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In Vitro Corm Development, Field Evaluation and Determination of Genetic Stability of Corm-derived Elite *Gladiolus* Germplasm

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ABSTRACT

An efficient protocol for *in vitro* corm production of *Gladiolus* (cv. 'Green Bay', 'Intrepid', 'Sabnam', 'White Friendship' and 'Red Ginger') was developed using liquid culture and coir as a matrix. The initial culture was established from the basal portions of the innermost leaves in Murashige and Skoog (MS) solid basal medium with 2.0 mg l^{-1} α-naphthaleneacetic acid (NAA). Adventitious shoot buds developed (MS supplemented with 0.2 mg l^{-1} NAA and 2.0 mg l^{-1} 6-benzyladenine) from responding callus (10-15 per callus culture) and elongated in solid MS with 0.5 mg l^{-1} NAA. High regeneration frequency of corms (98.90%) was established when individual plantlets were cultured in MS liquid medium supplemented with 6% sucrose and 0.5 mg l^{-1} NAA. *In vitro* corms were successfully cultivated in the field ('White Friendship' – high altitude cultivar; and 'Sabnam' – plain land cultivar) for two consecutive seasons and flowering was noted in the second generation of plants. Using RAPD profiles, the genetic fidelity of randomly selected *in vitro* and *ex vitro* corms of two cultivars ('White Friendship' and 'Sabnam') as well as that of their mother corms was compared.

Keywords: coir matrix, field performance, *Gladiolus hybridus*, *in vitro* corm, RAPD marker
Abbreviations: BA, 6-benzyladenine; **CTAB**, cetyl trimethyl ammonium bromide; **dATP**, deoxy adenosine triphosphate; **dCTP**, deoxy cytosine triphosphate; **dGTP**, deoxy guanosine triphosphate; **dTTP**, deoxy thymidine triphosphate; **MS**, Murashige and Skoog; **NAA**, napthalene acetic acid; **PCR**, polymerase chain reaction; **PPFD**, photosynthetic photon flux density; **RAPD**, random amplification of polymorphic DNA

INTRODUCTION

Gladiolus (hybrid cultivars of *G. hybridus* Hort.; type species: *G. communis* L.; Family: Iridaceae; commonly known as Gladiolus as well as Sword Lily) is a high value cut flower and an important species in floriculture for its attractive spikes. The current *Gladiolus* range aims to extend the vase life of flowers as well as to develop novel colors for better utilization of cultivars for floriculture (Kumar *et al*. 1999). However, the major constraint in commercial cultivation of *Gladiolus* is the non-availability of a large quantity of propagules and the slow rate of multiplication of cormels in hybrid cultivars (Singh and Dohare 1994). Furthermore, *Gladiolus* production also declines due to the incidence of *Fusarium* corm rot disease (*Fusarium oxysporium f. gladioli*) as well as other diseases (Sinha and Roy 2002; Roy *et al*. 2006). *In vitro* methodologies were adopted for the production of cormlets in different cultivars ('Eurovision' - Ziv 1989; 'Friendship' - Hussain *et al*. 1994; Dantu and Bhojwani 1995; 'Green Bay' - Sen and Sen 1995; 'Golden Wave' - Sinha and Roy 2002; 'Pacifica' - Roy *et al*. 2006; 'Pacific Pink', 'May Queen', 'Sharone' - Thun *et al*. 2008; Memon *et al*. 2010) to overcome the constraints in *Gladiolus* production; however, the *in vitro* corms produced in culture were not justified with spike production under *ex vitro* conditions (except for 'Friendship'; Dantu and Bhujwani 1995). The present investigation describes a suitable variety-independent protocol for *in vitro* corm production of five *Gladiolus* cultivars ('Green Bay', 'Intrepid', 'Sabnam', 'White Friendship' and 'Red Ginger') and assesses the productivity of corms of two cultivars ('White Friendship' and 'Sabnam' – economically important in the West Bengal plains) raised *ex vitro*. Corm clonal fidelity through random amplification of polymorphic DNA (RAPD) profiling was also assessed.

