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Indirect regeneration of long-term callus cultures in gladiolus: a protocol for stable genetic fidelity

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Gladiolus is an important and economically valued ornamental plant grown worldwide. One of the major challenges in its micropropagation is maintaining genetic stability during indirect regeneration and long-term callus maintenance. The objective of this study was to develop an optimized indirect shoot regeneration protocol for three commercial *Gladiolus* cultivars with consistent genetic traits. Callus initiated from the basal part of extended mother corm sprout (EMCS) explants in MS medium supplemented with 2 mgL⁻¹ 2,4-D, 2 mgL⁻¹ NAA and 1 mgL⁻¹ BAP. The synthesis of phenolic compounds was effectively controlled by the addition of 150 mgL⁻¹ ascorbic acid, 100 mgL⁻¹ citric acid, and 500 mgL⁻¹ activated charcoal. This medium led to an 80% decrease in the accumulation of phenolic compounds across all cultivars in comparison to the control. For shoot regeneration, calli which were maintained over the long term were transferred to MS medium supplemented with 2 mgL⁻¹ BAP, 2 mgL⁻¹ Kin and 0.25 mgL⁻¹ NAA. This significantly enhanced shoot regeneration percentage (95.55%) and number (39.44 shoots per explant). Additionally, cormel formation was significantly enhanced (16.66 cormels per explant) at the base of regenerated plantlets using MS medium containing 9% sucrose and 2 mgL⁻¹ indole-3-acetic acid, without any cormel formation in the control. Cormels were effectively acclimatized in the greenhouse with 100% survival rate. To demonstrate genetic stability, regenerated plantlets were evaluated by flow cytometry and Inter Simple Sequence Repeat (ISSR) markers, verifying their genetic identification with the mother plants. This study provides a reliable and scalable protocol for the commercial micropropagation of gladiolus, with promising applications in breeding programs that aim at transferring desirable traits such as disease resistance or specific floral features.

KEYWORDS

gladiolus micropropagation, genetic stability, flow cytometry, ISSR markers, cormel formation

1 Introduction

The gladiolus (*Gladiolus grandiflorus* L.), a well-known ornamental plant in the Iridaceae family, is prized for its attractive flower spikes and remarkable vase life. Gladiolus is a cross-pollinated, diploid species ($2n = 2x = 30$) (Azimi, 2020), with significant economic value evidenced by Flora Holland auctions, which generate 9 million euros annually (Sharma and Tripathi, 2008; Ramos-Garci et al., 2009; Azadi et al., 2016). The plant is known as the “queen of bulbous flowers” because of its tall, colorful flower stems, which come in a range of colors, including pink, red, purple, white, cream, and orange, as well as different flower shapes (Singh et al., 2018).

In their natural environment, gladiolus plants primarily reproduce through corms and cormels. Breeders often use seeds after hybridization to create new and improved cultivars (Swaroop et al., 2018). Despite their effectiveness, both approaches come with challenges, such as slow propagation rates, vulnerability to diseases, and limited genetic variation. Tissue culture has emerged as an effective method for the mass propagation and large-scale commercial production of ornamental plants, offering a promising solution to overcome existing limitations (Jaryal et al., 2025; Ntui et al., 2010; Kumar and Nandi, 2015; Nalousi et al., 2019). Both direct and indirect organogenesis have been investigated for effective micropropagation (Sinha and Roy, 2002; Wu et al., 2015; Kumar et al., 2024a). Indirect regeneration yet, has demonstrated advantages for both propagation and as a foundation for genetic engineering (Wu et al., 2015). A significant problem associated with indirect regeneration is the risk of somaclonal variation, which may result in genetic instability in the regenerated plants (Endemann et al., 2001; Ferreira et al., 2023; Krishna et al., 2016; Long et al., 2022).

Several factors contribute to this instability, including explant source (Dey et al., 2015), wounding during the culture process (Debnath and Ghosh, 2022), concentration of plant growth regulators (PGR) (Rakoczy-Trojanowska, 2002; Rai, 2021), *in vitro* environmental conditions (Polanco and Ruiz, 2002; Ferreira et al., 2023; Joshi et al., 2024), prolonged culture durations (Sun et al., 2013), and oxidative stress caused by free radicals (Krishna et al., 2016; Smulders and de Klerk, 2011). To mitigate these issues, reliable genetic stability evaluation methods are critical. Flow cytometry (FCM) is a rapid and efficient technique for evaluating ploidy levels in plants (Ochatt et al., 2011; Zafar et al., 2019). In addition, molecular markers such as Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeats (SSR), and Inter Simple Sequence Repeats (ISSR) are commonly used to confirm genetic stability in regenerated plants (Dey et al., 2015;

Nalousi et al., 2019; Singh et al., 2017). Of these, ISSR markers are distinguished for their high reproducibility and the ability to detect genetic variation without requiring species-specific primers (Faisal et al., 2014).

While significant progress has been made, comprehensive studies addressing the combination of long-term callus maintenance, high regeneration efficiency, and robust genetic stability assessment in gladiolus are insufficient. This study aims to fill this gap by developing an optimized protocol for indirect organogenesis using elongated mother corm sprout (EMCS) explants from three popular *Gladiolus* cultivars ‘Rose Supreme’, ‘Amsterdam’, and ‘Advance Red’ known for their vibrant colors (pink, white, and red). For the first time, the genetic stability of regenerated plants was evaluated using both FCM and ISSR markers, ensuring the reliability of the proposed protocol.

The optimized protocol offers numerous advantages, including the ability to maintain callus cultures for extended periods, achieve high shoot regeneration rates, and efficiently produce cormels. These attributes make the method suitable for both commercial micropropagation and genetic engineering applications aimed at enhancing disease resistance and other desirable traits in gladiolus.

Gladiolus (*Gladiolus grandiflorus*) is a cross-pollinating and diploid ($2n = 2x = 30$) plant (Azimi, 2020).

