Regeneration Response from Old Cell Suspension Cultures of Gladiolus

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Abstract

Conditions to obtain plantlets from older (than 36 months) cell suspension cultures (CSCs) of four *Gladiolus* cultivars ('Friendship', 'Peter Pears', 'Victor Borge' and 'Novalux') were optimized. To achieve this, slices of immature cormels were used as explants from which friable callus were produced *in vitro* on Murashige and Skoog (MS) basal medium containing 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzyleadenine (BA). CSCs were proliferated from this friable callus in the same medium, but in liquid form, i.e., free of gelling agent. Viable cells were visible under a microscope one week after the initiation of CSCs. At the initial stages of CSCs, two types of cells were noted: Type I (elongated, vacuolated cells with no starch content) and Type II (round and small compared to Type I cells). Plantlets were regenerated from the callus of all cultivars after 36 months the exception of 'Novalux'. A 15-30% packed cell volume of callus was ideal to obtain plantlet regeneration. Buds of newly regenerated plantlets transferred to cormlet induction medium (CIM), growth medium without hormone supplementations, elongated and proliferated but, when sub-cultured onto CIM containing 6% sucrose, only cormlets proliferated, the most prolific period being during the first 4-6 weeks after inoculation of buds onto CIM. 85-95% of cormlets germinated after breaking dormancy for 8 weeks at 4°C.

Keywords: Gladiolus, Callus, Cell suspension, Cormlet, Plantlet Regeneration

Abbreviations: CSC—cell suspension culture; MS—Murashige and Skoog; PCV—packed cell volume

1. Introduction

Gladiolus (genus *Gladiolus*; Iridaceae) is an economically important flowering plant. Many species of this genus are found in South Africa, Tropical Africa, Madagascar and Eurasia with modern *Gladiolus* considered to have been bred originally from only six species (Lewis *et al.* 1972). The current number of species in this genus is around 255 (Goldblatt and Manning 1999). Coetzee (2002) stated that the Netherlands earns more from South African flowers than South Africa earns from its gold. *In vitro* plant production technologies can be exploited to produce large numbers of valuable plant material within a comparatively short time span to produce disease-free germplasm, Pragya, *et al*. (2012) narrated that flower industry has become a part of the modern agriculture. With the improvement and living standards of the society, demand of the flowers grows and becomes the indispensable consumer goods in social life and daily life. Gladiolus callus and cell suspension cultures (CSCs) raised in the laboratory for mass propagation or for genetic

modification experiments usually lose their regeneration potential (i.e., totipotency) after only a few weeks. Thus, the present study aimed to establish an efficient method to enhance and maintain the regeneration potential of CSCs for a longer time-span and yield more plantlets

In *Gladiolus*, CSCs can be initiated from friable callus, which is soft and highly embryogenic. Regeneration through somatic embryogenesis has two main advantages: single cell origin and high rate of regeneration even in very old cultures. Innumerable useful variants coexist in a cell population that could theoretically be screened *in vitro*. Monocots used to be relatively difficult to manipulate in culture, although there has been much progress, particularly with crops of agronomic significance and ornamentals. Attempts have been made to establishment of cell-suspension, which regenerate plantlets. CSCs have been achieved in *Gladiolus* (Kamo *et al.* 1990; Remotti 1995). *In vitro* propagation has been reported for several *Gladiolus* cultivars (Simonsen and Hildebrand 1971; Wilfret 1971; Hussey 1977a; Ziv 1979; Bajaj *et al.* 1982; Logan and Zettler 1985; Dantu and Bhojwani 1987; Lilliein-Kipnis and Kochba 1987; Steinitz *et al.* 1991; Stefaniak 1994). Cormlet production from nodal buds and cormel tips *in vitro* has also been achieved (Arora *et al.* 1996), or from inflorescence tissue (Ziv *et al.* 1970). In *Gladiolus*, Nasir *et al.* (1996) and Jager *et al.* (1998) initiated callus from mature corm pieces containing buds and subsequently produced plantlets and cormlets from callus. Ahmad *et al.* (2000) were able to multiply cormlets. Pragya *et al.* (2010) reported successful rooting of in vitro produced microshoots of Gladiolus.

