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Nuclear 2C DNA and genome size analysis in somatic embryo regenerated gladiolus plants using flow cytometry

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Key words: callus, embryo maturation and germination, genetic stability, PGR.

Abstract: Gladiolus is a valuable asexually propagated plant of horticultural importance. Here, in the present communication, *in vitro* embryogenesis protocol and 2C DNA content of embryo regenerated plant are described. Callus was first induced from corms on 2, 4-D and NAA + BAP amended MS medium and the frequency was maximum (75.12%) in 0.5 mg/l NAA + 0.5 mg/l BAP added medium. The callus differentiated into embryos on 2, 4-D at variable numbers (3.13-5.32/callus mass); the addition of 1.0 mg/l BAP and 0.25 mg/l NAA was found very efficient in proliferating embryos (7.99/callus mass). Direct somatic embryos were also formed on corm surfaces on 2, 4-D (0.5-1.0 mg/l) amended medium at varying numbers. The embryos did not progress to maturity in same induction medium, so other PGR treatments containing GA_3 and ABA were added. The amendment of GA_3 was more responsive compared to ABA and 0.5-1.0 mg/l of GA_3 was identified as best effective treatment. The embryos showed a maximum of 62.15% maturity in 0.5 mg/l GA_3 added medium after 8th of culture. On BAP containing medium the mature embryos converted into plantlets and highest germination (42.65%) was noticed on 0.5 mg/l BAP added medium. The 2C DNA content of regenerated plant was measured by flow cytometry and was noted to be 1.34 pg. The somatic embryo derived plantlets are true-to-type, stable and grew normally in outdoor conditions, genome size is identical to corm derived gladiolus plants. This is the first flow cytometric DNA analysis description in somatic embryo regenerated gladiolus plant.

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All relevant data are within the paper and its Supporting Information files.

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1. Introduction

Ornamental is a major group of plants, comprises of dicot, monocot, gymnosperm, fern and other lower groups, grown in indoor-, outdoor decoration and for aesthetic values. These plants are mostly annual and perennials. A large number of ornamentals are used in cut-flower industry, some common examples are carnation, rose, dahlia, lilies, amaryllis etc. (Sarkar, 2010). Gladiolus, often called as sword lily, is a perennial bulbous flowering plant, belongs to the family Iridaceae. It comprises of about 260 species, of which 250 are native to African continent and some