2 Materials and methods

2.1 Plant materials and surface sterilization procedures

Gladiolus corms of three commercial cultivars including ‘Rose Supreme’ (RS), ‘Amsterdam’ (A), and ‘Advance Red’ (AR) were obtained from the research greenhouse of the Iranian Floriculture and Ornamental Plants Research Institute (Latitude: 33° 53’ N, Longitude: 50° 29’ E; altitude: 1,732 m; average temperature: 23.4°C; relative humidity: 57.1%). Uniform mother corm sprouts (3–3.5 cm diameter) were selected as explant sources. For sterilization, the corms’ surface scales were removed, and sprouts were divided into 1 cm² explants (Figure 1a). The explants were first washed under running tap water for 30 min with a mild detergent, followed by a 45-min heat treatment at 45°C in a water bath to eliminate internal contaminants. Surface disinfection was then performed by immersion in 70% (v/v) ethanol (1 min) and 1.5% (w/v) sodium hypochlorite (10 min). Finally, the explants were rinsed four times with sterile distilled water in a laminar airflow cabinet and cultured on MS medium supplemented with 0.5 mg L⁻¹ BAP for establishment.

2.2 Callus induction

Two weeks after the sprouts growth, EMCS explants (with an average length 50 mm) were transferred to the Murashige and Skoog (1962) (MS) medium supplemented with various

Abbreviations: 2,4-D, 2,4-Dichlorophenoxyacetic acid; BAP, 6-benzylaminopurine; EMCS, Elongated mother corm sprout; IAA, Indole-3-acetic acid; ISSR, Inter Simple Sequence Repeats; Kin, Kinetin; MS, Murashige and Skoog; NAA, Naphthalene acetic acid.

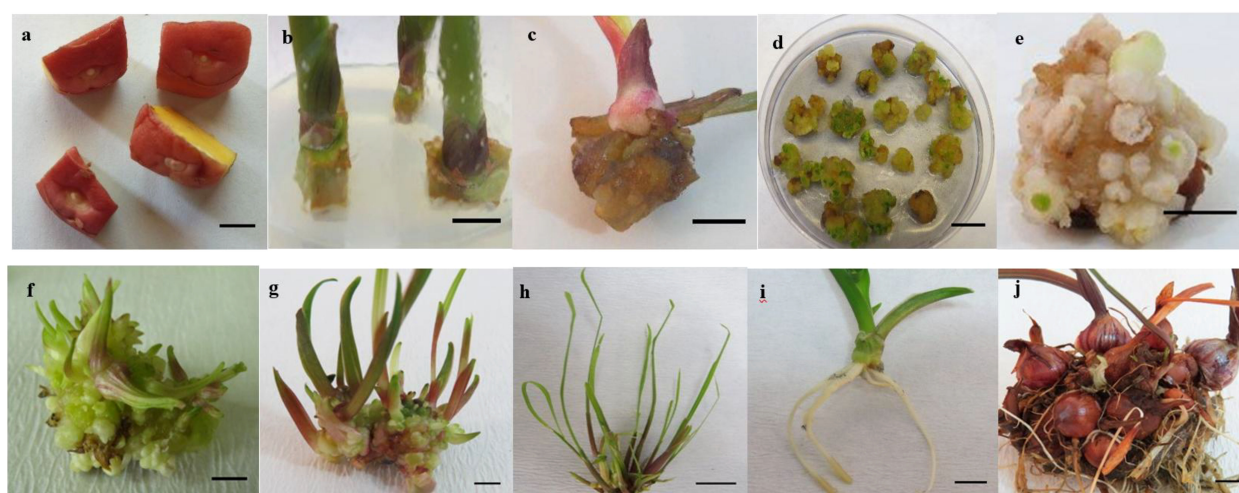


FIGURE 1

Flow diagram of indirect regeneration of *Gladiolus grandifloras* 'Advance red' from Elongated Mother Corm Sprout (EMCS) explants; (a) Slices of corm sprouts explant, (b) Establishment of EMCS explants, (c) Callus induction in bottom of the EMCS explants (in callus induction medium), (d) Long-term maintenance of callus, (e) Early regeneration steps (in shoot induction medium), (f) Shoot regeneration (in shoot induction medium), (g) Development of shoot (in shoot induction medium), (h) Multiple regenerated shoots (in shoot Proliferation medium), (i) Rooted plantlets (in root induction medium), (j) Cormel production (in cormel induction medium). Bar: 10 mm.

combinations of 2,4-D (0, 1, 2 and 3 mgL⁻¹), NAA (0, 1, 2 and 3 mgL⁻¹) and BAP (0, 1 and 2 mgL⁻¹) (Table 1).

2.3 Long-term maintenance of callus

The callus tissue was excised from the base of EMCS explants and subsequently transferred to MS medium supplemented with 0.5 mg L⁻¹ of 2,4-D. This medium also contained various concentrations of ascorbic acid (0, 100, and 150 mg L⁻¹), citric acid (0, 50, and 100 mg L⁻¹), and activated charcoal (0, 500, and 1000 mg L⁻¹) to reduce the production of phenolic compounds and facilitate long-term callus culture (Table 2).

2.4 Shoot regeneration of callus explants

In order to investigate the shoot induction potential from the long-term callus cultures (5 months), the callus was divided into equal pieces (2 cm²) and cultures on MS medium supplemented with various combinations of BAP (0, 1, 2 and 4 mgL⁻¹), Kin (0, 1, 2 and 4 mgL⁻¹) and NAA (0, 0.25 and 0.50 mgL⁻¹) (Table 3).

2.5 Formation of cormels at shoot base

Regenerated shoots were isolated and sub cultured on MS medium supplemented with 2 mgL⁻¹ BAP and 0.25 mgL⁻¹ NAA for shoot proliferation for six weeks. Proliferated shoots, averaging

60 mm in height were excised from the basal part and cultured on MS medium supplemented with various concentrations of sucrose (0, 3, 6 and 9%) and filter-sterilized IAA (0, 2 and 4 mgL⁻¹) along with 1000 mgL⁻¹ activated charcoal (Table 4) for 10 weeks.