MATERIALS AND METHODS

In vitro corm formation

The corms of five *Gladiolus* cultivars ('Green Bay', 'Intrepid', 'Sabnam', plain land cultivars; 'White Friendship', 'Red Ginger', high altitude cultivars) were collected from the Floriculture division, Bidhan Chandra Krishi Vishwavidyalaya (22° 56′ N, 88° 32′ E, 9.75 m above sea level), North 24 Parganas, West Bengal and were grown in earthen pots containing moist sand and were induced to sprout in a 16-h photoperiod (60 µmol m^{-2} s^{-1} photosynthetic photon flux density - PPFD). The basal portion of the innermost leaves (10-days old) was used as the explant. Explants were thoroughly washed with Tween 20 (0.1% v/v; Hi-media, Mumbai, India) for 15 min and surface sterilized with 0.1% (w/v) mercuric chloride (Merck, Mumbai, India) for 7 min and finally rinsed with sterile double distilled water four times. Sterilized explants were inoculated on agar (Hi-media, Type 1)-gelled (0.8%) Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962) supplemented with 3% sucrose (Merck). MS medium containing 2.0 mgl^{-1} α-naphthalene acetic acid (NAA; Hi-media) was incubated for 3 weeks to induce callus. The callus were then transferred onto MS medium supplemented with 0.2 mgl^{-1} NAA and 2.0 mgl^{-1} 6-benzyladenine (BA; Hi-media) to induce meristematic bud clusters from which multiple shoot clusters were induced on MS with 0.5 mgl^{-1} NAA. After shoot buds matured (clear shoot

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Original Research Paper

Table 1 Effect of matrix and concentration of sucrose on corm formation in cv. 'White Friendship' and 'Sabnam', in MS supplemented with 0.5 mgl^{-1} NAA, in light (growth period 12 weeks).

Sucrose concentration (%)	'White Friendship'				'Sabnam'			
	SAM		LMC		SAM		LMC	
	Frequency of corm formation (%)	Mean fresh weight of corm (mg) ± SE	Frequency of corm formation (%)	Mean fresh weight of corm (mg) ± SE	Frequency of corm formation (%)	Mean fresh weight of corm (mg) ± SE	Frequency of corm formation (%)	Mean fresh weight of corm (mg) ± SE
0.0	0	0	0	0	0	0	0	0
2.0	31.3	118 ± 6	36.5	220 ± 8	32.1	102 ± 6	35.6	241 ± 12
3.0	63.6	392 ± 12	65.7	416 ± 97	64.6	361 ± 72	66.1	491 ± 91
4.0	78.1	962 ± 129	80.1	1116 ± 176	78.9	999 ± 199	80.9	1206 ± 177
5.0	82.6	1836 ± 139	87.2	2116 ± 136	82.3	1915 ± 179	86.9	2301 ± 197
6.0	96.8	2676 ± 172	98.9	3452 ± 162	96.1	2606 ± 158	99.1	3512 ± 125
7.0	93.9	2431 ± 151	95.1	2616 ± 187	94.1	2461 ± 168	95.7	2676 ± 186
8.0	91.5	2199 ± 121	90.6	2216 ± 161	90.9	2019 ± 196	90.8	2303 ± 126
9.0	83.2	2016 ± 179	85.3	2113 ± 168	83.7	1919 ± 186	84.9	2119 ± 162
10.0	72.6	1705 ± 161	67.3	1686 ± 179	71.3	1792 ± 121	68.1	1502 ± 192
12.0	0	0	0	0	0	0	0	0
P value of χ^2 test of heterogeneity		<0.001		<0.001		<0.001		<0.001

and root system), plantlets were separated and maintained in the same medium for 3 to 4 weeks. The plantlets were subsequently placed in individual culture tubes containing MS liquid medium with sterile coir (coconut husk) as the matrix (Gagopadhyay *et al*. 2002) supplemented with 0.5 mgl^{-1} NAA and sucrose (0.0-10.0% and 12%) for the development of *in vitro* corms and roots, respectively. The cultures were incubated at two different temperatures (20 ± 2°C and 25 ± 2°C). Parallel sets of plantlets were also transferred to similar medium but gelled with agar instead of use of liquid medium with coir. The development of *in vitro* corms started within 4 weeks and matured after 12 weeks. Each treatment consisted of 15 to 20 replicas. The frequency of corm formation and diameter and fresh weight were recorded at maturation.

