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In vitro Regeneration of Gladiolus (*Gladiolus hybrida* L.): Optimization of Growth Media and Assessment of Genetic Fidelity

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A B S T R A C T

Gladiolus (*Gladiolus hybrida* L.) has been found as a potential cut flower cultivated world widely due to its attractive spikes and elegancy. The plant is propagated vegetatively through corms and cormels but more often its cultivation is hindered due to low multiplication rate of its corm and cormels. *Gladiolus* can grows through underground stems also, but it is more oftenly attacked by soil borne diseases. *In vitro* propagation techniques, assumes significance, especially for securing rapid multiplication of the novel cultivars using different explants sources and media. The *in vitro* regeneration of gladiolus cultivar *White prosperity* was achieved using shoot bud of cormels as an explant. The concentration and combination of plant growth regulators governed the regenerative capacity of explants. The BAP efficiently produced multiple shoots in gladiolus on B5 and MS media. The number of shoots varied from 1.3 to 3.0 shoots per explant on B5 media and 0.6 to 2.3 shoots per explant on MS media. After 30 days of incubation, the length of *in vitro* developed multiple shoots varied from 2.2 to 3.8cm in B5 media and 1.1 to 2.9cm in MS media. Also a monomorphic banding profile was obtained using Randomly Amplified Polymorphic DNA (RAPD) markers indicating that there was no genetic variation in *in-vitro* raised plants with respect to the mother plant when *in-vitro* regeneration was carried out. Hence, in vitro regeneration could be suggested for more efficient and cost effective mass propagation of *Gladiolus.*

Introduction

Gladiolus (*Gladiolus hybrida*) is a bulbous ornamental plant with great commercial importance in cut flower industry all over the world due to its magnificent and colorful spikes (Sinha *et al.*, 2002). The major gladiolus producing countries are the United States (Florida and California), Holland, Italy, France, Poland, Bulgaria, Brazil, India, Australia and Israel. In India, the major cut flowers grown are rose, tuberose and gladiolus (Singh *et al.*, 2010). Amongst the cut flowers, gladiolus occupied third position in terms of both area and production. Gladiolus is being cultivated in an area of 11660 ha in the India with an estimated production of 106 crore cut flowers (Kadam *et al.*, 2014). The major gladiolus producing states in the country are Uttar Pradesh, West Bengal, Odisha,

Chhattisgarh, Haryana and Maharashtra. Gladiolus is also grown in states like Uttarakhand, Karnataka, Andhra Pradesh and Sikkim (Kadam *et al.,* 2014). With the linking of India with global markets, international trade will assume considerable significance besides inducing changes in the domestic agricultural production scenario.

Gladiolus is propagated either by seeds, corm or by cormels. Although, seeds are an effective means of gladiolus propagation but seed-raised plants may not produce true-to-type population (Hussain *et al.,* 2001). In this way the conventional methods take about 8-12 years to produce sufficient number of corms of a variety for commercial cultivation (Dutta *et al.,* 2010). The conventional propagation of gladiolus in the field faces several problems due to the slow growth and low multiplication rate of cormel and disease attacks. The involvement of *Fusarium oxisporum* sp. *Gladioli* were also known to have impacts on the growth and survival rate of the seedlings (Dantu and Bhojwani, 1995). So the introduction of new varieties or virus/fungus-free planting material of gladiolus is difficult. Therefore, novel cultivars need to be rapidly mass multiplied by using these modern regeneration technologies in order to fulfill supply gap of huge demand of market.