2.6 Culture medium and conditions

In all experiments (except cormel production), the MS medium supplemented with 3% (w/v) sucrose and 7 gL⁻¹ agar. The pH of the medium was adjusted to 5.8 prior to autoclaving at 121°C and 1.5 kg cm⁻¹ pressure for 15 min. The explants were sub cultured every two weeks and were kept under 16-h light photoperiod at 25 ± 2°C. All chemicals used in this study were purchased from Duchefa Biochemie (Haarlem, The Netherlands).

2.7 Germination of cormels

In order to break the dormancy of regenerated cormels, they were stored at a low temperature (4 ± 1°C) in complete darkness for two months. Matured cormels were then planted in a sterilized substrate consisting of 50% cocopeat and 50% perlite. They were maintained under plastic bags in controlled conditions with the bags removed after two weeks. The germinated cormels were subsequently transferred to larger pots containing a sterile medium of coco peat and perlite mixed with sand (1:1). These pots were kept in a shaded greenhouse at 28 ± 2°C and 60 ± 5% relative humidity) for two weeks.

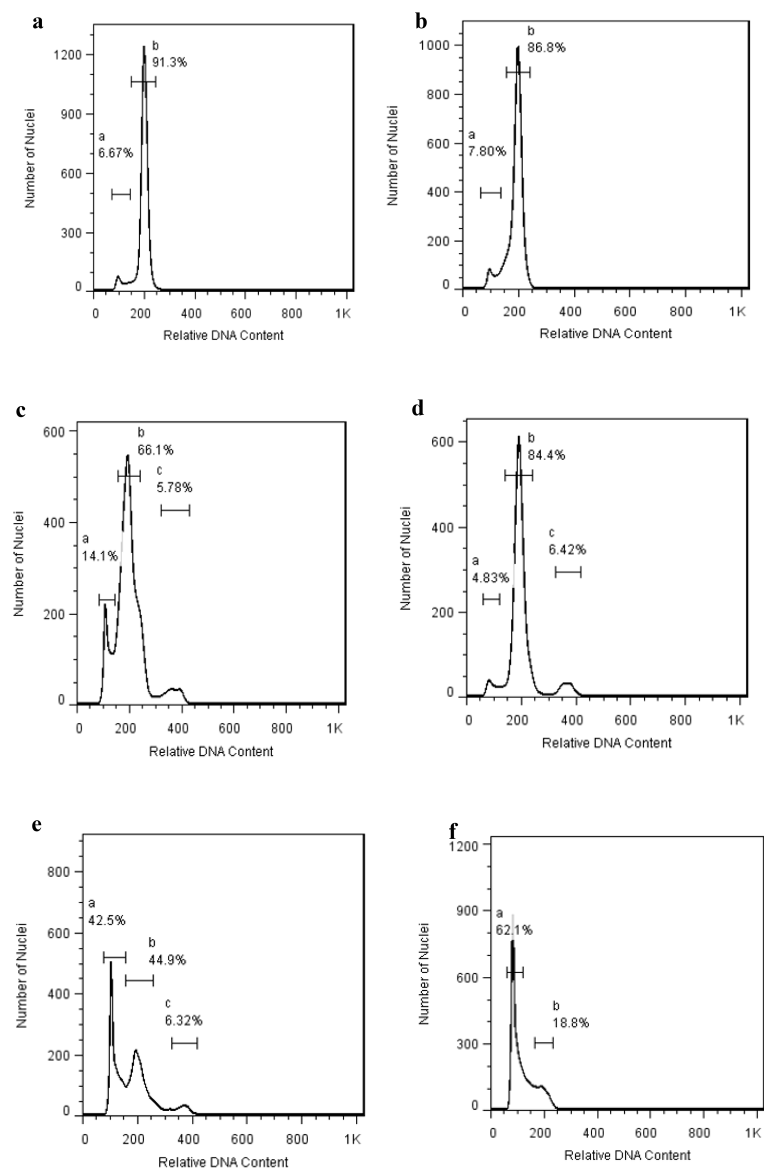


FIGURE 2
Flow cytometry histogram of *Gladiolus grandiflorus*; (a) *G. grandifloras* ‘Rose Supreme’ (mother plant). (b) *G. grandifloras* ‘Rose Supreme’ (*in vitro* regenerated plant). (c) *G. grandifloras* ‘Amsterdam’ (mother plant) (d) *G. grandifloras* ‘Amsterdam’ (*in vitro* regenerated plant) (e) *G. grandifloras* ‘Advance red’ (mother plant) (f) *G. grandifloras* ‘Advance red’ (*in vitro* regenerated plant) R1 R2 R3 MP C_ L.

2.8 Evaluation of genetic stability

2.8.1 Flow cytometry analysis

The Ploidy levels of leaves derived from mother plants and regenerated plants obtained through indirect shoot regeneration were assessed using ice-cold nuclei isolation buffer in flow cytometry (FCM) (Ebrahimzadeh et al., 2018). The mother plants served as control plants, and their nuclear DNA content were used as the reference comparison.

2.8.2 ISSR marker

100 mg of young leaves from both regenerated cormels (3 plantlets of each cultivar) and mother corms were used for genomic DNA isolation. The modified CTAB method (Murray and Thompson, 1980) was applied

TABLE 1 Effects of PGR combinations on callogenesis of EMCS explants in three *Gladiolus grandiflorus* cultivars after three weeks.