2. Materials and Methods

Experimentation was conducted on four cultivars of Gladiolus i.e. Friendship, Peter Pears, Victor Borge and Novalux. For this purpose immature cormels were harvested from plants when the crop was at flowering stage. The cormels were thoroughly washed using a liquid soap. Cormels were dehusked and soaked in 2% solution of Radomil Gold® 68 WP for four hours followed by thorough washing with distilled water. Cormels were further sterilized with 0.1% solution of Mercuric Chloride with one drop of Tween 20 surfactant for 2 minutes and rinsed thrice with distilled water under aseptic conditions (Nasir and Riazuddin 2008). The cormels were then cut into slices and cultured on callusing medium. Different media compositions that were used for various experiments are as follows:

1. For callus and cell suspension MS basal medium contained adenine Sulphate (30 µM), Thiamine HCL (3 µM), NaH_2PO_4 (580 µM), Casine Hydrolysate (1.0 g/l), 6-benzyleadenine (0.5 µM), Sucrose (30 g/l), and 2, 4-Dicholorophenoxy Acetic Acid (2 to 20 µM). Phytagel (2 g/l) was added to callus medium only and cell suspension medium was kept broth.
2. For shoot regeneration MS basal medium was supplemented with Adenine Sulphate (15 µM), Thiamine HCl (1.5 µM), NaH_2PO_4 (300 µM), BA (0.25 to 0.5 µM), and Phytagel (1.5g/l). Filter sterilized Zeatin (0.25 to 1.0 µM) was added after autoclaving sterilization.
3. Sucrose (30, 60 and 90 g/l) and Phytagel (1.5 g/l) were added to MS basal medium and used as cormlet supporting medium.

The pH of all media was adjusted at 5.7 using 1N hydrochloric acid (HCl) and 1N sodium hydroxide (NaOH) before sterilization.

2.1 Callusing, Cell Suspension and Plantlet Regeneration

For cell suspension preparation yellowish-nodular-friable callus was suspended into cell suspension medium in 250 ml Erlenmeyer flasks. One cm^3 piece of callus was used every time and the flasks were fixed into a shaker at 120 revolutions per minute. 10 ml fresh cell suspension medium was added to each flask culture every week until the volume reached 100 ml, then the suspensions were

sieved (through a 100µm pore size mesh) to separate the small cells and cell clumps. The aliquots containing small cells were re-suspended in two 250 ml flasks, each containing 40 ml of old suspension and 10 ml of fresh media. The sieved cell clumps were placed on MS callus medium. The embryogenic cell suspension cultures were found mature when volume of the suspended cells doubled after every fortnight. At this stage sieved cells were converted into cell clumps or small cell aggregates. And the suspension cultures required continuous sieving of these callus clumps. One ml of the sieved suspension was cultured into petri plates with 10 ml agar medium. After 3 weeks, the small cell aggregates and callus clumps developed into friable calli while the suspension cells were developed into small callus colonies. These calli were placed on callus medium for four weeks under dark and the developing calli were placed under defused light (500-600 lux) for another four weeks after culturing on Shoot medium for embryogenesis. All callus cultures like cell suspensions of Novalux were kept under dark; other regeneration cultures were kept under light (approximately 500-1000 lux) for four weeks and then shifted to full light (300-4000 lux, 24 $\pm$2°C), while all cell suspension cultures of Friendship, Peter Pears and Victor Borge were kept under diffused light. All other cultures were kept under 16-hour photoperiod illuminated with TL 40W/54 to maintain 3000-4000 lux. Packed Cell Volume (PCV), the ratio of the volume occupied by packed plant cells to the volume of the whole cell suspension culture medium measured after brief centrifugation, of the suspension culture. The suspended cells were poured into sterilized 50ml falcon tubes, firmly caped and sealed with Para-film. These tubes were fixed into J-20 rotor of the Beckman^{TM} Centrifuge. and thee suspension cultures were centrifuged at 1000g for 10 minutes at 20 °C, and percentage of Packed Cell Volume (PCV) and cell number (using hemocytometer) were calculated as described by Hall (1991). Cell viability of the suspension cells were visualized using Fluoriscein Diascetate/Phenosafranine as discribed by Widholm (1972).