Storage and field evaluation of *in vitro* raised corms

In vitro corms of 'White Friendship' and 'Sabnam' were air dried and stored in desiccators (25°C) for 30 days prior to sowing in the field. Corms from one-year-old plants were collected, desiccated for 60 days then sown again to raise the second generation of plants. The first and second generation of plants were compared with *in vivo* grown corms (mother plants) on the basis of different morphological parameters (**Table 3**) using a test of significance (student's *t*-test; Statistica 4.5).

Molecular evaluation of clonal fidelity of plants derived from *in vitro* corms using RAPD

To ensure the clonal fidelity (*in vitro*, *ex vitro* and mother corms of 'White Friendship' and 'Sabnam'), DNA was extracted from young leaves following a modified CTAB method (Rogers and Bendich 1988). The RAPD reaction in each sample was performed as per Williams *et al*. (1990) with 10 previously tested (Roy *et al*. 2006) oligonucleotide primers (Operon Technologies, Alameda, USA; OPB-01 to OPB-10). DNA was amplified in a Thermal Cycler (Perkin Elmer System-2400, Norwalk, CT, USA) with an initial denaturation of 120 s at 94°C, 45 cycles of 60 s at 94°C (denaturation), 60 s at 35°C (annealing) and 120 s at 72°C (extension), followed by a 300-s hold at 72°C to ensure that primer extension was complete. Each PCR reaction mixture (25 µl) consisted of 1X buffer, 0.2 mM dATP, dCTP, dGTP, dTTP (Roche, Indianapolis, USA), 2 mM $MgCl_2$ (Roche), 0.2 µM of primer, 100 ng of template DNA and 1U of *Taq* DNA polymerase (Roche). Amplified products were electrophoresed in a 1.8% agarose gel with an ØX174 *Hae*II digest (GENE India Pvt. Ltd., Bangalore), which served as the DNA molecular weight size marker.

Statistical analysis

X^2 test of heterogeneity (genetic experiment where consistency or otherwise of several group of data can be tested and it provides actual proportions of observed in each class; Datta 2006) was performed for different parameters (**Tables 1, 2**) to assess significant variations, if any, among the different sucrose concentrations as well as *Gladiolus* cultivars. Further, the test of significance (*t*-test, DF 28) between two means (each parameter of mother corm and micropropagated corm in both the cultivars were assessed in 1st and 2nd years; **Table 3**) was also conducted to estimate significant variation, if any.

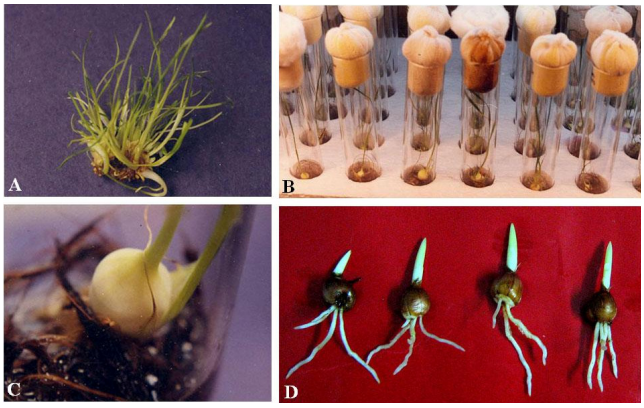

Fig. 1 Different stages of *in vitro* corm production of *Gladiolus* ('White Friendship'). (**A**) Multiple shoot cluster showing maximum number of shoots. (**B**) Mass induction of *in vitro* corms in liquid culture with coir matrix. (**C**) Magnified view of an *in vitro* corm. (**D**) Germinating *in vitro* corms.

RESULTS AND DISCUSSION

In vitro corm formation

Callus (creamish yellow and compact in nature) induction (on MS medium supplemented with 2 mgl^{-1} NAA) and proliferation (on MS medium supplemented with 0.2 mgl^{-1}

Table 2 Comparative study of *in vitro* corm induction and corm development of five *Gladiolus* cultivars.