PGRs (mg l ⁻¹)	Callogenesis (%)		
	R.S*	A.	A.R
2,4-D + NAA + BAP			
0 + 0 + 0	00.00 k	00.00 j	00.00 f
1 + 1 + 1	18.33 ± 1.45 i	08.33 ± 0.63 i	03.33 ± 0.43 f
1 + 1 + 2	11.66 ± 1.00 j	08.66 ± 0.77 i	07.66 ± 0.66 f
1 + 2 + 1	26.66 ± 2.02 gh	20.66 ± 0.76 gh	18.33 ± 1.15 e
1 + 2 + 2	21.66 ± 2.60 hi	17.66 ± 0.57 h	18.33 ± 1.52 e

(Continued)

TABLE 1 Continued

PGRs (mg l ⁻¹)	Callogenesis (%)		
	R.S*	A.	A.R
2,4-D + NAA + BAP			
1 + 3 + 1	31.66 ± 1.00 g	25.66 ± 1.20 gh	21.33 ± 0.33 e
1 + 3 + 2	26.66 ± 1.33 gh	28.33 ± 1.00 g	21.66 ± 1.00 e
2 + 1 + 1	48.33 ± 2.96 f	40.66 ± 1.45 f	42.33 ± 3.08 d
2 + 1 + 2	68.33 ± 2.18 cd	61.66 ± 4.40 c	61.66 ± 4.25 c
2 + 2 + 1	95.00 ± 2.96 a	85.00 ± 6.66 a	91.66 ± 1.85 a
2 + 2 + 2	76.66 ± 1.76 b	71.66 ± 3.33 b	73.33 ± 3.92 b
2 + 3 + 1	68.33 ± 0.88 cd	60.00 ± 4.93 c	66.66 ± 1.15 bc
2 + 3 + 2	61.66 ± 1.15 d	56.66 ± 2.08 cd	58.33 ± 2.00 c
3 + 1 + 1	70.00 ± 0.66 c	51.66 ± 3.78 de	65.00 ± 0.33 c
3 + 1 + 2	63.33 ± 1.52 cd	46.66 ± 1.33 ef	61.66 ± 3.88 c
3 + 2 + 1	68.33 ± 1.66 cd	45.88 ± 3.33 ef	66.66 ± 2.88 bc
3 + 2 + 2	65.33 ± 4.40 cd	45.00 ± 5.04 ef	66.66 ± 0.88 bc
3 + 3 + 1	55.00 ± 1.60 e	38.33 ± 4.40 f	38.33 ± 1.52 d
3 + 3 + 2	50.00 ± 1.45 ef	45.00 ± 1.00 ef	38.33 ± 1.15 d
Type of callus	compact	compact	friable
Color of callus	White -yellowish	white	white-brownish

In each column values with the same letters are not significantly different at 5% level using Duncan's multiple range tests.
Mean values ± standard error.
*R.S, A. and A.R refer to Rose supreme, Amsterdam and Advance red cultivars, respectively.

as described by Azadi et al., 2010. Ten Inter Simple Sequence Repeat (ISSR) markers were used to evaluate the genetic stability of the regenerated comels (Table 5). PCR amplifications were performed using a master mix (Master Mix RED, Ampliqon, Denmark) in 25 µl reaction mixture. The reaction was initiated with DNA denaturation at 94°C for 5 minutes, followed by 35 cycles of amplification (94°C for 30 s, 55°C for 30 s, 72°C for 30 s), and a final extension at 72°C for 10 minutes. After amplification, the PCR products were loaded onto a 1% agarose gel and electrophoresis at 85 V for 30 min.

2.9 Statistical analysis

The explants in the experiments were arranged in a completely randomized design (CRD) with three replications, each containing five explants. Data was analyzed using Duncan's multiple range test with SAS 9.1 software. Differences were significant at P values <0.05 or 0.01, compared to control values.

TABLE 2 Effects of various inhibitor combinations on phenolic compound reduction in three *Gladiolus grandiflorus* cultivars after 16 weeks.

Treatment (mgL ⁻¹)	Explant without phenolic compounds (%)		
	R.S*	A.	A.R
ascorbic acid + citric acid + activated charcoal			
0.0 + 0.0 + 0.0	00.00 e	00.00 d	00.00 d
100 + 100 + 0.00	30.00 ± 1.87 d	25.00 ± 2.66 c	20.00 ± 1.00 c
100 + 50 + 500	60.00 ± 2.08 c	55.00 ± 3.44 b	50.00 ± 2.00 b
150 + 100 + 500	90.00 ± 3.48 a	80.00 ± 3.92 a	70.00 ± 2.51 a
150 + 100 + 1000	70.44 ± 2.88 b	55.00 ± 4.25 b	48.33 ± 2.08 b

In each column values with the same letters are not significantly different at 5% level using Duncan's multiple range tests.
Mean values ± standard error.
*R.S, A. and A.R refer to Rose supreme, Amsterdam and Advance red cultivars, respectively.

3 Results and discussion

3.1 Effect of PGRs on callus induction

Three weeks after the EMCS explants were cultured on the callus induction medium, callus was initiated to form at the basal region of the explants (Figures 1b, c). Callus cultured on the media with various concentrations of PGRs displayed friable and proliferative characteristics. In contrast, the medium lacking PGRs did not induce any callus which indicates the essential role of PGRs in callus induction. The MS medium supplemented with 2 mgL⁻¹ 2,4-D+ 2 mgL⁻¹ NAA+ 1 mgL⁻¹ BAP exhibited the highest callogenesis across all three cultivars with average induction rate of 95.00, 85.00, 91.66% for R.S., A. and A.R. cultivars, respectively. Additionally the quality of the callus varied among the tested cultivars, as displayed in Table 1. Our findings indicate that the combination of two auxins (2 mgL⁻¹ 2,4-D + 2 mgL⁻¹ NAA) with BAP (1 mgL⁻¹) significantly enhanced callus formation (Table 1). Previous studies on gladiolus have highlighted the key factors influencing the callogenesis including the composition of the culture medium and PGRs, appropriate explant and genotype (Kamo, 1994; Kumar et al., 2024a, b; Mujib et al., 2016; Tripathi et al., 2017). In this study, all three cultivars exhibited optimal callogenesis with the same PGR composition using EMCS explants, suggesting that genotype did not significantly influence callus formation.

3.2 Effect of different treatments on long-term maintenance of callus

The calli lost their regenerative capacity two weeks after excision from the EMCS explant bases, primarily due to the

TABLE 3 The effects of PGRs combinations on plant regeneration from long term callus cultures in three *Gladiolus grandiflorus* cultivars after six weeks.