2.2 Acclimatization

The mature *in-vitro* produced cormlets were harvested and stored at 4°C for eight weeks to break dormancy. The germinating cormlets were transplanted in an autoclaved soil mixture, which was composed of silt, sand and leaf mold (1:1:1). Eight to ten cm long plants were transplanted to another media, where sand was omitted and additional 10g of NPK fertilizers (1:1:1) per 10 kg of the soil mixture was added. The germinating cormlets were transplanted in trays and covered with transparent polythene sheets to maintain maintain humidityrequired for cormlet germination and acclimatization. The developing plantlets were transferred to the field, with routine field practices of gladiolus field, in the month of October.

3. Results and Discussion

3.1 Callus Induction and Maintenance

The immature cormel slices of Friendship, Peter Pears, Victor Borge and Novalux when cultured on callus medium with various 2, 4-D concentrations started to swell and turned brown after 12-14 days. The cormel pieces of all the four Gladiolus cultivars responded in the presence of medium containing 0.5 µM BA and 2 µM to 18 µM 2, 4-D. All four cultivars produced friable, nodular, yellowish and embryogenic calli ranging from 16-96% on callus medium containing different concentrations of 2, 4-D. Friendship produced best embryogenic calli on callus medium containing 12-16 µM concentration of 2, 4-D, Peter Pears on 12-16 µM, Victor Borge on 8-12 µM, and Novalux on 14-18 µM (as shown in Figure 1A, 1B, 1C and 1D respectively). Although the best calli of all four cultivars were initiated on various callus medium containing 2, 4-D but medium containing 12, 10, 8 and 12 µM 2, 4-D were found best for maintenance of callus and suspension culture of Friendship, Peter Pears, Victor Borge and Novalux respectively. These media were found best for maintenance of various calli and suspension cultures of all four cultivars for more than three years without significant decrease in their regeneration potential. The direct formation of

embryos in the callus cultures at low frequency (4-12%) in the presence of 2, 4-D was noticed. These embryos also developed roots and emerged only from media containing 2-6 µM 2, 4-D. These embryos developed up to 4-6 weeks and died after sufficient accumulation of 2, 4-D. It was also noticed that these embryos only developed from the shoot buds present on the immature cormel slices (Table 1).