are to Eurasia (Ohri and Khoshoo, 1986). Beside its ornamental value, a few species are described to be wild, distributed in South Scandinavians Hill to the coast of Mediterranean sea. The *Gladiolus imbricatus,* one of the wilds, is known to be a rich source of vitamin C and minerals; the flowers are edible and its taste is very similar to lettuce (Elena *et al.,* 2012). The different species of *Gladioli* are also used as food plants by Lepidoptera larvae especially the Large Yellow Underwing. *Gladiolus* is primarily cultivated worldwide as cut flower because of its beautiful spike with moderate to long vase-life. The gladiolus flower spikes are long, one sided bearing variable flowers of attractive colours. The hybrid gladioli, in particular, demonstrate a wide range of colours that make the plant a success in cut flower market. The plant grow well in winter months, when temperature hovering around 10-25°C. The major gladiolus producing countries are Holland, Poland, Bulgaria, Romania, Israel, Australia, Denmark and US; in US, the annual sale of gladiolus is estimated to be around 370 million corms (Narain, 2004). In India, too, around 130.000 ha are under ornamental cultivation as the growth of floral market is high, upward and crossed 1000 crore mark, as per 2006 data (Singh, 2009, 2011). The gladiolus is predominantly distributed in Asia, South - and tropical Africa and in Europe (Goldblatt and Manning, 1998). Most species are diploid showing 2n = 30 chromosome number; a large number of species are tetraploid hybrids, exhibiting chromosome count of 60 (Krahulcová, 2003; Elena *et al.,* 2012). The various species of *Gladiolus* are propagated vegetatively by corms and cormlets separation, which is a very slow process while seeds are only used for raising new hybrid varieties (Memon, 2012). Beside conventional propagation, the *Gladiolus* is often multiplied by tissue culture in which leaf, nodal segment, apical shoot tip, inflorescence and corm pieces are cultured in various nutrient media (Xu *et al.,* 2009). Yelda and Bengi (2007) obtained profuse callus from leaf on 5.0 mg/l NAA added MS in dark condition. The MS amended with 1.0 mg/l BAP + 0.5 mg/l KIN + 0.5 mg/l NAA was found very effective in producing callus from cormlet (Pragya *et al.,* 2012). In *Gladiolus,* the plantlets are regenerated directly from cultured explants or plants arise de novo from callus following organogenesis and embryogenesis pathway (Stefaniak, 1994). Remotti (1995) obtained primary and secondary embryos in *Gladiolus x grandifloras* cv. Peter Pears from cell suspension, derived from cormlet embryogenic callus on 0.25 µM BAP or zeatin added MS medium. The incidences of somatic embryogenesis have also been reported in a number of other studies (Iantcheva *et al.,* 1999; Wu *et al.,* 2015) in which genetic transformation method was utilized to make the plant resistant to gray mold, dry rot, root rot and other plant diseases (Massey, 1928). Particle bombardment mediated genetic transformation was earlier attempted in improving traits such as shortening dormancy (Kamo and Joung, 2007; Kamo *et al.,* 2009). *Agrobacterium* mediated genetic transformation was also tried but was noted to be less efficient as fast regeneration protocols from transformed tissues are not fully optimized, which is a primary requirement for successful transformation studies (Wu *et al.,* 2015). Moreover, *Gladiolus* is a member of monocot, the tissue of monocotyledonous plant is noted to be less responsive/receptive in up taking foreign DNA during transformation mechanism (Smith and Hood, 1995), although there are some current exceptions (Zhao *et al.,* 2000; Zhang *et al.,* 2006). There are several factors, controlling the success of plant regeneration - the explant, medium and plant growth regulators (PGRs). The somatic embryogenesis way of plant regeneration has several advantages such as lower risk of somaclonal variation and higher rates of plant regeneration (Vergne *et al.,* 2010; Zhao *et al.,* 2013) and thus has been successfully practised in a variety of research programmes of biotechnological interest. Although *in vitro* embryogenesis has been reported in some specific gladiolus species, the plantlet recovery from embryos is still not too high. This is primarily because of non-synchronous embryo induction, and poor quality of embryos, preventing early maturation and subsequent germination in culture. Embryo (developed on explants directly) derived plantlets are true-to-type; genetic fidelity has often been maintained in somaclones (Jayanthi and Mandal, 2001). However, plants obtained from callus-mediated embryos demonstrate genetic and phenotypic variability and this instability increases with older culture of callus (Endemann *et al.,* 2001). The accumulation of higher levels of stress and the presence of PGRs are considered the signalling elements in inducing embryos and subsequent genetic variability in tissue culture raised plants (Clarindo *et al.,* 2008; Delporte *et al.,* 2013). Thus, there is an urge to check somaclones' fidelity in regenerated populations. A large number of conventional and modern techniques have been used recently to identify alteration in cultivating tissues and *in vitro* raised plants (Das *et al.,* 2013). Flow

cytometry, an important technique has frequently been used for measuring nuclear DNA content in plant cells and tissues (Zhang *et al.,* 2005). The technique offers simple, fast and precise way of determining 2C DNA in plant cells. It has several other fundamental and applied uses including the initiative of genome sequencing of known and unknown plant materials (Galbraith, 2009; Rewers *et al.,* 2012). In the present study, flow cytometric investigation was conducted to measure and compare the 2C DNA level of somatic embryo regenerated plantlets with naturally grown *Gladiolus,* which served as tissue source for *in vitro* study. The fast and large-scale plant regeneration was also described by studying the role of PGRs in developing callus and embryos and in the event of maturation and germination of embryo in gladiolus.

2. Materials and Methods

Plant material and cultural conditions

The gladiolus corms (*Gladiolus hybridus*) from Horticulture Society of India, Calcutta, were used as experimental material. First, the healthy gladiolus corms were selected and washed with cetrimide (a liquid detergent) for 10-15 min, followed by washing with running tap water for 30 min. This was followed by a treatment with 70% ethanol for 5 min, rinsed with distilled water for 3-4 times. Finally, the corms were surface sterilized with 0.05% $HgCl_2$ for 3 min, and washed 3-4 times with sterilized distilled water to remove the traces of mercuric chloride. The gladiolus corms were sliced into explants (8-10 mm), and were cultured in MS (Murashige and Skoog, 1962) amended with different concentrations of 2, 4-dichlorophenoxyacetic acid (2, 4-D), Indole acetic acid (IAA) and 1-naphthaleneacetic acid (NAA). The medium pH was adjusted to 5.7 before sterilization at 121°C. The cultures were kept at 25±2°C in a culture room under 16 h photoperiod provided by cool white fluorescent light of intensity of 40 Wm^{-2} S^{-1}.