PGRs (mg L ⁻¹)	Regeneration (%)			Number of shoot			Length of shoot (mm)		
BAP + Kin + NAA	R.S*	A.	A.R	R.S	A.	A.R	R.S	A.	A.R
0 + 0 + 0.00	00.00 j	00.00 k	00.00 j	00.00 j	00.00 i	00.00 j	00.00 k	00.00 g	00.00 h
1 + 1 + 0.25	41.66 ± 3.04 ef	36.66 ± 1.85 fg	43.33 ± 1.37 d-f	10.00 ± 1.54 hi	10.66 ± 0.68 i	12.66 ± 0.58 h	21.60 ± 1.50 j	20.00 ± 0.36 f	18.60 ± 0.63 g
1 + 1 + 0.50	23.33 ± 0.82 hi	21.66 ± 1.66 i	21.66 ± 1.27 hi	7.33 ± 1.24 i	8.00 ± 0.30 k	10.00 ± 0.38 i	20.60 ± 0.64 j	19.00 ± 0.45 f	18.30 ± 0.29 g
1 + 2 + 0.25	35.00 ± 2.23 fg	31.66 ± 3.33 gh	35.00 ± 2.00 fg	15.66 ± 1.53 fg	13.33 ± 0.84 gh	19.33 ± 0.63 f	34.30 ± 2.26 hi	35.30 ± 1.32 e	40.00 ± 0.50 de
1 + 2 + 0.50	30.00 ± 0.35 gh	30.00 ± 0.88 gh	28.33 ± 1.34 g-i	11.00 ± 0.57 h	11.66 ± 1.26 hi	15.66 ± 0.80 g	31.30 ± 1.77 i	33.30 ± 0.92 e	35.00 ± 0.19 f
1 + 4 + 0.25	45.00 ± 3.39 de	50.00 ± 1.20 de	50.00 ± 3.25 de	20.00 ± 0.35 e	18.66 ± 1.33 ef	19.00 ± 0.52 f	40.00 ± 1.01 g	43.30 ± 0.69 a-c	46.30 ± 0.73 bc
1 + 4 + 0.50	40.00 ± 0.93 ef	43.33 ± 1.52 ef	41.66 ± 0.50 ef	16.00 ± 1.53 fg	14.00 ± 0.58 g	16.33 ± 0.36 g	36.00 ± 1.22 h	40.00 ± 0.79 cd	41.30 ± 0.45 d
2 + 1 + 0.25	70.00 ± 4.19 b	65.00 ± 2.88 c	66.66 ± 1.49 bc	31.66 ± 1.42 c	30.00 ± 0.84 c	30.00 ± 1.17 bc	46.00 ± 0.88 de	45.60 ± 0.39 ab	48.00 ± 0.40 ab
2 + 1 + 0.50	53.33 ± 3.22 cd	55.00 ± 2.33 d	51.66 ± 1.00 d	21.66 ± 2.00 e	25.00 ± 1.26 d	20.00 ± 0.86 ef	40.60 ± 1.28 g	40.00 ± 0.80 cd	38.00 ± 1.16 e
2 + 2 + 0.25	98.33 ± 4.28 a	95.00 ± 2.88 a	93.33 ± 1.37 a	45.00 ± 2.62 a	38.33 ± 1.77 a	35.00 ± 0.52 a	51.60 ± 1.54 bc	43.30 ± 0.98 a-c	49.00 ± 2.67 b
2 + 2 + 0.50	76.66 ± 2.87 b	78.33 ± 4.40 b	71.66 ± 1.15 b	31.66 ± 1.73 c	30.00 ± 1.15 c	29.00 ± 2.08 c	50.00 ± 0.86 c	41.60 ± 0.71 bc	45.00 ± 0.68 c
2 + 4 + 0.25	70.00 ± 2.64 b	68.33 ± 3.71 c	60.00 ± 2.26 c	37.33 ± 1.20 b	32.00 ± 1.19 b	32.00 ± 1.24 b	55.00 ± 2.15 ab	43.30 ± 0.89 a-c	46.60 ± 1.62 bc
2 + 4 + 0.50	56.66 ± 3.54 c	46.66 ± 3.33 e	41.66 ± 3.54 ef	30.00 ± 1.45 c	25.33 ± 0.31d	23.33 ± 1.63 d	40.00 ± 0.28 g	40.00 ± 0.74 cd	38.30 ± 0.47 e
4 + 1 + 0.25	45.00 ± 1.36 de	31.66 ± 1.66 gh	23.33 ± 2.46 hi	25.00 ± 0.66 d	20.00 ± 1.45 d	21.66 ± 0.36 de	58.30 ± 1.66 a	47.60 ± 1.09 a	50.00 ± 0.67 a
4 + 1 + 0.50	35.00 ± 1.73 fg	26.66 ± 2.00 hi	20.00 ± 2.82 i	20.00 ± 1.05 e	18.00 ± 0.74 f	18.00 ± 0.54 fg	51.30 ± 0.50 bc	42.60 ± 0.76 bc	47.00 ± 0.40 bc
4 + 2 + 0.25	45.00 ± 1.45 de	33.33 ± 2.40 gh	35.00 ± 1.14 fg	18.66 ± 1.00 ef	16.00 ± 1.54 f	18.00 ± 1.22 fg	55.00 ± 1.37 ab	43.60 ± 0.53 a-c	48.00 ± 0.29 ab
4 + 2 + 0.50	38.33 ± 1.76 e-g	35.00 ± 2.88 gh	30.00 ± 0.37 gh	15.00 ± 0.69 g	11.66 ± 0.38 hi	13.33 ± 0.36 h	45.00 ± 1.28 ef	36.30± 1.08 de	40.60 ± 0.58 de
4 + 4 + 0.25	30.00 ± 0.76 gh	31.66 ± 2.51 gh	26.66 ± 2.15 g-i	13.00 ± 0.57 gh	10.00 ± 1.14 i	11.33 ± 0.32 hi	49.30 ± 1.61 cd	44.30 ± 0.49 a-c	47.30 ± 0.56 a-c
4 + 4 + 0.50	20.00 ± 0.44 i	19.33 ± 1.33 j	21.66 ± 1.08 hi	10.00 h ± 0.36 h	08.66 ± 1.04 jk	10.33 ± 0.80 i	41.60 ± 1.66 fg	40.00 ± 0.46 cd	42.00 ± 1.12 d

In each column values with the same letters are not significantly different at 5% level using Duncan’s multiple range tests.
Mean values ± standard error.
*R.S, A. and A.R refer to Rose supreme, Amsterdam and Advance red cultivars, respectively.

accumulation of phenolic compounds, which are known to damage callus tissue. It has been shown that the addition of phenolic inhibitors, along with darkness and low temperature, enhances callus viability in various plant species (Ko et al., 2009; Mingliang et al., 2011; Mohamed et al., 2018; Su et al., 2023).