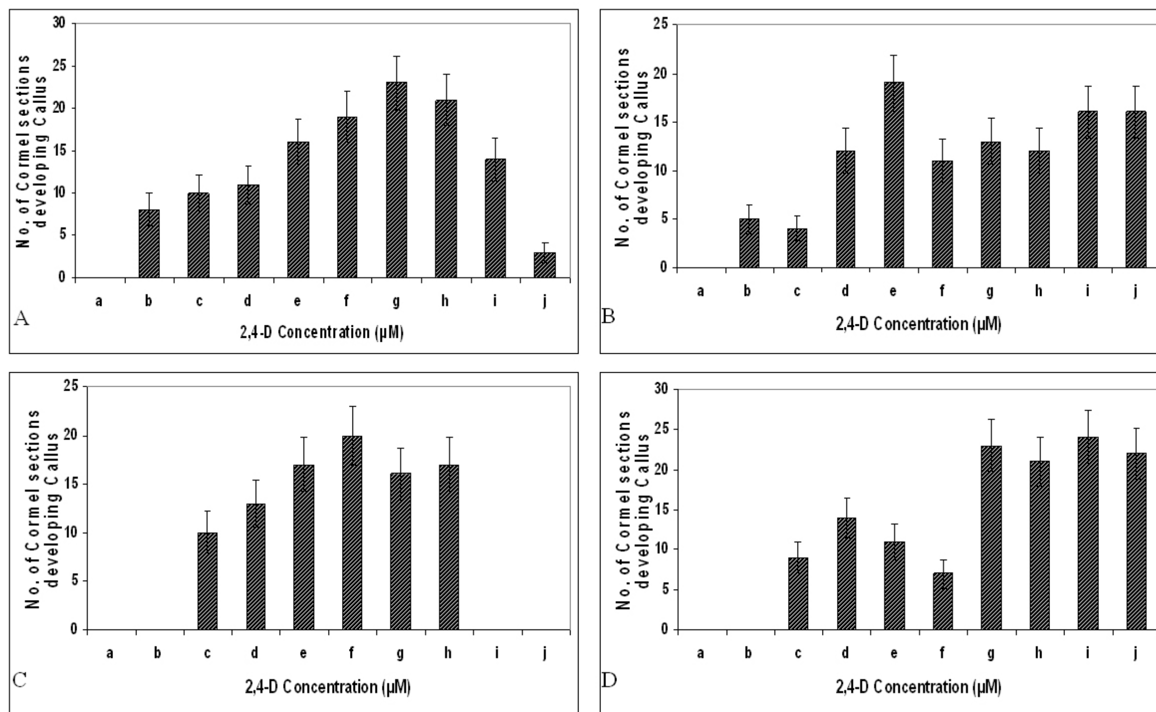

Figure 1. The influence of various concentrations of 2, 4-D on callus formation in Gladiolus Cultivars

A. Friendship B. Peter Pears C. Victor Borge D. Nouvalux

Table 1. Percentage initiation of callus from immature cormel slices

2.4, D Conc. µM	**Frequency of Callus Initiation (%) From Different Tissues**											
	Friendship			Peter Pears			Victor Borge			Novalux		
	Callus	Roots	Shoots	Callus	Roots	Shoots	Callus	Roots	Shoots	Callus	Roots	Shoots
00	0	0	0	0	0	0	0	0	0	0	0	0
02	0	0	0	0	8	8	0	0	0	0	4	4
04	32	4	4	20	0	0	0	8	8	0	12	12
06	40	0	0	16	0	0	40	0	0	36	0	8
08	44	0	0	48	0	0	52	0	0	56	0	0
10	64	0	0	76	0	0	68	0	0	44	0	0
12	76	0	0	44	0	0	80	0	0	28	0	0
14	96	0	0	52	0	0	64	0	0	92	0	0
16	84	0	0	48	0	0	68	0	0	84	0	0
18	56	0	0	64	0	0	0	0	0	96	0	0
20	12	0	0	64	0	0	0	0	0	88	0	0

Note: For each Treatment and Cultivar, 25 Immature Cormel Slices were cultured on five Petri Plates. Data was recorded for shoot emergence, root formation and percentage of callus induced.

Mature Gladiolus cormels have been recognized as suitable source for regenerable callus by many researchers (Kim *et al.* 1988; Kamo *et al.* 1990; Kamo 1994; Stefaniak 1994; Remotti 1995; Nasir *et al.* 1996) but most of the authors stressed that it is easier to obtain embryogenic callus from immature explants than from mature ones (Debergh *et al.* 1990; Hussey 1977a, b). Results of the presented research are in accordance with their findings. It was confirmed that an appropriate concentration of 2, 4-D in combination of BA was necessary for the initiation of friable callus from immature cormels slices; however, its requirements varied from cultivar to cultivar. It has been confirmed by Stefaniak, (1994) that the presence of 2, 4-D in the medium is necessary to induce the embyrogenic potency of Gladiolus. These findings are in contrast to Kamo *et al.* (1990) who reported that Peter Pears produced somatic embryos only on MS medium with NAA, but not with 2, 4-D. These results indicate that it is possible to obtain friable callus (20-95%) and establishment of cell suspension cultures that regenerate plants of the four Gladiolus cultivars including Peter Pears using 2, 4-D with BA in the medium. According to our results and that of Debergh *et al.* (1990) it is evident that development of embyrogenic callus from immature cormels of Gladiolus is of great importance to maintain its embroygenic potential for more than 3 years in cultures. Stefaniak (1994) have been successful to maintain the embryogenic potential of the callus derived from mature corms for more than 2 years. In the present study, it was found that immature cormels could maintain their regeneration potential for 3 years, except Novalux, which lost its regeneration potential by 77.7%, makes it feasible to plan long-term experiments for the biotechnological improvement of this cut flower. These findings are in contrast with Kamo (1995) who reported that theywere not able to consistently induce regenerable callus and the number of plants regenerated from the callus varied throughout the year, but many physiological factors of the plant could have affected regenerable callus induction. For example, the cormels used for callus induction have been in dormancy for various periods of time and under varying temperature regimes. These findings are in agreement with that of Stefaniak (1994) who reported that more than 2-year old callus of Gladiolus retained a very high regeneration capacity. The contradictory findings to that of Kamo (1995) might be due to the difference in the composition of hormones and explant source since they used mature cormels and did not use BA along with 2, 4-D. It was also found that regeneration ability of cell suspension derived calli was increased as compared with primary calli. This increase in the regenerable potential may be attributed to the increase in number of round cells in the suspension cultures. These findings are according to that by Remotti, (1995) who stated that due to differential sedimentation ability of the heavier round embryogenic cells which could be easily separated from the lighter elongated non-embryogenic cells in the cell suspension cultures after 2-3 months.