Therefore *in vitro* propagation techniques, assumes significance especially for securing rapid multiplication of the novel cultivars. Although there are several reports on *in vitro* propagation of gladiolus varieties, using different plant parts as explants, like shoot, bud and root, and various plant growth regulators such as 2,4-D, IAA, NAA and BAP (Misra *et al.,* 1999; Pathania *et al.,* 2001; Kumari *et al.,* 2005 and Roy *et al.,* 2006). The *in vitro* multiplication of gladiolus has been reported by using axillary buds (Begum *et al.,* 1995; Boonvanno *et al.,* 2000), shoot tip (Hussain *et al.,* 2001), cormels (Nagaraju *et al.,* 1995) and inflorescence axes (Ziv *et al.,* 2000). Moreover, successful protocols for *in vitro* corm formation (Dantu and Bhojwani, 1995; Sen *et al.,* 1995; Al-Juboory *et al.,* 1995), organogenesis and somatic embryogenesis (Remotti *et al.,* 1995; Kumar *et al.,* 2002) have been achieved also. However, in Gladiolus there is a clear scope for further refinement through *in vitro* culture methodology to acquire a higher number of shoots to complement traditional nursery methods (Hussain *et al.,* 2001). Another aspect of the current study was to check clonal fidelity between mother and *in vitro* regenerated propagules. Clonal multiplication is also the major concern for the horticulturist. There is a possibility that *in vitro* regenerated propagules exhibit somaclonal variations (Larkin *et al.,* 1981). This variation may be caused through pre-existing genetic variation occurred in the explant and the variation induced through *in vitro* cultures (Skirvin *et al.,* 1994). This variation is manifested in the form of DNA methylation, chromosome rearrangement and point mutation (Phillips *et al.,* 1994). Long duration of *in vitro* culture, alterations in auxin-cytokinin concentrations, explant source and the stress created by *in vitro* environment all together or independently may be responsible to induce somaclonal variation (Modgil *et al.,* 2005).

Oxidative stress is also produced by *in vitro* culture environment that leads to the production of free radicals within the cells and ultimately cause DNA damage (Jackson *et al.,* 1998). In order to assess clonal fidelity, *in vitro* regenerated propagules need to be thoroughly checked for their clonal characters. Various PCR based molecular techniques, Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeat (ISSR), Simple Sequence Repeats (SSR) and Restriction Fragment Length Polymorphism (RFLP) are nowadays more reliable for detection of clonal fidelity over morphological

and isozymic analysis in various micropropagated plants (Carvalho *et al.,* 2004; Martins *et al.,* 2004). Plant tissue culture offers a potential to deliver large quantities of disease-free, true-to-type healthy stock within a short span of time (Hussain *et al.,* 2001). The present study was undertaken for standardization of *in vitro* multiple shoot production protocol in gladiolus and to analyze the genetic stability of micropropagated plantlets using RAPD markers.

Materials and Methods

Procurement and preparation of explant

The healthy cormels of gladiolus cv. *White prosperity* were obtained from Sardar Vallabhbhai Patel University of agriculture and Technology, Meerut. The outer scale of cormels was removed and buds of cormels cut with the help of surgical blade. Then buds were washed with 3-4 drops of Twin-20 (liquid detergent) along with 0.1% bavistin followed by 70% ethanol for 4-5 minutes and 0.1 % $HgCl_2$ for 10 minutes. After each treatment, the buds were washed 3-4 times with sterile distilled water. Buds were dried using the blotting paper before inoculated on the media.

Growth media

MS medium (Murashige and Skoog, 1962) and Gamborg (B5) medium supplemented with 0 to 4.0 mg/l BAP was used for shoot regeneration. After regeneration *in vitro* grown shoots were transferred to the rooting medium. The media was supplemented with combination of 0 to 2.5mg/l NAA and 1.0mg/l BAP for root induction. 30g/L sucrose was used as carbon source and pH was adjusted 5.8. Agar-agar (0.75%) was added to solidify the media in culture tubes and jam bottles, each containing 50 ml of the medium. In order to increase the number of shoots per culture vessel, the explants were subcultured on the same medium.