For maintain the calli in a regenerable state, they were transferred to MS medium supplemented with phenolic inhibitors. The results demonstrated that the inclusion of 150 mg/L ascorbic acid, 100 mg/L citric acid, and 500 mg/L activated charcoal in the callus proliferation medium (containing 0.5 mg/L

TABLE 4 Effects of sucrose and IAA concentrations on cormel formation in three *Gladiolus grandiflorus* cultivars after 10 weeks.

Quantitative traits	Gladiolus cultivars	sucrose (%) + IAA (mgL ⁻¹)						
		0 + 0	3 + 2	3 + 4	6 + 2	6 + 4	9 + 2	9 + 4
Number of Cormel	R.S	00.00 d	12.66 ± 0.44 c	16.00 ± 0.36 b	14.66 ± 0.72 b	15.33 ± 0.57 b	18.66 ± 0.30 a	13.00 ± 0.50 c
	A.	00.00 e	11.66 ± 0.50 c	12.33 ± 0.60 bc	11.00 ± 0.57 d	13.00 ± 0.28 b	14.66 ± 0.50 a	12.66 ± 0.38 bc
	A.R	00.00 e	11.70 ± 0.44 d	14.00 ± 0.50 bc	12.66 ± 0.67 c	14.33 ± 0.70 b	16.66 ± 0.42 a	13.66 ± 0.33 c
Diameter of Cormel (mm)	R.S	00.00 f	12.50 ± 0.06 e	13.20 ± 0.14 d	13.50 ± 0.08 d	15.50 ± 0.22 c	17.50 ± 0.05 b	18.30± 0.10 a
	A.	00.00 f	10.00 ± 0.11 e	11.60 ± 0.40 d	11.70 ± 0.05 d	14.40 ± 0.18 c	15.10 ± 0.23 b	15.70 ± 0.14 a
	A.R	00.00 f	13.90 ± 0.09 e	16.20 ± 0.28 c	15.20 ± 0.05 d	16.30 ± 0.14 c	18.10 ± 0.12 b	19.20 ± 0.50 a
Fresh weight of Cormel (mg)	R.S	00.000 f	1700 ± 57.73 e	3030 ± 46.66 c	2300 ± 35.11 d	3140 ± 70.23 bc	3180 ± 57.73b	3800 ± 31.79 a
	A.	00.00 f	1460 ± 55.40 d	1890 ± 15.30 c	1160 ± 82.21 e	2400 ± 25.39 b	2000 ± 64.41 c	2770 ± 82.15 a
	A.R	00.00 f	2800 ± 64.71 e	3340 ± 66.09 c	3580 ± 25.69 b	3500 ± 36.24 b	3140 ± 68.64 c	4800 ± 28.59 a
Dry weight of Cormel (mg)	R.S	00.00 f	230.0 ± 8.81 e	440.0 ± 14.50 b	300.0 ± 5.77 d	460.0± 14.52 b	410.0 ± 20.81c	550.0 ± 23.33 a
	A.	00.00 e	200.0 ± 0.30 d	270.0 ± 5.77 c	250.0± 14.52 cd	320.0 ± 10.00 b	270.0± 14.00 c	390.0 ± 15.32 a
	A.R	00.00 e	400.0 ± 5.77 d	470.0 ± 6.66 bc	520.0 ± 8.81 a	510.0 ± 10.00 ab	430.0 ± 7.92 cd	560.0 ± 6.79 a

In each row values with the same letters are not significantly different at 5% level using Duncan’s multiple range tests. Mean values ± standard error. *R.S, A. and A.R refer to Rose supreme, Amsterdam and Advance red cultivars, respectively. 1000 mgL⁻¹ of activated charcoal were added to all treatments.

TABLE 5 Nucleotide sequences of ISSR primers used in genetic stability analysis.

