

**STUDIES ON SOMATIC EMBRYOGENESIS AND PRODUCTION
OF SYNTHETIC SEEDS IN *GLADIOLUS* CULTIVARS**

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STUDIES ON SOMATIC EMBRYOGENESIS AND PRODUCTION OF SYNTHETIC SEEDS IN *GLADIOLUS* CULTIVARS

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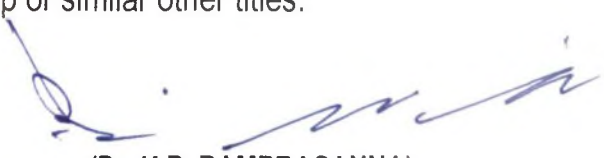
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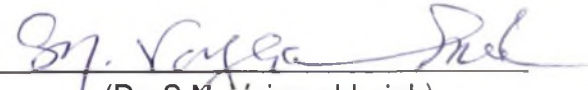
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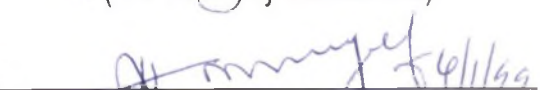

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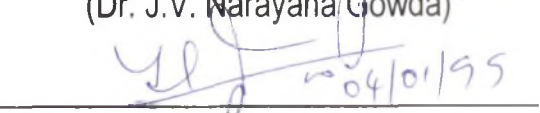
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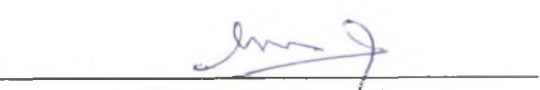
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INTRODUCTION

I. INTRODUCTION

The technique of plant cell, tissue and organ culture, collectively called plant tissue culture, has progressed from a remote possibility to a fast expanding reality in the areas of both plant propagation and crop improvement. The concept of 'totipotency', proposed by Haberlandt (1902), was successfully tested experimentally by obtaining whole plants from single cells *in vitro*. This was achieved in stages starting from excised root cultures, meristems, callus and single cell cultures. While callus, cell and protoplast cultures have served as the basic tools in genetic engineering and plant breeding, the culture of meristems and other *in vitro* methods have wider applications in propagation of disease free plants and germplasm conservation.

The culture of embryo, ovary and ovule as well as *in vitro* pollination and fertilization are recognized as potential techniques to overcome morphological and physiological sterility and incompatibility problems in plant breeding. Haploids derived from anthers, isolated microspores and protoplast - culture under *in vitro*, have served as the basic tools in genetic engineering. The other notable contribution from the field of plant tissue culture towards crop improvement is the isolation, culture and fusion of single cells or protoplasts which have their special significance in cell modification and somatic hybridization.

The contributions of tissue culture technology to the field of plant propagation are especially notable. The first success by Morel and Martin (1952) in obtaining virus free dahlias through meristem culture and then the discovery of the regulatory

mechanism of auxins and cytokinins in organ formation by Skoog and Miller (1957) and later, to achievements of Morel (1960) in mericlone orchids laid a firm foundation to commercialization of the tissue culture technology. Today, this technology has emerged as a viable alternative to the conventional vegetative propagation with definite and indispensable advantages.

Meristem culture was one of the first and is still a most widely practiced procedure in commercial propagation of ornamentals. The organized adventitious shoots arising from meristem culture have distinct advantages, as they have generally shown satisfactory clonal stability among regenerates. However, because shoots must be separated normally from the parental tissue, a process which involves decision making, it often proves unsuitable for rapid mechanical subculture or harvest, resulting in slowing down to some extent the process and increasing the cost of production. Therefore, callus and suspension cultures, which lend themselves readily to automated subculture, and may be induced to regenerate plants by either organogenesis or embryogenesis, have gained increased importance. Although, somaclonal variations often reported in some crop plants impose restrictions, yet the process will be a boon in a large number of horticultural crops which exhibit a high level of genetic stability. Regeneration of plants through somatic embryogenesis in certain species has direct application in propagation of elite and unique plants. Even if somoclonal variation occurs, it can be used to create novelty and to develop new variants, particularly in ornamentals.

Somatic embryogenesis is a unique process in plants, through which morphologically and functionally normal embryos, and indeed, whole plants are often

produced from undifferentiated cells in culture. Since the early description of somatic embryogenesis in carrot cells (Steward *et al.*, 1958), this concept has been recognized as the potential pathway for mass multiplication by regeneration of plants from cell culture systems and also as an important model for studying early regulatory morphogenic events in plant embryogenesis.

The rate of proliferation and development of asexual embryos *in vitro* via somatic embryogenesis has led to the speculation that these embryos could be transformed into seeds if a synthetic seed coat is applied. The somatic embryos enclosed in a protective seed coat, termed as 'synthetic seed' or 'artificial seed' have been proposed as a low cost high volume propagation system (Redenbaugh *et al.*, 1986). The distinct advantage of production of synthetic seeds is the application of conventional seed handling technique for the delivery of somatic embryos with the sole objective of producing 'clonal seeds' at the cost comparable to true seeds. The other advantage is the utility of this technique in germplasm conservation as an alternate approach to the field gene banks.

Gladiolus (*Gladiolus sp.*), belonging to the family Iridaceae, is one of the most popular bulbous flower plants grown in many parts of the world. Its magnificent spike with variety of colours has made it attractive for use as cutflower and garden display purposes. Commercially, it ranks next only to tulips in Holland and other countries. In India, *Gladiolus* has undoubtedly emerged as the best bulbous flower in recent years. Attracted by its good keeping quality, easy marketability and remunerative returns, many growers have taken to *Gladiolus* cultivation and thus the demand for its planting material has considerably increased.

Gladiolus is propagated mainly through corms/cormels. However, due to slow and low rate of multiplication, it takes years to obtain enough planting material, thereby hampering commercial cultivation of this important flower. There are attempts to maximize the production of propagules under both *in vitro* and *in vivo* conditions. But commercially, a viable process for its mass multiplication is yet to be developed.

Although somatic embryogenesis has been reported in several ornamental species, information on perfection of this process for producing high quality somatic embryos as a means of rapid clonal propagation is very scanty in *Gladiolus*. Also, there are no reports on production of synthetic seeds in *Gladiolus*, either as a means of somatic embryo delivery or for germplasm conservation. Keeping this in view, a programme of studies *in vitro* was carried out in some elite *Gladiolus* hybrids, with the following objectives:

1. Investigating the response of explant to growth regulators *in vitro*
2. Optimization of callus growth in cultures.
3. Investigating the influence of growth regulators on the morphogenic response in callus cultures.
4. Standardization of protocol for somatic embryogenesis, and
5. Optimization of encapsulation techniques for production of synthetic seeds.

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

In the middle of the twentieth century, an experimental tool became available to dissect out individual cell, tissue or organ from the plant system and culture them under direct experimental control, free from the complex interactions occurring in the intact plant. Such a technique later came to be known as 'plant tissue culture' and was based on the concept of 'totipotency', which is inherent in the cell theory of Schleiden (1838) and Schwann (1839). Today, consequent upon several pioneering research efforts, plant cell, tissue and organ culture technology has emerged as an important tool in both basic studies, as well as in commercial applications.

The early history

The early history of plant tissue culture can be traced back to 1902, when Haberlandt, a German botanist made a historic, but then an unsupported claim that, one day it should be possible to rear plants from isolated cells. He introduced the concept of totipotency, viz. that all living cells containing a normal complement of chromosomes capable of regenerating into an entire plant. However, he was not successful in proving his logical thinking as he had used mature mesophyll cells as the culture material, apparently overlooking the fact that mature mesophyll cells are highly differentiated with their meristematic activity in obedience.

Prompted by the logical insight of Haberlandt, his students and contemporaries made several attempts between 1902 and 1934 to induce isolated cells into regenerating tissue masses by cell division. But, the success was very

limited. White (1934), after assessing the reasons for the previous failures, came out with a successful demonstration on the possibility of growing excised tomato root tips *in vitro* for indefinite periods. Further, independent studies by Gautheret (1939), Nobecourt (1939) and White (1939) demonstrated that cells in culture can be made to proliferate continuously into unorganized masses of tissue, called 'callus'. These revelations laid a sound base for artificial culture of plant parts even before the knowledge of plant growth regulators. However, the discovery of cytokinins by Miller *et al.* (1955) followed by the classical elucidation that the organogenesis of shoots and roots in culture cells could be manipulated by changing the concentration of auxin-cytokinin in the nutrient media (Skoog and Miller, 1957) ushered a new era in the field of plant tissue culture.

The other very important facts often cited as milestones in the history of plant tissue culture are the elimination of viruses through meristem culture in dahlias (Morel and Martin 1952), isolation and culture of single cells (Muir *et al.*, 1954), somatic embryogenesis (Steward *et al.*, 1958 and Reinert, 1959), enzymatic isolation of protoplasts (Cocking, 1960), the regeneration of plants from single cells (Vasil and Hildebrandt, 1965), anther culture (Guha and Maheswari, 1966), variability of cells in culture (Lutz, 1969) and development of homozygous diploid from microspore culture (Nitsch, 1974).

Thus, the technique of plant tissue culture, as a tool in plant biology, has come to stay either in fundamental or applied research. While the earlier researchers explored the technique for tackling the problems of academic interest, the later scientists started using it in various applied fields such as hybridization,

variety development, rapid clonal propagation of disease free plants and germplasm conservation.

The present review is focused on regeneration of plants through organogenesis and somatic embryogenesis with special emphasis on callus mediated morphogenesis. The review also deals with the strategies and prospects of synthetic seed production as a means of somatic embryo delivery system. Although micro propagation studies have been reported in *Gladiolus*, there is not much information on regeneration of plants through callus mediated somatic embryogenesis. Therefore, the review also deals with other related bulb and corm producing ornamentals to cover various aspects of organogenesis and embryogenesis. With respect to synthetic seed production, the review is focused entirely on other crops, as this aspect has not been reported in *Gladiolus*.

Morphogenesis

Explants from various tissues of the plant when cultured on a nutrient medium *in vitro*, respond mainly by :

- a) formation of adventitious shoots and roots separately, and
- b) formation of adventitious embryos that are structurally similar to the embryos found in true seeds (Murashige, 1974).

The nature of morphogenesis is regulated by the kind of stimulus brought about by endogenous growth substances within the plant tissues or the exogenous growth regulating substances added to the culture media. Generally, a high concentration of cytokinins causes bud initiation; whereas, high levels of auxins and

a low concentration of cytokinins promote abundant cell proliferation with the formation of callus. Often, auxin alone is used to initiate callus. On the other hand, auxin alone or along with a very low concentration of cytokinin is responsible for the induction of root primordia (Flick *et al.*, 1983).

Hicks (1980) described that morphogenesis can occur in either of the two distinct ways :

- a) direct morphogenesis, wherein new organs are formed from the differentiated cells of an explant without the proliferation of undifferentiated tissue , and
- b) indirect morphogenesis, wherein the new organs are formed from unspecialised and undifferentiated cells of callus tissues or suspension cultures.

Organogenesis

Many plant species often produce adventitious shoot buds directly on explants from a variety of organs including petioles, stems, roots, cotyledons and other storage organs. These shoots are excised and transferred to rooting medium to induce rooting. Formation of roots directly on non-root explants is relatively rare, although it is possible as observed by Kartha *et al.* (1974) in *Brassica* and Rao *et al.* (1973) in *Petunia*.

In most bulbous and corm producing ornamentals, adventitious shoots and roots are often produced directly on explants. They are later excised and induced to form storage organs like bulblets or cormlets before transfer *extra vitrum*. In certain

bulbous species of *Lilium* and *Iris*, bulblets are also produced directly on bulb scales *in vitro* (George, 1993).

Liu and Burger (1986) cultured pedicel sections taken from the flowers of *Lilium longiflorum* on a modified MS medium containing various concentrations of cytokinins and auxins and obtained the maximum number of adventitious shoots at a hormonal combination of 5 μ M BAP and 2 μ M NAA. A gradient in the formation of buds in the pedicel was observed with the section nearest the receptacle forming the maximum number especially when the section was placed upside down on the culture medium. IAA and IBA, both at 10 μ M, were most effective in stimulating adventitious roots on *in vitro* derived shoots.

Linde *et al.* (1986), cultured bulbscales of bulbous *Iris* separated from the bulb sections with pieces of basal bulb tissue attached in half strength MS macro and micro nutrients, 100mg/l mesoinositol, 0.04mg/l aneurin HCl and 1% agar with pH 6.0 in dark at 20°C and obtained adventitious plantlets. These plantlets in tubes when subjected to 5°C for 4 weeks and later replaced to 20°C for another four weeks, produced bulblets. They suggested to subculture the originally formed plantlets on the same medium containing 0.3mg/l of both NAA and BAP for increasing the propagation factor.

Huang and Liu (1989) developed a method for clonal multiplication of *Lycosis aurea* through differentiation of adventitious shoots directly from the explants of bulb scale segments. They induced the explants to undergo organogenesis by placement in darkness on MS medium, supplemented with 30mg/l each of NAA and BAP for the

first 3 months. The swollen explants were then transferred to a second medium with 3mg/l NAA and 10mg/l BAP and given illumination for differentiation of adventitious buds and emergence of shoots. The shoots were multiplied by transferring groups of three or more to fresh medium at 4 week intervals. To obtain plants, individual shoots were rooted in another medium with 3 mg NAA/l. The method is projected to yield at least 30,000 plants per bulb in the first year.

In *Tulipa gesneriana*, culture of floral stem explants of cultivar Lucky Strike, Lustige Witwe and Gander in MS medium supplemented with 0.0053 m mol NAA, 0.0044m mol BAP and 0.014 m mol 2ip in darkness at 19-20°C, resulted in production of adventitious shoots (Le, 1989). More adventitious buds were produced when the source of the explant were grown in darkness particularly in case of Lustige Witwe and Gander. Some adventitious buds subcultured without growth substances for 9 weeks at 5°C and then subjected to 25°C, produced bulbilis.

Linde *et al.* (1988) obtained adventitious shoots in Dutch *Iris* by culturing basal part of the shoot on MS basal medium, containing 0.1 mg NAA and 0.5mg BAP per litre at 15° C with a multiplication factor of 4 in a 7 week cycle. The explant shoots thus formed were stimulated to produce bulbs by growing them on MS medium without BAP for 6 weeks at 15°C, then for 8 weeks at 5° C followed by culture at 20°C in darkness.

In *Gladiolus* 'hybridus' varieties, terminal/axillary buds taken from corm sections and cultured on MS medium supplemented with BAP at 3mg/l resulted in development of adventitious buds. The newly developed buds were transferred to

fresh medium with 1.0 mg BA/l and maintained for further proliferation of adventitious shoots. The propagation coefficients for most of the varieties ranged from 10 to 15. The medium free from BAP supplemented with activated charcoal at 5g/l and IAA at 0.1mg/l was used for rooting (Rumynin *et al.*, 1990).

The effect of *in vitro* culture conditions on direct regeneration and growth of *Hyacinthus orientalis* bulbs was investigated by Takayama *et al.* (1991). The bulblet regeneration was stimulated on MS solid medium containing combinations of 4.4 - 44 μ M BAP and 0.54 - 5.4 μ M NAA, but their growth was slow. However, when cultures were transferred to liquid medium and placed on a rotary shaker, the growth of bulblets was markedly stimulated.

Formation of adventitious shoots from corm sections of *Gladiolus* was reported by De Bruyn and Ferreira (1992). They cultured explants on MS medium and found that the adventitious shoot formation was best in *G. daleni* with the addition of 0.5mg/l BAP and in *G. tristis* with 1-2mg/l BAP. Shoots of *G. daleni* produced corms *in vitro*, but few were produced from *G. tristis*. Both shoot and corm production were optimal when 60-90g/l sucrose was added to the medium and cultures were incubated at 15°C compared to 20 or 24°C.

Somatic embryogenesis

Somatic embryogenesis is the process where the vegetative cells organise themselves into compact cell masses and pass through the characteristic embryological stages similar to the stages in development of zygotic embryos viz. globular, heart and torpedo stages and develop into bipolar embryos. This process

occurs naturally in a wide variety of species from both reproductive tissues such as the nucellus and synergid cells, and somatic tissues such as leaf lamina. Raghavan (1976), Tisserat *et al.* (1979) and Vasil and Vasil (1980) described the spontaneous occurrence of somatic embryogenesis *in vivo*. However, currently, somatic embryogenesis is best known as a pathway for induced regeneration from *in vitro* tissue culture, occurring either indirectly from callus suspension or protoplast culture, or directly from cells of an organised structure such as the stem segment of zygotic embryo (Zimmerman, 1993).

The hypothesis of somatic embryogenesis, advanced by Sharp *et al.* (1980) and further elaborated by Evans *et al.* (1981) and Sharp *et al.* (1982), suggests that direct embryogenesis in culture proceeds from cells which were already determined for embryogenic development prior to explanting. They are pre-embryogenic determined cells (PEDCs) requiring only growth regulators or favourable conditions to allow release into cell division and expression of embryogenesis. In contrast, indirect embryogenesis requires predetermination of differentiated cells, callus proliferation and the development of the embryogenically determined state. For these induced embryogenically determined cells (IEDCs), growth regulators are required not only for re-entry into mitosis, but also for determination of the embryogenic state. Once the cells are determined for embryogenesis, there are no fundamental differences in behaviour between those which were predetermined before explanting and those in which the embryogenic state required induction *in vitro*. Plantlets are then produced directly by following the full embryogenic pathway as a co-ordinated group of embryogenic determined cells.

Formation of somatic embryos, directly on explant-tissues without any intervening callus phase was observed on those explants that were physiologically very juvenile, like pollen microspores within the anther and tissues of all or part of the ovary, ovule, zygotic embryo or young seedlings (Smith and Krikorian, 1988).

Maheswaran and Williams (1985) cultured immature zygotic embryos of *Trifolium repens in vitro* in the presence of 0.05mg/l BAP and produced somatic embryoids directly without an intervening callus phase, through proliferation of the cells of the hypocotyl epidermis. Control embryos grown for 2-7 days on EC 6 basal medium without BAP showed a single epidermal layer and five to six layers of cortical cells. Cell divisions in the epidermal layer were regularly anticlinal. In the presence of 0.05 mg/1 BAP, after 2 days in culture, occasional periclinal and irregular divisions were seen in epidermal cells. Some epidermal regions appeared to become more than one cell thick as a result of periclinal divisions in a number of neighbouring cells. These centres of irregular proliferation became localised to produce slight swellings on the hypocotyl surface and subsequently, structures resembling multicellular globular zygotic proembryos. The pattern of divisions in cells of hypocotyl epidermis suggested the initiation of embryoids from both single cell and a group of cells. Further development gave rise to 'heart', 'torpedo' and cotyledonary stages similar to zygotic embryogenesis.

Maheswaran and Williams (1986) reported direct secondary somatic embryogenesis from immature sexual embryos of *Trifolium repens* cultured *in vitro*. The primary somatic embryoids derived from immature sexual embryos of *Trifolium repens* gave rise to secondary embryoids through multicellular proliferation of

superficial cells on the lower surface of the axis and cotyledons in EC 6 basal medium containing 1-2 mg/l BAP. At 0.05 mg/l and lower levels of BAP, the isolated primary somatic embryoids continued development to give single plantlets. At 1,2 and 2.05 mg/l BAP, growth of the main axis was suppressed, and the secondary embryoids were initiated over the entire lower surface of the explant in contact with the medium. Embryoid formation was more prolific at 2mg/l BAP than at 1.0mg/l. At 2.05 mg/l BAP, morphological abnormalities were more frequent, apparently as a result of broad or fasciated initiation sites. The results of the study also revealed that secondary embryogenesis differs from primary embryogenesis in requiring a substantially higher concentration of BAP for initiation, and in the occurrence of initiation sites over the entire lower surface of the embryoid, rather than just the hypocotyl region. The important feature of the secondary embryogenesis system that the authors described here is the ease with which embryoids could be separated from the parent tissue without damage to the axis or root pole, and their consequent rapid normal establishment on transfer to hormone-free medium. The system also provides a means for rapid cloning with minimal cellular de-stabilization and de-differentiation as the process of initiation of embryogeny at both primary and secondary stages is predominantly multicellular.

Kreuger *et al.* (1995) described a method for large scale production of somatic embryos of *Cyclamen persicum* in liquid medium by avoiding callus phase. There are five steps involved, namely, initiation of embryogenic cell lines, proliferation of pro-embryogenic masses (PEMs) on an auxin containing medium, development of somatic embryos on hormone free medium with high osmolarity, germination and subsequent plantlet formation. Cell lines were initiated by culturing

any other explant, the seedling tuber, directly in B5 liquid medium with 25 μ M 2,4-D and 5 μ M kinetin. Explant density, hormone concentrations and subculture regime were identified as important parameters for obtaining embryogenic cell lines. The rate of uptake of the hormones 2,4-D and kinetin influenced the formation of PEMs. Highly embryogenic cell lines were obtained only when PEMs had formed within 5-7 weeks. PEMs were proliferated for at least 24 months and could be isolated from each sub-culture for the production of somatic embryos. A high sucrose content (175mM) in the development medium, without hormones, ensured efficient embryo development from PEMs. A subsequent sub-culture in low sucrose concentration (58mM) induced formation of tuber, thus promoting germination.

Callus induction, growth and organogenesis

Bulb and corm producing ornamentals give rise to highly regenerative callus which is reported to maintain genetic stability over long periods. The shoots regenerated from callus were found to produce bulb/corm at their base and these gave rise to plants which were diploid, with no apparent phenotypic variation (Davies and Griffiths, 1971 and Hussey and Wyvill, 1973 in *Freesia* and Hussey, 1977 in *Liliums*).

Callus cultures are generally initiated from bulb scales, stem/corm tissues, leaves, flower pedicels, anthers and flower buds of bulb and corm producing species on MS medium supplemented with mild to strong auxins. The most commonly used auxins for both callus initiation and subsequent growth are 2,4-D and NAA (Sheridan, 1968 and Hussey, 1977 in *Liliums*; Ziv and Halevy, 1972 in *Gladiolus* and Hussey, 1977 in *Freesia*).

In *Cyclamen persicum*, Loewenberg (1969) initiated callus from 4mm corm cubes on a medium containing 1.0mg/l IAA, 1.0mg/l kinetin and 67.5mg/l adenine. Shoots and roots were regenerated sporadically from the callus over several years. However, Geier *et al.* (1979) used sections of young leaves to induce callus on medium supplemented with 1.0mg/l NAA and 1.0mg/l BAP and 0.49 mM adenine sulphate. This callus was capable of forming adventitious shoots in the primary and first subculture, but the organogenic capacity of the callus declined in subsequent subcultures.

