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Full Length Research Paper

Enhancement of *in vitro* micro corm production in *Gladiolus* using alternative matrix

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An efficient *in vitro* protocol for mass propagation of *Gladiolus* was developed. Basal portions of the innermost leaves responded readily in culture. Ten to fifteen adventitious shoot buds emerged from every responding explant through direct organogenesis, which proliferated in agar-gelled MS basal medium supplemented with NAA (0.002 mM) and BA (0.009 mM). Large number of micro corms was produced in the plantlets on transfer to MS liquid medium supplemented with 176 mM sucrose and 0.002 mM NAA using coir as an alternative matrix. The micro corms grew in size under field condition for two consecutive seasons for flowering. The identical isozymic, RAPD and ISSR profiles of randomly selected micro corms and corms obtained from the field with respect to mother corm population indicated a near full-proof and cost effective micropropagation protocol of *Gladiolus*.

Key words: alternative matrix, genetic fidelity, *Gladiolus*, isozyme, ISSR, micro corms, RAPD.

INTRODUCTION

The exuberance of colourful spikes of *Gladiolus* is a delight in any floral bouquet. It is an all-time favorite for the flower lovers in flower shows and is a specialty for the growers aiming at cut flower business on a commercial scale (http://muextension.missouri.edu/xplor/agguides/-hort/g06620.htm). The species and varieties of it are numerous since the genus *Gladiolus* includes 180 species with more than 10,000 cultivars (Sinha and Roy, 2002). The numbers are rising every year through hybridization, with the aim of extending vase life, producing novel colours, floret arrangement on the spikes and to prolong the flowering period (Kumar et al., 1999).

Since *Gladiolus* perennates by underground stems or corms, the infection by soil-borne fungus, *Fusarium oxy-sporum* f. sp. *gladioli* causing severe loss and damage are a major bottleneck to its mass propagation (http://www.aboutgardens.com/Plants/Bulbs/Gladiolus/). With the growing symbiosis between plant breeders and biotechnologists, disease resistant varieties of *Gladiolus* developed through plant transformation will be a reality in near future and some reports are already available (Kamo, 1997; Kamo and Blowers, 1999; Löffler et al., 2000). However, few improvisation of the state-of-the-art technology is still needed since callus based indirect regeneration system has been used in genetic modification programme (Löffler et al., 2000). A direct regeneration protocol would have been a better proposition for the recovery of higher number of successful transformants. This paper, hence, aims at development of an efficient direct mode of regeneration of *Gladiolus* as a prelude to transformation and also simultaneous mass propagation of *in vitro* cormlets in relatively quick time minimizing the cost and hassles of otherwise till now sophisticated plant tissue culture practices. Furthermore, the aspect of clonal fidelity of the *in vitro* propagated corms and cormlets has also taken in consideration utilizing isozymic analysis and PCR-based techniques like RAPD and ISSR.

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Abbreviations: NAA, 1-Napthylacetic acid; BA, 6-Benzylaminopurine; RAPD, Random Amplified Polymorphic DNA; ISSR, Inter Simple Sequence Repeat.

MATERIALS AND METHODS

Tissue culture

Aseptic culture of *Gladiolus* was initiated from healthy corms of white flowered variety '*pacifica'* (Figure 1a), procured from Suttons Seed Company, Kolkata. The corms were germinated in pots with moist sand. Basal portions of innermost leaves after 10 days of sprouting were used as explants. The explants were thoroughly washed with few drops of Tween 20 and surface sterilized with 0.1% (w/v) mercuric chloride for 7 min followed by rinsing with double distilled sterile water for four times and established in MS basal medium (Murashige and Skoog, 1962) supplemented with 0.55 mM myoinositol, 88 mM sucrose and 0.01 mM NAA (1-napthylacetic acid). Media were gelled with 0.75% agar (Difco Bacto). The explants started to swell within 30 days and those sub cultured to MS medium were supplemented with 0.002 mM NAA and 0.009 mM BA (6-benzylaminopurine) where visible multiple shoot bud initiation started around the edges of cut surfaces of the explants within another 30 days. Free hand sections of the responding explants were done to study the mode of regeneration. After sufficient maturation of the shoot buds, the plantlets were separated and maintained in the same medium for two more sub cultural passages (one passage of 30 days). The plantlets were subsequently placed individually in culture tubes containing MS liquid medium with sterile coir, coconut husk, as matrix (Gangopadhyay et al., 2002) supplemented with 176 mM sucrose and 0.002 mM NAA for development of *in vitro* cormlets and roots. Parallel sets of plantlets were also transferred to glass jars in similar medium but gelled with agar instead of liquid medium with coir.