Induction of callus and embryogenic tissue

Corm pieces were cultured on MS, amended with 3% sucrose, 100 mg/l inositol and 2, 4-D, IAA and NAA at different concentrations (0.25, 0.5, 1.0 and 2.0 mg/l) for callus induction. BAP (0.5 and 1.0 mg/l) was also used in combination with NAA (0.5, 1.0 mg/l) in separate experiments. Within 7-10 days, brown yellowish callus was formed from the cut end of corms, which later engulfed the whole surface. The same level of PGRs was also used for callus maintenance. The induced callus transformed into embryogenic callus, which showed small, globular or 'nearly globular' or elongated, cylindrical structures on callus surface. These embryo structures were visible with naked eye. The effectiveness of PGRs and their levels were optimized by scoring callus induction percentage. The ineffective PGR treatments were discontinued.

Somatic embryo differentiation and proliferation

Embryogenic callus (about 50 mg) was cultured on MS amended with 2, 4-D (0.5, 1.0 and 2.0 mg/l), NAA (0.5, 1.0 and 2.0 mg/l) and BAP (0.5, 1.0 and 2.0 mg/l) separately for embryo differentiation and proliferation. In other experiments, NAA (0.25, 0.5 mg/l) in combination with BAP (1.0, 2.0 mg/l) were also used. The embryo numbers increased with time and these cultures were maintained by regular sub-culturing at interval of 3-4 weeks. The callus showing embryogenesis (the percentage) and embryo numbers (number/callus mass) were recorded at periodic intervals.

Maturation of embryos

The developed embryos on callus were placed on a medium, containing all the essential components of MS, vitamins, inositol but without any PGRs for maturation of embryos. In other experiments, the MS was additionally added with abscisic acid (ABA) at 0.25, 0.5, 1.0 mg/l and gibberellic acid (GA_3) at 0.25, 0.5, 1.0 mg/l. In both GA_3 and ABA added medium, the somatic embryos transformed into green and elongated structures (maturing embryos), which germinated into plantlets later. The percentage of embryos showing maturation was recorded.

Embryo germination and plant recovery

Apparently green somatic embryos were separated and cultured on medium, added with various concentrations of BAP (0.5, 1.0, 2.0 mg/l) either alone or with NAA (0.25, 0.5 mg/l). In another set of experiments, the embryos were cultured on maturation medium for germination in which best maturation had noticed. A separate control medium (without PGR) was also prepared to make the comparison. The number of somatic embryos germinated and developed into plantlets (root and epicotyl development) were recorded. As the germinated plants had very little roots, plants were incubated in root promoting PGRs like Indole-3-butyric acid (IBA). Roots were formed at variable numbers soon after IBA treatment. Embryo regenerated plantlets were transferred to 250 ml conical flask containing ½ MS + 3% sucrose but without PGR and kept for a week for acclimatization.

Transplantation of plants

Finally, the plantlets were removed from the conical flasks, were transferred to small coffee cups filled with sterilized soil rite and sand (1:1), and wrapped with pored polythene bags for maintaining humidity. Plants were later transplanted to plastic pots (15 cm), kept for 3 weeks in incubation at room temperature of 25±2°C with 16 h photoperiod, provided by cool white fluorescent illumination. Finally, plants were transferred to plastic pots (15 cm) filled with soilrite and sand (1:1) in outdoor condition.

2C DNA and flow cytometry

Corm derived gladiolus plants, grown naturally in garden and somatic embryo regenerated plants were used for 2C DNA determination study. Leaf samples from both sources were processed according to the Dolezel *et al*. (2007) method. About 1.0 cm² of *Gladiolus* young leaf and *Pongamia pinnata*, 2C= 2.51 pg DNA (in which 2C DNA analysis study was optimized in the laboratories of our University) were harvested the day of analysis and chopped in 0.5 ml Otto I buffer (0.3% citric acid monohydrate, 0.05% NP-40), 50 µM propidium iodide and 100 µM RNAse (Sigma-Aldrich, USA) following Choudhury *et al.* (2014) method. The homogenate was filtered through a 100-µm nylon filter, and was analyzed by a (CFM) BD FACS Calibur (BD Biosciences, San Jose, CA, USA) flow cytometer. The 2C nuclear DNA level of this experimental material was determined as follows:

$$\text{2C DNA of } G.\,hybridus = \frac{2.51 \times \text{Mean position of } \frac{G0}{G1} \text{ peak of } G.\,hybridus}{\text{Mean position of G0/G1 peak of } P.\ pinnata}$$