In *Lilium longiflorum*, Sheridan (1968) initiated callus from sections cut from the top of stems on Linsmaier and Skoog (LS) medium containing 2 mg/l IAA. Shoots were produced when the callus was subcultured to a shaken liquid or agar-solidified medium without regulants. Konchak and Rodeva (1986) studied the effects of NAA, Kinetin or BA each at 0.0 - 5.0 mg/l on bulblet regeneration from bulb scale explants of the cv. Yellow Blaze in LS medium. NAA at 0.5mg/l stimulated regeneration, whereas the presence of 0.5mg/l kinetin or 0.1mg/l BA in the nutrient medium stimulated callus production. In contrast, Priyadarshi and Sen (1992) induced callus on sections of young bulb scales of *Lilium longiflorum* by culturing on MS medium supplemented with 1.0mg/l 2, 4-D and 0.25mg/l BAP, in the dark. The callus was multiplied on the same medium containing 1.0mg/l NAA and 0.25mg/l BAP and gave rise to plantlets when transferred to the BAP omitted medium under light. In this study the chromosome analysis of callus maintained for 3 years did not reveal any marked variation in chromosome morphology, thus confirming the stable nature of callus for long term culture. The callus also maintained high degree of

regenerating capacity along with rapid growth during the culture period of 3 years. Mizuguchi *et al.* (1994) induced white callus from mother bulb scales of *Lilium japonicum* on MS medium containing various concentrations of NAA and BAP. The best white callus growth was obtained with the combination of 0.01 PPM NAA and 1.0 PPM BAP. Adventive globular buds formed on the callus on growth regulator free medium after 3 weeks in culture and bulblets developed from the adventitious buds after 6 weeks. The combination of 1.0 PPM NAA and 0.1 PPM BAP promoted the growth of white callus and the formation of abnormal bulblets.

Gu and Gao (1987) studied the effects of plant growth regulators on callus induction, maintenance and organogenesis from bulb segments of *Narcissus tazetta*. Calluses initiated from bulb scale segments of *Narcissus tazetta* var. *Chinensis* on MS medium containing 1.0mg/l BAP and 0.1mg/l 2,4-D, were transferred to MS medium either without growth regulators or containing 1.0mg/l BAP or 0.1 - 0.5 mg/l NAA for obtaining adventitious shoots.

Callus cultures were derived from flower stem explants and bulb scales in *Tulipa gesneriana* cv. Red Metador by Kim *et al.* (1989). On MS medium with 5 PPM NAA and 0.05 PPM BAP, callus formation of flower stem explant was better in 16 hours light than in 8 hours light or continuous light or darkness. The highest percent of callus formation occurred on MS medium containing 10 PPM NAA and or no BAP. Cold treatment of bulbs for upto 120 days reduced the percentage callus formation of bulb scales, particularly at longer day lengths, although callus initiation occurred more rapidly.

In *Gladiolus*, callus capable of regenerating adventitious shoots has been obtained from several explants. Ziv *et al.* (1970) obtained callus from 3-4mm sections of young inflorescence stems, cut before the inflorescence begins to emerge. The sections were incubated at 25°C in the light, distal side downwards on semi solid MS medium containing 0.7 mM adenine sulphate, 5-10 mg/l NAA and 0.5mg/l kinetin to induce callus formation. Explants showing proliferation, or callus were subcultured to the same medium containing 0.5mg/l kinetin or 0.25-1.0 mg/l BAP for adventitious root, shoot and cormlet formation. The cormlets formed *in vitro* can be planted in soil or used to start new cultures (Hussey, 1978). Corm explants of cvs. Firebrand and Topaz formed callus in about 40 days on MS medium containing 10 PPM 2,4-D (Kim *et al.*, 1988). On solid MS medium containing 1.0 PPM kinetin, 2-3% sucrose and 0.5 - 1.0 percent agar, fresh callus gave the best regeneration into plantlets under a 16 hour photoperiod. Roots and shoots were formed after 20 and 30 days, respectively. With callus older than 6 months, a cytokinin free medium was more successful for producing plantlets. Kamo *et al.* (1990) cultured inflorescence stalk of *Gladiolus* cv. Blue Isle and Hunting Song on MS basal medium supplemented with 53.6 µM NAA and obtained compact and non friable callus; whereas, cormel slices and intact plantlets of cultivars Peter Pears, Rose Supreme and Jenny Lee formed friable callus when cultured on MS basal medium supplemented with 2,4-D.

Differences in *Gladiolus* cultivars in the formation and growth of callus *in vitro* was investigated by Kim and Lee (1993). They induced callus from corm tissue of cultivars High Style, Hunting Song, Fire Brand, Topaz, Red Beauty, Victor Borge, True Love, Spic and Span, White Prosperity, Nova Lux and White Friendship on MS

medium containing 10mg/l 2,4-D. The induction rate ranged from 21.5 to 100 per cent in 26 days depending on the cultivar. Subculture of the callus on 2,4-D supplemented medium was possible only with the cultivars Fire Brand, Topaz, True Love, and Spic and Span. However, the authors could maintain uniform callus only in cv. Topaz. The optimum concentrations of 2,4-D in the first and second subcultures were 0.5 and 0.1 to 1.0mg/l, respectively. The growth of callus in subculture followed the typical sigmoid curve, with a 2 week lag phase and a stationary phase after 6 weeks.

Influence of additional nitrogen and phosphate on the growth of callus was studied by Halesh (1995) in *Gladious* cv. Local Yellow and Black Jack. The addition of casein hydrolysate 2.50 mg/l, with urea 340mg/l to MS medium containing 2,4-D at 2.0 mg/l and BAP or kinetin at 0.5 mg/l, the growth of callus was maximum in both the cultivars.

Somatic embryogenesis in callus cultures

With the development of cell suspension culture techniques (Muir *et al.*, 1954), the phenomenon of somatic embryogenesis was discovered in suspension cultures (Steward *et al.*, 1958) and in callus cultures on agar medium in carrot (Reinert, 1959). These original descriptions have still remained as the primary experimental system for studying somatic embryogenesis in a variety of agronomically and horticulturally important plants. Currently, this unique developmental potential has been recognised both as an important pathway for the regeneration of plants from cell culture systems and as a potential model for studying

early regulatory and morphogenic events in plant embryogenesis (Zimmerman, 1993).

Large scale production of somatic embryos and plants from these can be very advantageous when dealing with desired genotypes or traits which are difficult to multiply (Kreuger *et al.*, 1995). As both the growth of embryogenic cells and subsequent development of somatic embryos can be carried out in a liquid medium, it is possible to combine somatic embryogenesis with engineering technology to create large scale mechanised automated culture systems. Such systems are capable of producing propagules repetitively with low labour inputs (Razdan, 1993).

The model system most frequently used for somatic embryogenesis is carrot (*Daucus carota L.*), which is easily induced to form somatic embryos from cultured callus cells by a relatively simple manipulation of the culturing conditions. This model generally involves :

1. the establishment of a callus cell line from small pieces of explant involving auxin,
2. the selection of an embryogenic subpopulation of the cultured cells,
3. the removal of auxin from the culture medium, and
4. the dilution of the cells to a relatively low density and development into somatic embryos (Zimmerman, 1993).

The overall embryogenic potential of a culture is highest when the culture is relatively young and resides primarily within a sub-cultured population that has been termed 'proembryogenic masses' (PEMs). Halperin (1966) and Williams and Maheswaran (1986) defined PEMs as cell clumps, able to produce somatic embryos

and consisting of small, dense, highly cytoplasmic cells, the embryogenic cells and having a meristem like morphology.

Embryogenic cell lines can also be established by putting the explant directly into a liquid medium containing low concentrations of an auxin. From the explant many cells are released into the medium and after 1-2 weeks, the so called PEMs appear. The PEMs in suspension cultures will not develop into embryos due to the presence of an auxin and the high cell density, but can proliferate to form large number of PEMs. After the transfer to hormone free medium and dilution of the culture, the PEMs will develop into somatic embryos (De Vries *et al.*, 1988). However, indirect formation of somatic embryos, from callus or suspension cultures, is observed more frequently than direct embryogenesis.

Somatic embryos closely resemble that of zygotic embryos, both morphologically and developmentally. Zimmerman (1993) summarised the stages of embryo development taking embryogenesis in carrot as model. The embryos of the first recognizable stage, the globular stage, generally grow out of the small cell clusters of meristematic cells (pro-embryos) within 5 to 7 days after the cells are shifted to auxin free medium. After 2 to 3 more days of isodiametric growth, the globular stage is followed by an oblong stage, which signals the shift from isodiametric to bilaterally symmetrical growth and the beginning of the heart stage. The globular to heart transition is clearly marked by the outgrowth of the two cotyledons, the elongation of the hypocotyl, and the beginning of the development of the radical. These processes continue through the torpedo and plantlet stages and by 2.5 to 3 weeks after induction, plantlets can be identified that contain green

cotyledons, elongated hypocotyls, and developed radicals with clearly differentiated root hairs. These plantlets continue to grow either in liquid culture or they can be transplanted to solid medium for regeneration of whole plants.

Many methods have been described for the development of somatic embryos via indirect embryogenesis. Merkle *et al.* (1990) reported that in any method described, the development of embryos and plantlet regeneration invariably involved 3-4 steps depending upon the purpose and the nature of the process. George (1993) described these steps as:

1. initiation of callus growth on a primary medium containing high concentrations of auxin,
2. callus, usually without any obvious sign of embryogenesis, subcultured to a second or third medium each containing a progressively reduced concentration of auxin,
3. embryogenic callus, and or small somatic embryos, finally moved to a medium containing no auxin to encourage embryo growth, and
4. conversion to plantlets.

Mc William *et al.* (1974) studied the initiation of embryoids in suspension cultures of carrot. The embryoids were initiated at or near the surface of characteristic cellular aggregates (embryogenic clumps) and were released from aggregates as free floating structures capable of further development into plantlets. Embryoid development was promoted by transfer from a medium containing 2,4-D to one from which it was omitted. The study of the structure of the embryogenic clumps in these two media, by the thin film section technique, revealed that the

embryoids were seen to arise from individual superficial cells in the 2,4-D omitted medium. But it has not been possible to distinguish, in advance of embryogeny, such cells from within the superficial cell layers. The fragmentation of the proliferating clumps in 2,4-D containing medium and the release of embryoids into the 2,4-D omitted medium was promoted by some of the superficial cells becoming free of starch and undergoing pronounced enlargement.

Ho and Vasil (1983) reported that the suspension culture established from calli derived out of young leaves of sugarcane contains both embryogenic and non-embryogenic cells. The embryogenic cells were small, richly cytoplasmic and actively dividing with a prominent nucleus and conspicuous starch grains. The non-embryogenic cells were large, vacuolated and often elongated with sparse starch grains, which did not show morphogenic competence. The procedure that the authors described for elimination of non-embryogenic cells from the suspension culture is as follows. After the vigorous shaking of the suspension culture, large and denser clumps of embryogenic cells sank to the bottom of the flask within a few seconds, while the vacuolated non-embryogenic cells remained in the upper portion. The middle part of the suspension consisting of small clumps of embryogenic cells, were used as inoculum for subculture. The resulting cultures comprised predominantly of embryogenic cells which showed morphogenic competence.

Ammirato (1988) reported that abscisic acid (ABA), a naturally occurring plant growth regulator, exerts a number of striking effects on the development of somatic embryos grown in suspension cultures. When added at moderate concentrations to populations of proembryos, ABA permits normal

embryogenic growth and maturation, but inhibits diversions from this pathway. It prevents the inhibition and growth of new embryogenic centres and the formation of cotyledons and shoots. In fully formed embryos, it inhibits their germination and conversion into plantlets. ABA also influences cellular differentiation, affecting both anatomy and biochemistry. Its role is to permit the normal progression of the embryogenic programme. With ABA, somatic embryos closely resemble zygotic embryos in both structure and behaviour. Practically, the manipulation of endogenous or exogenous ABA levels in populations of somatic embryos increases the frequency of embryos that reach maturity and facilitates their manipulation in large scale propagation and mechanised delivery.

The observations of Buchheim *et al.* (1989) demonstrated that the somatic embryos required maturation before acquiring the capacity for vigorous conversion and plantlet development in soyabean. Somatic embryos induced by 2,4-D have released 2,4-D from the conjugated state even after transfer to 2,4-D free medium. Since compounds with high auxin activity inhibit somatic embryo development effectively, the continued presence of 2,4-D, therefore, not only resulted in low conversion frequency but also in development of morphologically abnormal somatic embryos. Maturation of somatic embryos on MS basal medium supplemented with activated charcoal and 0.28 molar sucrose for a period of 8 weeks exhibited vigorous, high frequency development of plantlets. This was due to absorption of residual 2,4-D by activated charcoal in the maturation medium. Desiccation of immature somatic embryos under controlled humidity regimes also resulted in increased frequency of conversion of immature somatic embryos. The desiccation under 75 per cent and 93 per cent RH, produced a dramatic increase in conversion

than at 40 per cent RH. The desiccation requirement of immature somatic embryos for increased conversion was compared with the similar requirements for conversion of immature zygotic embryos.

Chee (1990) reported that in cucumber, callus induced from hypocotyl explant on MS medium showed different morphogenesis. The tissue developed on the surface of the explants, where the explant was in contact with the medium, was translucent, smooth and gelatinous. The upper part of the gelatinous tissue contained small putative embryogenic tissues, which were found to consist of cytoplasmically dense, multi cellular aggregates, resembling proembryogenic masses. These aggregates later organised into embryos with high recovery of fertile plants. The embryogenic potential of the gelatinous callus tissue was not lost with successive subcultures on induction medium for at least 6 months. In contrast to gelatinous tissue, callus that was hard and nodular or white and friable, was not found morphogenically competent.

In *Freesia refracta*, somatic embryogenesis was obtained by transferring pale yellow, translucent, nodular calluses induced from epidermal cells of young inflorescence on MS medium containing 2.0 mg IAA, 0.5 mg BAP and 0.5 mg NAA/l onto a modified N6 medium containing 2.0 mg IAA and 3.0 mg BAP/l. All the regenerated plants were morphologically normal, flowered normally and had a normal chromosome number of $2n = 22$ (Wang *et al.*, 1990).

Plant regeneration, via *in vitro* somatic embryogenesis from root culture of some rhizomatus *Iris* was studied by Laublin *et al.* (1991). Root and leaf pieces from *in vitro* grown plants of several genotypes of 3 rhizomatus *Iris* species were cultured

in vitro. Callus induction occurred only on root cultures incubated under low light intensity (35 mol/ m /sec.) on induction medium containing 2,4-D (4.5 or 22.5 μ M), NAA (5.4 μ M) and kinetin (0.5 μ M). Somatic embryos developed after transfer of callus onto 4 regeneration media containing 9 or 22 μ M BAP or 5 μ M kinetin with 2 μ M TIBA or 9 μ M BAP with 4 μ M TIBA. Plantlets were obtained from these embryos, with varied degree of success. Genotypic differences were also found, in both callus induction and somatic embryo formation.

Bach (1992) studied the induction of somatic embryogenesis and regeneration of plantlets in *Freesia hybrida* cultivars. Embryogenic callus was initiated on MS medium in cultures of corm apical meristems of the cultivars Blue Heaven and Yellow Sea as well as from mature zygotic embryos of Super Freesia Blue. The explants formed embryogenic structures (average 20 per cent) in the presence of picloram or 2,4-D, but picloram was superior to 2,4-D both for induction and maintenance of callus or subsequent regeneration of plants. The per cent differentiation of embryogenic structures was better in the zygotic embryo explants than in the meristems, but the ability to develop mature embryos was similar for the two primary types of explant. Embryo development took place when the embryonic tissue was transferred to MS medium containing low concentration of auxins. Maturation of somatic embryos was stimulated by cytokinins (BAP). An average germination of 5-10 per cent occurred following transfer of somatic embryos to an auxin free solid medium.

Frey *et al.* (1992) followed two protocols for embryo initiation and development while studying somatic embryogenesis in carnation. They include,

semisolid callus induction medium (CIM), followed by transfer to semisolid or liquid embryo production medium (EPM) or liquid CIM followed by liquid EPM. Between the two, the optimum protocol for the induction of somatic embryos happened to be the initiation of callus in liquid basal MS medium supplemented with 3.0 μM 2,4-D followed by transfer to liquid basal medium lacking 2, 4-D for embryo development. There was no evidence that cytokinin, in either liquid or semisolid CIM, improved embryo formation. Somatic embryos originated from single cells and early embryonic development proceeded continuously via globular, heart shaped and torpedo stages, but clearly developed apical or root meristems were not always formed.

Lelien-Kipnis *et al.* (1992) investigated proliferation and regeneration of *Nerine* in liquid culture. Inflorescence derived explants were cultured on MS medium supplemented with 2, 4-D and BAP (each at 10 μM). Callus like tissue interspersed with nodular regions as well as direct organogenesis was observed at the junction between pedicel and peduncle. Subculture of the nodular tissue in liquid medium, supplemented with 0.25 μM NAA, 10 μM BAP and 2.5 PPM paclobutrazol in flasks, resulted in proliferation of compact but friable meristematic clusters from which plantlet regeneration was very sporadic. However, exclusion of paclobutrazol from the proliferation medium resulted in the development of proembryogenic masses. Differentiation of somatic embryos was enhanced by 2ip and these germinated in growth regulator free medium. A conversion rate of 40 - 50 plantlets per 3g of embryogenic tissue was obtained. Plantlets developed, rooted and formed bulblets on a medium containing 6 per cent sucrose and a low concentration of NAA.

In *Gladiolus*, somatic embryogenesis and plantlet regeneration was reported by Stefaniak (1994). Corm slices, young leaf bases, and plantlets without roots of 4 *Gladiolus hortulans* cultivars were placed on MS medium supplemented with 2, 6 or 10 mg 2,4-D or NAA/l. Friable embryogenic callus and somatic embryos were obtained from cv. Blue Isle, Jenny Lee and Rose Supreme when 2 mg 2,4-D/l was used and from Peter Pears with 10 mg NAA/l. Somatic embryos transferred on MS medium free from hormones regenerated into plantlets. For all cultivars, a high percentage of corm slices produced embryogenic callus that regenerated plantlets, when compared with other explant types. All plantlets obtained through embryogenesis did not differ phenotypically from the parental clones. The embryogenic friable callus has been maintained for over 2 years in culture and retained a very high regeneration capacity.

Gonsalves *et al.* (1995) regenerated six squash cultivars via somatic embryogenesis. Somatic embryogenesis was initiated in induction medium containing 22.62 μ M 2, 4-D and embryos were germinated in maturation medium containing 0.27 μ M NAA and 0.23 μ M kinetin. Plants were elongated and rooted on basal medium without hormones. The determination of relative production of plants from cotyledons that were kept on induction medium for different time periods indicated that all explants produced somatic embryos after 11 to 17 weeks on induction medium. However, plantlet production was optimal with explants kept on induction medium for 13 weeks for cv. Seneca Zucchini and for 15 weeks for cv. Caserta Inbred 557311, producing an average of 4.5 and 9.3 plants per explant, respectively.

Jain *et al.* (1996) studied somatic embryogenesis from callus and suspension culture of *Gladiolus*. Callus and cell suspensions from different explants viz. leaf bases, peduncle, corm slices and leaf tips of two cultivars (American Beauty, Wedding Bouquet) were established on MS modified media. Among the explants used, the callus produced from *in vitro* regenerated plantlets was found to be highly embryogenic. MS media supplemented with 2 mg/l 2, 4-D, 1 g/l casein hydrolysate and 1 mg/l thiamine HCl, supported good callus growth. Callus was maintained on MS medium containing 10 mg/l NAA and 0.5 mg/l kinetin for the purpose of suspensions. Suspensions were established on MS media containing various concentrations and combinations of kinetin and BAP. Liquid MS medium supplemented with 0.2 mg/l BAP produced embryogenic suspension. The somatic embryos readily germinated and formed plantlets when transferred to the solid MS medium, supplemented with 1 mg/l kinetin and 0.1 mg/l NAA. Plantlets were rooted on the regeneration medium itself.

Synthetic seed

The seed containing the new plant in miniature, represents the ultimate convenience for crop production when compared with any other form of propagule. Equipped with structural and physiological devices, the seed is well suited for simplified handling and storage and serves as the easiest unit for dispersal (Bewley and Black, 1985). However, it would be advantageous to combine the structural and functional efficiency of seed with the clonal plant derived via *in vitro* somatic embryogenesis for various other applications. The field that seeks the use of somatic embryos as functional seed is termed as 'Synthetic Seed Technology' (Gray,

1987) and the somatic embryos engineered in a synthetic capsule is termed as synthetic or artificial seed (Gray, 1990).

The concept of artificial seed appeared in print almost 20 years after the discovery of somatic embryogenesis in 1958 (Murashige, 1978). However, the concept was mentioned only briefly and no results appeared in the literature prior to a report on encapsulation of alfalfa and celery somatic embryos, with natural hydrogels such as calcium alginate (Redenbaugh *et al.*, 1987). Kitto and Janick (1985a) reported coating clumps of carrot somatic embryos and shoots in a wafer disk, but presented data only on embryo 'survival'. Their encapsulation methods deviated from the concept of true seed analog, which is a single somatic embryo encapsulated in a synthetic seed coat with the embryo germinating and becoming a complete plant (Redenbaugh *et al.*, 1987).

The embryos of the zygotic seed is surrounded by nutritive and protective tissues that facilitates the embryos to remain their metabolic activities for a very long time. Beyond protection and nutrition, such tissues also create barriers to regulate gas exchange. Oxygen availability, which is limited by these tissues, influence embryo respiration, regulates embryo development and prevents precocious germination (Carman, 1988). In contrast, somatic embryos are subjected to whatever gas regime is present in their surrounding culture vessel. The availability of oxygen and other gases depends on subculture interval whether or not the vessel is tightly sealed as well as gas usage and evolution by other associated tissues such as substanding tissues.

Nutritive tissue that directly encases the embryo provide the food reserves to the embryo until the embryo becomes a self sufficient and autotrophic organism. In endospermic seeds, endosperm performs a nutritive function and all substances required for the growth by the developing embryo must either pass through the suspensor or be absorbed from endosperm by the body of the embryo (Bewley and Black, 1985). In many other seeds, nucellar tissue also performs a nutritive function. In non endospermic seeds, mature embryo itself contains adequate storage reserves that provide energy for germination. Thus the zygotic embryo is provided with distinct and specific environments; whereas, somatic embryos develop in a highly unorganised manner on or within callus cultures or immersed in liquid medium. Often suspension is the only link between the embryo and growth medium.

In zygotic seed, the protective tissue (seed coat) besides providing protection from the disease causing organisms and mechanical stress, also exerts a physical influence on embryo development that alters morphology. The physical pressure exerted by the seed coat may also contribute to normal development of the embryo, attainment of maturity and onset of dormancy. In addition to these, the seed coat is possessed with some degree of impermeability to water and gases, including oxygen, and exert regulatory influence on metabolism and growth of the embryo (Bewley and Black, 1985). The somatic embryos that develop and germinate in a different environment, are deprived of the complex and the controlled nutritive regime as well as the protective devices attached to zygotic embryos. As a result, dehydration and senescence occurs immediately after the somatic embryos are separated from the nurse culture. Therefore, in order to provide certain aspects of

complex seed environment, the somatic embryos are encapsulated with synthetic seed coat and make them analogous to true seed (Fujii *et al.*, 1987).

Redenbaugh (1993a) reported that the synthetic seed coat used in encapsulation must serve as synthetic endosperm containing carbon sources, nutrients, growth regulators, antimicrobial agents, etc. The synthetic seed coat must be non damaging to the embryo, should protect the embryo from mechanical damage during handling and allow germination and conversion to occur, without inducing undesirable variation.