Development of micro corms started within 45 days and those started maturing within 90 days. Cultures were kept under 16 h photoperiod (40-80 µmol $m^{-2}s^{-1}$) at 25°C±1°C and 78% relative humidity.

Matured and dried microcorms were taken and the roots were separated out. Micro corms thus harvested from both agar-gelled and liquid medium containing coir were categorized into different size groups. For microcorms obtained from the agar-gelled medium were grouped into two sizes. Size I ranged from 4.0 to 6.0 mm and size II ranged from 6.1 to 10.0 mm in diameter. Microcorms of liquid medium containing coir were grouped into three size groups. Size I ranged from 8.0 to 14.0 mm, size II ranged from 14.1 to 20.0 mm and size III ranged from 20.1 to 25.0 mm in diameter. The microcorms were stored in desiccators for 30 days prior to sowing in the field. The corms from the plants grown in the field after first season were collected, desiccated for another 60 days and sown again in the following season and the plants emerging out bloomed. All experiments were repeated thrice with three replicates for each treatment.

Isozymic analyses

Isozymic analyses of four enzymes–esterase (EST, E.C.3.1.1.1), peroxidase (PRX, E.C.1.11.1.7), acid phosphatase (ACP, E.C.3.1.3.2) and α–amylase (E.C.3.2.1.1) were done following standard protocol (Das and Mukherkee, 1997) after extraction of 2 g of meristematic portions of random samples (three in each case) of mother corms, tissue culture derived microcorms and corms collected from the field after one and two seasons. The protein content was estimated by the Folin-phenol method (Lowry et al., 1951). Densitometric scan of the gels was done using Biorad Gel Documentation System (Gel Doc 1000, version 1.5).

RAPD and ISSR analyses

Experimental materials similar to isozymic analysis were also considered for RAPD and ISSR analysis. DNA was extracted by CTAB method (Rogers and Bendich, 1998). DNA concentration in the samples was adjusted to 25 mg dm^{-3} for PCR reaction in each sample. RAPD analysis was performed according to the method of Williams et al. (1990) using ten oligonucleotide (decamer) primers, OPA 01–OPA 05 and OPB 01–OPB 05 (Operon Tech., Alameda, USA). Amplifications were carried out in a Thermal Cycler (Perkin Elmer System – 2400, Norwalk, CT, USA) with an initial denaturation of 120 s at 94°C and temperature profile of each cycle was, 60 s denaturation at 94°C, 60 s annealing at 35°C and 120 s for extension at 72°C. Reaction continued for 45 cycles followed by 300 s hold at 72°C to ensure that the primer extension was completed. PCR reaction mixture of 0.025 cm^3 consisted 1X buffer, 0.2 mM dATP, dCTP, dGTP,dTTP, 2 mM $MgCl_2$, 0.2 µM of primer, 100 ng of template DNA and 1 unit of Taq DNA polymerase (Roche).