Statistical analysis

The data on the effects of PGRs on callus induction, embryogenesis and embryo numbers were analysed and expressed as mean ± standard error. Each set of experiments consisted of three replicates and each experiment was repeated twice. The flow cytometry experiment was performed by randomly selecting regenerated plants. The presented mean values were separated using Duncan's Multiple Range Tests at $p \leq 0.05$.

3. Results

Induction of callus and callus biomass growth

Corms were used as explant on various auxin amended MS medium; the explant started to swell and callus was formed (Fig. 1 a) form the cut ends later. Three different auxins and their concentrations were tested of which 1.0 mg/l 2, 4-D showed highest callus induction percentage (72.66%), followed by the treatment with 0.5 mg/l 2, 4-D, in which 67.85% of cultured explants callused (Table 1). Other two

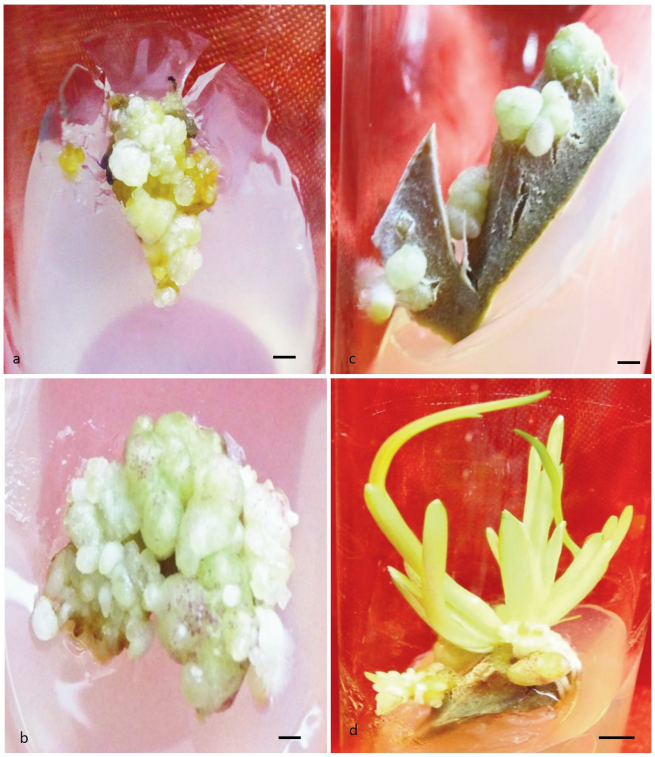

Fig. 1 - a) Corm callus induced in 2,4-D added MS medium; b) Numerous somatic embryos developed on callus; c) Directly induced somatic embryos on corm surface and d) Somatic embryo regenerated plantlets (bars: a-c: 2 mm; d: 0.5 cm).

Table 1 - Callus induction frequency in *Gladiolus*. Corm explants was cultured on MS medium, added with 2, 4-D, IAA, and NAA alone and BAP and NAA in below indicated combinations

2,4-D (mg/l)	IAA (mg/l)	NAA (mg/l)	Callus induction (%)
0.25	0.0	0.0	48.25±4.44 f
0.5	0.0	0.0	67.85±4.65 c
1.0	0.0	0.0	72.66±5.76 b
2.0	0.0	0.0	58.64±3.87 e
0.0	0.25	0.0	0.0 g
0.0	0.5	0.0	0.0 g
0.0	1.0	0.0	0.0 g
0.0	2.0	0.0	0.0 g
0.0	0.0	0.25	0.0 g
0.0	0.0	0.5	0.0 g
0.0	0.0	1.0	0.0 g
0.0	0.0	2.0	0.0 g
	BAP (mg/l)		
	0.5	0.5	75.12±5.55 a
	1.0	0.5	63.16±3.89 d
	0.5	1.0	64.66±4.85 d