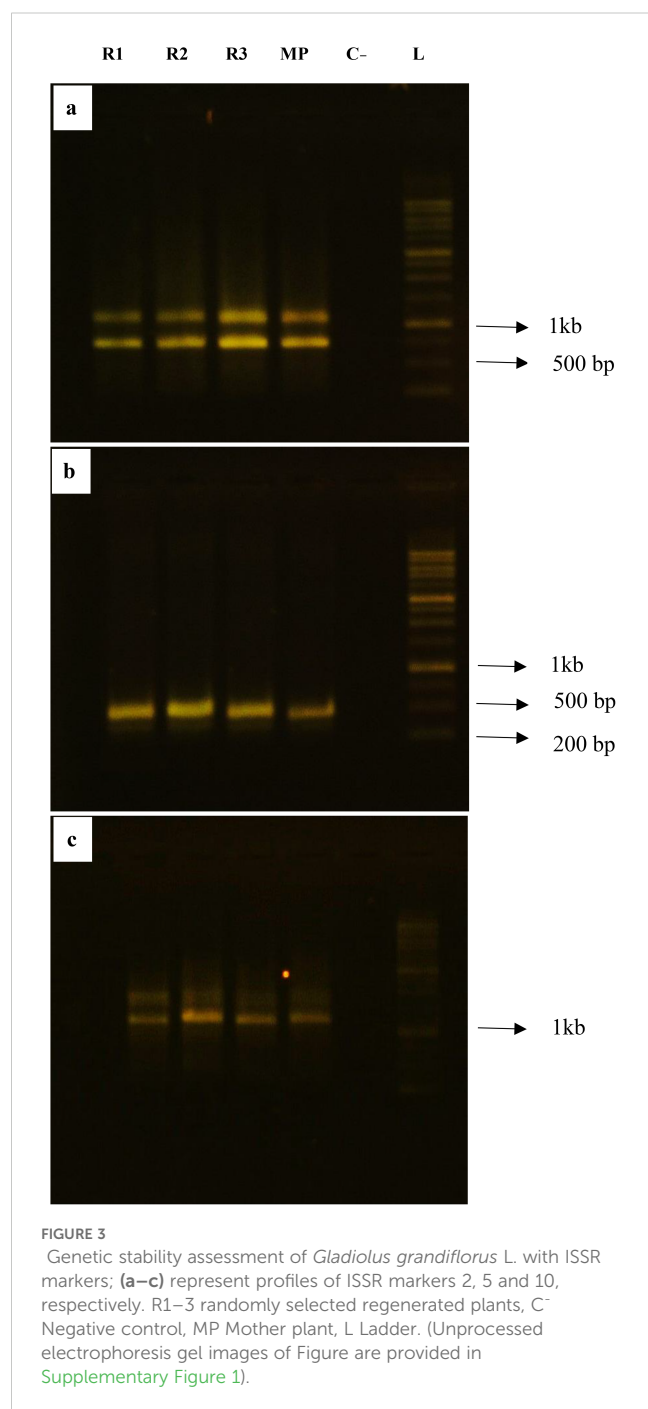
Primers	Primer code	Primer sequence (5' to 3')
ISSR-1	1	AGAGAGAGAGAGAGAGC
ISSR-2	4	CTCTCTCTCTCTTG
ISSR-3	5	GAGAGAGAGAGAGAGATG
ISSR-4	12	CACACACACACAAC
ISSR-5	14	CAC ACA CAC ACA GG
ISSR-6	21	GAGAGAGAGAGAGAGAC
ISSR-7	22	ACACACACACACACACC
ISSR-8	25	ACACACACACACACACYG
ISSR-9	26	CACACACACACACACART
ISSR-10	29	TCCTCCTCCTCCTCCRY

2,4-D) significantly improved callus quality (Figure 1d) and effectively controlled phenolic compound production. This treatment resulted in a marked increase in the percentage of viable calli with no phenolic compound production (with averages of 90.00%, 80.00%, and 70.00% for R.S, A., and A.R cultivars, respectively) (Table 2). Furthermore, the calli retained their regenerative potential for up to one year. This long-term callus culture enables consistent corm production throughout the year which is a critical factor for commercial applications. Additionally,

it provides a reliable system for *in vitro* breeding programs (De Klerk, 2012).

3.3 Effect of PGRs on shoot induction

In the present study, shoot regeneration was successfully induced after six weeks of sub culturing calli (five months old) onto the shoot induction medium (Figures 1e–g). MS medium supplemented with 2 mg/L BAP, 2 mg/L Kin, and 0.25 mg/L NAA resulted in the highest regeneration rates (average of 98.33%, 95.00%, and 93.33% for R.S, A., and A.R cultivars, respectively). This medium also yielded the greatest shoot number per explant, with averages of 45.00, 38.33, and 35.00 shoots for the R.S, A., and A.R cultivars, respectively. However, the longest shoot with averages of 58.30, 47.60 and 50.00 mm for R.S, A. and A.R respectively, obtained in the medium supplemented with high concentrations of BAP (4 mgL⁻¹ BAP + 1 mgL⁻¹ Kin + 0.25 mgL⁻¹ NAA). Regeneration through callogenesis is primarily influenced by the quality of callus, the composition, as well as the concentration of cytokines, and its efficiency tend to decreases over time (Memon, 2012). Both direct and indirect regeneration methods have been reported for gladiolus tissue culture (Xu et al., 2009; Bera et al., 2015; Kumar et al., 2024a, b). Indirect organogenesis not only enhances the rate of reproduction but also significantly reduces production costs and time (Azadi et al., 2017; Kumar et al., 2018; Lalthafamkimi et al., 2022).



3.4 Effect of sucrose and IAA on cormel formation

Plantlet clusters were cultured on cormel production media (Figure 1h). In all treatments, root formation occurred first (Figure 1i), followed by cormel initiation after three weeks. By the 10th week, cormels had formed in all plantlets (Figure 1j).

The maximum number of cormels per plantlet (with an average of 18.66, 14.66, 16.66 cormels for R.S, A. and A.R cultivars,

respectively) was obtained in MS medium supplemented with 9% Sucrose + 2 mgL⁻¹ IAA + 1000 mgL⁻¹ activated charcoal. However, the maximum diameter (with an average of 18.30, 15.70, 19.20 mm for R.S, A. and A.R cultivars, respectively), fresh weight (with an average of 3800, 2770, 4800 mg for R.S, A. and A.R cultivars, respectively) and dry weight (with average of 550, 390, 560 mg for R.S, A. and A.R cultivars, respectively) of cormels were obtained in MS medium supplemented with 9% sucrose + 4 mgL⁻¹ IAA + 1000 mgL⁻¹ activated charcoal (Table 4).

The type of PGRs, concentration of salts, sucrose, and activated charcoal are effective in rooting and cormel formation in gladiolus (De Bruyn and Ferreira, 1992; Nagaraju et al., 2002; Sinha and Roy, 2002; Kumar et al., 2011). Different auxins such as NAA, IBA and IAA have the significant impact on rooting and cormel formation (Dantu and Bhojwani, 1995; Tripathi et al., 2017; Naik et al., 2024). Increasing the concentration of sucrose (4 - 10%) improved the formation of storage organs (Kumar et al., 1999; Sinha and Roy, 2002; Ascough et al., 2009; Kumar et al., 2011). Activated charcoal can also promote growth, adsorption of ions and PGRs, darkening of culture media, and creation of similar conditions as the soil does in nature (Nhut et al., 2001; Thomas, 2008; Martins et al., 2024).

