study, the “true-to-type” nature of the regenerant was verified through flow cytometry because no ploidy alteration was found because of the close resemblance of G0/G1 histogram peaks in all cultures, including the mother specimen of *G. grandiflorus*.

Because the outcomes of a single approach cannot be fully assured, an additional method (marker-based PCR analysis) was used in this investigation. In contrast to the conventional dominant marker (RAPD), a gene-specific marker (SCoT) has received much attention because of its potential to identify clonal differences even at very low levels (Gautam and Bhattacharya 2021). Furthermore, it plays an important role in assessing the genetic stability

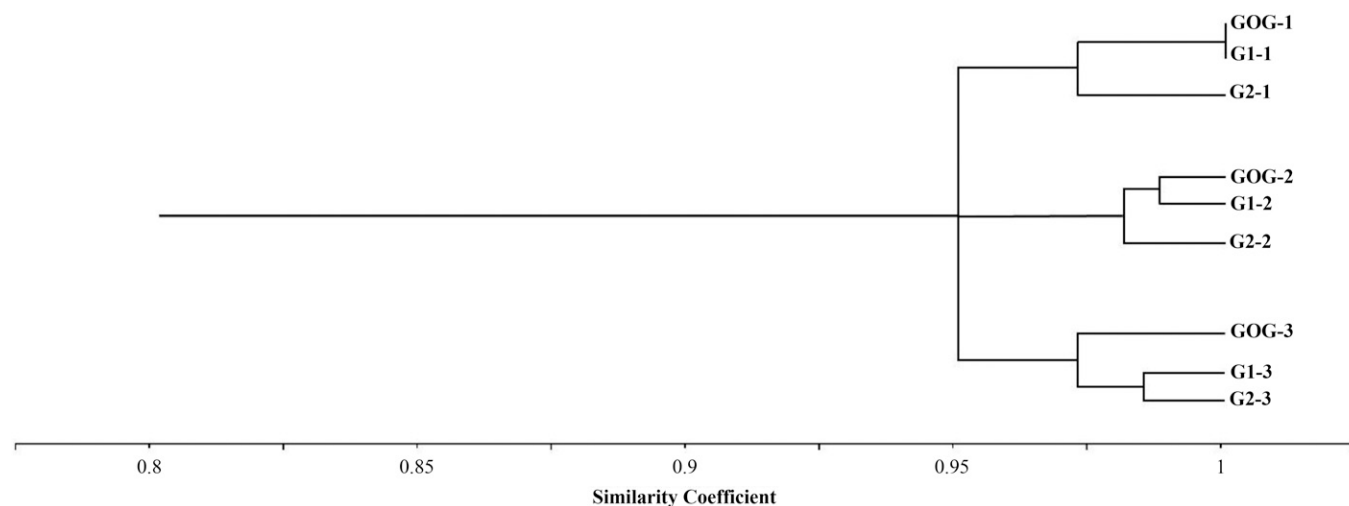


Fig. 8. Dendrogram displaying the clustering analysis of the field-grown (GOG) and in vitro-derived plants through direct organogenesis (G1) and somatic embryogenesis (G2).

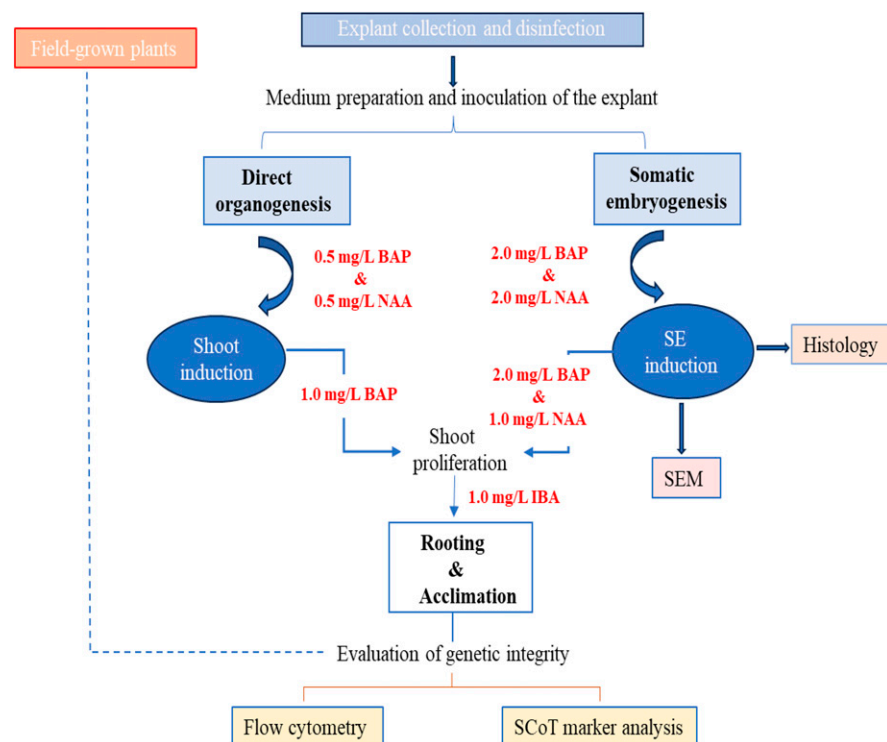


Fig. 9. Schematic representation depicting in vitro regeneration protocol; scanning electron microscopy, histological investigation, and evaluation of genetic stability of *G. grandiflorus*.

of in vitro-propagated plants. Six SCoT primers (SCoT\_1, SCoT\_3, SCoT\_5, SCoT\_11, SCoT\_17, and SCoT\_33) produced the expected outcomes (monomorphic bands among direct, somatic embryo-derived, and the mother plantlet), with an average genetic similarity of 95.13% across all plant samples. This is the first study to use the SCoT marker approach to evaluate genetic uniformity in *G. grandiflorus*; however, other methods have been used for *G. grandiflorus* and *G. hybrida* for the same purpose (Hiremath 2019; Kumar et al. 2018; Mousavimatin et al. 2025). To evaluate the genome integrity of tissue culture-raised plants, a SCoT marker-assisted genetic assessment has been used across various plant species, including *Prunus salicina* cultivars Santa Rosa and Frontier (Thakur et al. 2021) and *Nothapodytes foetida* (Attaluri and Dharavath 2025).

In conclusion, this study established an efficient in vitro regeneration system for *G. grandiflorus* via direct organogenesis and direct somatic embryogenesis using corm explants. Histology and SEM techniques confirmed the origin and development of the somatic embryos. Flow cytometry and the DNA-based SCoT marker technique ensured the genomic integrity of the regenerants, thereby confirming the true-to-type nature of the in vitro-raised plantlets. Healthy and fully developed plantlets were successfully acclimatized to the outdoor environment. This study provided uniform, disease-free, and economically viable plants with minimal somaclonal variations and enabled the large-scale production of *G. grandiflorus*. To the best of our knowledge, this is the first detailed report of high-frequency direct somatic

embryogenesis and genetic fidelity assessment using SCoT markers.

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