Values are expressed as means ± standard errors of three replicates of two experiments; within each column means followed by the same letter are not significantly different at p≤0.05 according to Duncan's Multiple Range Test (DMRT).

auxins i.e. NAA and IAA were observed to be non-responsive when used alone. With BAP (0.5 mg/l), NAA was very active in producing callus, maximum efficient concentration being 0.5 mg/l NAA, followed by 1.0 mg/l NAA. Corm induced callus was cultured on a variety of 2,4-D added concentrations (0.25, 0.5, 1.0 and 2.0 mg/l) for fast growth of callus and the moderate level was observed to be very efficient in producing good callus biomass growth. At the end of 12th weeks culture, 1.12 g of callus biomass was observed in 1.0 mg/l 2,4-D added medium (Fig. 2), next effective concentration was 0.5 mg/l 2,4-D in which callus biomass was 0.91 g, little more of biomass (0.84 g) recorded at 2.0 mg/l 2,4-D.

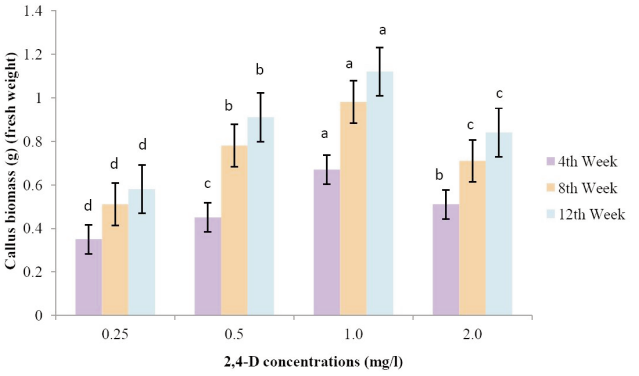

Fig. 2 - Callus biomass growth on MS medium, added with various concentrations of 2, 4-D. Values are means ± standard deviation of three replicates. Within each bar, means followed by the letter, are not significantly different at p≤0.05 according to DMRT.

Direct and indirect somatic embryogenesis

In order to study plant regeneration, hard compact callus was placed on a variety of PGR amended medium and within a few weeks of incubation, callus started to differentiate and produced somatic embryos at variable numbers (Fig. 1 b). Three different concentrations were tested, of which 1.0 mg/l 2, 4-D was observed to be very efficient in which 79.13 percentage embryogenesis with an average of 5.34 embryos/callus mass was noted. Equally effective 2,4-D concentration was 0.5 mg/l that showed good embryo numbers and embryogenesis percentage (Table 2). Unlike 2,4-D, NAA - another important auxin was noted to be inactive in inducing embryos. BAP, a member of cytokinin, was however, noted to be active in inducing embryo, although low in number with lower rate, most efficient concentration was 0.5 mg/l BAP in which 15.45 embryogenesis percentage and 2.93 embryo numbers were noted. The addition of NAA in BAP amended medium improved embryo numbers and embryogenesis percentage. The best treatment identified was 0.5 mg/l NAA and 2.0 mg/l BAP, which showed 69.18 embryogenesis percentage with 12.13 embryo numbers; next best treatment was 0.25 mg/l NAA and 1.0 mg/l BAP in which a mean of 7.99 embryo number/callus mass was noticed. The numbers of embryos however, increased with time. Beside callus induced embryos, direct embryo formation on corm surface (Fig. 1 c) was also noticed at lower frequency. The embryos were roundish, oval, elliptical in structure, often had white cotyledonary apex with yellowish radicle primordia at other ends.

Table 2 - Embryogenesis percentage and somatic embryo numbers in Gladiolus callus, MS was amended with below indicated PGR concentrations and combinations. Data were scored after 4 weeks of culture

2,4-D (mg/l)	NAA (mg/l)	BAP (mg/l)	Embryogenesis (%)	Embryo numbers/ callus mass
0	0.5	0	0.0 g	0.0 h
0	1	0	0.0 g	0.0 h
0	2	0	0.0 g	0.0 h
0	0	0.5	15.45±2.12 e	2.93±0.79 f
0	0	1	14.61±2.42 e	2.38±0.16 f
0	0	2	8.66±1.73 f	1.96±0.10 g
0	0.25	1	80.15±6.66 a	7.99±1.21 b
0	0.5	1	70.12±5.22 c	6.46±1.76 c
0	0.5	2	69.18±3.44 c	12.13±2.28 a
0.5	0	0	75.42±6.43 b	5.32±0.87 d
1	0	0	79.13±5.38 a	5.34±2.10 d
2	0	0	39.33±3.58 d	3.13±1.06 e

Values are expressed as means ± standard errors of three replicates of two experiments; within each column means followed by the same letter are not significantly different at p≤0.05 according to Duncan's Multiple Range Test (DMRT).