Two non-anchored oligonucleotide ISSR primers, $(CAG)_5$ and $(GACA)_4$ (synthesized by Isogen) were used for amplifications (Bornet and Branchard, 2001). To optimize the reaction conditions, several PCR parameters were tested including DNA concentration (0.5-150 ng/reaction, 14 values), primer concentration (10-500 pmol/reaction, 9 values), $MgCl_2$ concentration (0-10 mM, 11 values), dNTP concentration (20-700 mM each, 8 values), Taq DNA polymerase (0.5-2 units/reaction, 5 values), and number of cycles (15-40, 11 values). Reactions without DNA were used as negative controls. The optimum annealing temperature was determined for each ISSR primer from a minimum of 5 temperatures. Amplifications were carried out in two thermocyclers (MJ Research and Perkin Elmer) with an initial denaturation of 90s at 94°C and temperature profile of each cycle was 60 s denaturation at 94°C, 60 s annealing at specific temperature (as standardized for each primer) and 240s for extension at 72°C. Reaction continued for 27 cycles followed by 420s hold at 72°C to ensure that the primer extension reaction was completed. The best patterns were amplified in the presence of 12 ng genomic DNA, 100 pmol of primer, 2.5 mM of $MgCl_2$, 200 µM of each of the four dNTPs, 1.25 units of Taq polymerase, 1x enzyme buffer and 27 PCR cycles. Of all the PCR parameters, the annealing temperature was found to be the most crucial and stringent and those are as follows: 60°C for $(CAG)_5$ and 50°C for $(GACA)_4$. Amplified products were electrophoresed in 1.8% agarose gel with DNA molecular weight marker X (0.07–12.2 kbp), Boehringer Mannheim, GmbH, Germany as size markers.

Statistical analysis

Standard errors of the means were calculated and 'Student's t-test' was performed to check the level of significance of the differences between the weight of 50 microcorms grown on agar-gelled and liquid medium with coir due to two concentrations of sucrose.

RESULTS AND DISCUSSION

Basal portions of the innermost leaves (1 cm x 0.75 cm) of the newly sprouted *Gladiolus* plants responded readily in culture and almost ten to fifteen adventitious shoot buds emerged in every responding explant (Figure 1b). *Gladiolus*, being a plant of commercial importance, almost every portion of it has been tried as explants and

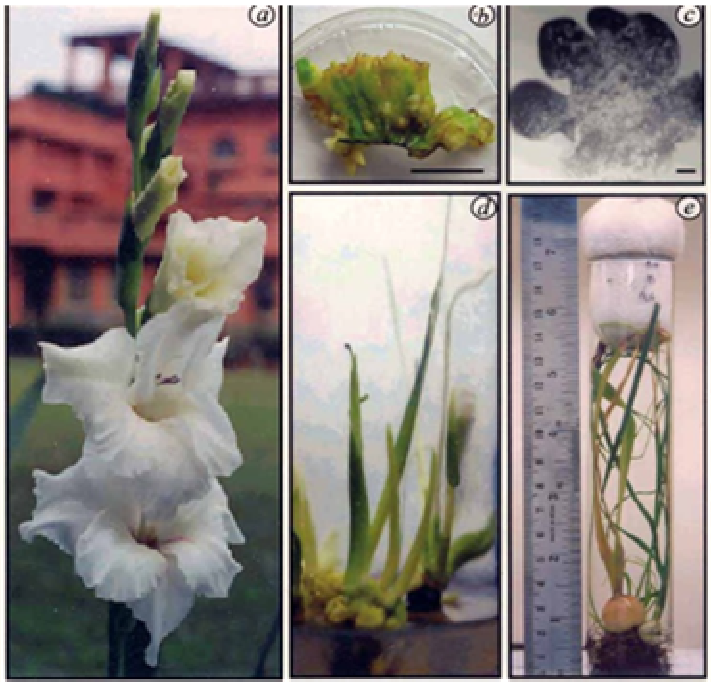

Figure 1. Micropropagation of *Gladiolus* variety '*pacifica*': a–flower in full bloom b-photomicrograph of adventitious shoot bud proliferation from explant; c–free hand section of responding explant showing direct organogenesis; d–shoot proliferation in agar-gelled medium; e–development of micro corm in liquid medium with coir.

Table 1. Effect of matrix and concentration of sucrose in media on number of micro corm production per plant.