DNA extraction and PCR amplification conditions

The genomic DNA was isolated from *in vitro* raised plantlets by Murray and Thompson, (1980) method. Clonal fidelity of *in vitro* raised regenerants was tested by using 10 RAPD markers (Table 1) (Williams *et al.,* 1990; Zietkiewicz *et al.,* 1994). PCR amplifications were carried out in a total volume of 20µl containing 1µl of genomic DNA (25ng/µl) as template, 2.0µl of 10x Taq buffer, 0.6µl of 10mM dNTP, 1.0µl of 10mM primer, 0.5µl of 1U/µl Taq polymerase and 14.9µl sterile water. PCR amplification was performed in a DNA thermal cycler (Gene Amp PCR system 9700, Applied Biosystems, CA, USA). The initial DNA denaturation at 94°C for 4 minute, followed by denaturation at 92°C for 1minute, annealing at 37°C was done and 2 minute extension at 72°C, with a final extension at 72°C for 7 minute. Reaction was continued for 40 cycles and the samples were then electrophoresed on 2% agarose gel.

Data scoring and analysis

The scoring of bands was done on the basis of their presence (‘1’) or absence (‘0’). The genetic associations were evaluated by calculating the Jaccard’s similarity coefficient for pair-wise comparisons based on the proportion of shared bands produced by the primers. The similarity matrix was subjected to the cluster analysis of unweighted pair group method with arithmetic averages (UPGMA) and a dendrogram was generated by using NTSYS-pc version 2.1 software (Rohlf, 2000). Data were subjected to analysis of variance for a factorial experiment. Critical differences (CD) were calculated to determine the statistical significance of different

treatment means. Consistent, well-resolved fragments in the size range of 100 bp to 3000bp were manually scored.

Results and Discussion

In vitro propagation technique by using shoot buds as explants from gladiolus cormel was carried out on different media in order to develop a cost-effective method for clonal production of gladiolus. The present study on "*In vitro* regeneration of gladiolus (*Gladiolus hybrida*): optimization of growth media and assessment of genetic fidelity**"** was carried out on cultivar *White prosperity.*

Shoot organogenesis

Multiplication is usually achieved through excessive shoot proliferation and there after transfer of *in vitro* developed shoots to rooting media. The organogenesis of shoots can be obtained in two different ways either through direct development of shoots from different explant sources, such as cormel's shoot tip cultures or by shoot development through callus phase. Various stages/sizes of any explant might have different regenerative capacity and this regenerative capacity is much dependent upon the concentration and combination of plant growth regulators (Memon *et al.,* 2013). In present study BAP produced efficient number of shoots in gladiolus and it was found as a potent cytokinin. The number of regenerated shoot found to be varying with the mean 1.3 to 3.0 and 0.6 to 2.3 shoot per explant in B5 and MS media respectively. Induction of shoot bud was observed within 7-8 days in B5 media 10-11 days in MS media (Fig. 1). Maximum length and numbers of shoots were found in both medium when it was supplemented with 1.0mg/l of BAP, while minimum at 0.5mg/l of BAP. The shoot length and number of shoots were maximum in B5 media and MS media with the mean value 3.8000d, 3.0333d and 2.9000e, 2.3000f respectively, while minimum were recorded at 0.5 mg/l BAP with the mean value 0.500, 0.2666 and 1.1033, 0.6000b respectively (Table 1). The low concentration of BAP (1 mg/l) produces more number of shoots (upto 16 per culture vessel) from cormels (Aftab *et al.*, 2008). Grewal *et al.*, (1995) obtained single shoot per explant on MS medium supplemented with 1mg/l BAP in cultivars viz. *Mayur*, *Sylvia*, *Spic* and *Span*, whereas 14-20 shoot primordial obtained within 4 weeks when cultured on MS medium with 5mg/l BAP. Higher dose requirement of BAP was recognized as to be genotype dependent (Hussain *et al.,* 2001).

The differences in *in-vitro* response might be due to cormel size or varietal differences as the effect of concentration and combination of PGR varied with variety and explant size. Top section of cormel showed better potential for efficient shoot regeneration with BAP supplementation (4mg/l). Better shoot induction (upto 89%) was observed with top slice of cormel (dia. 1.0 to 1.5cm) in response to MS medium containing 4 mg/l KIN rather than BAP (Babu and Chawla, 2000).