Somatic embryo maturation and germination, and the role of various PGRs

Embryos were cultured on medium amended with GA_3 and ABA at different concentrations for embryo maturation. The application of GA_3 was more efficient in comparison with ABA treatment (Table 3). In 0.5 mg/l GA_3 amended medium, 54.55% embryo maturation was noticed after 4 weeks, which increased to 62.15% after 8 of incubation. At the same level of ABA (0.5 mg/l), the embryo maturation percentage was low (18.75%) and with culture time the maturation percentage marginally improved i.e. 20.15% was noted after 8th weeks. Higher concentration of GA_3 and ABA reduced embryo maturation frequency quite sharply.

Well matured somatic embryos, grew well in maturation medium but the germination frequency was low. The best maturation conditions i.e. the medium amended with 0.5 mg/l GA_3 and 0.5 mg/l ABA

Table 3 - Influence of GA3 and ABA on somatic embryo maturation in Gladiolus. Data were scored after 4th and 8th weeks of culture

MS+ PGR (mg/l)	Maturation after 4 weeks (%)	Maturation after 8 weeks (%)
Control	4.21 f	6.30 f
GA_3 (mg/l)		
0.25	30.65±2.11 c	38.60±3.33 c
0.5	54.55±3.88 a	62.15±4.87 a
1	42.62±2.66 b	48.62±4.88 b
ABA (mg/l)		
0.25	12.15±1.86 e	16.65±2.11 e
0.5	18.75±2.01 d	20.15±1.89 d
1	13.12±1.12 e	15.34±1.78 e

Values are expressed as means ± standard errors of three replicates of two experiments; within each column means followed by the same letter are not significantly different at p≤0.05 according to Duncan's Multiple Range Test (DMRT).

showed very poor level of embryo germination (Table 4) i.e. 8.50 and 4.84% respectively. So other PGR combinations were tested for better and early plantlets recovery from somatic embryos. Three different concentrations of BAP were added in MS and the germination ability was observed (Table 4). In 0.5 mg/l BAP amended medium the maximum somatic embryo germination percentage (42.65%) was noticed, followed by a treatment with 1.0 mg/l BAP (31.85%), while higher level (2.0 mg/l) declined somatic embryo germination (18.16%) rate. The optimized BAP (0.5 mg/l) level with NAA was also similarly tested to improve embryo germination and a moderate germination percentage (33.25-33.25%) was noted in those PGR treatments. The somatic embryo regenerated plantlets (Fig. 1 d) grew well in culture, but did not have strong root systems always, so a medium amended with root promoting PGRs were added. Although in almost all treatments, roots were formed at variable numbers, IBA (0.5, 1.0, 2.0 mg/l) was observed to be very effective in inducing roots (data not shown). The plants were finally transferred to outdoor conditions.

Table 4 - Somatic embryo germination in various PGRs added medium in Gladiolus. Data were scored after 6 weeks of culture

MS+ PGR (mg/l)		germination (%)
Control		0.0 g
GA_3 (mg/l)		
0.5		8.50±0.89 e
1		6.66±1.11 f
ABA (mg/l)		
0.5		4.84±1.02 g
1		4.68±1.82 g
BAP (mg/l)		
0.5		42.65±2.67 a
1		31.85±1.89 c
2		18.16±1.22 d
BAP	NAP	
0.5	0.25	33.25±2.23 b
0.2	0.25	31.66±2.24 c

Values are expressed as means ± standard errors of three replicates of two experiments; within each column means followed by the same letter are not significantly different at p≤0.05 according to Duncan's Multiple Range Test (DMRT).

Genome size analysis of gladiolus

For the analysis of genome size, the 2C DNA content of somatic embryo regenerated plantlets was measured and was compared with naturally grown gladiolus using flow cytometric method. Nuclear homogenate of juvenile leaves from naturally grown and somatic embryo regenerated plants were used for flow cytometry and the obtained results are presented in figure 3. The somatic embryo regenerated plant of gladiolus showed 2C DNA content of 1.34 pg while corm grown plant obtained naturally had 2C DNA content of 1.29 pg. The 2C nuclear DNA content values of both the two sources are nearly the same that suggests no major alteration in genome size in embryo regenerated plants when compared with corm derived gladiolus. The genetic stability is thus maintained in the somatic embryo regenerated population.