Two types of artificial seeds have been reported; hydrated and desiccated. Hydrated artificial seeds consist of somatic embryos individually encapsulated in a hydrogel such as sodium alginate. They are produced by mixing the embryos with sodium alginate and dropped into calcium salt solution to form calcium alginate capsules. The capsules are then washed and planted (Redenbaugh *et al.*, 1986). The desiccated artificial seeds are produced by coating the somatic embryos in polyoxyethylene glycol. The coated mixture is allowed to dry for several hours on a Teflon surface in a sterile hood. The dried mixture is then placed on culture medium *in vitro*, allowed to rehydrate and then scored for embryo survival (Kitto and Janick, 1985a). Redenbaugh (1990) observed that the desiccated artificial seeds had other problems that needed resolution. The desiccation process itself damages the embryos. One exception to this was reported by Gray (1987), who found that desiccation appeared to release grape somatic embryos from a quiescent state and increase conversion (20% to 28% for desiccated embryos stored 7 to 21 days and 5% for non-desiccated embryos).

Hydrogel encapsulated or hydrated synthetic seeds are the most commonly produced synthetic seeds and the most commonly used hydrogel is the sodium alginate because of its gelatin properties, ease of use, less viscosity and low cost (Redenbaugh *et al.*, 1987). The production of hydrated synthetic seeds is through a simple direct process, wherein the somatic embryos are mixed with sodium alginate and dropped into complexing bath containing calcium chloride using a dropper or pipette. Each drop containing a embryo at the centre will form into a calcium alginate capsule or bead around the embryo. The formation of the bead is due to complexing of sodium alginate when mixed with di or trivalent metal cations to form calcium alginate via formation of ionic bonds between carboxylic acid groups of the guluronic acid molecules of the alginate. The sodium alginate will stabilize and remain stable at room temperature and does not require heat to produce gel. The hardness of the capsule is a function of the guluronic and mannuronic acid ratio and the complexing time. Minimum complexing time in calcium chloride is 15 minutes, otherwise, the centre of the capsule will still be liquid. The capsule hardness of 0.5 to 2.0 kg breaking pressure per capsule was found to allow germination, while providing sufficient integrity so that the capsules could be handled on a routine basis without damage or breakage. The size of the capsule can be controlled by the viscosity of the sodium alginate and by the inside diameter of the nozzle used to form the drops (Redenbaugh, 1993b).

Redenbaugh *et al.* (1987) reported that the alginate encapsulated seeds of celery, an albuminous species should contain a synthetic endosperm to provide nutrients and carbon source necessary for germination and conversion. Whereas,

alfalfa, a non albuminous crop should need no endosperm. However, several experiments conducted to test conversion frequencies for both alfalfa and celery synthetic seeds showed that, in general conversion frequencies of encapsulated somatic embryos were less than that for non encapsulated embryos. Similarly, the conversion frequencies for both alfalfa and celery synthetic seeds in an *in vivo* environment resulted in much lower conversion frequencies (7% and 10% respectively) as compared to *in vitro* frequencies. The storage life of the encapsulated somatic embryos was found to be quite short with the alginate encapsulation protocol in alfalfa. There was a significant decrease in subsequent conversion after 7 days, whereas the conversion frequencies of naked embryos were only slightly diminished over a 49 day period. The inhibition of embryo respiration was quoted as the possible reason for decreased conversion frequencies.

Datta and Potrykus (1989) studied the conversion frequencies of encapsulated and non encapsulated somatic embryos of barley (*Hordeum vulgare*) both at fresh and after 6 months storage. The somatic embryos obtained from microspore explants without intervening callus phase were encapsulated using sodium alginate and calcium chloride and were allowed to germinate directly on modified FW medium or stored at 4°C in the dark in sterile petri dishes. The germination test was also performed for non encapsulated embryos derived fresh as well as from 6 months stored ones under identical conditions. The comparison of conversion frequencies of encapsulated and non encapsulated somatic embryos revealed that the germination response was more vigorous for encapsulated embryos than that of non encapsulated embryos. The conversion frequency expressed in terms of germination per cent and seedling development was maximum

with 80 per cent of artificial seeds germinating, compared to 62 per cent of non encapsulated under fresh culture. In contrast, the non encapsulated embryos stored under identical conditions were dead within 2 weeks. However, the encapsulated embryos germinated well with a germination of 37.5 per cent even after 6 months of cold storage.

The effect of hardening treatments to increase the survival of synthetically coated asexual embryos of carrot was investigated by Kitto and Janick (1985b). Hardening carrot embryos by exposure during the induction phase to high inoculum density (0.8g as compared to 0.4g/25ml), high sucrose (12% as compared to 2%), chilling (4°C the last 3 days of a 2 week embryo induction phase otherwise carried out at 29°C) or pre treated with abscisic acid (ABA) at 10^{-6} M increased survival of embryos after polyox encapsulation, compared to non hardened embryos. Although individual hardening treatments increased embryo survival, the combinations of ABA with high inoculum density, high sucrose or chilling resulted in lower embryo survival. The possible explanation for the absence of additivity is that the asexual embryos of carrot treated with ABA in combination with another ABA regime have supra-optimal ABA levels.

Fujii *et al.* (1989), reported that the establishment of plants from artificial seeds may be increased by bringing improvement in maturation of somatic embryos. Maturation of somatic embryos on a medium containing ABA prior to encapsulation increased plant establishment to 80 per cent when they were planted directly into a soil potting mixture under non sterile conditions, without any exogenous nutrients in growth chambers.

Chetia and Devi (1996) studied the regeneration potential of orchid species from artificial seeds. Artificial seeds were developed by encapsulating protocorms with sodium alginate matrix in three orchid species, namely *Cymbidium longifolium*, *Renanthera imschootiana* and *Agrostophyllum myrianthum* and cultured in Nitsch medium and 0.7 per cent agar along with non encapsulated protocorms. Artificial seeds showed higher germination compared to non encapsulated in both the media. Artificial seeds of *C. longifolium* showed maximum germination in both Nitsch (86%) and 0.7 per cent agar medium (80%) after 5 weeks of culture. Whereas, *A. myrianthum* showed the least germination of 63.33 per cent in Nitsch medium and 60 per cent in 0.7 per cent agar medium after 7 weeks. Though germination was slightly lower in agar medium, the authors claimed that the agar medium could be efficiently used for germination of orchid artificial seeds of orchids from the point of economy. Reculture of artificial seeds stored at room temperature and 4°C at 15 days interval showed decline in germination with increase in duration of storage. A low germination (10-20%) was observed after 45 days of storage at room temperature. However, about 50 per cent germination can be maintained upto 60 days by storing at 4°C which will facilitate transportation of germplasm and thereby help in commercial propagation of orchids.

MATERIAL AND METHODS

III. MATERIAL AND METHODS

The present investigations were carried out in the plant tissue culture laboratory of the Department of Horticulture, Government of Karnataka, Lalbagh, Bangalore. The broad outlines about the materials used and methodologies adopted are described hereunder.

Plant material

The corms of elite *Gladiolus* cultivars namely Priscilla, Victor Borge and Rose Supreme, obtained from Flamingo International, Holland through Kumar Gen-Tech and Tissue Cultures Co., Pune, were utilized as explant material. The salient features of the cultivars selected for the study are as follows :

Cultivar	Flower Colour	Days to flower	Corm multiplication ratio
Priscilla	Light pink edged with lilac-pink and yellow centre.	65-70	1: 2.15
Victor Borge	Vermilion-red	60-65	1: 1.50
Rose Supreme	Rose with creamy green throat	70-75	1: 2.60

Preparation of explant

Initially tunics and other appendages were removed from the corms. The corms were washed thoroughly under running tap water and soaked in 0.2 per cent

carbendazim solution for 3 hours. Further, they were also washed with double distilled water (DDW) thoroughly and the tissue explants, each having one shoot bud, were removed from these corms. The explants were treated with 0.1 per cent streptomycin for 15 min. and 0.1 per cent mercuric chloride for 5 min. sequentially and were repeatedly rinsed with sterile water. The explants were further trimmed to approximately 0.75 cm³ and used for inoculations. All operations were carried out under aseptic conditions.

Culture media

Murashige and Skoog's (1962) medium, which is being used for *in vitro* studies in general among horticultural crops was used. The composition of MS medium is presented in Table 1.

Stock solutions

Stock solutions were prepared by classifying different constituents of the MS (1962) nutrients into various groups. Generally, the nutrients were weighed individually and dissolved in double distilled water one by one under each group (Table 1), while, the stocks of organics like thiamine HCl, pyridoxine HCl and nicotinic acid were prepared separately and used at the time of preparation of the media. Other organics like myoinositol and glycine were weighed separately and incorporated into the medium directly.

The stocks of 3-indolebutyric acid (IBA), 1-napthalene acetic acid (NAA), 2,4-dichlorophenoxy acetic acid (2,4-D) were prepared by dissolving 100 mg chemical in

Table 1. Murashige and Skoog's medium (1962) : Composition and preparation.

Ingradients	Final concentration (mg/l)	Weight of salts in stock soln.	Volume made up with D.W (ml)	Aliquots use medium (ml)
A. Nitrates				
NH ₄ NO ₃	1650.00	41.250g		
KNO ₃	1900.00	47.500g	500	20
B. Sulphates				
MgSO ₄ 7H ₂ O	370.00	9.25 g		
MnSO ₄ 4H ₂ O	22.30	557.50mg		
ZnSO ₄ 7H ₂ O	8.60	215.00mg	250	10
CuSO ₄ 5H ₂ O	0.025	2.50mg	100	1
C. Halides				
CaCl ₂ 2H ₂ O	440.00	11.00 g		
KI	0.83	20.75mg	250	10
CoCl ₂ 6H ₂ O	0.025	2.50mg	100	1
D. Borate and molybdate				
H ₃ BO ₃	6.20	155.00mg		
Na ₂ MoO ₄ 2H ₂ O	0.25	6.25mg	250	10
E. Phosphate				
KH ₂ PO ₄	170.00	4.25 g	250	10
F. Iron				
Na ₂ EDTA	37.30	1.492 g		
FeSO ₄ 7H ₂ O	27.80	1.112 g	200	5
G. Vitamins and other organics				
Thiamine HCl	0.10	100.00mg	100	0.1
Pyridoxine HCl	0.50	100.00mg	100	0.5
Nicotinic acid	0.50	100.00mg	100	0.5
Glycine	2.00	2.00mg	5	5
Myoinositol	100.00	100.00mg	100	100

Sucrose : 30 g/l. Agar : 7.5 g/l. pH : 5.8

a few drops of ethyl alcohol and the final volume was made up to 100ml by adding double distilled water. Similarly, stocks of 6-benzylaminopurine (BAP) and 6-furfurylaminopurine (kinetin) were prepared by dissolving 100 mg chemical in minimal quantity of 0.1 N HCl and the final volume was made up to 100 ml by adding double distilled water in a volumetric flask. All the stock solutions were stored in a refrigerator (4-5°C) and made use of in preparation of the medium.

Preparation of the medium

Sucrose at 30g/l was first dissolved in double distilled water and transferred to a volumetric flask. Appropriate amounts of stock solutions (Table 1) containing media constituents including organic supplements were pipette out into the sucrose solution. The growth regulators were added as per treatment under each experiment. The final volume of the medium was made up by adding double distilled water. The pH was adjusted to 5.8 by adding 0.1 N HCl or 0.1 N NaOH. Agar at the rate of 7.5g/l was added as the gelling agent. The medium was dispensed into test tubes or conical flasks, as the case may be. The culture vessels were plugged with cotton plugs or polyurethane caps and sterilized by autoclaving at 121°C and 1.2kg/cm² pressure for 15 minutes. The vessels were stored under dust-free conditions before inoculating.

Culture incubation

The cultures were incubated in controlled environment chamber maintained at a temperature of 26±2 °C and relative humidity (RH) of 60 per cent. The light was provided at 1500 Lux under a photoperiodic regime of 16 hours light and 8 hours

dark. To provide adequate aeration in liquid cultures, the flasks containing the cultures were placed on a horizontal rotary shaker and were agitated continuously at 150 rpm for the required duration.

Experimental series

The experiments conducted during the course of the study were broadly classified into five groups. They were:

1. Response of explants to growth regulators *in vitro*.
2. Growth and maintenance of callus cultures.
3. Regeneration response of callus.
4. Somatic embryogenesis in callus cultures, and
5. Production of synthetic seeds and their viability.

The details of the treatments imposed, specific procedures adopted, culture conditions provided and observations recorded under each experiment are presented as follows :

Response of explants to growth regulators

Experiment 1. Effect of 6-benzylaminopurine (BAP) and kinetin on sprouting of shoot bud explant.

The objective of this experiment was to maximise growth/production of young meristems suitable to be used as explants for callus initiation on medium containing different levels of BAP and kinetin.

The treatmental details of the experiment are as follows :

Sl.No.	Treatment	Sl.No.	Treatment
1.	Control 0.0	5.	Kinetin 2.0
2.	BAP (mg/l) 2.0	6.	4.0
3.	4.0	7.	6.0
4.	6.0		

Culture details

Culture medium : MS (1962). pH : 5.8

Explant : Shoot buds along with corm tissue. Culture vessels : Baby jars.

Volume of the medium : 40ml/culture. No. of replications : 6

No. of explants/replication : 24. Culture period : 20 days.

Observations

i. No. of explants responded / culture. ii. No. of sprouts produced/culture.

Experiment 2. Effect of interaction between 2,4-D and BAP on callus formation in meristem explants.

To induce callus from the young meristems, the interaction effect of 2,4-D and BAP was studied under this experiment.

The treatmental details of the experiment are as follows :

Sl.No.	Treatment		Sl.No.	Treatment	
	2,4-D	BAP		2,4-D	BAP
	(mg/l)	(mg/l)		(mg/l)	(mg/l)
1.	2.0	0.0	7.	6.0	0.2
2.	4.0	0.0	8.	8.0	0.2
3.	6.0	0.0	9.	2.0	0.5
4.	8.0	0.0	10.	4.0	0.5
5.	2.0	0.2	11.	6.0	0.5
6.	4.0	0.2	12.	8.0	0.5

Culture details

The experiment was conducted in two stages. In stage I, young meristems proliferated on MS medium containing 4.0 mg/l kinetin and in stage II, the cultures from stage I, were used as explants.

Culture medium : MS (1962). pH : 5.8

Volume of the medium : 16 ml/culture.

Culture vessels : Test tubes.

No. of replications : 6

Culture period : 30 days in both stages.

Inoculum : 250 ± 10 mg/culture.

Observations

Stage I: The response was measured by the degree of expansion of explants and scored as: very poor (+), poor (+ +) and good (+ + +).

Stage II: Callus induction was measured by determining the fresh weight of harvested callus (g/culture).

Experiment 3: Effect of interaction between NAA and BAP on callus formation in meristem explants.

The interaction effect of NAA and BAP on callus induction was studied with different treatment combinations.

The treatmental details are as follows :

Sl.No.	Treatment			Sl.No.	Treatment		
	NAA	+	BAP		NAA	+	BAP
		mg/l				mg/l	
1.	2.0		0.0	7.	6.0		0.2
2.	4.0		0.0	8.	8.0		0.2
3.	6.0		0.0	9.	2.0		0.5
4.	8.0		0.0	10.	4.0		0.5
5.	2.0		0.2	11.	6.0		0.5
6.	4.0		0.2	12.	8.0		0.5

Culture details

This experiment was also conducted in two stages. Similar to the previous experiment, the young meristems that were proliferated on MS medium containing 4.0 mg/l kinetin was used as explant material in stage I. In stage II, the cultures of stage I were continued with the same treatment combinations to induce callus.

Medium : MS (1962). pH : 5.8

Culture vessels : Test tubes. Volume of the medium : 16 ml/culture.

Explant : Stage I - Young meristems. Stage II - Inoculum from stage I.

Culture period : 30 days in both the stages.

No.of replications : 6 Inoculum : 250 ± 10 mg/culture.

Observations

Stage I: Response was measured by the degree of expansion of explants and scored as: very poor (+), poor (+ +) and good (+ + +).

Stage II: Callus induction was measured by determining the fresh weight of harvested callus (g/culture) from the pre weighed explant.

Growth and maintenance of callus cultures

Experiment 1. Effect of 2,4-D, BAP and kinetin on growth and survival of callus in cultures.

The objective of this experiment was to optimize the concentration and combination of growth regulators for further maintenance of the induced callus. Therefore, the effect of different levels of 2,4-D in combination with BAP and kinetin on callus proliferation and survival was tested.

The treatmental details are as follows :

Sl.No.	Treatment		Sl.No.	Treatment	
	2, 4-D	+ BAP		2, 4-D	+ kinetin
	(mg/l)			(mg/l)	
1.	0.5	0.0	10.	0.5	0.2
2.	0.5	0.2	11.	0.5	0.5
3.	0.5	0.5	12.	1.0	0.2
4.	1.0	0.0	13.	1.0	0.5
5.	1.0	0.2	14.	2.0	0.2
6.	1.0	0.5	15.	2.0	0.5
7.	2.0	0.0			
8.	2.0	0.2			
9.	2.0	0.5			

Culture details

The callus induced on MS medium with 4.0 mg/l NAA and 0.2 mg/l BAP was used as inoculum for this experiment.

Culture medium : Murashige and Skoog (1962). pH : 5.8

Volume of the medium : 16ml/culture. Culture vessels : Test tubes.

Inoculum size : 250 ± 10 mg/culture. Culture period : 30 days.

No. of replications : 9

Observations

Callus growth (g/culture) and Callus survival (days to senescence).

To determine the growth of the callus, weight of the harvested callus was recorded from the pre weighed inoculum. The survival of the callus was decided by the number of days in which the callus remained healthy on the culture medium.

Experiment 2. Patterns of growth in the callus of *Gladiolus* cultivars.

The objective of this experiment was to find out the varietal differences in the patterns of growth, if any among the cvs. Priscilla, Victor Borge and Rose Supreme.

Culture details

Prewriteghed (200 mg/culture) calluses of the three cultivars were grown on MS medium containing 1.0 mg/l 2,4-D and 0.2 mg/l BAP. Twenty seven tubes were maintained for each variety. Callus from three number of tubes was harvested at 5 days interval and its fresh weight determined. Samples of pre weighed fresh tissues were dried at 50 °C for 5 hours and their dry weights determined. The growth of callus on the basis of fresh and dry weights per culture for each of the three cultivars was plotted against culture duration (40 days), to determine the patterns of growth.

Observations

- i. Callus fresh weight (mg/culture), and
- ii. Callus dry weight (mg/culture)

Experiment 3 . Effect of different sources of nitrogen on growth and survival of callus in cultures.

The objective of this experiment was to evaluate the influence of different sources of nitrogen in culture medium on callus proliferation and survival. The callus induced on MS medium containing 1.0mg/l 2,4-D and 0.2mg/l BAP was used as inoculum for this experiment.

The details of the experiment are as follows :

Sl.No.	Treatment
1.	Control
2.	NH ₄ SO ₄ (100mg/l)
3.	NH ₄ SO ₄ (200mg/l)
4.	NH ₄ NO ₃ (100mg/l)
5.	NH ₄ NO ₃ (200mg/l)
6.	Casein hydrolysate (100mg/l)
7.	Casein hydrolysate (200mg/l)
8.	NH ₄ SO ₄ (100mg/l)+casein hydrolysate(100mg/l)
9.	NH ₄ NO ₃ (100mg/l)+casein hydrolysate(100mg/l)
10.	NH ₄ SO ₄ (100mg/l)+NH ₄ NO ₃ (100mg/l)
11.	NH ₄ SO ₄ (100mg/l)+NH ₄ NO ₃ (100mg/l)+casein hydrolysate(100mg/l)
12.	NH ₄ SO ₄ (100mg/l)+NH ₄ NO ₃ (100mg/l)+casein hydrolysate(200mg/l)

Culture details

Culture medium : MS(1962)with 1.0mg/l 2,4-D and 0.2 mg/l BAP. pH : 5.8

Volume of the medium : 16ml/culture. Culture Vessels : Test tubes.

Inoculum : 250 $\pm$ 10 mg/culture. Culture period : 30 days. No. of replications : 9

Observations

- i. Callus growth (g/culture), and ii. Callus survival (days to senescence)

Regeneration response of callus

Experiment 1. Effect of interaction between BAP and NAA on morphogenic response of callus.

The objective of this experiment was to induce proembryogenic masses (PEMs) from the calluses of cultivars Priscilla, Victor Borge and Rose Supreme on MS medium containing different levels of BAP and kinetin.

The following are the treatment and other culture details

Sl.No.	Treatment		Sl.No.	Treatment	
	BAP	NAA		BAP	NAA
	(mg/l)			(mg/l)	
1.	0.0	0.0	10.	1.0	0.0
2.	0.0	0.5	11.	1.0	0.5
3.	0.0	1.0	12.	1.0	1.0
4.	0.2	0.0	13.	2.0	0.0
5.	0.2	0.5	14.	2.0	0.5
6.	0.2	1.0	15.	2.0	1.0
7.	0.5	0.0			
8.	0.5	0.5			
9.	0.5	1.0			

Culture details

The callus maintained on MS medium containing 1.0 mg/l 2,4-D and 0.2mg/l BAP was subcultured on MS media containing different concentrations and combinations of NAA and BAP and the responses were observed at 3 stages viz. development of proembryogenic masses, transformation of proembryogenic masses into somatic embryos and development of adventitious shoots / roots.

Culture medium : MS (1962). pH : 5.8

Volume of the medium : 16 ml/culture. Culture vessels : Test tubes.

Inoculum : 250 ± 10 mg/culture. Culture period : 60 days. No. of replications : 6

Observations

i. Proembryogenic masses. The degree of response was scored as:

Nil (-), very poor (+), poor (+ +) and good (+ + +).

ii. No. of somatic embryos per culture. iii. No. of adventitious shoots /culture.

The first observation was made on 60 th day after inoculation for the induction of proembryogenic masses (PEMs). The second observation after 45 days of inoculation for transformation of PEMs into somatic embryos and the third observation after 60 days of inoculation for development of adventitious shoots.

Experiment 2. Effect of interaction between kinetin and NAA on morphogenic response of callus.

The objective of this experiment was also to induce proembryogenic masses from the callus of the cvs. Priscilla, Victor Borge and Rose Supreme on MS medium containing different levels of kinetin and NAA.

The treatmental details of the experiment are as follows:

Sl.No.	Treatment		Sl.No.	Treatment	
	Kinetin	NAA		Kinetin	NAA
	(mg/l)	(mg/l)		(mg/l)	(mg/l)
1.	0.2	0.0	7.	1.0	0.0
2.	0.2	0.5	8.	1.0	0.5
3.	0.2	1.0	9.	1.0	1.0
4.	0.5	0.0	10.	2.0	0.0
5.	0.5	0.5	11.	2.0	0.5
6.	0.5	1.0	12.	2.0	1.0

Culture details

The callus maintained on MS medium containing 1.0 mg/l 2,4-D and 0.5 mg/l BAP was used as the inoculum.

Culture medium : MS (1962). pH : 5.8

Volume of the medium : 16ml/culture. Culture vessels : Test tubes.