Matrix	Concentration of sucrose (mM)	Number of plants (%)			
		Without Microcorm	1-2 Micro-corms/plant	2-4 Micro-corms/ plant	4-6 Microcorms/ plant
Agar	88	21.90	25.71	34.28	18.09
	176	15.46	17.52	43.29	23.71
Coir	88	7.60	10.86	53.26	28.26
	176	0	10.90	60.90	28.18

differential responses have been attained (Zaidi et al., 2000). The basal most portions of the innermost leaves as used in the present study was most responsive in terms of adventitious multiple shoot bud formation since explant tried beyond this region up on the leaves did not either result in uniform response or showed callus induction. Histological observation revealed direct organogenic nature of response (Figure 1c). Indirect mode of shoot organogenesis, via callusing though is in literature (Sinha and Roy, 2002; Kumar et al., 1999) but the direct one, as observed in the present study is definitely of advantage in case of maintaining clonal purity. The proliferation of shoot buds was dependant upon sequential transfer of the responding explants from auxin rich medium (NAA alone, 0.01 mM) to medium supplemented with low concentration of NAA (0.002 mM) and added cytokinin, in form of BA (0.009 mM). Otherwise, the shoot buds tended to coalesce giving rise ultimately to callus. Clearly separable shoot buds developed into individual plantlets in the last mentioned medium (Figure 1d), where it was maintained for two subcultural passages prior to experimentation on *in vitro* cormlet production.

Osmoticum in form of higher concentration of sucrose (176 mM) along with supplementation of medium with auxin alone (NAA, 0.002 mM) was found to be beneficial for micro corm production since large number of micro corms were produced, especially in liquid medium with coir as the matrix (Figure 1e). Microcorms developed also in agar-gelled medium but the former system was found to be more efficient (Table 1-2). Firstly because, there was no need for regular sub cultural practice as it was attained simply by addition of sterile liquid medium apart from other ready advantages of using eco friendly

Table 2. Effect of matrix and concentration of sucrose in media on mean weight ± S.E. of micro corms.

Groups of micro corms[1]	Weight of 50 Micro corms (gm)			
	Agar		Coir with liquid medium	
	88 mM Sucrose	176 mM Sucrose	88 mM Sucrose	176 mM Sucrose
I	2.25±0.187	3.25±0.216*	5.20±0.178	12.5±0.512**
II	6.0±0.178	8.75±0.265**	17.70±0.256	42.5±0.450**
III	-	-	33.33±0.33	80.0±0.368**

[1]based on diameter, mentioned in the text; * $p<0.05$, ** $p<0.01$

Figure 2. a-b – Mass propagation of *in vitro* micro corms; c – d – corms in field condition after first (c) and second (d) season

biodegradable matrices like coir and *Luffa*-sponge in plant tissue culture practices as reported by the present group in a number of plant systems (Gangopadhyay et al., 2002; 2004a). The promoting effect of high concentration of sucrose in microcorm formation, however, has been reported earlier (Steinitz, et al., 1991) and it showed its significant effect irrespective of matrices, agar or coir in the present study too as revealed by 'Student's t-test' (Table 2). The size and number of microcorm production *en masse* in liquid medium with coir supplemented with 176 mM sucrose as obtained in the present study (Figures 2a,b) probably supersedes all other available literature on *in vitro* corm production in *Gladiolus*, indicating ready commercialization prospect of this simple and cost effective protocol.

Assurance of clonal fidelity is another important aspect of any successful micropropagation protocol prior to commercialization. PCR-based molecular markers have largely been employed in recent times for various purposes (Hao et al., 2002; Fu et al., 2003) and the present group itself has been successful in ascertaining clonal purity of micropropagated plants with respect to mother, in a number of plant materials (Gangopadhyay et al., 2003; 2004a; 2004b) using molecular techniques. Similar endeavor was also taken for this material and RAPD and ISSR profiles of randomly selected samples (three in each case) of mother corms, tissue culture derived microcorms and corms collected from the field after first and second seasons (Figure 2c,d) were examined simultaneously. For the sake of brevity profile of only one material from each of the four types of samples has been presented. The totally identical DNA profiles of all the microcorms and corms obtained from the field after first and second season with respect to mother corm population after PCR with ten random (RAPD, result of four primers are being presented, Figure 3a) and two ISSR primers (Figure 3b) probably indicate a near full-proof micropropagation protocol of *Gladiolus*. RAPD profiles of *Gladiolus* cultivars differing in sensitivity to *Fusarium oxysporum* f. sp. g*ladioli* though have been reported (Dallavalle et al., 2002) but this is probably the first report of ISSR fingerprinting of *Gladiolus*.