One of the possible reasons for successful regeneration might be the presence of growing point (meristematic tissues) in the cormel or the direct contact of physiological base of the cormel top section with the media which further increased the absorption area for nutrient uptake. Regarding bottom section of cormels, most of the cultures exhibited mortality where the physiological base of the bottom section was on the nutrient medium and the cut surface on upper side. The large cut surface might be the reason of death of explants due to oxidative stress as there might be a possibility of free radical generation that led to activation of peroxidases, catalase and SOD enzymes (Olmos *et al.,* 1994). Transverse slices of cormel showed no regeneration (Emek and Erdag, 2007).

Table.1 Shoot induction in B5 and MS medium

S. No.	B5 medium			MS medium	
	Concentration of BAP (mg/l)	No. of shoots per explant	Shoot length per explant (cm)	No. of shoots per explant	Shoot length per explant (cm)
1	-	0.26± 0.08 a	0.50± 0.05 a	-	-
2	0.5	1.30± 0.11b	2.23 ± 0.04 b	0.6 ± 0.05 b	1.10 ± 0.12 b
3	1.0	3.03± 0.27 d	3.80 ± 0.04 d	2.30 ± 0.11 f	2.90± 0.11 e
4	1.5	2.0± 0.13 c	2.50 ± 0.57 c	1.23 ± 0.12c	2.10± 0.05 cd
5	2.0	2.13± 0.08 c	2.30 ± 0.57 e	1.40 ± 0.05	2.23 ± 0.0333
6	3.0	1.50± 0.11 b	2.60 ± 0.57 c	1.70 ± 0.11 e	2.30 ± 0.11 d
7	4.0	2.23 ± 0.12 c	3.10 ± 0.11 d	1.60± 0.05 de	2.0 ± 0.05 c
CD- CV-		0.44 14.14	0.20 4.65	0.28 12.68	0.26 8.15

*Each treatment consists of 3 replicates. *Means followed by the same letters (a,b,c) are not significantly different (p <0.05) using Duncan New Multiple Range Test (DMRT's test).

Table.2 Root induction in B5 and MS medium

S. No.	Concentration of NAA (mg/l) along with BAP (1mg/l)	B5 Medium		MS Medium	
		Root Length	Number of roots	Root Length	Number of roots
1	0.0	0.3 ± 0.12 a	2.0± 0.54 a	0 ± 0.0 a	0 ± 0.0 a
2	0.1	0.8 ± 0.11 b	3.0± 0.54 a	0± 0.0 a	0± 0.0 a
3	0.5	1.4 ± 0.07 c	5.0± 0.31 b	0.36 ± 0.16 b	2.0± 0.44 b
4	1.0	2.2 ± 0.12 d	5.4± 0.40 b	0.76 ± 0.09 c	2.0± 0.54 b
5	1.5	3.2 ± 0.10 f	6.0± 0.44 b	0.96 ± 0.09 cd	3.0± 0.54 bc
6	2.0	2.8 ± 0.07 e	5.0± 0.44 b	1.40 ± 0.09 e	4.0± 0.44 e
7	2.5	2.4±0.08 d	3.0± 0.44 a	1.16 ± 0.09 d	3.2± 0.37 bc
	CD- CV-	0.27 11.21	1.33 24.31	0.27 30.83	1.17 44.48

*Each treatment consists of 3 replicates. *Means followed by the same letters (a,b,c) are not significantly different (p <0.05) using Duncan New Multiple Range Test (DMRT's test).

Fig.1 A. shoot induction on B5 media, B. shoot induction on MS media, C and D. root induction, E. subcultured growth of shoot on B5 media and F. subcultured growth on MS media