Inoculum : 250 ± 10 mg/culture. No. of replications : 6 Culture period : 60 days.

Observations

Observations made were similar to the previous experiment.

i. Proembryogenic masses (PEMs): The degree of response was scored as:

Nil (-), very poor (+), poor (+ +) and good (+ + +).

ii. No. of somatic embryos per culture. iii. No. of adventitious shoots/culture.

Somatic embryogenesis in callus cultures

Experiment 1. Effect of BAP and kinetin on transformation of proembryogenic masses into somatic embryos and plantlets.

The objective of this experiment was to induce the processes of transformation of proembryogenic masses into somatic embryos and plantlets.

The treatmental details of the experiment are as follows :

Sl.No.	Treatment	Sl.No.	Treatment
1.	BAP mg/l 0.2	5.	Kinetin (mg/l) 0.2
2.	0.5	6.	0.5
3.	1.0	7.	1.0
4.	2.0	8.	2.0
		9.	Control 0.0

Culture details

The proembryogenic masses (PEMs) developed on MS agar medium containing 0.5mg/l BAP and 1.0mg/l NAA for a period of 30 days were used as inoculum in this experiment.

Culture medium: MS (1962). pH : 5.8 Volume of the medium : 16ml/culture.

Culture vessels : Test tubes. Inoculum: 250 ± 10 mg/culture .

No. of replications: 6. Culture duration: 40 days.

Observations

- i. No. of somatic embryos /culture on 20th day after transfer from embryo induction medium.
- ii. No. of plantlets/culture on 40th day after transfer from embryo induction medium.
- iii. Length of plantlets (cm) iv. No. of roots/culture.

Experiment 2. Effect of pre incubation on transformation of proembryogenic masses into somatic embryos and plantlets.

This experiment was conducted to induce further the conversion of proembryogenic masses (PEMs) into somatic embryos by incubating the PEMs for a short period of 10 days with different levels of BAP and kinetin followed by transfer to hormone-free half strength MS medium.

The details of the treatment and culture conditions are as follows :

Sl. No.	Treatment (Pre incubation)	Sl. No.	Treatment (Pre incubation)
1.	BAP (mg/l) 0.2	5.	Kinetin (mg/l) 0.2
2.	0.5	6.	0.5
3.	1.0	7.	1.0
4.	2.0	8.	2.0
		9.	Control 0.0

Culture details

Incubation medium: MS with growth regulators as per treatment. Development medium: Half strength MS free from growth hormones. Culture vessels: Test tubes.

No. of replications : 6 Inoculum : 250 ± 10 mg/culture.

Culture duration: Incubation medium : 10 days. Development medium : 30 days.

Observations

- i. No. of somatic embryos /culture on 10th day after transfer from incubation medium.
- ii. No. of plantlets/culture on 30th day after transfer from incubation medium.
- iii. Length of plantlets (cm), and iv. No. of roots/culture.

Experiment 3. Effect of culture media and duration on somatic embryo-genesis and plantlet production.

The objective of this experiment was to define suitable embryo induction medium and also duration of culture to maximise embryo proliferation and plantlet production.

The details of the experiment are as follows:

Sl. No.	Treatment Days in (EIM)	Sl. No.	Treatment Days in (EDM)
	Agar (EIM) - Agar (EDM)		Liquid (EIM) - Agar (EDM)
1.	0	8.	5
2.	5	9.	10
3.	10	10.	15
4.	15	11.	20
5.	20	12.	25
6.	25	13.	30
7.	30		

Culture details

The calluses of Priscilla and Victor Borge maintained on MS medium containing 1.0 mg/l 2,4-D and 0.5 mg/l BAP were cultured simultaneously on agar and liquid media, both containing MS salts with 0.5 mg/l BAP and 1.0 mg/l NAA, for a period of 30 days. The conical flasks containing liquid cultures were placed on horizontal rotary shaker at 150 rpm. During the period of incubation, cultures were drawn from both the media and subsequently cultured on agar medium containing MS salts with 0.2 mg/l kinetin.

Embryo induction medium(EIM): MS +0.5mg/l+BAP+ 1.0mg/l NAA.

Embryo development medium (EDM) : MS + 0.2 mg/l kinetin.

Culture vessels : Agar culture - Test tubes. Liquid culture : 250 ml flasks.

No. of replications : 6 Inoculum in agar medium : 250 ± 10 mg/culture.

Inoculum in liquid medium : 750 mg/culture following transfer to 3 culture tubes containing agar medium.

Observations

- i. No. of somatic embryos/culture on 20 th day after transfer from embryo induction medium to embryo development medium.
- ii. No. of plantlets/culture and No. of roots /culture were recorded on 40 th day after transfer from embryo induction medium to embryo development medium.

Experiment 4. Effect of culture media on embryo morphology and plantlet production.

The objective of this experiment was to define culture medium optimum for embryo induction, conversion and plantlet production .

The details of the experiment are as follows :

Sl. No.	Treatment
1.	Agar embryo induction medium (EIM) for 30 days transferred to agar embryo development medium (EDM).
2.	Liquid embryo induction medium (EIM) for 25 days transferred to agar embryo development medium (EDM).
3.	Liquid embryo induction medium (EIM) for 25 days transferred to liquid embryo development medium (EDM) for 20 days and later transferred to plantlet production medium (PLM).

Culture details

The callus of the cv. Victor Borge from maintenance medium was cultured simultaneously on agar and liquid induction media (MS + 1.0 mg/l NAA + 0.5 mg/l BAP) for a period of 30 days and 25 days, respectively and transferred subsequently to agar development medium. In another treatment, callus cultured in liquid induction medium for a period of 25 days was transferred to liquid embryo development medium for a period of 20 days and subsequently transferred to agar plantlet production medium.

Embryo induction medium (EIM) : MS + 0.5 mg/l BAP+1.0mg/l NAA

Embryo development medium (EDM) : MS + 0.2mg/l Kinetin

Plantlet production medium (PLM) : Half MS free from hormones.

Culture vessels: Agar culture - Test tubes. Liquid culture - 250ml conical flasks.

No. of replications : 6 Inoculum in agar medium : 250 ± 10 mg/culture.

Inoculum in liquid medium: (i) 750 mg/l culture followed by transfer to culture tubes containing agar embryo development medium in treatment 2.

(ii) 750mg / culture followed by transfer entirely to a flask containing liquid embryo development medium and subsequently transferred to 3 culture tubes containing plantlet production medium.

Observations

- i. No. of embryos /culture, embryo length (mm) and thickness (mm) were recorded on agar EDM on 20th day after transfer from agar/liquid EIM in case of treatment 1 and 2 and in liquid EDM on 20th day after transfer from liquid EIM in case of treatment 3.
- ii. No. of plantlets/culture were recorded on agar EDM on 40th day after transfer from agar/liquid EIM in case of treatments 1 and 2 and on agar PLM on 20th day after transfer from liquid EDM in case of treatment 3.

Experiment 5. Stages in somatic embryogenesis and the role of protein synthesis on somatic embryogenesis and plantlet production.

The objective of this experiment was to observe closely the embryological stages involved in differentiation and development of somatic embryos and also to examine the role of protein synthesis in regulation of the process of embryogenesis by incorporating cycloheximide (CH), a protein synthesis inhibitor into embryo induction medium.

Treatment details

Set I : 5, 10, 15, 20, 25 and 30th day in liquid embryo induction medium without CH.

Set II: Incorporation of CH on 5, 10, 15, 20, 25 and 30th day after inoculation into liquid embryo induction medium.

Culture details

To observe the embryological stages periodically, samples of the culture at different events of morphogenesis were drawn and examined under microscope by taking sections wherever required. Photographs of all the stages of embryo development were taken.

To examine the role of protein synthesis in the process of somatic embryogenesis, the effect of cycloheximide, on embryo development and plantlet production was tested. This experiment was conducted in two sets. In first set, the callus from maintenance medium was cultured on liquid induction medium for a period of 30 days. During the induction period, cultures were drawn periodically at every 5 days interval and transferred to agar development medium. To another set, cycloheximide at a concentration of 0.5 per cent was incorporated at an interval of 5 days from the day of inoculation of callus and all the cultures were continued for a period of 30 days. Later, the cultures were transferred to agar development medium. Observations were made for embryo development and plantlet production in both the sets and the results were compared.

Embryo induction medium: MS + 0.5 mg/l BAP + 1.0 mg/l NAA.

Embryo development medium : MS + 0.2 mg/l kinetin.

Volume of the liquid induction medium : 80 ml/culture.

Volume of the agar development medium : 16 ml/culture.

Culture duration: Without cycloheximide: As per treatment.

With cycloheximide : 30 days (cycloheximide incorporated at 5 days interval and continued up to 30 days). No. of replications : 6

Observations

- i. No. of embryos matured on development medium on 20th day after transfer from induction medium.
- ii. No. of plantlets produced /culture on development medium on 40th day after transfer from induction medium.

Production of synthetic seeds and their viability

Experiment 1. Effect of hydrogel encapsulation on survival of callus, shoot buds and somatic embryos under different storage temperatures.

The objective of this experiment was to understand the role of hydrogel encapsulation on survival of different material viz. callus, shoot buds and somatic embryos derived *in vitro* under different storage temperatures.

Experimental details

Sodium alginate at 4 per cent and calcium chloride at 100 mM were used as the encapsulating gel and the complexing agent, respectively. The procedure adopted for encapsulation was as follows:

Callus bits, adventitious shoot buds and somatic embryos obtained *in vitro* were mixed aseptically with sodium alginate and dropped into a beaker containing calcium chloride using a pipette, in such a way that each drop contained a callus bit or adventitious shoot bud or somatic embryo at the centre. The complexing time allowed was a minimum of 15 minutes. Later, the capsules were rinsed with sterile

distilled water and stored in sterile petri plates. These petri plates were covered with para film on their sides.

The petri plates containing the encapsulated capsules were stored in culture room at $26 \pm 2^{\circ}\text{C}$ under continuous dark and in refrigerator at $5 \pm 1^{\circ}\text{C}$ for a period of 30 days. The survival of callus, shoots buds and somatic embryos were tested on the day of encapsulation and on 3, 6, 10, 15 and 30th day after encapsulation by drawing samples from both the storage conditions. Simultaneously, non encapsulated materials were also stored under similar conditions and tested for their survival.

The survival of encapsulated callus, shoot buds and somatic embryos was assessed by subjecting to the Tetrazolium test (Lakon, 1949). The encapsulated callus/adventitious shoots/somatic embryos were soaked in 2,3,5 triphenyl tetrazolium chloride solution (1%) for a period of 1 hour and observed for development of a red coloured compound (formazan). Such of the calluses/adventitious shoots/somatic embryos within the capsules that had taken more than 75 per cent red colour, were considered as viable and the rest as dead.

Observations

- i. No. of surviving encapsulated callus, adventitious shoot buds and SEs.
- ii. No. of surviving non encapsulated callus, adventitious shoot buds and SEs.

Experiment 2. Effect of different additives on contamination, survival and germination of encapsulated somatic embryos.

The objective of this experiment was to develop a suitable methodology for production of hydrated synthetic seeds free of contamination.

The treatments imposed and other details of the experiment are as follows:

Sl.No.	Treatment
1.	Control : Encapsulating SEs with sodium alginate, complexed with CaCl ₂ both nonsterilised.
2.	Addition of 0.1% carbendazim to sodium alginate, complexed with CaCl ₂ both nonsterilized.
3.	Addition of 0.1% streptomycin to sodium alginate, complexed with CaCl ₂ both nonsterilized.
4.	Addition of 0.1% carbendazim and 0.1% streptomycin to sodium alginate, complexed with CaCl ₂ both nonsterilized.
5.	Encapsulating SEs with sodium alginate gel, complexed with CaCl ₂ both sterilized.
6.	Encapsulating SEs with sodium alginate containing half MS basal nutrients complexed with CaCl ₂ both sterilized.

Experimental details

The synthetic seeds were produced by encapsulating the somatic embryos with sodium alginate gel (4%) and complexing with calcium chloride (100 mM). Before mixing the SEs with the alginate gel, various additives were added into the gel as per treatments. The encapsulating gel and complexing agents were sterilized or non sterilized as the case may be. The encapsulated embryos were plated on germination medium in sterile petri plates and the plates were incubated in the culture room.

Encapsulation gel :Sodium alginate (4%). Complexing agent: CaCl₂(100mM)

Germination medium : Half strength MS basal. pH : 5.8 No. of replications : 6

Germination conditions : Temperature : 26 ± 2 °C. Light/dark period : 16/8 Hrs.

Observations

- i. Number of capsules contaminated with both bacteria and fungus.
- ii. Number of capsules contaminated with only fungus.
- iii. Number of capsules contaminated with only bacteria.
- iv. Number of encapsulated embryos survived.
- v. Number of encapsulated embryos germinated.

Experiment 3. Effect of hardening treatments on survival of hydrogel encapsulated somatic embryos.

This experiment was aimed at increasing the survival of encapsulated somatic embryos by subjecting them to different hardening treatments before encapsulation.

The details of the treatments imposed are as follows:

Sl.No.	Treatment
1.	Encapsulation of SEs immediately after harvest from development medium.
2.	Encapsulation of SEs after subjecting them to desiccation under a laminar airflow cabinet for 1 hour.
3.	Addition of $1\mu\text{M}$ ABA to liquid development medium 6 days prior to embryo harvest and immediate encapsulation.
4.	SEs subjected to chilling @ 5°C for 3 days before encapsulation.
5.	Addition of $1\mu\text{M}$ ABA to the liquid development medium 6 days before harvest and subjecting SEs to chilling @ 5°C for 3 days before encapsulation.

Experimental details

The encapsulated embryos were stored in sterile petri plates for a period of 30 days. The capsules were drawn on 0, 5, 15 and 30th day of storage and tested for their survival by Tetrazolium test.

Encapsulation gel : Sodium alginate (4%) sterilized.

Complexing agent : CaCl_2 (100mM) sterilized.

Storage conditions : Temperature : 26 ± 2 °C, under continuous dark.

No. of replications : 6

Observations

Number of encapsulated somatic embryos survived after 30 days.

Statistical analysis

The data recorded from all the experiments were statistically analysed by following the completely randomised design (CRD) as described by Panse and Sukhatme (1978) and the level of significance was expressed at 5 % alpha level.

EXPERIMENTAL RESULTS

IV. EXPERIMENTAL RESULTS

The results of the studies on somatic embryogenesis and production of synthetic seeds in *Gladiolus* cultivars are presented in this chapter.

Response of explants to growth regulators *in vitro*.

Three experiments were conducted in succession to study the response of explants to different growth regulators to maximize *in vitro* sprouting and to induce callus.

1. Effect of 6-benzylaminopurine (BAP) and kinetin on sprouting of shoot bud explants in *Gladiolus* cultivars.

The shoot bud explants along with corm tissue of the cvs. Priscilla, Victor Borge and Rose Supreme were cultured on MS medium containing different levels of BAP and kinetin. The response of explants to produce multiple sprouts differed significantly with the type of hormone and its concentration. The response was better at all levels of BAP and kinetin, when compared with the control and the maximum response was noticed under the influence of kinetin at 4.0 mg/l in all the cultivars. Among the cultivars, the highest response to produce multiple shoots came from Rose Supreme followed by Priscilla and Victor Borge. The mean number of explants responded out of 24 explants incubated were 23.83, 23.50 and 22.67 in Rose Supreme, Priscilla and Victor Borge, respectively. The response under control was low, as only 22.0, 20.17 and 20.0 out of 24 explants responded in Rose Supreme, Priscilla and Victor Borge, respectively.

Generally, sprouting of explants was better on a kinetin medium unlike the BAP medium and the number of sprouts per explant was greater in the medium containing 4.0mg/l kinetin in all the cultivars. Among the cultivars, the production of sprouts per explant was highest in Rose Supreme (7.67) compared to Priscilla (6.17) and Victor Borge (5.17). Sprouting was minimum (1.0) under control in all the cultivars (Table 2).

2. Effect of interaction between 2,4-D and BAP on callus formation in meristem explants of *Gladiolus* cultivars.

Culturing of young meristems on MS medium fortified with 2,4-D and BAP in different combinations and concentrations did not induce any callus even after 30 days of inoculation. The explants showed mere expansion in their sizes and the degree of expansion varied with the hormonal levels and their combinations (Plate 1). While the response of Priscilla was good on media supplemented with 6 and 8 mg/l 2,4-D and 0.5mg/l BAP, Victor Borge responded better on media containing 6 and 8 mg/l 2,4-D with either 0.2 or 0.5 mg/l BAP. The hormonal combination of 6.0mg/l 2,4-D and 0.2 mg/l BAP was found to be optimum in Rose Supreme.

The explants with different degrees of expansion under stage-I, when transferred to media containing the identical combinations and concentrations of growth hormones showed callus induction (Plate 2). The callus development was significantly higher on medium fortified with 2.0 mg/l 2,4-D and 0.2 mg/l BAP in Priscilla (0.85 g/culture) and Victor Borge (0.60 g/culture), whereas 4.0 mg/l 2,4-D with 0.2 mg/l BAP supported higher callus development in Rose Supreme (0.61 g/culture). The results are presented in Table 3.

Table 2. Effect of 6 - benzylaminopurine (BAP) and kinetin on sprouting of explants in *Gladiolus* cultivars.

Sl. No.	Treatment	Cultivars					
		Priscilla		Victor Borge		Rose Supreme	
		No. of Explants responded	No. of Sprouts per explant	No. of Explants responded	No. of Sprouts per explant	No. of Explants responded	No. of Sprouts per explant
1.	Control	20.17	1.00	20.00	1.00	22.00	1.00
2.	BAP(mg/l) 2.0	21.83	1.67	20.83	1.50	22.50	2.17
3.	4.0	22.83	4.67	22.50	3.00	23.33	6.67
4.	6.0	23.00	4.83	22.33	3.50	22.83	6.83
5.	Kinetin(mg/l) 2.0	22.00	2.33	21.67	2.00	23.00	2.67
7.	4.0	23.50	6.17	22.67	5.17	23.83	7.67
8.	6.0	23.17	6.00	22.67	4.83	23.00	7.50
CD (0.05 P)		1.01	0.66	1.71	0.77	0.89	0.65
SEm(±)		0.71	0.47	1.21	0.55	0.63	0.46

Culture medium : Murashige and Skoog (1962). pH : 5.8

Volume of the medium : 40 ml/culture. Explant : Shoot bud with corn tissue.

No. of replications : 6 No. of explants/replication : 24.

Culture conditions : Temperature : $26 \pm 2^\circ\text{C}$. Light/ dark period : 16/8 Hrs.

Culture period : 20 days.

Table 3. Effect of interaction between 2,4-D and BAP on callus formation in meristem explants of *Gladiolus* cultivars.

Sl.No.	Treatment 2,4 - D + BAP (mg/l)	Stage - I			Stage - II		
		Response-Enlargement of explant			Response-Callus initiation (g/culture)		
		Priscilla	Victor Borge	Rose Supreme	Priscilla	Victor Borge	Rose Supreme
1.	2.0 0.0	++	+	+	0.65	0.37	0.43
2.	4.0 0.0	++	++	++	0.39	0.27	0.23
3.	6.0 0.0	++	++	++	0.30	0.00	0.00
4.	8.0 0.0	+++	++	++	0.00	0.00	0.00
5.	2.0 0.2	+	+	++	0.85	0.60	0.57
6.	4.0 0.2	++	++	++	0.59	0.29	0.61
7.	6.0 0.2	++	+++	+++	0.39	0.22	0.45
8.	8.0 0.2	++	+++	++	0.31	0.00	0.37
9.	2.0 0.5	++	++	+	0.60	0.25	0.50
10.	4.0 0.5	++	++	++	0.33	0.00	0.45
11.	6.0 0.5	+++	+++	++	0.37	0.00	0.38
12.	8.0 0.5	+++	+++	++	0.42	0.00	0.30
CD (0.05 P)		-	-	-	0.04	0.02	0.05
SEm(±)		-	-	-	0.03	0.02	0.03

Culture medium : Murashige and Skoog (1962). pH : 5.8

Volume of the medium : 16 ml/culture. No. of replications : 6

Culture conditions : Temperature : 26 ± 2 °C. Light/dark period : 16/8 Hrs.

Culture period : 30 days in both stages. Inoculum : 250 ± 10 mg/culture.

Explant: Stage - I : Young meristems. Stage - II : Inoculum from Stage - I.

Response : very poor (+), poor (++), good (+++).

Plate 1. Enlargement of meristem explants on MS medium containing 2,4-D + BAP
and NAA + BAP

A. 2,4-D + BAP (mg/l)

1.	6.0	0.2
2.	6.0	0.5
3.	8.0	0.2
4.	8.0	0.5

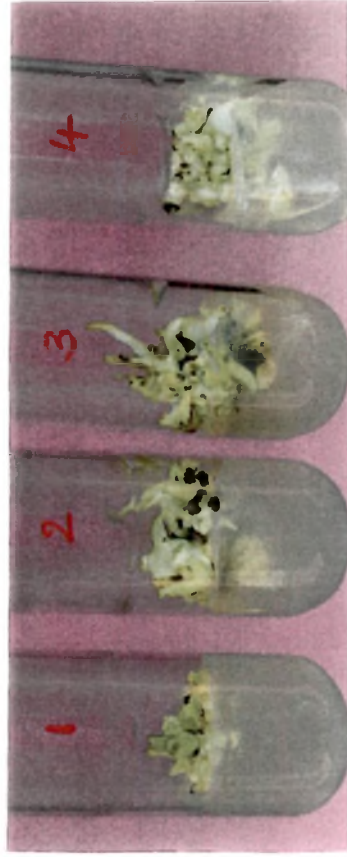
B. NAA + BAP (mg/l)

1.	6.0	0.2
2.	6.0	0.5
3.	8.0	0.2
4.	8.0	0.5

A



Priscilla



Victor Borge

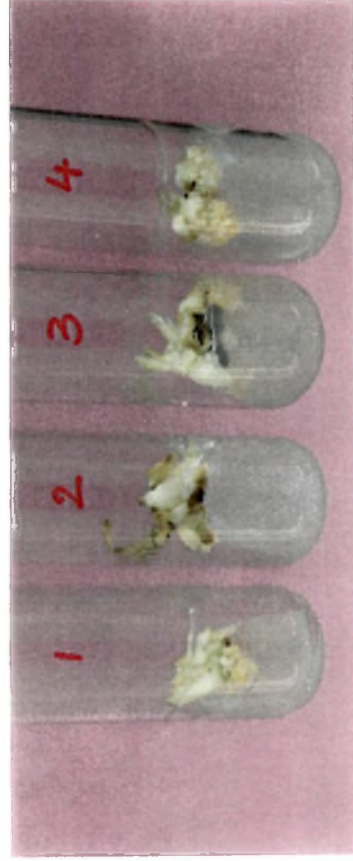


Rose Supreme

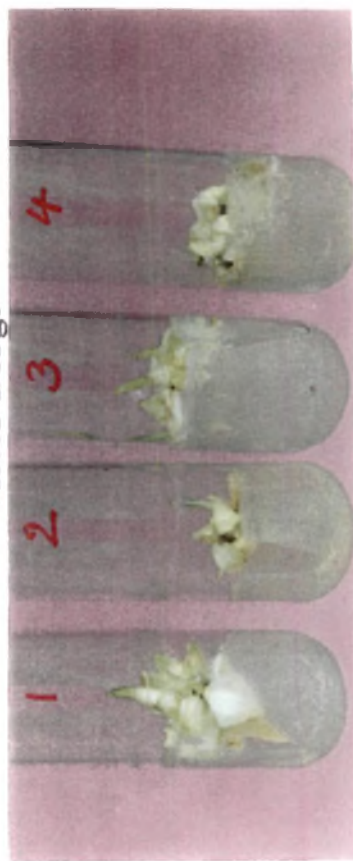
B



Priscilla



Victor Borge



Rose Supreme

Plate 1

Plate 2. Callus initiation from enlarged meristem explants on MS medium containing 2,4-D + BAP

	2,4-D	+	BAP (mg/l)
1.	2.0		0.0
2.	4.0		0.0
3.	6.0		0.0
4.	8.0		0.0
5.	2.0		0.2
6.	4.0		0.2
7.	6.0		0.2
8.	8.0		0.2
9.	2.0		0.5
10.	4.0		0.5
11.	6.0		0.5
12.	8.0		0.5



1 2 3 4



5 6 7 8



9 10 11 12

Priscilla



1 2 3 4



5 6 7 8



9 10 11 12

Victor Borge



1 2 3 4 5 6 7 8 9 10 11 12

Rose Supreme
Plate 2

3. Effect of interaction between NAA and BAP on callus formation in meristem explants of *Gladiolus* cultivars.

The fact that the 2,4-D and BAP combinations tested in the previous experiment were not very effective in promoting callus initiation, prompted to probe the effect of NAA and BAP combinations for promoting better formation of callus .