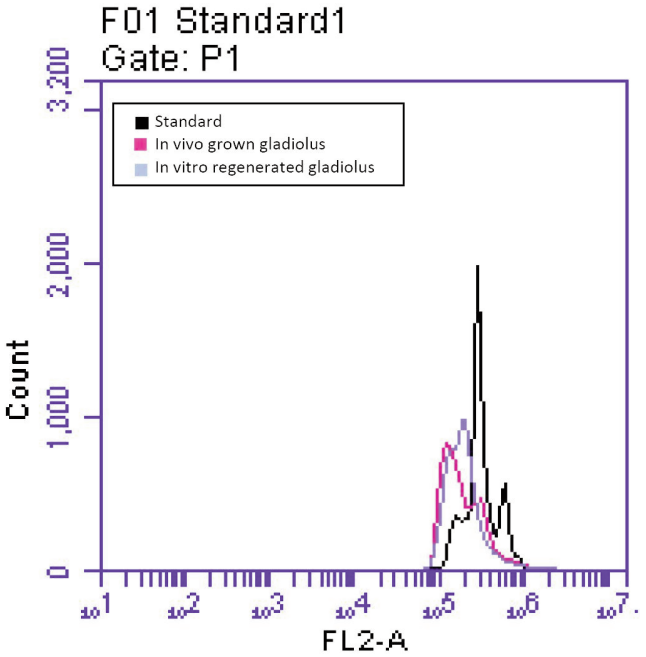

Fig. 3 - Histogram of relative 2C DNA content of corm grown (field) and somatic embryo regenerated gladiolus with standard *Pongamia pinnata* plant.

4. Discussion and Conclusions

In the present study, plant regeneration through somatic embryogenesis and 2C DNA content of regenerated plant were investigated in *Gladiolus*. Callus was first induced from corm on 2, 4-D added MS in which low to moderate doses were observed to be very effective. The amendment of auxins especially 2, 4-D was found very active in inducing callus in a number of investigated plants including gladiolus (Kamo and Joung, 2007; Su *et al.,* 2009; Feher, 2015). On 2, 4-D added medium, embryos were differentiated on callus, and thus the origin of embryos are indirect in gladiolus. This callus mediated embryo induction incidence has been noted in a number of investigated ornamentals of horticultural importance (Jiang and Zhang, 2007; Gow *et al.,* 2009).The process of in vitro embryogenesis is often controlled by a variety of PGRs and its analogues. The impact of 2, 4-D in inducing callus and somatic embryos are reported widely in literature (Gaj, 2004; Feher, 2015). At lower level, 2, 4-D activates *yucca* (*YUC)* gene, which helps in synthesizing IAA and its transport in developing embryos in cultivated tissues (Su *et al.,* 2009; Bai *et al.,* 2013). The application of 2, 4-D also induces stress in culture, which provokes vegetative cells to acquire embryogenic state (Gliwicka *et al.,* 2013), although the exact molecular mechanism influencing this transition is still not understood clearly.

Although the embryo formation was more frequent on 2, 4-D added medium, the addition of NAA and BAP at various levels was noted to be equally responsive in inducing callus and in promoting embryo numbers in gladiolus, this is in agreement with Stefaniak (1994) findings where NAA was identified to be more effective in inducing embryos compared to other auxins tested. The same level of NAA with BAP was earlier reported to be very effective in a number of bulbous/ tuberous ornamental monocots (Mujib *et al.,* 2006; 2008). Similar use of NAA with BAP was noted to be very beneficial for shoot formation in gladiolus, both during direct organogenesis and callus mediated regeneration (Ascough *et al.,* 2009; Memon, 2012). Wu *et al.* (2015) obtained globular somatic embryos on embryogenic callus on 3.0 mg/l TDZ and 0.2 mg/l BA amended MS without any requirement of NAA. Yelda and Bengi (2007) earlier reported somatic embryo induction on 0.1 mg/l BAP added MS medium, very similar to BAP induced embryo induction noted in our observations. Thus, for acquiring somatic embryo, the cytokinin signalling is also equally important, often sufficient as against common perception of auxins, which induce embryos in culture (Mujib *et al.,* 2016). Cytokinin induced somatic embryo formation has been observed in a number of plants and was reported (Iantcheva *et al.,* 1999). Although the molecular role of cytokinin in triggering *in vitro* embryogenesis is not fully elucidated, cytokinins induced *Wuschel* (*WUS*) gene expression and transcription factor synthesis at early embryogenesis and shoot apical meristem development time were reported in several studied organisms (Su *et al.,* 2009; Gordon *et al.,* 2009; Wang and Chong, 2016). The clear demarcation and establishment of shoot and root apical meristem are important steps in embryogenesis/somatic embryogenesis (Scheres, 2007). Although the role of auxin and auxin-cytokinin interaction in determining shoot apical meristem is known, cytokinin mediated root apical meristem development needs further investigation. Su *et al.* (2012) however, indicated that cytokinin induces the synthesis of *Arabidopsis response regulator*, ARR7 and ARR15, the two essential transcription factors, for root apical meristem development during somatic embryogenesis.