3.2 Cell Suspension

The friable callus of each cultivar established cell suspensions in their respective medium. It was observed that PCV of Friendship was significantly high as compared with other three cultivars (Figure 2A). One week after initiation of suspension cultures only few cells were released and visualized under microscope. These cells were found viable as assessed with 0.01% final concentration of Fluorescein Diacetate Dye (FDA). At initial stages of suspension cultures, two types of cells were observed. The first cell type consisted of elongated, vacuolated and with no starch contents, these cells disappeared after 4-5 weeks (Figure 3A). The percentage of the elongated cells after two weeks was 75-80 of the total cells. The second type was round in shape and small in size compared to first type of cells. The viable round cells after two weeks were 20-25%. These cells contained dense cytoplasm, apparent nucleus and were rich in starch and plastid. These cells were found highly embryogenic when compared with primary callus. After 6-8 weeks these cells increased in number and after 16 weeks only round cells were found in the suspension cultures (Figure 3B). The PCV of the suspended cells increased up to 8 months and then the PCV started decreasing without sieving even after addition of fresh medium. The PCV was effectively maintained by regular addition of fresh medium and frequent sieving to remove old callus clumps.

After eight weeks of suspension initiation, cell aggregates and callus clumps increased in number. The degeneration process of these callus clumps started after exposure in prolonged suspension cultures without sieving. Cell suspension cultures of Novalux released excessive amount of phenolic compounds as compared with other cultivars. These resulted in frequent degeneration of the suspension cultures of Novalux. This problem was overcome by regular addition of fresh medium and frequent sieving to remove dead callus clumps.