Similar to the 2,4-D experiment, there was no callus development under stage-I and the response was only mere expansion (Plate 1). While the response of Priscilla was better on medium containing 6 and 8 mg/l NAA with 0.5 mg/l BAP, the response of Victor Borge was better at 6 and 8 mg/l NAA with either 0.2 or 0.5 mg/l BAP. However, Rose Supreme responded better in medium containing 6 and 8 mg/l NAA without the supplement of BAP.

The explants from Stage-I under various degrees of expansion when transferred to Stage-II medium containing the identical levels of NAA and BAP, resulted in development of callus (Plate 3). The callus initiation was highest on medium supplemented with 4.0 mg/l NAA and 0.2 mg/l BAP in both Priscilla (1.10g/culture) and Victor Borge (0.85 g/culture). The next highest callus development was noticed on medium containing 2.0 mg/l NAA and 0.2 mg/l BAP in Priscilla (0.96 g/culture) and Victor Borge (0.58 g/culture). In Rose Supreme, the medium containing 6.0 mg/l NAA and 0.2 mg/l BAP supported better callus development (1.07 g/culture), followed by the medium with 4.0 mg/l NAA and 0.2 mg/l BAP (0.98g/culture). The results are shown in Table 4.

Table 4. Effect of interaction between NAA and BAP on callus formation in meristem explants of *Gladiolus* cultivars.

Sl. No.	Treatment NAA + BAP (mg/l)	Stage - I			Stage - II		
		Response -Enlargement of explant			Response-Callus initiation(g/culture)		
		Priscilla	Victor Borge	Rose Supreme	Priscilla	Victor Borge	Rose Supreme
1.	2.0	0.0	+	+	0.00	0.49	0.00
2.	4.0	0.0	+	++	0.00	0.49	0.00
3.	6.0	0.0	+	+++	0.00	0.59	0.59
4.	8.0	0.0	++	+++	0.00	0.00	0.40
5.	2.0	0.2	++	++	0.96	0.58	0.73
6.	4.0	0.2	++	++	1.10	0.85	0.98
7.	6.0	0.2	++	++	0.82	0.34	1.07
8.	8.0	0.2	++	++	0.68	0.30	0.58
9.	2.0	0.5	+	+	0.36	0.29	0.47
10.	4.0	0.5	++	++	0.84	0.56	0.56
11.	6.0	0.5	+++	++	0.86	0.44	0.58
12.	8.0	0.5	+++	+++	0.42	0.28	0.51
CD (0.05 P)		-	-	-	0.06	0.04	0.05
SEm(+)		-	-	-	0.04	0.06	0.03

Culture medium : Murashige and Skoog (1962). pH : 5.8.
Volume of the medium : 16 ml/culture. No. of replications : 6
Culture conditions : Temperature : $26 \pm 2^{\circ}\text{C}$. Light /dark period : 16 Hrs.
Culture period : 30 days in both stages. Inoculum : 250 ± 10 mg/culture.
Explant : Stage I - Young meristems. Stage II - Inoculum from Stage I.
Response : very poor (+), poor (++), good (+++).

Plate 3. Callus initiation from enlarged meristem explants on MS medium containing
NAA + BAP

	NAA	+	BAP (mg/l)
1.	2.0		0.0
2.	4.0		0.0
3.	6.0		0.0
4.	8.0		0.0
5.	2.0		0.2
6.	4.0		0.2
7.	6.0		0.2
8.	8.0		0.2
9.	2.0		0.5
10.	4.0		0.5
11.	6.0		0.5
12.	8.0		0.5



1 2 3 4



5 6 7 8



9 10 11 12

Priscilla



1 2 3 4



5 6 7 8



9 10 11 12

Victor Borge



1 2 3 4

5 6



7

8

9

10



11

12

**Rose Supreme
Plate 3**

Growth and maintenance of callus cultures

Three experiments were conducted under this series to optimize culture medium for growth and maintenance of callus.

1. Effect of 2,4-D, BAP and kinetin on growth and survival of callus in *Gladiolus* cultivars.

The callus induced on MS medium supplemented with 4.0 mg/l NAA and 0.2 mg/l BAP, was subcultured after 30 days on medium containing 2,4-D with different BAP or kinetin at different combinations and observed significant differences on growth and survival of callus with respect to both hormonal combinations and cultivars (Table 5)

In Priscilla and Rose Supreme, the growth of callus in the form of callus fresh weight was significantly better with 2,4-D and BAP when compared with 2,4-D and kinetin combinations. This can be observed from the results that the growth of callus was highest (1.47 g/culture) both in Priscilla and Rose Supreme at 1.0 mg/l 2,4-D and 0.2 mg/l BAP. The growth of callus at 1.0 mg/l 2,4-D and 0.2 mg/l kinetin although highest (1.32 and 1.33 g/culture respectively for Priscilla and Rose Supreme) but was significantly lower compared to the corresponding levels of 2,4-D and BAP. Similar results were also observed in Victor Borge. The growth of callus at 1.0 mg/l 2,4-D and 0.2 mg/l BAP was 1.35 g/culture compared to 1.32 g/culture at the corresponding levels of 2,4-D and kinetin. Therefore, callus was maintained on MS medium containing 1.0mg/l 2,4-D and 0.2 mg/l BAP for future experiments.

Table 5. Effect of 2,4-D, BAP and kinetin on growth of callus in *Gladiolus* cultivars.

Sl. No.	Treatment	Callus weight (g /culture)			Days to senescence		
		Priscilla	Victor Borge	Rose Supreme	Priscilla	Victor Borge	Rose Supreme
1.	2,4 - D + BAP (mg/l)						
2.	0.5	1.07	0.98	1.12	41	37	42
3.	0.5	1.35	1.33	1.30	41	38	43
4.	0.5	1.41	1.26	1.44	43	38	45
5.	1.0	1.23	1.20	1.25	40	37	41
6.	1.0	1.47	1.35	1.47	42	37	45
7.	1.0	1.38	1.26	1.35	41	37	43
8.	2.0	1.25	1.19	1.32	37	36	38
9.	2.0	1.29	1.08	1.48	36	34	37
	2.0	1.19	1.22	1.23	35	34	36
10.	2,4 -D + kinetin (mg/l)						
11.	0.5	1.22	1.22	1.24	41	38	43
12.	0.5	1.15	1.19	1.26	41	38	43
13.	1.0	1.32	1.21	1.33	41	37	43
14.	1.0	1.26	1.32	1.24	41	37	42
15.	2.0	1.19	1.23	1.12	36	35	37
	2.0	1.16	1.25	1.15	36	35	35
CD (0.05 P)		0.04	0.04	0.03	-	-	-
SEm(+)		0.03	0.03	0.02	-	-	-

Culture medium : Murashige and Skoog (1962). pH : 5.8

Volume of the medium : 16 ml/culture. No. of replications : 9

Culture conditions : Temperature : 26 ± 2 °C. Light /dark period : 16/ 8 Hrs.

Culture period : 30 days. Inoculum : 250 ± 10 mg/culture.

The survival of callus in maintenance medium was determined by recording the number of days the callus remained healthy. It was observed that the browning and senescence of callus occurred more quickly with higher levels of 2,4-D. However, no significant differences were seen with respect to BAP or kinetin levels. At any given hormonal combination, callus of Rose Supreme remained healthy for longer period followed by Priscilla. The callus of Victor Borge was the least survived. On the medium containing 1.0 mg/l 2,4-D and 0.2 mg/l BAP, that supported maximum callus growth, the survival of callus was for a period of 45, 42 and 37 days respectively in Rose Supreme, Priscilla and Victor Borge.

2. Patterns of growth in the callus of *Gladiolus* cultivars.

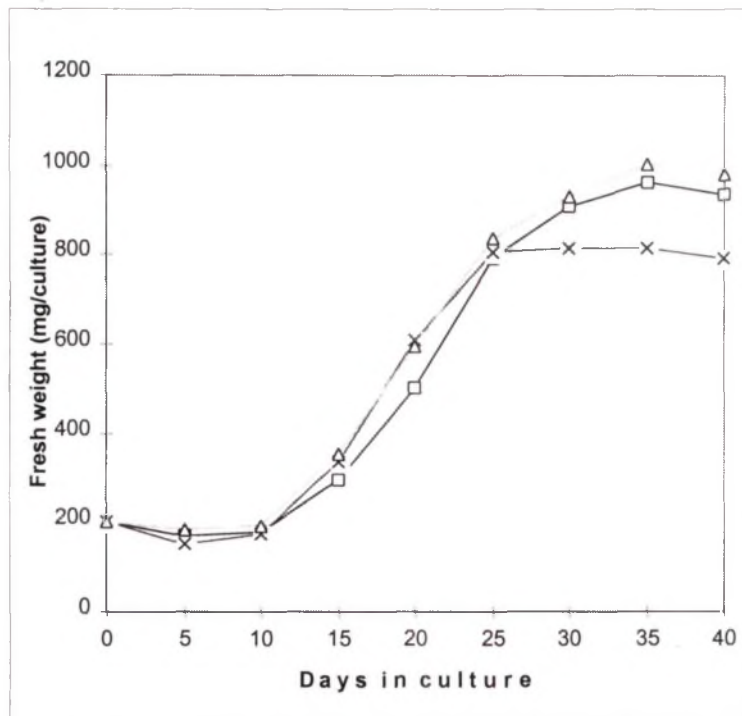
Though it was found that the growth of callus in all the 3 cultivars was better on the medium containing 1.0mg/l 2,4-D and 0.2 mg/l BAP, still the rate of growth differed from each other. Therefore, it was thought that the study of growth patterns would be interesting, hence the experiment was conducted. The callus growth curves on fresh weight basis during the culture passage of 40 days revealed an initial lag phase of growth for a period of 10 days in all the cultivars. The callus fresh weight decreased from the initial weight of 200 mg/culture to 180.67, 175.33 and 193.67 mg/culture respectively for Priscilla, Victor Borge and Rose Supreme. The lag phase was succeeded by a sudden exponential increase in the callus weight. On the 15th day after inoculation, the growth of callus increased, respectively, to 293.50, 336.17 and 353.00 mg/culture in Priscilla, Victor Borge and Rose Supreme. This trend continued and the maximum growth was recorded on the 30th day in Victor Borge (816.50 mg/culture) and on the 35th day both in Priscilla (961.0 mg/culture)

and Rose Supreme (1002.17 mg/culture). Then the growth declined on the subsequent days. On the 40th day, the callus fresh weights recorded were 793.67, 934.17 and 980.0 mg/culture in Victor Borge, Priscilla and Rose Supreme, respectively (Fig. 1A).

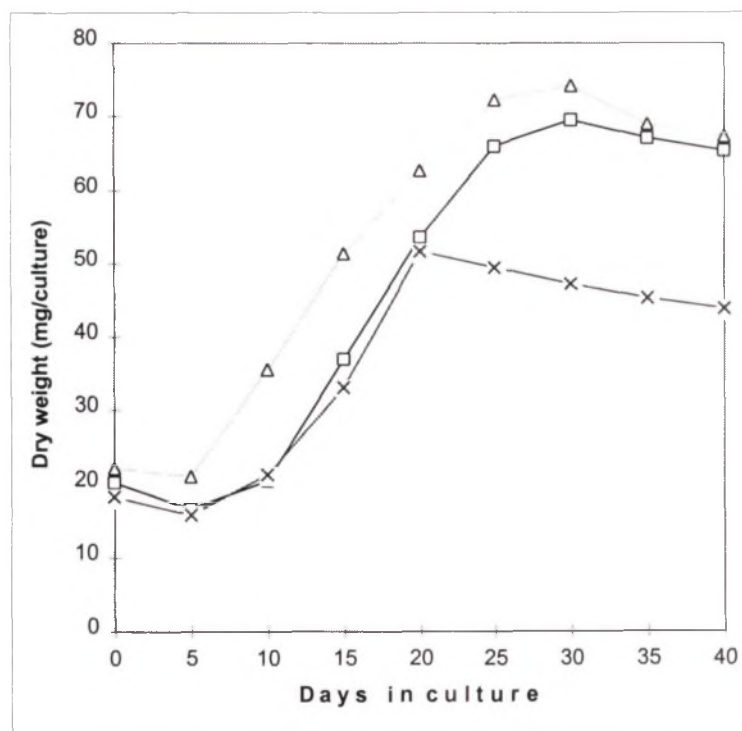
A similar trend was also noticed in the growth of callus on dry weight basis (Fig. 1B). The callus dry weights decreased from 18.17, 20.17 and 22.0 mg/culture to 15.67, 16.67 and 21.00 mg/culture respectively in Victor Borge, Priscilla and Rose Supreme on the 5th day after inoculation. However, the callus dry weight increased thereafter rapidly and the highest accumulation of dry matter was noticed on the 20th day in Victor Borge (51.67 mg/culture) and on the 30th day in both Priscilla (69.50 mg/culture) and Rose Supreme (74.33 mg/culture).

3. Effect of different sources of nitrogen on growth and survival of callus in *Gladiolus* cultivars.

This experiment was conducted to test the effect of incorporation of additional nitrogen on callus growth in the maintenance medium. Therefore, the treatments comprising various nitrogen supplements at different concentration and combinations were tested on the growth of callus. The results of the study indicated that the growth of callus fresh weight basis increased significantly with the addition of NH_4NO_3 when compared with the control and other treatments. The growth of callus in Rose Supreme was highest (1.85 mg/culture) in medium containing 100 mg/l NH_4NO_3 , whereas, in cvs. Priscilla and Victor Borge, the growth was greater in medium containing 200 mg/l NH_4NO_3 . Yet, the values were lower than those of Rose Supreme (1.59 g/culture and 1.27 g/culture). NH_4SO_4 and casein hydrolysate,



A. Callus fresh weight



B. Callus dry weight

□ Priscilla, x Victor Borge, Δ Rose Supreme

Fig 1. Patterns of growth in the callus of *Gladiolus* cultivars

either alone or in combination, inhibited the growth in general. However, additional supply of casein hydrolysate (100mg/l) in Priscilla (1.37 g/culture) and casein hydrolysate(100 mg/l) with NH_4SO_4 (100mg/l) in Rose Supreme (1.66 g/culture) favoured better callus growth unlike in control.

The callus continued to grow and remained healthy for longer duration in all the cultivars with additional NH_4NO_3 into the maintenance medium when compared with control or other nitrogen supplements. On medium supplemented with 200 mg/l NH_4NO_3 , the callus remained viable for longer duration and callus browning and senescence occurred only on 47th, 46th and 41st day after inoculation in Rose Supreme, Priscilla and Victor Borge, respectively. The survival of callus was for a short period on medium containing the combination of NH_4SO_4 (100 mg/l), NH_4NO_3 (100 mg/l) and casein hydrolysate (200 mg/l), wherein the senescence occurred on 35th, 36th and 34th day after inoculation in Rose Supreme, Priscilla and Victor Borge, respectively. The results are presented in Table 6 and illustrated in Plate 4.

However, the medium with additional nitrogen supply was not used either for maintenance of callus or for further experiments as the presence of the additional nitrogen is likely to influence the process of somatic embryogenesis in future experiments.

Regeneration response of callus

1. Effect of interaction between BAP and NAA on morphogenic response in calluses of *Gladiolus* cultivars.

The callus maintained on medium (MS + 1.0 mg/l 2,4-D + 0.2 mg/l BAP) when transferred to MS medium containing different concentrations and

Table 6. Effect of different sources of nitrogen on growth of callus in *Gladiolus* cultivars.

Sl. No.	Treatment	Callus weight (g/culture)			Days to senescence		
		Priscilla	Victor Borge	Rose Supreme	Priscilla	Victor Borge	Rose Supreme
1.	Control	1.15	1.15	1.28	41	39	45
2.	NH ₄ SO ₄ (100mg/l)	1.09	1.13	1.31	40	37	43
3.	NH ₄ SO ₄ (200mg/l)	0.83	0.88	1.10	39	35	41
4.	NH ₄ NO ₃ (100mg/l)	1.30	1.21	1.85	44	40	45
5.	NH ₄ NO ₃ (200mg/l)	1.59	1.27	1.23	46	41	47
6.	Casein hydrolysate (100mg/l)	1.37	1.03	1.00	36	37	37
7.	Casein hydrolysate (200mg/l)	1.07	0.94	1.16	37	34	36
8.	NH ₄ SO ₄ (100mg/l)+Casein hydrolysate (100mg/l)	0.94	0.73	0.87	39	35	35
9.	NH ₄ SO ₄ (100mg/l)+Casein hydrolysate (100mg/l)	1.09	1.10	1.66	42	40	40
10.	NH ₄ SO ₄ (100mg/l)+ NH ₄ NO ₃ (100mg/l)	0.81	0.86	1.06	39	34	37
11.	NH ₄ SO ₄ (100mg/l)+NH ₄ NO ₃ (100mg/l)+ Casein hydrolysate (100mg/l)	1.06	0.81	0.96	37	36	41
12.	NH ₄ SO ₄ (100mg/l)+ NH ₄ NO ₃ (100mg/l)+ Casein hydrolysate (200mg/l)	0.80	0.89	1.17	36	34	35
	CD (0.05 P)	0.04	0.06	0.09	-	-	-
	SEm(+)	0.03	0.04	0.06	-	-	-

Culture medium : Murashige and Skoog (1962) containing 2,4 -D (1.0mg/l) and BAP (0.2mg/l).

Volume of the medium : 16 ml/culture. No of replications : 9 pH : 5.8

Culture conditions : Temperature : 26 ± 2 °C. Light/dark period :16/8 Hrs.

Culture period : 30 days. Inoculum : 250 ±10 mg/culture.

Plate 4. Callus growth on MS medium containing 1.0 mg/l 2,4-D + 0.2mg/l + BAP
with and without nitrogen supplements

A . Growth of callus without nitrogen supplements.

B . Growth of callus with nitrogen supplements.

1. NH_4SO_4 (200 mg/l)
2. NH_4NO_3 (200 mg/l)



A

PR : Priscilla
RS : Rose Supreme
VB : Victor Borge



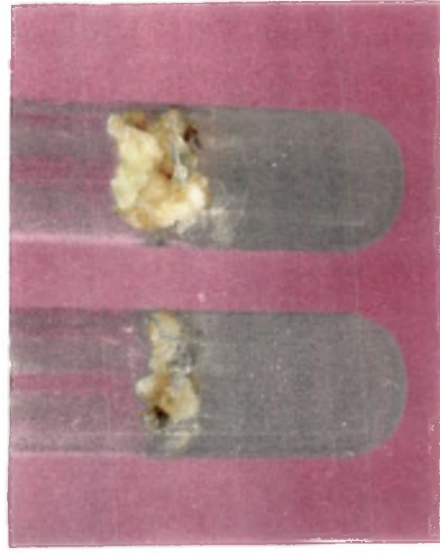
1 2

Priscilla



1 2

Rose Supreme



1 2

Victor Borge

B

combinations of BAP and NAA, morphogenic response was elicited and the response varied with the auxin-cytokinin balance and the duration of culture (Plate 5). In stage I, after 30 days of inoculation, the callus differentiated into proembryogenic masses (PEMs) and the degree of differentiation was highest at an auxin-cytokinin ratio of 1.0 : 0.5 in the culture medium. The PEMs were composed of small and compact cell aggregates, characterized by active cell divisions and presence of dense cytoplasm. The PEMs were studded with asynchronously developing embryoids. The next best development of PEMs was seen on medium containing 1.0 mg/l NAA and 0.2 mg/l BAP (Table 7).

The PEMs with various stages of differentiation when continued on their respective media, were transformed into somatic embryos in certain auxin-cytokinin combinations only. After 45 days of inoculation, transformation of PEMs into somatic embryos was maximum with hormonal combination of 1.0 mg/l NAA and 0.5 mg/l BAP in all the cultivars. Among the cultivars, the highest transformation was seen in Victor Borge (9.67 No./culture) followed by Rose Supreme (5.17 No./culture) and Priscilla (2.17 No./culture). The next best conversion of PEMs into SEs was seen in Rose Supreme (5.0 No./culture) on medium containing 0.5 mg/l each of NAA & BAP followed by Priscilla (1.5 No./culture) on medium containing 1.0 mg/l each of NAA and BAP.

It was also observed from the results that certain concentrations of cytokinin (BAP) favoured regeneration of adventitious shoots. When the inoculum was continued on the respective medium upto 60 days, medium containing 2.0 mg/l BAP elicited regeneration of highest number of adventitious shoots in Rose Supreme

Table 7. Effect of interaction between BAP and NAA on morphogenic response in calluses of *Gladiolus* cultivars.