Gladiolus cultivars	% Corm induction	Mean diameter (mm)	Mean fresh weight of corm (mg) ± SE
'White Friendship'	98.9	19.6	3452 ± 162
'Red ginger'	95.3	18.1	3221 ± 113
'Green bay'	92.5	15.6	3152 ± 103
'Interpaid'	96.4	17.2	3257 ± 132
'Sabnam'	99.1	19.8	3512 ± 125
P value of χ^2 test of heterogeneity		<0.05	<0.05

Table 3 Comparison between *in vitro* corm raised and control plants of *Gladiolus* cultivars cv. 'White Friendship' and 'Sabnam' under field conditions.

Parameter	Generation No.	'White Friendship'		'Sabnam'	
		Control	Micropropagated	Control	Micropropagated
No. of leaves	1st	7.2 ± 0.2	5.1 ± 0.2	8.3 ± 0.2	5.2 ± 0.1
	2nd	7.2 ± 0.2	7.3 ± 0.1	8.3 ± 0.2	8.2 ± 0.2
Height of the last formed leaf from ground (cm)	1st	58.2 ± 2.6	41.3 ± 1.3	72.5 ± 2.1	54.5 ± 1.1
	2nd	58.2 ± 2.6	55.3 ± 1.7	72.5 ± 2.1	68.5 ± 3.1
Breadth of 3rd leaf (cm)	1st	3.3 ± 0.3	2.1 ± 0.1	3.5 ± 0.2	2.4 ± 0.2
	2nd	3.3 ± 0.3	3.1 ± 0.4	3.5 ± 0.2	3.4 ± 0.4
% plants flowering	1st	100	59	100	42
	2nd	100	100	100	100
No. of days for appearance of spike since germination	1st	99.5 ± 3.2	05.2 ± 1.1	99.7 ± 2.1	107.2 ± 1.1
	2nd	99.5 ± 3.2	99.2 ± 4.1	99.7 ± 2.1	99.7.2 ± 4.1
Height of the spike base of the first floret to tip (cm)	1st	43.4 ± 2.6	30.1 ± 1.3	37.9 ± 2.7	30.2 ± 1.2
	2nd	43.4 ± 2.6	40.1 ± 4.3	37.9 ± 2.7	37.2 ± 3.2
No. of florets	1st	14.5 ± 2.1	9.1 ± 0.8	15.8 ± 1.1	9.5 ± 0.5
	2nd	14.5 ± 2.1	14.1 ± 2.8	15.8 ± 1.1	15.5 ± 1.5
Colour of flowers	1st and 2nd	Snow white	Snow white	Aconite violet	Aconite violet
Diameter of harvested corm (g)	1st	4.5 ± 0.3	3.1 ± 0.2	5.9 ± 0.2	3.6 ± 0.3
	2nd	4.5 ± 0.3	4.4 ± 0.2	5.9 ± 0.2	5.6 ± 0.3
Weight of corm (g)	1st	45.2 ± 6	15.3 ± 3	52.5 ± 2	24.5 ± 3
	2nd	45.2 ± 6	45.3 ± 3	52.5 ± 2	53.5 ± 3

Means followed by same letter do not differ significantly at $P < 0.05$. For all quantitative parameters the variations were significant ($P < 0.05$ to 0.01) in the 1st generation but were non-significant ($P > 0.05$) in the 2nd generation.

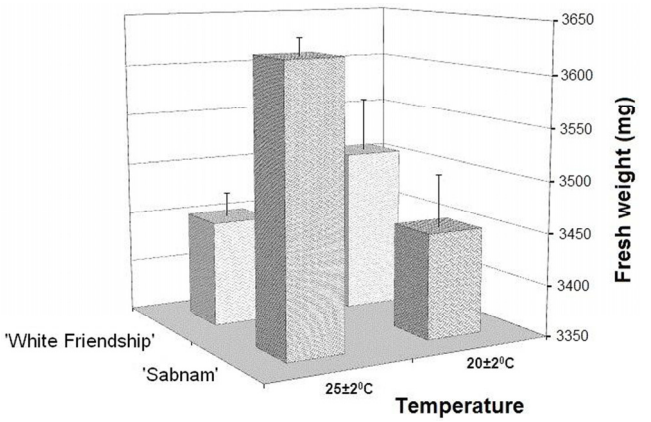

Fig. 2 The effect of temperature on *in vitro* corm formation of two *Gladiolus* varieties ('White Friendship' and 'Sabnam'). Bars represent mean of 15 observations.