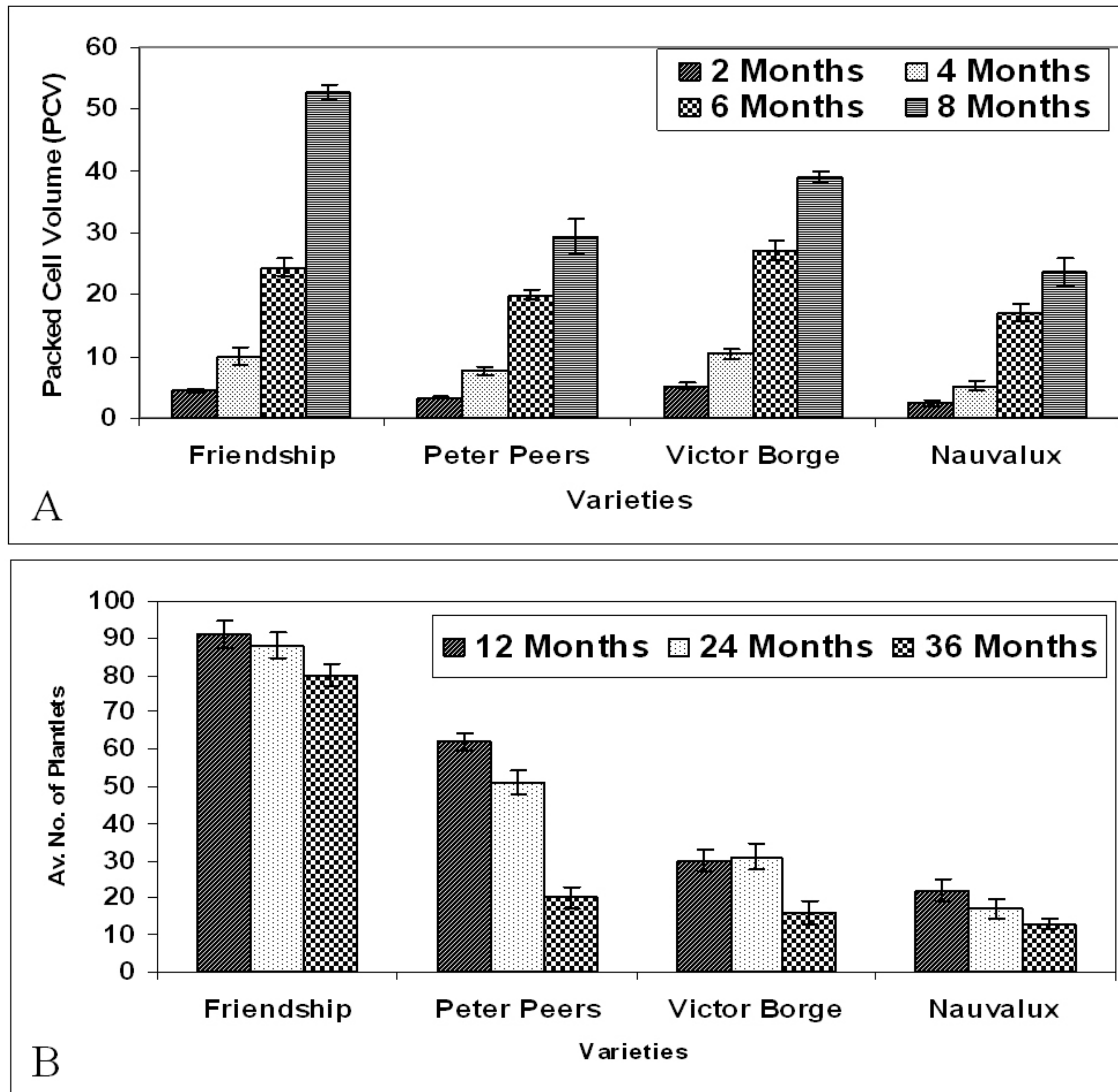

Figure 2. A. Packed Cell Volume (PCV) of the four Gladiolus Cultivars.
B. Regeneration Response of Calli of four Cultivars derived from Immature cormel slices