Sl. No.	Treatment BAP + NAA (mg/l)	Stage - I Proembryogenic masses (PEMs) (30 days after inoculation)				Stage - II Somatic embryos (Nos./culture) (45 days after inoculation)				Stage - III Adventitious shoots (Nos./culture) (60 days after inoculation)			
		Priscilla	Victor Borge	Rose Supreme		Priscilla	Victor Borge	Rose Supreme		Priscilla	Victor Borge	Rose Supreme	
1.	0.0	-	-	+		0.00	0.00	0.00		0.00	0.00	0.00	
2.	0.0	+	++	++		0.00	0.00	0.00		0.00	0.00	0.00	
3.	0.0	+	++	++		0.00	0.00	0.00		0.00	0.00	0.00	
4.	0.2	+	-	-		0.00	0.00	0.00		9.50	10.50	2.17	
5.	0.2	+	+	+		0.00	0.00	3.83		1.17	0.00	2.83	
6.	0.2	++	++	++		0.33	0.00	1.83		0.83	0.00	8.67	
7.	0.5	-	+	+		0.00	0.00	0.00		6.00	0.00	7.50	
8.	0.5	+	+	++		0.00	0.00	5.00		0.00	3.83	2.33	
9.	0.5	+++	+++	+++		2.17	9.67	5.17		0.00	0.00	0.00	
10.	1.0	+	-	-		0.00	0.00	0.00		5.17	0.00	6.12	
11.	1.0	+	+	+		0.00	0.00	0.00		3.67	0.00	4.50	
12.	1.0	++	++	+		1.50	0.00	0.00		2.17	0.00	1.33	
13.	2.0	+	-	-		0.00	0.00	0.00		0.00	0.00	31.17	
14.	2.0	+	+	+		0.00	0.00	0.00		2.50	0.00	0.00	
15.	2.0	-	-	++		0.00	0.00	1.17		2.00	0.00	3.33	
CD(0.05 P)		-	-	-		0.28	0.44	0.91		1.03	0.51	1.80	
SEm(+)		-	-	-		0.20	0.31	0.65		0.73	0.36	1.27	

Culture medium : Murashige and Skoog (1962) . pH : 5.8

Volume of the medium : 16 ml/culture. No. of replications : 6 Inoculum : 250 $\pm$ 10 mg/culture.

Culture conditions : Temperature : 26 $\pm$ 2 °C. Light /dark period : 16 /8 Hrs.

Culture duration : Stage I : 30 days after inoculation - observation for proembryonic masses(PEMs).

Stage II : 45 days after inoculation - observation for somatic embryos (SEs)

Stage III : 60 days after inoculation - observation for adventitious shoots.

Response : Nil (-), very poor (+), poor (++) and good (+++).

Plate 5. Morphogenic response of callus on MS medium containing BAP and NAA

	BAP	+	NAA (mg/l)
1.	0.0		0.0
2.	0.0		0.5
3.	0.0		1.0
4.	0.2		0.0
5.	0.2		0.5
6.	0.2		1.0
7.	0.5		0.0
8.	0.5		0.5
9.	0.5		1.0
10.	1.0		0.0
11.	1.0		0.5
12.	1.0		1.0
13.	2.0		0.0
14.	2.0		0.5
15.	2.0		1.0



1 2 3 4 5



6 7 8 9 10



11 12 13 14 15

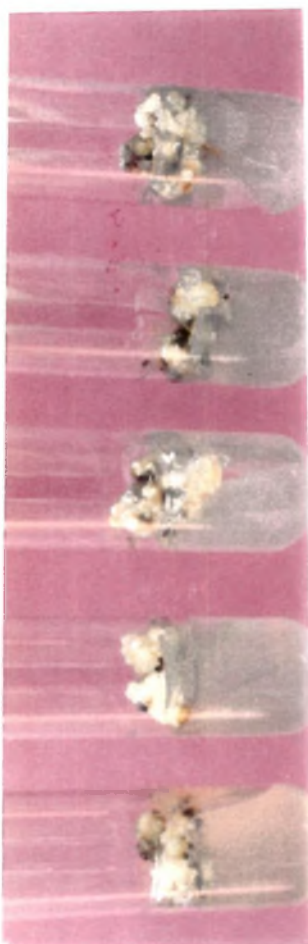
Priscilla



1 2 3 4 5



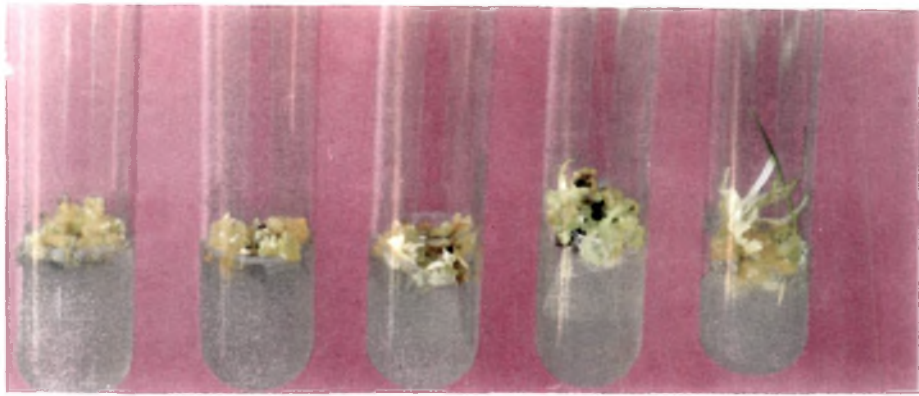
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11 12 13 14 15

Victor Borge

Plate 5



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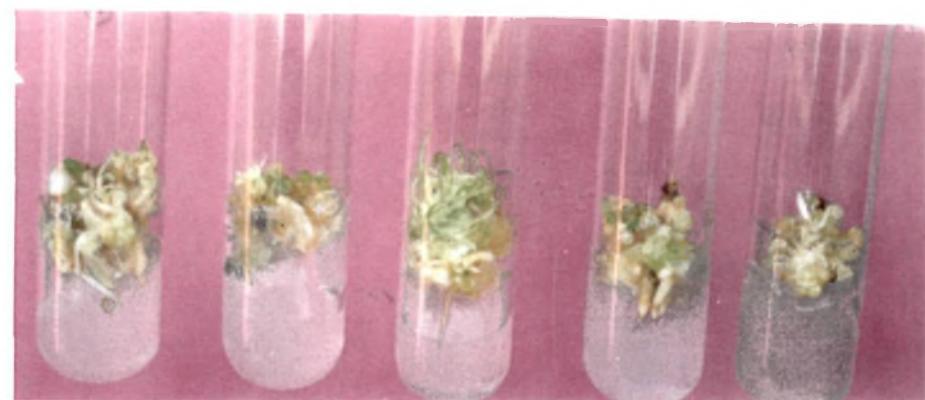
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Rose Supreme

Plate 5

(31.17 No./culture), whereas the medium containing only 0.2 mg/l BAP favoured regeneration of highest number of adventitious shoots in Priscilla (9.50 No./culture) and Victor Borge (10.50 No./culture).

2. Effect of interaction between kinetin and NAA on morphogenic response in calluses of *Gladiolus* cultivars.

The significance of this experiment was to test the further possibility of increasing differentiation of PEMs and transformation of PEMs into somatic embryos (SEs) with different concentrations and combinations of kinetin and NAA in the culture medium. However, except at 1.0 mg/l NAA with 0.2 or 0.5 mg/l kinetin, that promoted differentiation of callus into PEMs in Victor Borge, none of the other combinations was found favourable either for Victor Borge or other two cultivars under the study (Table 8 and Plate 6). The conversion of PEMs into SEs was also low when compared to NAA and BAP combinations. Nevertheless, kinetin alone at 0.5 mg/l elicited highest regeneration of adventitious shoots in Priscilla (20.67/culture) followed by 1.0 mg/l in case of Victor Borge (20.0/culture). In Rose Supreme, the medium containing 0.5 mg/l NAA and 0.5 mg/l kinetin supported regeneration of highest adventitious shoots (15.50/culture).

Somatic embryogenesis in callus cultures

As many as five experiments were conducted in succession under this series to standardize the protocol for somatic embryogenesis and plantlet production.

Table 8. Effect of interaction between kinetin and NAA on morphogenic response in calluses of *Gladiolus* cultivars.

Sl. No.	Treatment Kinetin + NAA (mg/l)	Stage-I Proembryogenic masses (PEMs) (30 days after inoculation)			Stage -II Somatic embryos (Nos./culture) (45 days after inoculation)			Stage -III Adventitious shoots (Nos./culture) (60 days after inoculation)		
		Priscilla	Victor Borge	Rose Supreme	Priscilla	Victor Borge	Rose Supreme	Priscilla	Victor Borge	Rose Supreme
1.	0.2	-	+	+	0.00	0.00	0.00	8.17	0.00	0.00
2.	0.2	+	+	++	0.00	0.00	0.00	5.00	5.17	0.00
3.	0.2	+	++	++	0.00	5.67	2.00	0.00	0.00	3.67
4.	0.5	-	-	+	0.00	0.00	0.00	20.67	14.00	9.50
5.	0.5	+	+	+	0.00	0.00	0.00	4.00	0.00	15.50
6.	0.5	++	++	++	0.00	0.00	2.67	0.00	0.00	0.67
7.	1.0	-	-	+	0.00	0.00	0.00	11.50	20.00	13.50
8.	1.0	+	+	++	1.33	2.33	6.17	2.00	11.50	1.67
9.	1.0	++	+	+	1.17	2.83	0.00	0.00	7.17	0.00
10.	2.0	-	-	-	0.00	0.00	0.00	9.83	4.67	1.50
11.	2.0	+	+	+	0.00	0.00	0.00	0.00	0.00	0.00
12.	2.0	+	-	+	0.00	0.00	0.00	3.50	1.33	0.00
CD (0.05 P)		-	-	-	0.17	0.75	0.61	1.64	1.70	1.22
SEm(+)		-	-	-	0.12	0.53	0.43	1.16	1.20	0.86

Culture medium : Murashige and Skoog (1962). pH : 5.8

Volume of the medium : 16 ml/culture. No. of replications : 6 Inoculum : 250 ± 10 mg/culture.

Culture conditions : Temperature : $26 \pm 2^\circ$ C. Light/dark period : 16/8 Hrs.

Culture duration : Stage I : 30 days after inoculation - observation for proembryonic masses (PEMs)

Stage II : 45 days after inoculation - observation for somatic embryos (SEs).

Stage III : 60 days after inoculation - observation for adventitious shoots.

Response : Nil (-), very poor (+), poor(++) and good (+++).

Plate 6. Morphogenic response of callus on MS medium containing kinetin and NAA.

	kinetin	+	NAA (mg/l)
1.	0.2		0.0
2.	0.2		0.5
3.	0.2		1.0
4.	0.5		0.0
5.	0.5		0.5
6.	0.5		1.0
7.	1.0		0.0
8.	1.0		0.5
9.	1.0		1.0
10.	2.0		0.0
11.	2.0		0.5
12.	2.0		1.0



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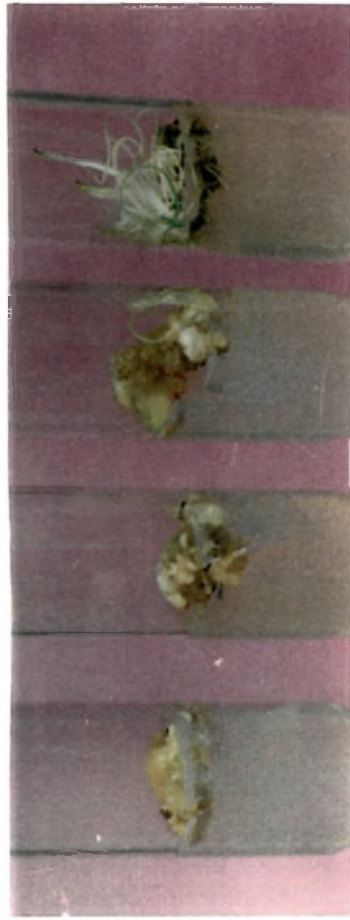


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9 10 11 12

Priscilla



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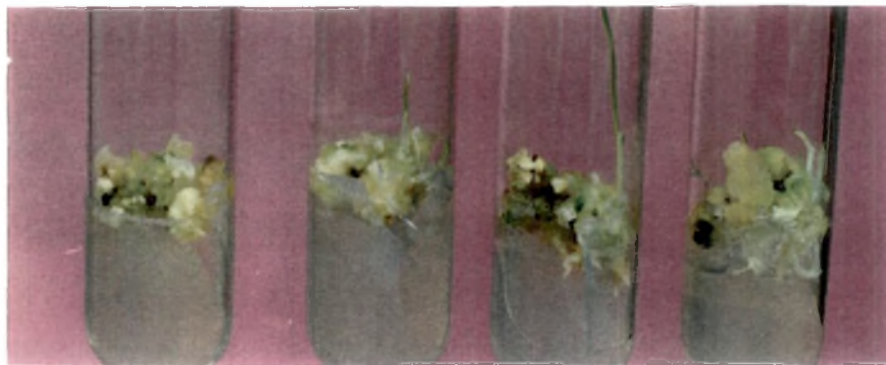


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9 10 11 12

Victor Borge

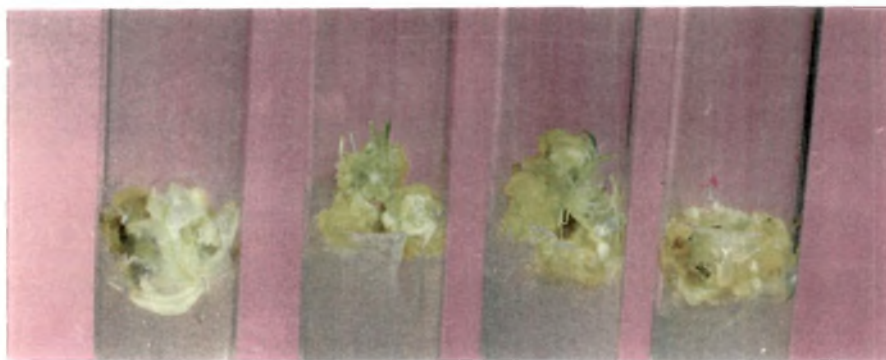


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Rose Supreme

Plate 6

1. Effect of BAP and kinetin on transformation of proembryogenic masses into somatic embryos and plantlets in *Gladiolus* cultivars.

The very fact that the PEMs obtained on morphogenic medium (MS+1.0 mg/l NAA + 0.5 mg/l BAP) were not transformed into somatic embryos (SEs) and plantlets to a desired level when continued on the same medium even upto 45 days prompted to conduct this experiment. The PEMs obtained on induction medium were transferred to MS medium containing different levels of BAP and kinetin and observed for somatic embryo development and plantlet production. It can be observed from the results (Table 9) that the transformation of PEMs into SEs and plantlets was highly encouraging at lower concentrations of both kinetin and BAP. At 0.2 mg/l kinetin, the number of SEs developed per culture was significantly high in both Priscilla (76.50) and Victor Borge (74.50) when compared to other levels of kinetin or BAP. The next best development was seen on medium containing BAP at 0.2 mg/l with the development of 55.83 and 57.33 embryos/culture, respectively, for Priscilla and Victor Borge. In control, wherein the PEMs were directly transferred from induction medium to MS medium free from hormones, there was no development of embryos (Plate 7).

The production of plantlets per culture was also maximum on medium containing 0.2 mg/l kinetin. Among the cultivars, more number of plantlets was produced under Victor Borge (51.0/culture) followed by Priscilla (44.67/culture). However, the mean length of plantlets was highest with kinetin at 0.2 mg/l in both Victor Borge (4.65 cm) and Priscilla (4.25 cm).

Table 9. Effect of BAP and kinetin on transformation of proembryogenic masses (PEMs) into somatic embryos (SEs) and plantlets in *Gladiolus* cultivars.

Sl. No.	Treatment	Somatic embryos (Nos./ culture)		Plantlets (Nos./ culture)		Length of plantlets (cm)		Roots (Nos./culture)	
		Priscilla	Victor Borge	Priscilla	Victor Borge	Priscilla	Victor Borge	Priscilla	Victor Borge
1.	BAP (mg/l)	0.2	55.83	57.33	38.17	41.83	2.76	2.56	6.9
2.		0.5	15.33	8.33	5.00	4.50	1.20	1.43	0.0
3.		1.0	6.33	6.00	3.67	3.33	0.97	1.09	0.0
4.		2.0	2.17	3.17	0.83	0.50	0.26	0.32	0.0
5.	Kinetin (mg/l)	0.2	76.50	74.50	44.67	51.00	3.40	3.27	10.0
6.		0.5	51.67	53.67	25.33	23.50	4.25	4.65	0.0
7.		1.0	13.17	24.50	6.50	9.83	3.19	3.09	0.0
8.		2.0	2.33	2.33	0.67	1.17	1.23	2.30	0.0
9.	Control		0.00	0.00	0.00	0.00	-	-	-
CD (0.05 P)		5.04	4.87	3.78	3.95	0.60	0.26	-	-
SEm(±)		3.56	3.44	2.67	2.79	0.43	0.19	-	-

Culture medium : Murashige and Skoog (1962). pH : 5.8

Volume of the medium : 16ml/culture. No. of replications : 6.

Culture conditions : Temperature : $26 \pm 2^{\circ}\text{C}$. Light /dark duration : 16/8 Hrs.

Inoculum : PEMs from embryo induction medium (MS + 0.5 mg/l BAP +1.0mg/l NAA) for 30 days.

Inoculum size : 250 ± 10 mg/culture.

Observations : Embryo development : 20th day after transfer from induction medium.

Plantlet production : 40th day after transfer from induction medium.

Plate 7. Transformation of PEMs into SEs and plantlets on MS medium containing BAP and kinetin.

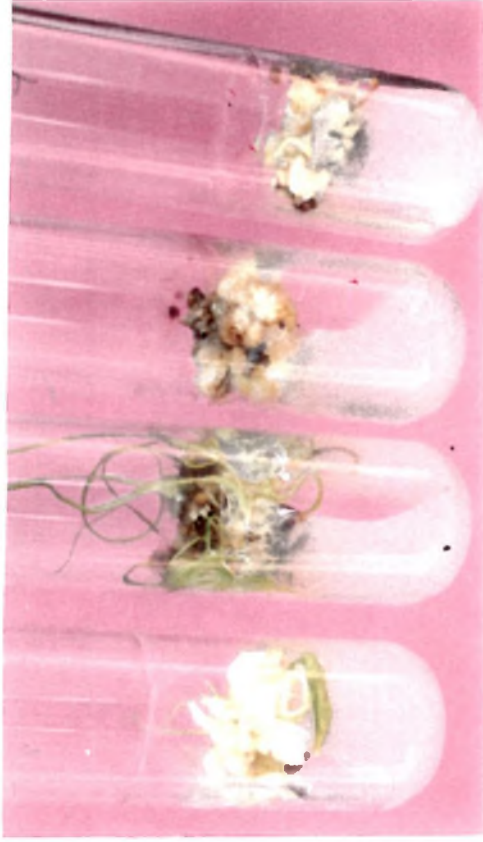
1.	BAP (mg/l)	0.2
2.		0.5
3.		1.0
4.		2.0
5.	kinetin (mg/l)	0.2
6.		0.5
7.		1.0
8.		2.0
9.	Control (No growth hormones)	

A . Development of SEs and plantlets on treatment medium after 30 days.

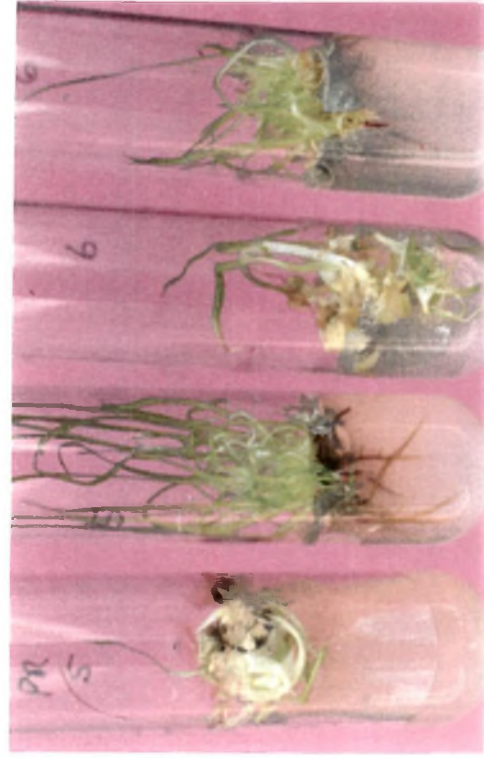
B . Development of SEs and plantlets on half strength MS medium after pre incubation in treatment medium for 10 days.



1A 1B 2A 2B



3A 3B 4A 4B



5A 5B 6A 6B

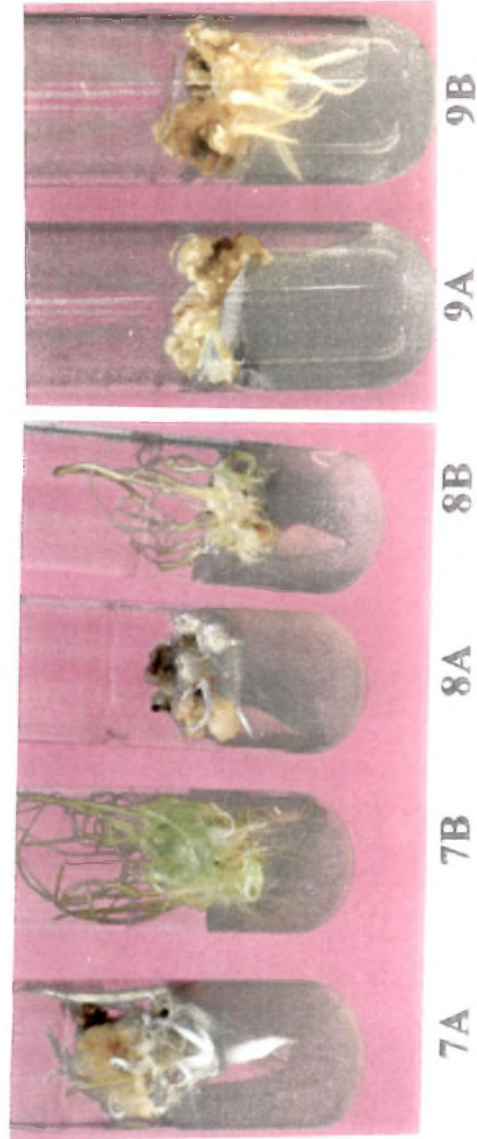
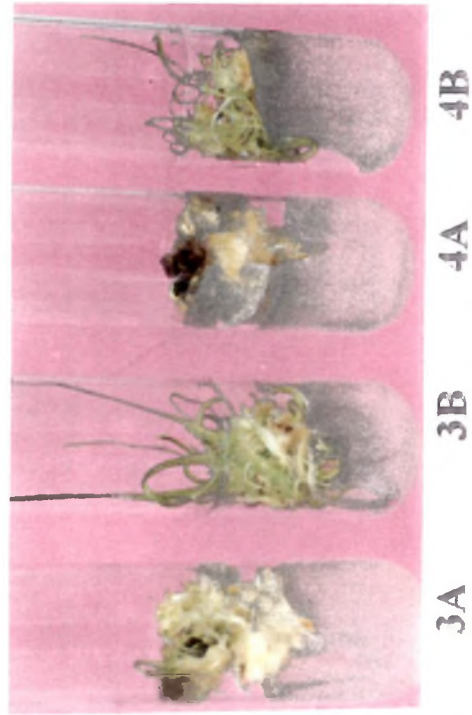
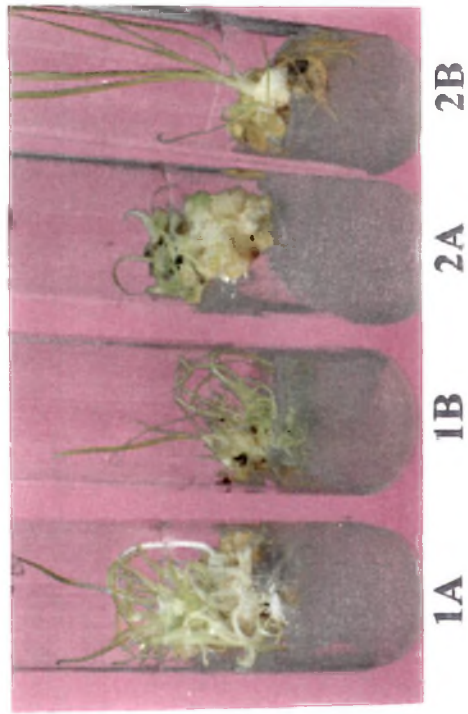


7A 7B 8A 8B



9A 9B

Priscilla
Plate 7



Victor Borge

Plate 7

2. Effect of pre incubation on transformation of proembryogenic masses into somatic embryos and plantlets in *Gladiolus* cultivars.