Induced embryos did not grow fast therefore other PGR treatments were added to improve embryo quality, which facilitates *in vitro* plant regeneration. In the present study, various concentrations of GA_3 and ABA were used, both influenced embryo maturation but the role of GA_3 was more profound compared to ABA. The observation is very similar to Vieitez findings (1995) in which poor role of ABA on embryo maturation was noted. ABA induced improved embryo maturation was, however, reported in other groups of plants including model *Arabidopsis* (Maruyama *et al.,* 2007; Bai *et al.,* 2013). In contrast, the incorporation of GA_3 facilitated embryo maturation by growing embryo size and by synthesizing more chlorophyll, which help in building protein, lipid, triglycerides and other energy reserves necessary during seed germination (Santos-Mendoza *et al.,* 2008). The combination of GA_3 and ABA has also been reported to activate *leafy cotyledon, LEC* and *FUS3* gene expression, help making energy reserves essential for seed/embryo germination (Braybook and Harada, 2008). Embryo maturation and germination steps are both very important in somatic embryo based plant propagation. Mature somatic embryos were transferred to a medium primarily amended with BAP or with NAA for plantlet formation. Within a few days, leaflets were coming out from coleoptiles and later roots were developed. The use of BAP alone or with NAA was earlier noted

to be very effective for conversion of plantlets from somatic embryos in other groups of plants (Mujib *et al.,* 2013). The regenerated shoots were transferred to IBA added medium for better induction of roots as the embryo derived shoots had with less developed roots. IBA promptly induced roots in gladiolus, like many other investigated materials (Mujib *et al.,* 2008). In gladiolus, it appears therefore that various PGRs control *in vitro* embryogenesis and plant regeneration and their requirement varies considerably. The embryo forming ability from callus in gladiolus was high and reproducible, which could efficiently be exploited in developing transgenics.

The plants obtained from callus through organogenesis and embryogenesis demonstrate genetic variation and these changes are identified by traditional and modern techniques (Das *et al.,* 2013). Here, in *G. hybridus*, plants were developed from callus via somatic embryogenesis pathway; the callus and the embryos are influenced by PGRs and stresses, both affect cell cycle/DNA synthesis. Thus, a study of regenerated plant status was indeed necessary. We analysed 2C DNA content in gladiolus plants, derived from corm and somatic embryo by flow cytometry. The level of 2C DNA of somatic embryo regenerated and corm obtained plants are the same in gladiolus. The amount of nuclear DNA was unchanged and the genetic fidelity was maintained. Due to its precision, the flow cytometric technique has widely been used in genetic stability studies (2C DNA analysis) in a number of investigated materials including *in vitro* regenerated plants (Loureiro *et al.,* 2007; Sliwinska and Thiem, 2007). The *in vitro* cultural condition, however, cause genetic irregularities in cultivating tissues (Giorgetti *et al.,* 2011); using flow cytometry this alteration can easily be identified (Dolezel *et al.,* 2007). Despite of the large number of applications and its ease, the 2C-DNA estimation has not been conducted widely, rather restricted to only about 2% of the flowering angiosperms (Galbraith, 2009). Thus, any attempt with obtained 2C value, even from *in vitro* raised culture, will significantly enrich C-value database in angiosperm. Similarly, the development of embryogenesis protocols discussed here can also be used in preparing synthetic seeds for clonal propagation and for conservation of important gladiolus germplasm.

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