Under natural conditions, one mother corm typically produces approximately 1–2 corms and 5–50 cormels depending on the genotypes (Pragya et al., 2012). Cormels produced under such condition require 3–4 growth seasons to reach the standard size necessary for producing flowering spike. Therefore, they need several years to produce a commercial colony (Bajaj et al., 1983). However, regenerated plantlets *in vitro* require 16 to 24 weeks for cormel formation and development (Steinitz and Lilien-kipnis, 1989). In the present study, we observed a high yield of cormels in approximately ten weeks, resulting in a complete protocol cycle of around 20 weeks. This represents a substantial reduction in time compared to conventional methods, highlighting the efficiency of the proposed approach.

3.5 Germination and acclimatization of cormel

Acclimatization and transfer of *in vitro* regenerated plantlets to an ex-vitro environment with minimal cost, and high survival rates are critical steps for successful propagation (Chandra et al., 2010). In geophytes, the production of storage organs provides an effective strategy for ensuring high adaptation rates. However, these storage organs often exhibit dormancy, which can be overcome using various strategies, such as exposure to low temperature, darkness, gibberellic acid (GA₃), or specific medium compositions (Kumar and Raju, 2007; Padhi et al., 2018; Kumari et al., 2024). In this study, the produced cormels were removed from culture media, washed thoroughly and dried at room temperature for two weeks. In order to break the dormancy of the cormels, they were placed at low temperature (4 °C) in darkness for two months. The cormels were then cultured in disinfected substrate consisting of 50% coco peat +

TABLE 6 ISSR marker analysis of regenerated *Gladiolus* plantlets.

Size of amplicons (bp)	Total bands	Polymorphic band number	Monomorphic bands	Primers
500-1000	8	0	2	ISSR-2
500	4	0	2	ISSR-5
1000	4	0	2	ISSR-10
–	16	0	6	Total

50% perlite. All cormels successfully germinated (100%) and survived upon transfer to greenhouse condition.

3.6 Assessment of genetic stability

One of the key features of an effective plant regeneration protocol is its ability to produce healthy, true-to-type plantlets in short time (Mujib et al., 2016). In this study, we evaluated the genetic stability of regenerated gladiolus plantlets using two techniques: flow cytometry (FCM) and inter-simple sequence repeat (ISSR) markers. FCM analysis showed that the DNA ratios of regenerated plants (Figures 2b, d, f) were stable and matched those of the mother plants (Figures 2a, c, e). For the ISSR analysis, we screened the regenerated plantlets using ten primers (Table 5). Three of these primers produced consistent, monomorphic banding patterns across all plants (Figures 3a–c, Supplementary Figure 1), confirming that the regenerated gladiolus plantlets were genetically stable (Table 6).

Genetic stability is a crucial for successful plant regeneration, as somaclonal variation can lead to undesirable changes in plant characteristics (Mujib et al., 2016). FCM analysis revealed complete nuclear DNA content stability between mother plants and their *in vitro*-regenerated counterparts (Figure 2). The 2C DNA content values were determined to be 14.32 ± 0.18 pg for ‘Rose Supreme’, 14.15 ± 0.22 pg for ‘Amsterdam’, and 14.41 ± 0.15 pg for ‘Advance Red’, with no significant differences ($p > 0.05$) between source plants and regenerants. These measurements correspond to the G0/G1 peaks shown in Figure 2 panels (a–f), confirming the diploid nature ($2n = 2x = 60$) of all analyzed plants. The consistent DNA ploidy levels across all cultivars demonstrate the genomic stability of our regeneration protocol.

To further validate genetic stability at the molecular level, ISSR markers were employed. These markers are highly reproducible, cost-effective, and capable of detecting genetic variations without requiring species-specific primers (Abouseada et al., 2023; Bhatia et al., 2011). The monomorphic banding patterns we observed (Figure 3a–c; Supplementary Figure 1) are consistent with findings from other species, such as apple (Bisht et al., 2024), Muehlenbeckia platyclade (Badhepuri et al., 2024), Rhododendron formosum (Marwein et al., 2024) and Polianthes tuberosa (Naloussi et al., 2019). In a recent study, alternative molecular marker such as Sequence-Related Amplified Polymorphism (SRAP) has also been employed to assess the genetic diversity and stability among *Gladiolus hybridus* L. cultivars with high accuracy (Jadhav et al., 2025).

3.7 Conclusion

Plant tissue culture has become an incredibly useful method for rapid production of genetically identical plants. However, it faces challenges, particularly the phenolic compounds accumulation and the risk of somaclonal variation. In this study, we successfully achieved notable improvements over traditional methods, which often take more than a year to complete, by efficiently controlling the production of phenolic compounds, creating long-term callus cultures, and cutting the production time to just 20 weeks. To verify the genetic stability of the regenerated plantlets, we utilized ISSR marker which is a reliable PCR-based method along with FCM for the detection of genetic variations. Our analysis confirmed that the regenerated plants grown in greenhouse conditions were genetically stable, demonstrating the effectiveness of the protocol. This protocol has significant potential for commercial gladiolus production, as it enables the rapid and reliable propagation of genetically identical plants. Moreover, it prepares a robust foundation for genetic transformation studies that aimed at improving desirable traits. Further research based on this protocol is already continuing, focusing on the genetic engineering of gladiolus for enhancing its ornamental and commercial value.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

SM: Project administration, Writing – original draft, Data curation. SNM: Methodology, Writing – review & editing. LS: Formal Analysis, Validation, Writing – review & editing. PA: Conceptualization, Data curation, Methodology, Resources, Supervision, Writing – review & editing.

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Conflict of interest

The author SM is CEO of Fartak Oxin Baft Knowledge Based Company.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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The author(s) declare that Generative AI was used in the creation of this manuscript. This manuscript was edited with

limited use of Chat GPT (GPT-4-) solely to refine sentence structure. The authors meticulously reviewed and verified the content to ensure accuracy and scientific integrity.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fhort.2025.1571042/full#supplementary-material>

SUPPLEMENTARY FIGURE 1

Raw data of electrophoresis. Pictures show unprocessed electrophoresis gel images of Figure 3.

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