The primitive effect of BAP and kinetin at lower concentrations in transformation of PEMs into somatic embryos (SEs) and plantlets, lend significance to the idea that the incubation of PEMs under BAP and kinetin for a short period and then transfer to hormone free medium could further enhance the transformation process. Therefore, the experiment involving incubation of PEMs under different levels of BAP and kinetin for a period of 10 days and later transferred to half strength MS medium for further development of embryos was conducted. The transformation of PEMs into SEs and plantlets was significantly higher in Priscilla (77.5 No./culture) when incubated at 0.2 mg/l kinetin followed by incubation at 0.5 mg/l (57.0 No./culture). But in case of Victor Borge, incubation at higher level of kinetin (1.0 mg/l) resulted in development of 72.0 embryos per culture (Table 10, Plate 7). However, the medium containing BAP at 1.0 mg/l favoured higher development of somatic embryos in both Priscilla (37.17 No./culture) and Victor Borge (68.83 No./culture). The number of plantlets produced and number of roots initiated per culture including the mean length of plantlets were also found to be maximum under the treatment that promoted higher development of somatic embryos.

3. Effect of culture media and duration on somatic embryogenesis and plantlet production in *Gladiolus* cultivars.

The speculation that the process of somatic embryogenesis could be further enhanced in liquid medium, gave confidence to the conduct of this experiment. The callus maintained on MS medium supplemented with 1.0 mg/l 2,4,-D and 0.2 mg/l BAP was incubated simultaneously on agar and liquid embryo induction media (EIM)

Table 10. Effect of pre incubation on transformation of proembryogenic masses into somatic embryos and plantlets in *Gladiolus* cultivars.

Sl. No.	Treatment (pre incubation)	Somatic embryos (Nos./ culture.)		Plantlets (Nos./ culture)		Length of plantlets (cm)		Roots (Nos./ culture)	
		Priscilla	Victor Borge	Priscilla	Victor Borge	Priscilla	Victor Borge	Priscilla	Victor Borge
1.	BAP (mg/l)	28.33	49.67	12.83	32.17	4.78	3.77	13.67	15.67
2.	0.5	16.33	11.67	7.50	7.50	3.27	7.35	15.33	26.17
3.	1.0	37.17	68.83	18.67	41.50	4.82	5.31	12.50	13.83
4.	2.0	0.00	21.50	0.00	11.50	0.00	3.04	3.17	7.00
5.	Kinetin(mg/l)	77.50	33.83	42.50	16.00	5.32	3.40	21.33	13.17
6.	0.5	57.00	24.00	27.33	11.00	1.30	6.44	17.00	2.83
7.	1.0	5.17	72.00	2.33	51.17	3.54	7.51	9.50	47.83
8.	2.0	2.33	15.67	1.33	8.17	0.92	3.88	2.00	13.33
9.	Control	0.00	0.00	0.00	0.00	0.00	0.00	1.17	9.00
CD (0.05 P)		4.82	6.48	4.05	4.60	0.44	0.46	2.97	3.77
SEm (±)		3.40	4.58	2.86	3.26	0.31	0.33	2.10	2.66

Pre incubation medium : MS (1962) containing different levels of BAP and kinetin as per treatment . pH : 5.8

Development medium : Half strength MS (1962) free from growth hormones. Volume of the medium : 16 ml/culture.

No. of replications : 6 Culture conditions : Temperature : $26 \pm 2^{\circ}\text{C}$. Light /dark period : 16/8 Hrs.

Inoculum in pre incubation medium: PEMs from induction medium (MS + 0.5mg/l BAP + 1.0 mg/l NAA) for 30 days.

Inoculum in development medium : PEMs pre incubated in MS medium containing different levels of BAP and kinetin for 10 days.

Inoculum size : $250 \pm 10\text{mg/culture}$.

Observations : Somatic embryo development : 10 th day after transfer from pre incubation medium.

Plantlet production : 30 th day after transfer from pre incubation medium.

both containing MS nutrients accomplished with 0.5 mg/l BAP and 1.0 mg/l NAA. The cultures were drawn periodically at 5 days interval and cultured on agar embryo development medium (EDM) containing 0.2 mg/l kinetin. The results are shown in Table 11 and illustrated in Plate 8. There was no development of SEs upto 10 days from agar EIM-agar EDM in both Priscilla and Victor Borge. But, the number of embryos produced per culture increased significantly with increase in incubation period after 15 days and the maximum production was recorded on the 30th day in both Priscilla (50.50) and Victor Borge (65.0). However, in liquid EIM, the process of embryogenesis had set in much earlier. In Victor Borge, the process initiated on 5th day with the production of 2.0 embryos per culture and on 10th day in Priscilla with the production of 9.33 embryos per culture and the process reached its peak on the 25th day in Victor Borge (99.67 No./culture) and on the 30th day in Priscilla (105.0 No./culture). The production of embryonic plantlets per culture were highest from agar EIM- agar EDM on 30th day with the development of 26.0 and 34.17 plantlets, respectively, in Priscilla and Victor Borge. But from liquid EIM - agar EDM, the plantlet production per culture was highest on the 25th day in Victor Borge (58.0) and on the 30th day in Priscilla (62.50). Under both the culture media, the number of roots developed per culture increased with increase in culture duration and the maximum was reached on the 30th day.

4. Effect of culture media on embryo morphology and plantlet production in *Gladiolus* cv. Victor Borge.

The higher rate of somatic embryogenesis and plantlet production obtained from liquid embryo induction medium (EIM) - agar embryo development medium (EDM) led to believe that further manipulations in the embryo induction system could

Table 11. Effect of culture media and duration on somatic embryogenesis and plantlet production in *Gladiolus* cultivars.

Sl.No.	Treatment Days in EIM	Somatic embryos (Nos./culture)		Plantlets (Nos./culture)		Roots (Nos./culture)	
		Priscilla	Victor Borge	Priscilla	Victor Borge	Priscilla	Victor Borge
1.	Agar (EIM) - Agar (EDM)						
2.	0	0.00	0.00	0.00	0.00	0.00	0.00
3.	5	0.00	0.00	0.00	0.00	0.00	0.00
4.	10	0.00	0.00	0.00	0.00	0.00	0.00
5.	15	1.17	3.33	0.00	0.67	0.00	0.00
6.	20	7.83	13.33	3.50	4.33	3.17	1.83
7.	25	11.17	17.50	4.50	7.83	4.50	11.33
	30	50.50	65.00	26.00	34.17	28.50	31.83
	CD (0.05 P)	3.19	2.81	2.12	2.23	1.87	1.68
	SEm (+)	2.26	1.99	1.50	1.58	1.32	1.19
8.	Liquid (EIM) - Agar (EDM)						
9.	5	0.00	2.00	0.00	0.00	0.00	0.00
10.	10	9.33	14.83	0.00	3.83	1.67	3.00
11.	15	11.67	57.17	1.50	24.67	2.50	12.67
12.	20	25.50	66.50	7.33	30.50	13.67	14.00
13.	25	34.00	99.67	16.17	58.00	18.67	36.17
	30	105.00	59.00	62.50	34.33	33.00	36.67
	CD (0.05 P)	6.21	6.46	4.17	5.53	3.42	2.92
	SEm (+)	4.39	4.57	2.96	3.91	2.41	2.06

Embryo induction medium (EIM) : MS(1962) + 0.5 mg /l BAP +1.0 mg /l NAA. pH : 5.8

Embryo development medium (EDM) : MS(1962)+ 0.2 mg/l kinetin. No. of replications : 6.

Volume of the medium : Agar : 16ml/culture. Liquid : 80ml/culture.

Culture conditions : Temperature : 26 ± 2 °C. Light/dark period : 16/8 Hrs.

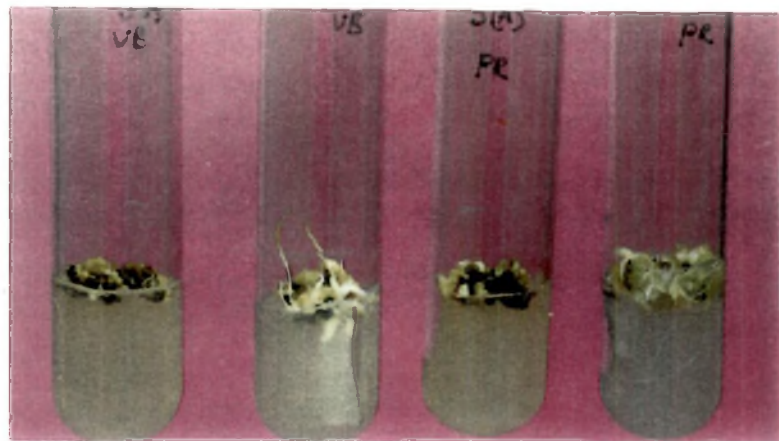
Inoculum : Agar medium : 250 ± 10 mg/culture. Liquid medium : 750 mg/culture following transfer to 3 tubes of agar EDM.

Observations : Embryo development : 20 th day after transfer from EIM to EDM.

Plantlet production : 40 th day after transfer from EIM to EDM.

Plate 8. Development of SEs and plantlets from PEMs obtained by incubation in agar/liquid embryo induction media for different days

Treatments	Days in embryo induction medium	Culture medium	Cultivar
1AV	5	Agar	Victor Borge
1LV	5	Liquid	Victor Borge
1AP	5	Agar	Priscilla
1LP	5	Liquid	Priscilla
2AV	10	Agar	Victor Borge
2LV	10	Liquid	Victor Borge
2AP	10	Agar	Priscilla
2LP	10	Liquid	Priscilla
3AV	15	Agar	Victor Borge
3LV	15	Liquid	Victor Borge
3AP	15	Agar	Priscilla
3LP	15	Liquid	Priscilla
4AV	20	Agar	Victor Borge
4LV	20	Liquid	Victor Borge
4AP	20	Agar	Priscilla
4LP	20	Liquid	Priscilla
5AV	25	Agar	Victor Borge
5LV	25	Liquid	Victor Borge
5AP	25	Agar	Priscilla
5LP	25	Liquid	Priscilla
6AV	30	Agar	Victor Borge
6LV	30	Liquid	Victor Borge
6AP	30	Agar	Priscilla
6LP	30	Liquid	Priscilla
7V (Control)	-	-	Victor Borge
7P (Control)	-	-	Priscilla

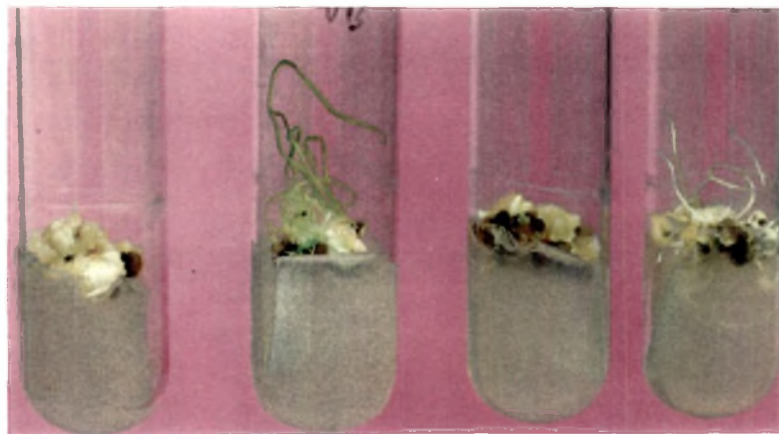


1AV

1LV

1AP

1LP

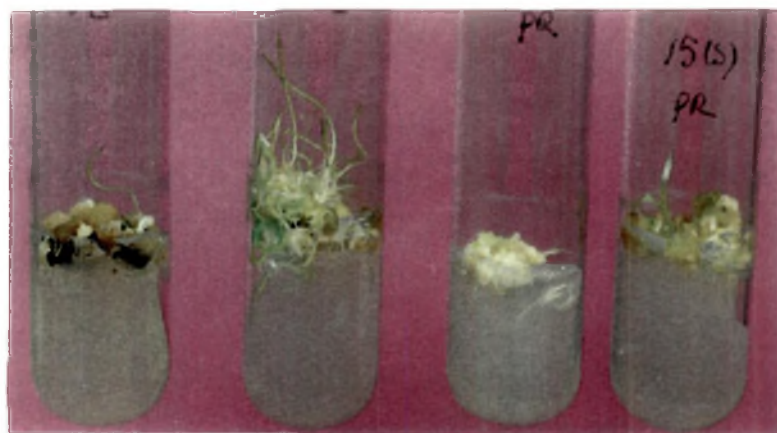


2AV

2LV

2AP

2LP



3AV

3LV

3AP

3LP

A : Agar Culture
L : Liquid Culture

V : Victor Borge
P : Priscilla

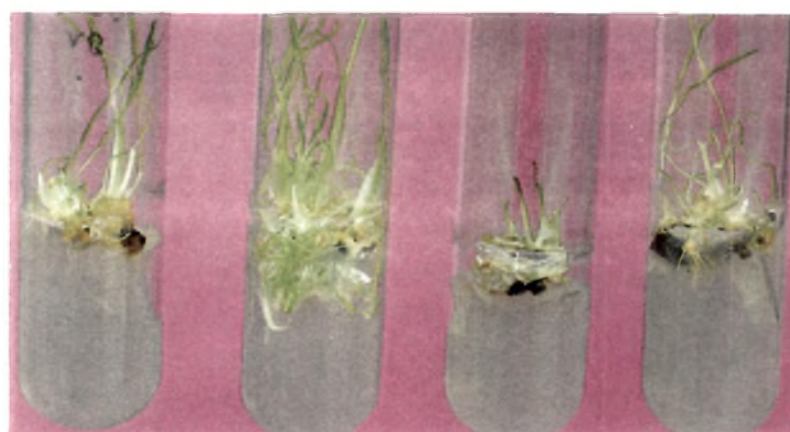


4AV

4LV

4AP

4LP



5AV

5LV

5AP

5LP



6AV

6LV

6AP

6LP

7V

7P

A : Agar Culture
L : Liquid Culture

V : Victor Borge
P : Priscilla

improve embryo morphology and plantlet production. Therefore, in this experiment the PEMs of Victor Borge induced from liquid EIM for a period of 25 days were transferred to liquid EDM to facilitate embryo development. The somatic embryos under various stages of development on pro embryogenic tissues were transferred to agar half strength plantlet elongation medium (PLM) free from hormones for plantlet production. The morphological characters of the embryos arising from this system were compared with that of the embryos arising from agar (EIM) - agar (EDM) and liquid (EIM) - agar (EDM) and the observations are shown in Table 12 and illustrated in Plate 9. The rate of embryogenesis and embryo morphological characters in terms of length and thickness of the embryos differed significantly with the system adopted. The number of embryos developed per culture was significantly higher (125.67) from the liquid EIM - liquid EDM - agar PLM system, when compared to other two systems namely agar EIM - agar EDM (65.0) and liquid EIM - agar EDM (99.67). The embryos arising from agar EIM - agar EDM were short and thin (3.03 mm in length and 1.15 mm in thickness), compared to the embryos developed from liquid EIM-liquid EDM-agar PLM (4.87 mm in length and 1.97 mm in thickness). The number of plantlets obtained from liquid EIM - liquid EDM - agar PLM was also highest (75.83/culture) and they were more vigorous compared to other two systems. The number of plantlets/culture was 60.83 from liquid EIM - agar EDM and least from agar EIM- agar EDM (25.17). The embryos developed on agar EIM - agar EDM were more fragile, whereas the embryos obtained from liquid EIM- liquid EDM were more sturdy and vigorous.

The embryonic plantlets initiated roots within 15 days and produced cormels *in vitro* after 90 days on MS medium containing 0.5 mg/l IBA and 6 per cent sucrose.

Table 12. Effect of culture media on embryo morphology and plantlet production in *Gladiolus* cv. Victor Borge.

Sl.No.	Treatment	Somatic Embryos (Nos/culture)	Length of SEs (mm)	Thickness of SEs (mm)	Plantlets (Nos./ culture)
1.	Agar (EIM) - Agar (EDM) (30 days)	65.00	3.03	1.15	25.17
2.	Liquid (EIM) - Agar (EDM) (25 days)	99.67	3.48	1.33	60.83
3.	Liquid (EIM)-Liquid (EDM)-Agar (PLM) (25 days)	125.67	4.87	1.97	75.83
	CD (0.05 P)	13.66	0.51	0.32	8.84
	SEm(+)	9.66	0.36	0.23	6.25

Embryo induction medium (EIM) : MS (1962) + 0.5 mg/l BAP + 1.0 mg/l NAA. pH : 5.8

Embryo development medium (EDM) : MS (1962) + 0.2 mg/l kinetin.

Plantlet production medium (PLM) : Half strength MS (1962) free from hormones.

Volume of the medium : Agar : 16 ml /culture. Liquid : 80 ml / culture. No. of replications : 6

Culture conditions : Temperature 26 ± 2 °C. Light/dark period : 16/8 Hrs.

Inoculum in liquid medium : 750 mg / culture following transfer to 3 culture tubes containing agar EDM/PLM.

Inoculum in agar medium : 250 + 10 mg/culture.

Observations :Embryo development : On agar (EDM) on 20 th day after transfer from agar /liquid (EIM) in case of treatments 1 & 2.

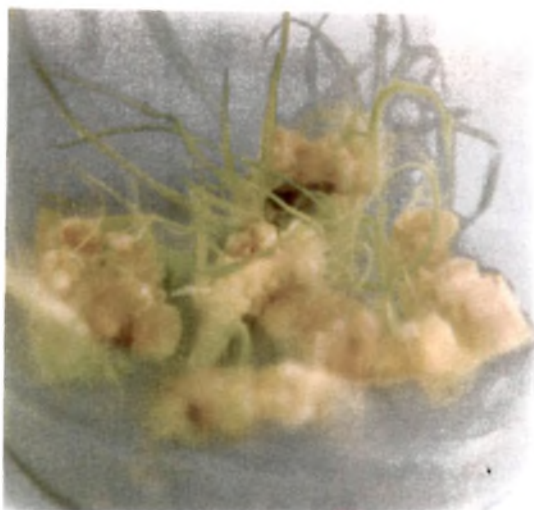
On liquid (EDM) on 20 th day after transfer from liquid (EIM) in case of treatment 3.

Plantlet production : On agar (EDM) on 40 th day after transfer agar/liquid (EIM) in case of treatments 1&2.

On agar (PLM) on 20 th day after transfer from liquid (EDM) in case of treatment 3.

Plate 9. Development of somatic embryos from agar and liquid culture systems
cv. Victor Borge.

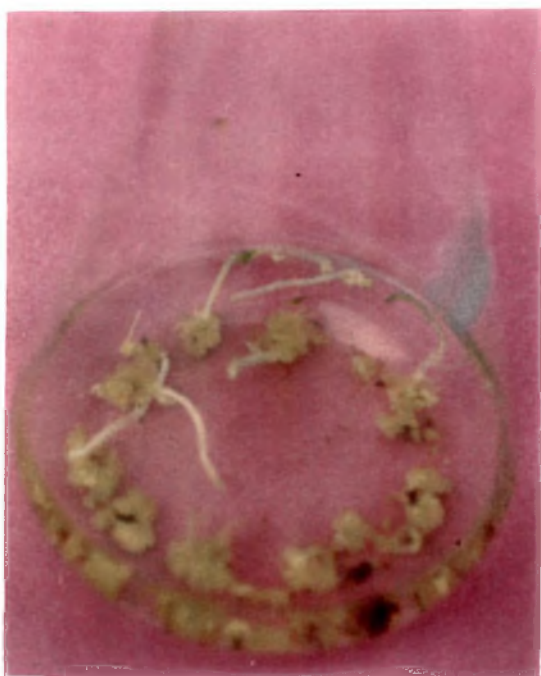
- A . Development of SEs from agar EIM - agar EDM system.
- B. Close view of SEs developed from agar EIM - agar EDM system.
- C. Development of SEs from liquid EIM - liquid EDM system.
- D. Close view of SEs developed from liquid EIM - liquid EDM system.



A



B



C



D

These cormels produced flowering size corms in the second season. These observations are based on the experiments conducted separately to induce *in vitro* rooting and field planting of the *in vitro* produced cormels.

The sequence of steps involved in somatic embryogenesis and plantlet production in cv. Priscilla, Victor Borge and Rose Supreme, is illustrated in plate 10 and the sequence of steps involved in development of flowering plants from cormels produced via *in vitro* somatic embryogenesis is illustrated in plate 11.

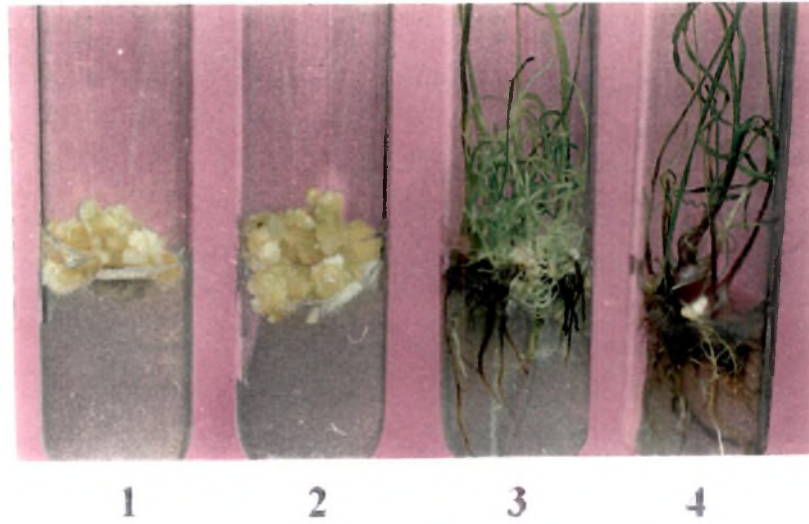
5. Stages in somatic embryogenesis and the role of protein synthesis on embryo development and plantlet production in *Gladiolus* cv. Victor Borge.

This experiment was conducted in two sets. In the first set, the creamy white callus of the cv. Victor Borge, subcultured on maintenance medium, was transferred to liquid embryo induction medium (MS + 1.0mg/l NAA + 0.5 mg/l BAP) and incubated for 30 days. Cultures were drawn from this set at 5 days interval and observed for development of proembryogenic masses and somatic embryos. Simultaneously, the cultures were transferred to agar embryo development medium for maturation of somatic embryos and production of plantlets. To the second set, cycloheximide (CH) was added at 5 days interval and continued upto 30 days and later transferred to agar embryo development medium and observed for the recovery of somatic embryos and plantlets.