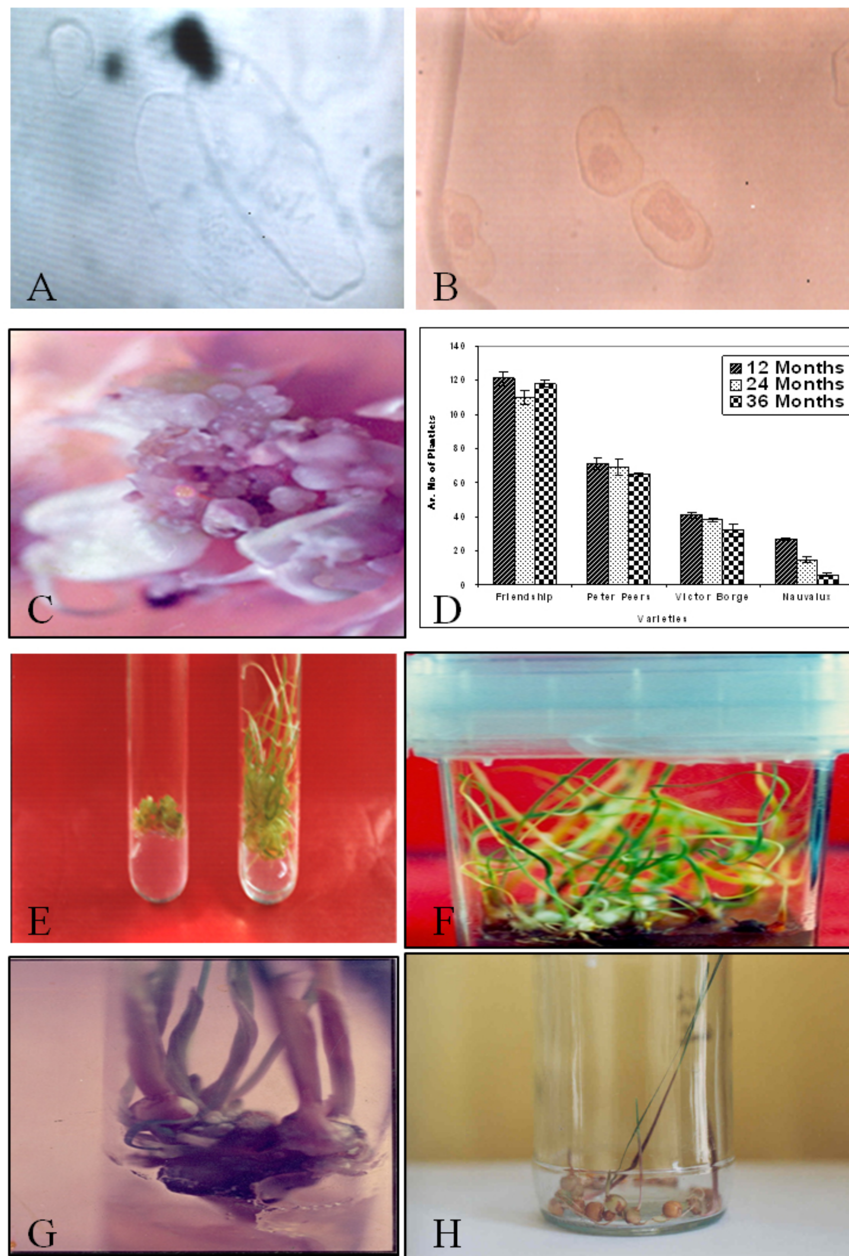

Figure 3. A. Elongated cells found at initial stage of suspension culture;

B. Uniform cells round in shape, capable to regenerate;

C. Embryogenesis and regeneration of plantlets from the calli derived from immature cormel slices;

D. Regeneration response of calli of four cultivars derived from immature cormel slices**;**

E. Direct high frequency *in-vitro* production of cormlets form gladiolus calli;

F & G. *In-vitro* initiation of cormlets from gladiolus plantlets;

H. Germination of in-vitro produced cormlets.

Shillito *et al.* (1989) have reported initiation of friable type of calli in maize from immature embryos at a low frequency (0.2%). Friable callus of Gladiolus has been shown to respond like type II callus, as described for cereals and grasses. Suspension cultures started with this type of callus had 1) round cells and 2) elongated cells. A similar type of cells in the cell suspension cultures has been reported (Vasil, and Vasil, 1981; Zaghmout, and Torello, 1989; Remotti, 1995). We noticed that round type of cells were able to multiply in the cell suspension and maintained their embryogenic potential for more than three years, while the elongated cells were unable to survive for longer period in the same suspension conditions. These findings are in agreement with that of Remotti, (1995), he stated that friable callus of Gladiolus did not always release embryogenic cells. It was possible to enhance the chances of long term cultures by selecting and enriching for embryogenic cell. Frequent sub-culturing, periodic sieving through 100µm pore size mesh and removal of lighter floating non-embryogenic cells led to dominate heavier round embryogenic cells in the cell suspension cultures.

It was found that round embryogenic cells contained small vacuols, a rich cytoplasm, starch, their shapes and contents varied from cultivar to cultivar. These findings correlate with the results of Schwendiman *et al.* (1988) who observed that presence of starch is often related to embryogenic cells and it is generally considered to be an indicator of development towards somatic embryos.

We succeeded to maintain the cell proliferation and regeneration capability of cell suspension cultures for more than 3 years by regular sieving, addition of fresh medium as well as measurement and maintenance of PCV (figure 2B). These results are comparable with the findings of (Nielsen and Knudsen, 1993) on Kentucky Blue Grass, and (Remotti, 1995) on Gladiolus, in that the mature suspension cultures did not have a distinct lag phase.