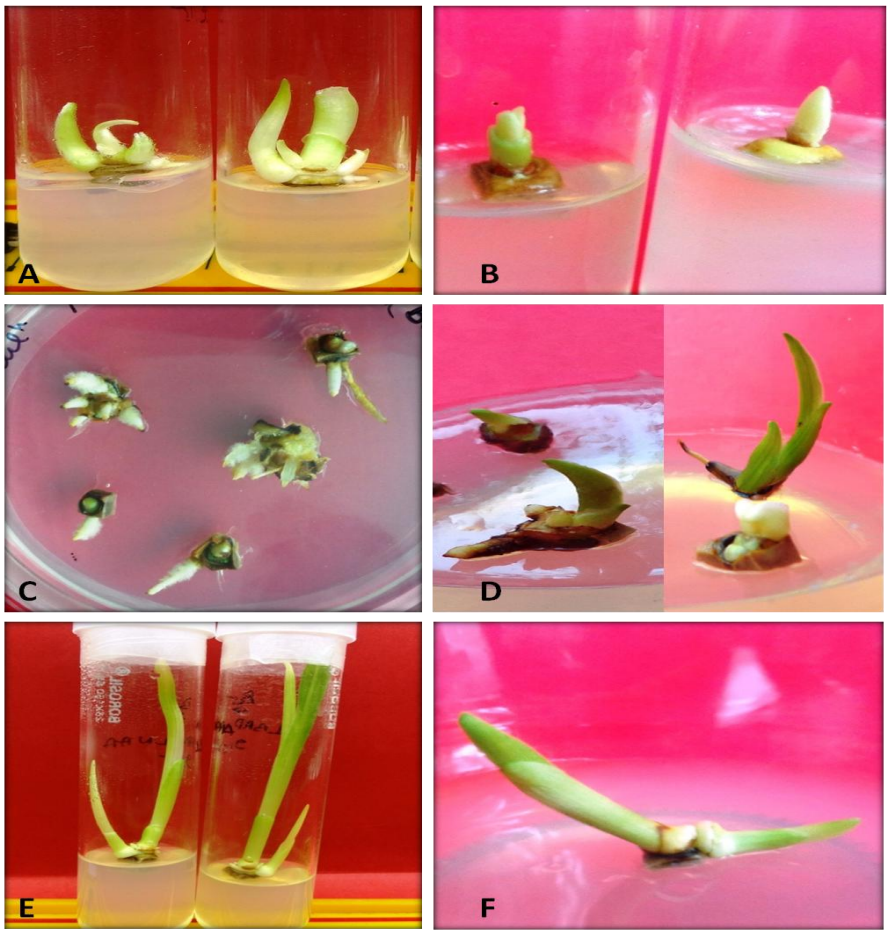
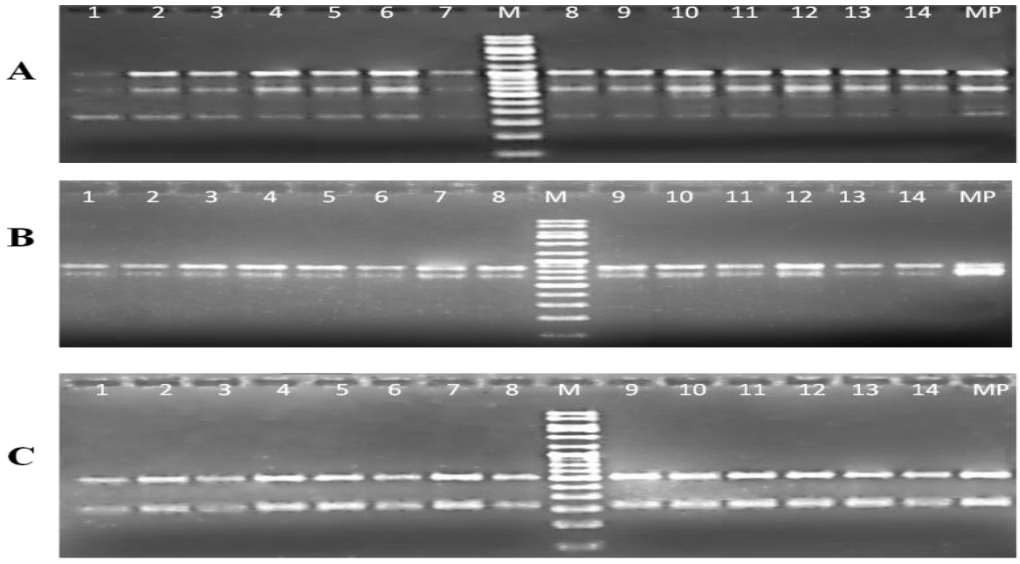