Cost effectiveness is a major checkpoint in any commercial micropropagation programme and assurance of clonal fidelity through any PCR-based detection system definitely appears to be self-contradictory. Efforts, hence, were made to use relatively cheaper profiling technique. Isozymes, though belonging to the second generation of molecular markers (Zeidler, 2000), were considered to be the right candidate for this purpose as it is a cheaper proposition than PCR-based fingerprinting. Four widely used isozymes, viz. esterase; peroxidase,

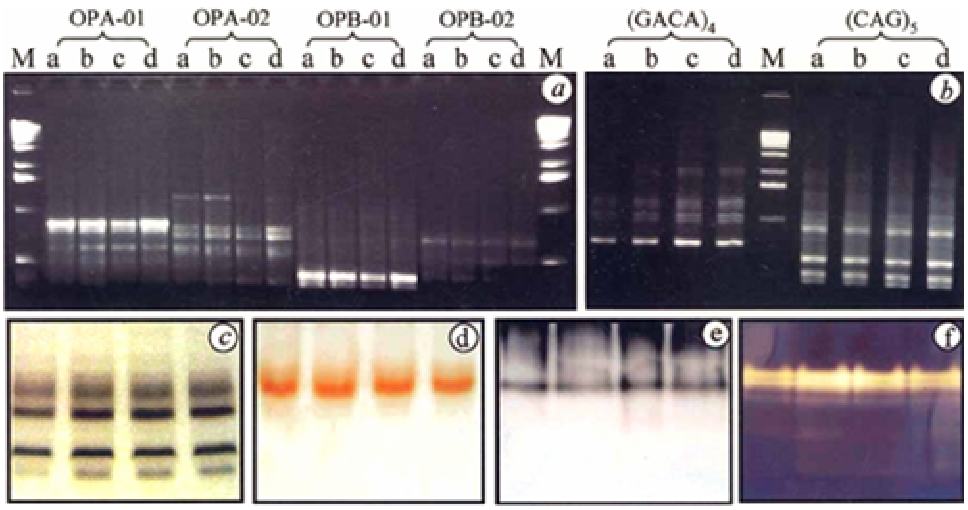

Figure 3. a - RAPD profiles of corms and micro corms of *Gladiolus* variety 'pacifica': Mother (a), micro corms from culture tubes (b), corms from field after first (c) and second (d) season with OPA-01, -02 and OPB-01, -02 primers. M – DNA molecular weight marker X, 0.07 – 12.2 kbp, Boehringer Mannheim, GmbH. Germany.
b – ISSR profiles of corms and micro corms of *Gladiolus* variety 'pacifica': Mother (a), micro corms from culture tubes (b), corms from field after first (c) and second (d) season with $(GACA)_4$ and $(CAG)_5$ primers. M – DNA molecular weight marker X, 0.07 – 12.2 kbp, Boehringer Mannheim, GmbH. Germany.
c – f - Isozymic profiles of (esterase – c; peroxidase – d; acid phosphatase - e; and α – amylase - f) corms and micro corms of *Gladiolus* variety 'pacifica': Lanes from left to right in each gel represent mother, micro corms from culture tubes, corms from field after first and second season.

acid phosphatase and α-amylase were studied in similar experimental design as done in case of RAPD and ISSR. Of the four isozymes analyzed, only esterase showed high polymorphism while the other three was low in polymorphic band (Figures 3c-f) but interestingly enough, tissue culture derived microcorms, recovered from field and also the mother population all showed identical banding profiles in all the four isozymes thus serving the purpose of assurance of clonal fidelity in relatively low cost. Esterase profiling has previously been used to study the genetic stability of micropropagated banana clones (Ramalakshmi Dutta, et al., 2003/4) in field condition with success.

Upgradation of existing methodology is a continuing practice for any commercially important plant and *Gladiolus* is no exception to that. Apart from this relatively straightforward and cost effective protocol of mass propagation of *in vitro* microcorms of *Gladiolus*, the present study also opens up an efficient way of regeneration of this commercially important material by direct shoot bud organogenesis and the work on *Agrobacterium*-mediated transformation of *Gladiolus* exploiting this protocol to recover large number of putative transgenics is presently underway.

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