3.3 Plantlet Regeneration

Plantlets were regenerated from all four cultivars of immature cormel slices went through a callus phase (Figure 3C). After three years plantlet regeneration ability of Friendship, Peter Pears, Victor Borge was least effected, whereas, Novalux lost its 77.70 % regeneration ability after 3 years. Other three cultivars maintained their regeneration potential even after 36 months but the regeneration ability of Friendship was significantly high as compared with other three cultivars (Figure 3D). The combination of auxins that resulted in optimal plant regeneration differs for each cultivar. All callus types of Friendship regenerated highest number of plants as compared with Peter Pears, Victor Borge and Novalux. The calli derived from cell suspension with 15-30% PCV were found highly embryogenic for regeneration of highest number of plantlets (Figure 3C&D). Longitudinal structures about 1 mm in length were formed within 3-4 weeks in all types of calli. Calli proliferated quickly during the process of embryogenesis under defused light and after 6-8 weeks the rate of further proliferation decreased significantly, however, the plantlet regeneration process continued and new plantlets were initiating depending upon the availability of fresh medium. Plantlet regeneration from callus of Gladiolus on MS-medium with various growth regulators has been reported by many researchers (Kamo, 1994; Stefaniak, 1994). Remotti, and Löffler, (1995) reported NAA as effective at inducing regenerable callus from corm slices of Gladiolus as 2, 4-D or Picloram. Emek and Erdag (2007) reported somatic embryogenesis from leaf explants of Gladiolus. In the present study, we observed that plantlet regeneration from friable type of calli could be achieved at high frequency in the presence of BA and Zeatin at various concentrations and combinations, depending upon the requirements of the cultivars. It has been reported that optimum concentration of BA required for shoot proliferation varies from cultivars to cultivars (Hussey 1977a; Dantu and Bhojwani 1987; Grewal *et al.* 1990; Piriyakumari and Sheela 2005). Erdag *et al.* (2009) reported *in vitro* somatic embryogenesis from cormel-derived callus cultures of gladiolus. This may be attributed to difference of endogenous level of hormones among various Gladiolus cultivars. Developing shoots produced on Shoot induction medium were separated and cultured on hormones free MS-basal medium, showed elongation and roots were also formed. The individual

shoots along with sufficient roots were transferred to soil, but survival was difficult even under controlled conditions. Dantu, and Bhojwani, (1987) also observed that shoot elongation occurred only when cytokinins were omitted or reduced to low level. They also reported that transplantation and establishment of *in-vitro* produced Gladiolus plantlets is not always easy. In addition, Lilliein-Kipinis and Kochba (1987) reported serious problems in acclimatization of *Gladiolus* plantlets.

3.4 *In-Vitro* Cormlet Production

As better survival of plantlets in soil is of ultimate importance, efforts were mainly focused on the production of *in-vitro* cormlets, which showed higher survival rate in the soil under open field conditions. The frequency and weight of all cormlets showed identical response in the presence of hormone free medium containing 6% sucrose. The developing shoots of all cultivars were transferred to the medium containing 3-9% sucrose, when the plantlet height was about 4-6 cm in length. Each shoot started growing and developed normal root system. Freshly regenerated plantlets from all calli, when transferred to the hormone free medium containing 3% sucrose, started elongation and proliferation of buds (Figure 3F) whereas, after sub-culturing of growing plantlets in the hormone free medium containing 6% sucrose cormlet development was significantly increased (Figure 3G). It was also noted that shifting of three week old developing embryogenic calli of longitudinal structures ionto cormlet formation media with 6% sucrose proliferated directly into cormlets (Figure 3E). Calli proliferated quickly during the process of embryogenesis under diffused light and after 6-8 weeks the highest frequency of proliferation of plantlets was noted during the first 4-6 weeks of shifting from regeneration medium (containing hormone) to the cormlet induction medium (hormone free). Sucrose concentration of 6% in the hormone free medium was found best for healthier cormlet production (Figure 3G). The higher sucrose level more than 6% did not further improve the cormlet size, whereas, lower sucrose level than 6% led to smaller cormlets. The cormlets showed 85-95% germination after breaking dormancy at 4^0C for 8 weeks (Figure 3H). Ziv *et al.* (1970) reported the poor survival of *in-vitro* produced plantlets of *Gladiolus*.

We noticed that Gladiolus shoots produce in the medium containing cytokinins under *in-vitro* conditions could be effectively stimulated to produce cormlets in the respective medium containing various sucrose concentrations. The 6% sucrose concentration was found suitable for production of healthier cormlets. Dantu and Bhojwani (1987); Grewal *et al.* (1990) reported that high sucrose level at 9-12 % did not improve the cormlet size whereas lowering the level of sucrose to 3 %led to smaller cormlets. *In-vitro* produced cormlets showed upto 98% germination in the field conditions after 8 weeks of storage at 4^oC for breaking cormlet dormancy. Ahmad *et al.* (2000) have achieved 90-95% germination of cormlets under soil conditions.

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