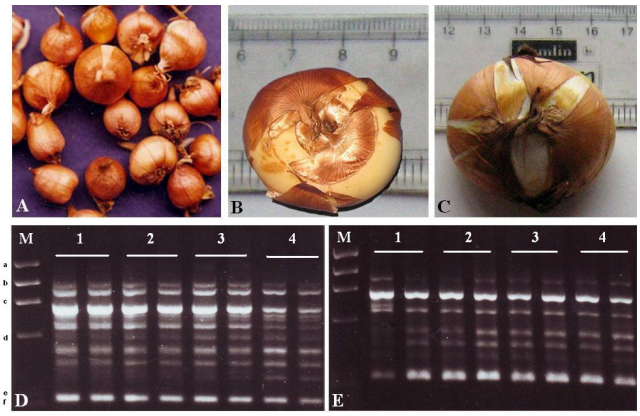

Fig. 3 *In vitro* raised corms of *Gladiolus* ('White Friendship') and the corm after harvest along with their RAPD profile. (**A**) *In vitro* corm of *Gladiolus* after maturation. (**B**) Corm harvested from 1st generation. (**C**) Corm harvested from 2nd generation. (**D, E**) RAPD profiles (1); tissue culture-derived corms (2); corms collected from field after 1st generation (3); 2nd generation (4) mother variety 'White Friendship' with OPB-08 (D) and 'Sabnam' with OPB-04 (E). M – DNA molecular weight marker (ØX174 *Hae*II digest; a: 1353 bp, b: 1078 bp, c: 872 bp, d: 603 bp, e: 310 bp, f: 281/271 bp).

NAA and 2.0 mg l^{-1} BA) were possible in agar-gelled MS media. Shoot buds were visible after 21 days and these developed into individual plantlets just after a single sub-culture (**Fig. 1A**).

Osmoticum in the form of a higher concentration of sucrose (6%), together with auxin (NAA, 0.5 mgl^{-1}), was beneficial for *in vitro* corm production, especially in liquid medium with coir as the matrix (**Fig. 1B, 1C**). *In vitro* corms raised in semi-solid agar medium were less effective (**Table 1**). In the coir matrix system, *in vitro* corm size was enhanced after the addition of fresh liquid medium at 4-week intervals. Sub-culturing during *in vitro* corm formation was somewhat difficult due to mechanical injury of the corms as was also reported earlier (Ziv 1989; Hussain *et al.* 1994; Dantu and Bhojwani 1995; Sen and Sen 1995). The diameter (15.6-19.8 mm) and fresh weight (3.15-3.51 g) of *in vitro* corms was significant ($P < 0.001$) among the cultivars (**Table 2**). The frequency of corm production as well as mean fresh weight of the corms raised *in vitro* varied significantly ($P < 0.001$) among the sucrose concentrations in both cultivars and in both media (**Table 1**). However, liquid medium with coir matrix supplemented with 6% sucrose and 0.5 mg l^{-1} NAA probably supersedes all other available reports on *in vitro* corm production in *Gladiolus* (Ziv 1989; De Bruyn and Ferreira 1992; Steinitz *et al*. 1991; Hussain *et al.* 1994; Sen and Sen 1995; Dantu and Bhojwani 1995; Sinha and Roy 2002; Nhut *et al*. 2004; Mokshin *et al*. 2006; Roy *et al.* 2006; Thun *et al.* 2008; Memon *et al.* 2010). A flow chart of a cultivar-independent protocol for *in vitro* corm production in *Gladiolus* has been presented for effective utilization (**Fig. 4**).