Fig.2 RAPD primers, (A) OPA 09, (B) OPA 01 and (C) OPA 10 profiling pattern



Root development

In vitro grown multiple shoots were subculture for root induction on both B5 and MS medium enriched with various concentrations of NAA along with BAP. The culture was incubated at $25^0C \pm 2^0C$ with 16/8 hr light/dark regime under fluorescent light. Data was observed after four weeks of subculture. The root regeneration has been found to be varying with the mean value 2a to 6b and 0a to 4c root per explant in B5 and MS media respectively. Induction of roots from plantlets was observed within 6-9 days in B5 media 7-11 days in MS media (Figure 1). Maximum length and numbers of roots were found in B5 media when it was supplemented with 1.0mg/l of BAP and 1.5mg/l NAA, whereas maximum length and numbers of roots were found in MS media when it was supplemented with 1.0 mg/l of BAP and 2 mg/l NAA. While minimum length and numbers of roots were found at 1.0 mg/l BAP 0.1 mg/L NAA in both media. The root length and number of roots were maximum in B5 media as compared to MS media (Table 2). Highest number of roots (upto 24) were also recorded in cv. "*Peach blossom"* on MS medium containing 1mg/l NAA (Priyakumari and Sheela, 2005). It has also been previously reported that very poor response was obtained in case of root initiation on MS medium containing IBA or NAA while sucrose concentration show positive effect on the rooting response and quality of roots in different cultivars (Kumar *et al.*, 1999).

Genetic fidelity

Plants regenerated from adventitious buds around axillary buds or from other well developed meristematic tissue showed the lowest tendency of genetic variation (Rout *et al.,* 1998; Joshi and Dhawan, 2007), whereas plants derived from callus as compared to those raised from embryogenic tissues showed more variations (Al-Zahim *et al.*, 1999; Yang *et al.,* 1999). Previous reports also suggested that even plants derived from organized meristems are not always genetically true to type in many crops (Devarumath *et al.,* 2002). Hence, it becomes imperative to regularly check the genetic purity of the micro-propagated plants in order to produce clonally uniform progeny while using different techniques of micro-propagation. The presence or absence of variations during *in vitro* propagation depends upon the source of explants and method of regeneration (Goto *et al.,* 1998). The sub- and supra-optimal levels of plant growth substances, especially synthetic ones, have also been associated with somaclonal variation (Martin and Pachathundkandi, 2006). Even at optimal levels, long-term multiplication often may lead to somaclonal or epigenetic variations in micro-propagated plants thus questioning the very fidelity of their clonal nature. During this study total 35 distinct bands produced in fourteen *in vitro* regenerated clones and one mother plant. A total number of 525 bands were generated by all primers showed monomorphic banding pattern. The number of scorable bands for each primer varied from 2 (OPA10 and OPA 01) to 5 (OPA 15) with an average of 3.5 bands per primer. Primer OPA 01 produced two bands with the length 350bp and 450bp (Figure 2), primer OPA 10 also produced two bands, 160 bp and 300bp long (photo plate 2.C). Three bands were produced by four primers i.e. OPA 04, OPA 07, OPA 09 and OPA 16. The primer OPP 05 produced four bands vary from 170bp to 300bp. Three primers OPA 15, OPA 17 and OPA 19 produced the five distinct bands with a length of 80 to 340bp, 125 to 520bp and180bp to 580bp respectively in each clone along with mother (Fig. 2). Thus *in vitro* regenerated plants shows genetic similarity with their mother plant. Clones derived from cormel's shoot tip explants were however true to their

type, one leaf-derived clone showed genetic variation (Bhatia *et al.,* 2010).

The findings of Potter and Jones, (1991) state that somaclonal variations are associated with regeneration from undifferentiated tissues and plants regenerated from existing meristems are genetically stable. These findings support the fact that meristem-based micro-propagation system is much more stable genetically than those in which regeneration occur via callus phase.

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