The effect of temperature was evaluated in two *Gladiolus* varieties ('White Friendship' and 'Sabnam') (**Fig. 2**). 'White Friendship' responded better at a lower temperature (3.52 ± 0.120 g at 2°C) than 'Sabnam' (3.31 ± 0.1 g) with respect to mean corm weight. Conversely, 'Sabnam' required higher temperatures to form larger corms (25 ± 2°C; 3.51 ± 0.1 g) than 'White Friendship' (3.45 ± 0.1 g), although none of these results were statistically evaluated.

Field performance of *in vitro* corm raised plants

The corms that formed *in vitro* (**Fig. 3A**) and grown under field conditions yielded smaller corms than control (control was referred to as the mother stock which was grown under and maintained in *in vivo* conditions) after the 1st generation (for all quantitative parameters the variations were significant; $P < 0.05$ to 0.01) while 2nd generation corms (raised from 1st generation corms) in both control and *in vitro* were nearly identical, as evident from non-significant ($P > 0.05$)

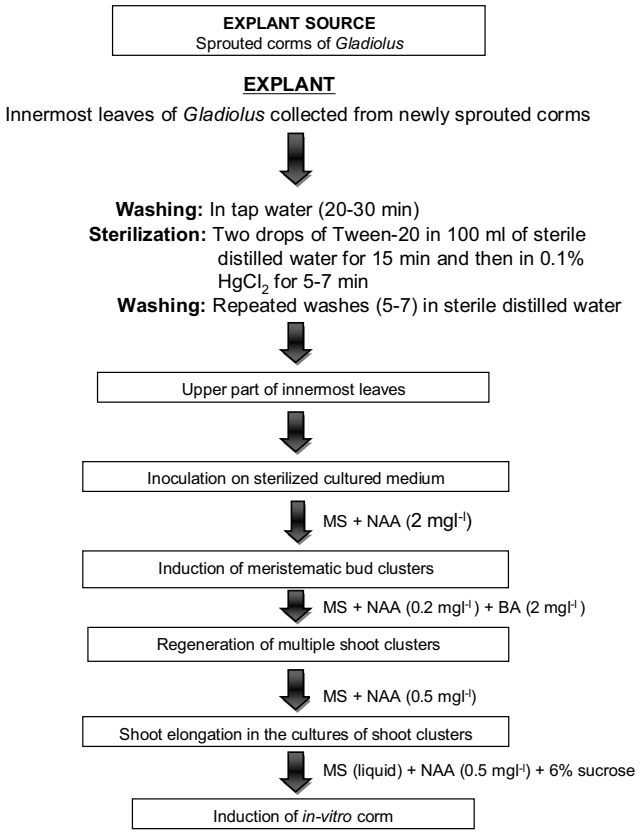

Fig. 4 Flowchart of cultivar-independent protocol for *in vitro* corm formation of *Gladiolus* cultivars.

variations for different quantitative parameters under study (**Table 3**). The 1st generation of plants raised from *in vitro* corms showed poor vigour possibly due to nutritional deficiency, as was suggested earlier (Wilfret 1980; Halevy 1985). The germination frequencies of the corms raised *in vitro* in plastic trays with moist sand and those in the field (100%, germination within 15-20 days; **Fig. 1D**) also appeared the same for both generations. Results also indicated that flower colour of plants raised from *in vitro* corms were comparable to the control.

Clonal fidelity among plantlets raised from *in vitro* corms

Ensuring clonal fidelity is another important aspect of any successful micropropagation protocol prior to commercialization. PCR-based molecular markers have previously been used with success to assess clonal fidelity in *Gladiolus* (Roy *et al*. 2006). In the present investigation, RAPD profiles of randomly selected samples (sprouted leaves) of mother corms, tissue culture-derived corms and corms collected from the field after the 1st (**Fig. 3B**) and 2nd (**Fig. 3C**) generation were examined simultaneously. Identical DNA profiles (**Fig. 3D-E**) for all samples were observed, suggesting genetic fidelity.

CONCLUSION

The present study offers an efficient micropropagation protocol (**Fig. 4**) for *in vitro* corm production of *Gladiolus* that is cultivar-independent and demonstrates the efficiency and stability of micropropagated corms of two cultivars as evaluated under field conditions.

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