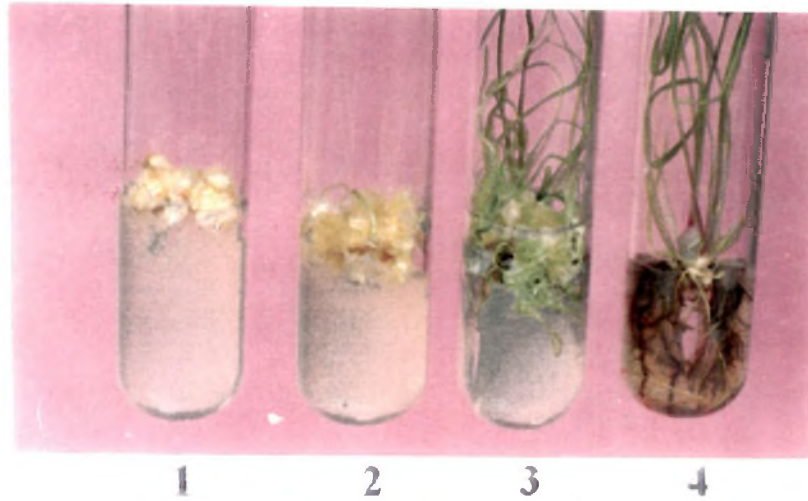
In the first set without CH, after 5 days of incubation, the creamy white callus started developing into yellow and friable embryogenic clusters. By 25th day, maximum development of embryogenic cell clusters was observed. The microscopic

Plate 10. Developmental stages of somatic embryos and plantlets.

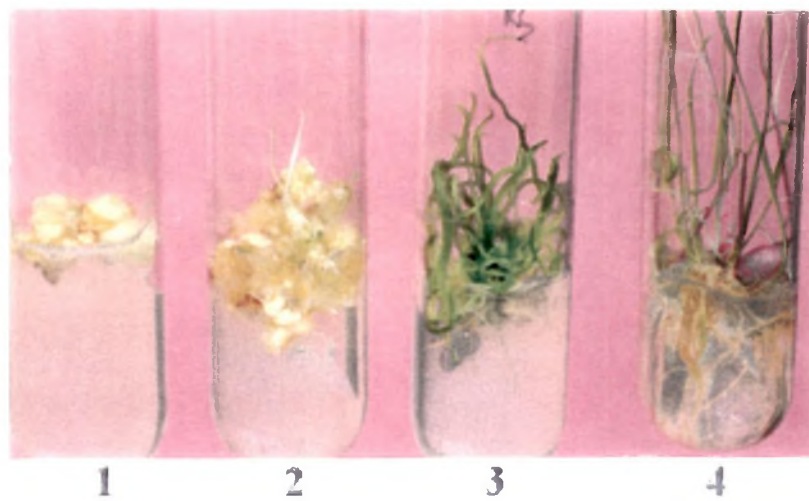
1. Growth of callus on maintenance medium (MS + 1.0 mg/l 2,4-D + 0.2 mg/l BAP)
2. Induction of pro embryogenic masses (PEMs) on embryo induction medium (MS + 1.0 mg/l NAA + 0.5 mg/l BAP)
3. Embryo germination and plantlet production on embryo development medium (MS + 0.2 mg/l kinetin)
4. Rooting and cormel induction on half strength MS + 0.5 mg/l IBA with 6 per cent sucrose.



Priscilla



Victor Borge



Rose Supreme

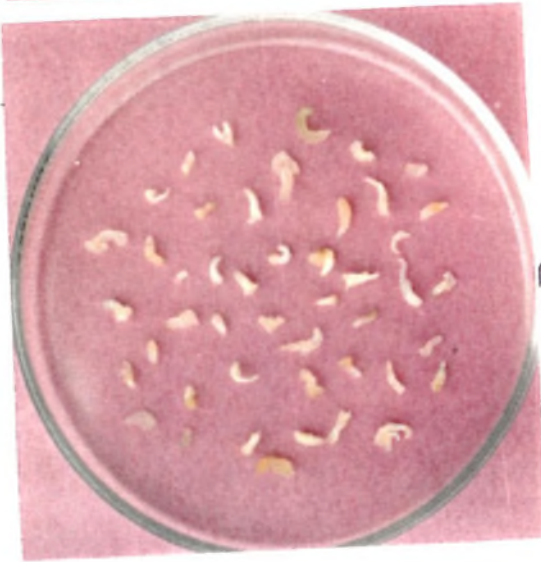
Plate 11. Sequence of the steps involved in development of flowering stage plants from cormels produced via *in vitro* somatic embryogenesis.

- A . Development of somatic embryos on embryo development medium
- B . Isolated somatic embryos
- C . Germination of isolated embryos
- D . Rooting and plantlet establishment
- E . Cormel production
- F . First generation plants in pots
- G . Second generation plants (flowering) in pots

Plate 11



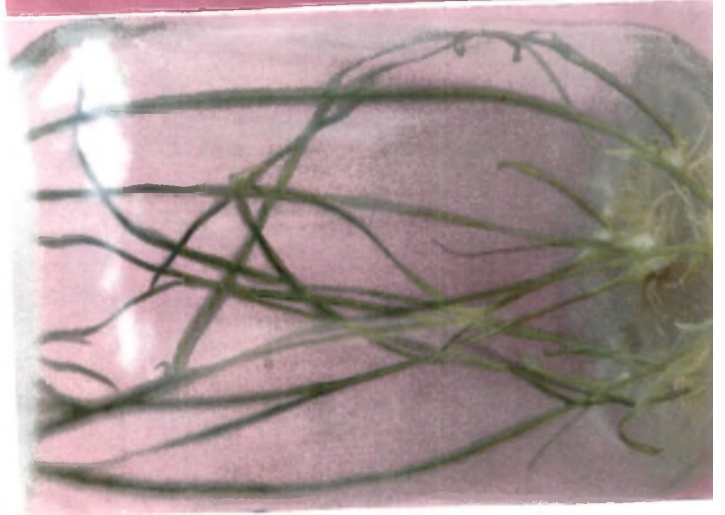
A



B



C



F



G

observation of the embryogenic events revealed the development of globular to heart shaped embryos between 7-10 days and late torpedo stage between 12-15 days and advance stage embryos after 15-20 days. The different stages of embryo development are illustrated in Plate 12.

The embryogenic clusters comprising proembryogenic masses and somatic embryos at various stages of development in set I, when transferred to agar embryo development medium at 5 days interval, showed maturation of few somatic embryos (4.33Nos./culture) from the culture incubated for only 5 days. Thereafter, increased recovery of somatic embryos was noticed and the highest number of somatic embryos (95.83/culture) was recorded in the culture incubated for 25 days and on the 30th day the recovery declined. The plantlet production was also highest from the culture incubated in embryo induction medium for 25 days. In contrast to this set, the incorporation of CH to the embryo induction medium, adversely affected the recovery of somatic embryos and plantlet production. The cultures to which CH was incorporated on the 10th and 15th day after incubation, did not yield any somatic embryos, whereas from the culture to which CH was incorporated on the 20th day, resulted in production of only 14.67 somatic embryos/culture as against the recovery of to 64.17 somatic embryos/culture when there was no CH. Although, highest number of 73.17 somatic embryos/culture was recorded in the culture to which CH was added on the 30th day, the recovery was significantly lower when compared to the maximum recovery of 95.83 somatic embryos/culture under first set without CH. The results are presented in Table 13 and Fig. 2 and illustrated in plate 13.

Plate 12. Morphogenic events of somatic embryogenesis.

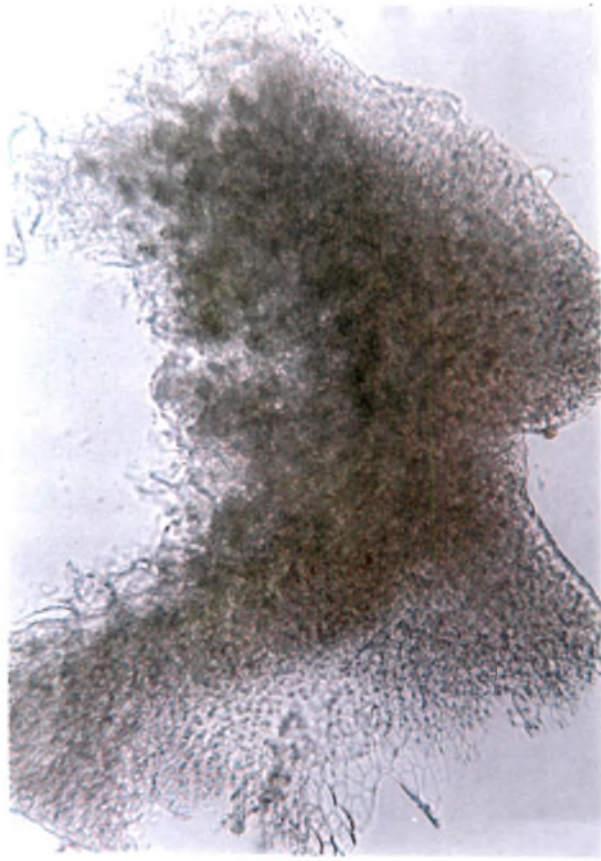
- A . Creamy white callus with undifferentiated cell clusters
- B . Differentiation of cell clusters by enlargement and cell wall thickening
- C . Cross section of the embryogenic cell clusters
- D . Development of globular and heart shaped embryos
- E . Enlargement of heart stage to torpedo stage
- F . Embryo like structure with shoot apex
- G . Development of a typical somatic embryo
- H . Shoot and root organogenesis



A



B



C

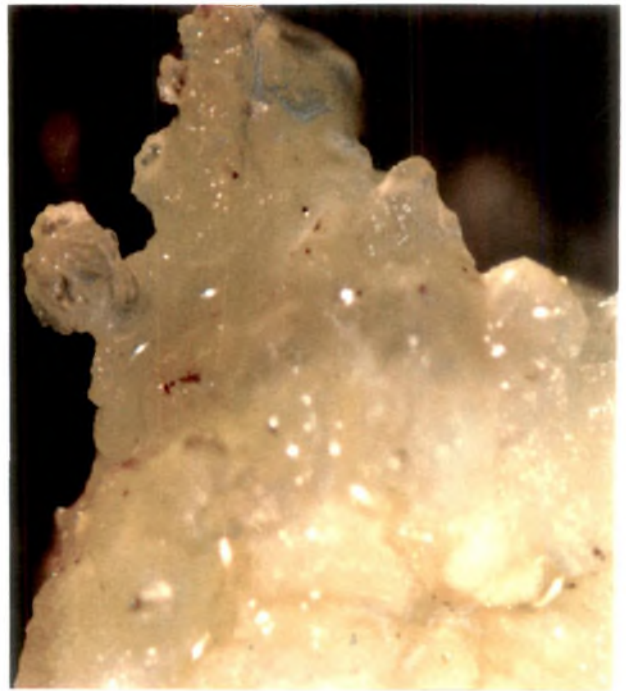


D

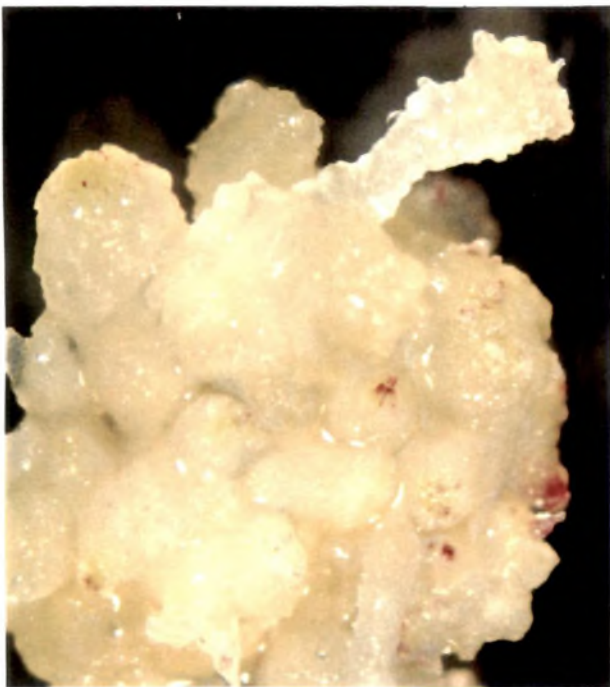
Plate 12



E



F



G



H

Table 13. Effect of cycloheximide (CH) regimes on somatic embryogenesis and plantlet production in *Gladiolus* cv. Victor Borge.

Sl. No.	Treatment Days in liquid induction medium without CH or Days (after inoculation) on which CH was incorporated and continued upto 30 days	Somatic Embryos (Nos/culture)		Plantlets (Nos./culture)	
		Without CH	With CH (0.1%)	Without CH	With CH (0.1%)
1.	5	4.33	0.00	0.00	0.00
2.	10	15.33	0.00	7.17	0.00
3.	15	51.83	0.00	24.67	0.00
4.	20	64.17	14.67	27.83	3.83
5.	25	95.83	24.83	52.53	12.67
6.	30	57.00	73.17	20.33	27.50
	CD (0.05 P)	7.06	5.00	5.11	3.03
	SEm(+)	5.00	3.53	3.61	2.14

Embryo induction medium : Liquid MS (1962) + 0.5 mg/l BAP + 1.0 mg/l NAA . pH : 5.8

Embryo development medium : Agar MS (1962) + 0.2 mg/l Kinetin.

Volume of the medium : Liquid induction medium : 80 ml/culture. Agar development medium : 16 ml/culture.

Inoculum : 750 mg/culture in liquid induction medium following transfer to 3 culture tubes containing agar development medium.

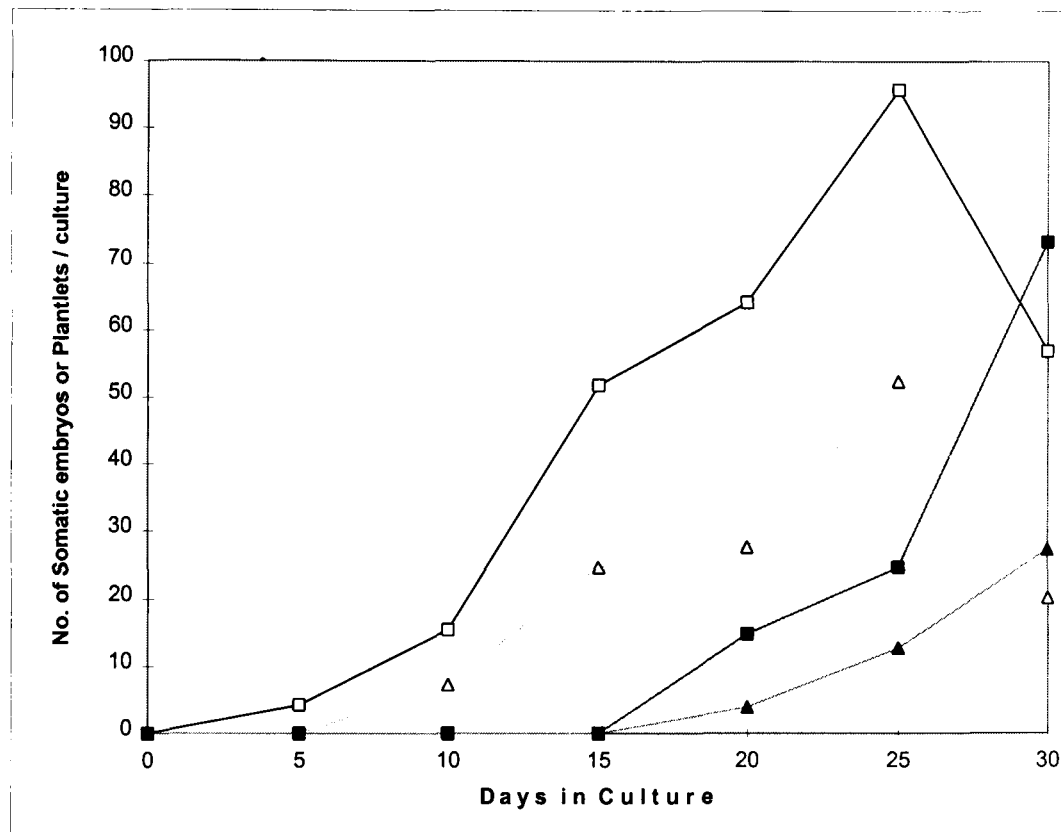
Culture duration : Without CH : As per treatment.

With CH : 30 days (cycloheximide added at 5 days interval and continued up to 30 days.)

Culture conditions : Temperature 26 ± 2°C. Light /dark period : 16 /8 Hrs. No. of replications : 6.

Observations:Embryo development : On 20 th day after transfer from induction medium.

Plantlet production : On 40 th day after transfer from induction medium.



- Somatic embryos without cycloheximide
- Somatic embryos with cycloheximide
- △-- Plantlets without cycloheximide
- ▲-- Plantlets with cycloheximide

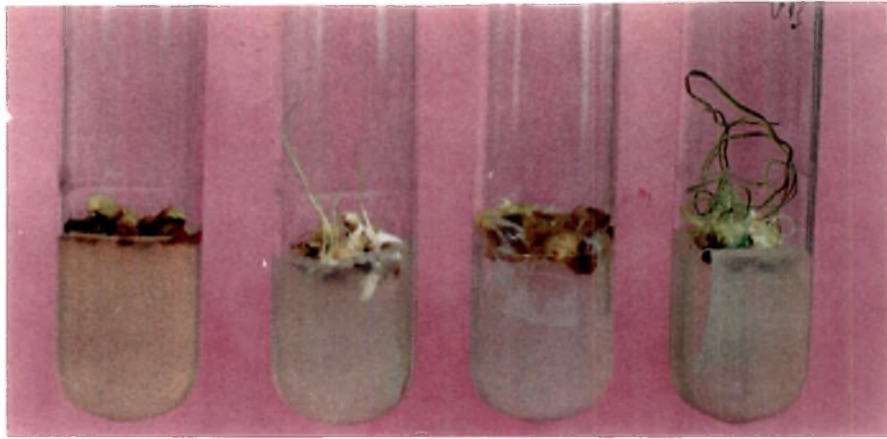
Fig 2. Effect of cycloheximide (CH) regimes on somatic embryogenesis and plantlet production in *Gladiolus* cv. Victor Borge.

Plate 13. Development of somatic embryos and plantlets with and without cycloheximide (CH).

Duration in liquid induction medium with and without cycloheximide

1. 5 days
2. 10 days
3. 15 days
4. 20 days
5. 25 days
6. 30 days

- A . With cycloheximide
B . Without cycloheximide

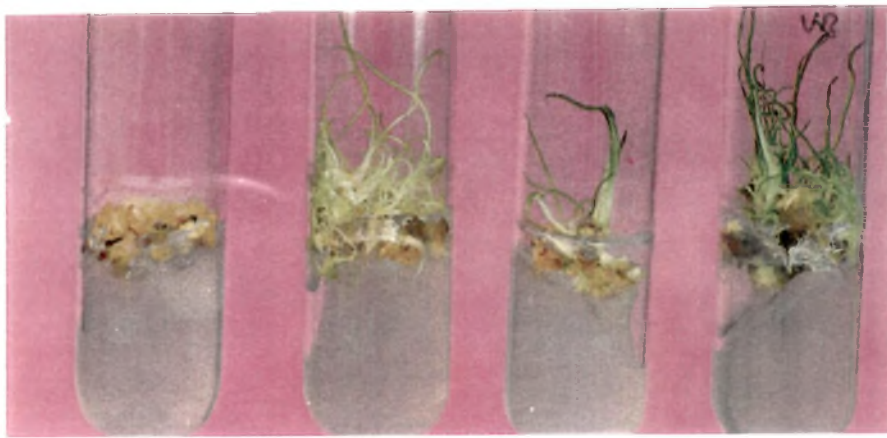


1A

1B

2A

2B

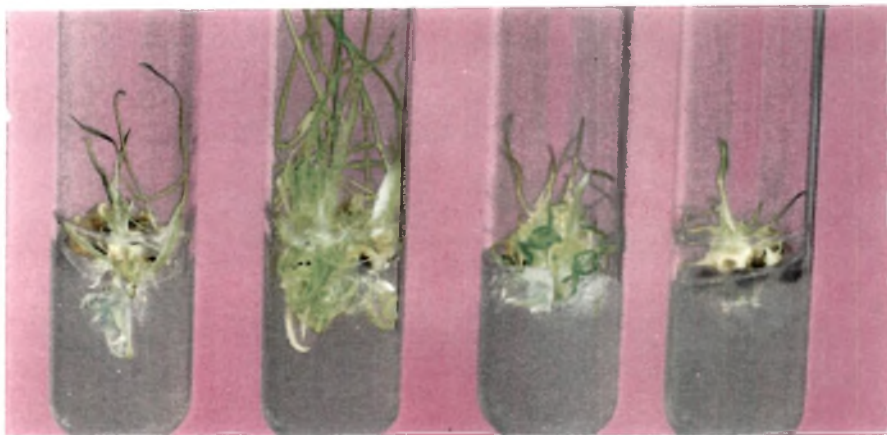


3A

3B

4A

4B



5A

5B

6A

6B

Plate 13

Production of synthetic seeds and their viability

1. Effect of hydrogel encapsulation on survival of callus, shoot buds and somatic embryos under different storage temperatures in *Gladiolus* cv. Rose Supreme.

The survival of both encapsulated and nonencapsulated material namely callus, adventitious shoot buds and somatic embryos, decreased significantly with increased storage periods at both the storage temperatures ($26 \pm 2^\circ\text{C}$ and $5 \pm 1^\circ\text{C}$) under dark. However, the reduction in viability was more conspicuous in non encapsulated material in contrast to encapsulated material at both the temperatures. Against the storage of 20 embryos, the mean number of embryos that survived after 10 days at 26°C storage temperature, was only 0.50 without encapsulation when compared with 5.33 of encapsulated embryos. Similarly, at 5°C , the encapsulated somatic embryos survived better (11.17) in contrast to non encapsulated (1.17) after 10 days. Similar trends were also observed with callus and adventitious shoot buds. Among the 3 types of materials encapsulated, adventitious shoot buds survived better and SEs were the least survived. After 15 days of storage at 26°C , the mean number of encapsulated adventitious shoot buds that survived was 6.50, when compared with only 4.33 in callus and 1.17 in somatic embryos. Between the two storage temperatures, survival of both encapsulated and non encapsulated material was better under 5°C compared to 26°C . At 5°C , the encapsulated SEs survived upto 30 days (3.83) whereas at 26°C , none of the embryos survived (Table 14). The illustrations of the encapsulated somatic embryos, adventitious shoot buds and callus are presented in plate 14 and the viability of encapsulated somatic embryos, adventitious shoot buds and callus before and after storage is presented in plate 15.

Table 14. Effect of hydrogel encapsulation on survival of callus, shoot buds and somatic embryos under different storage temperatures in *Gladiolus* cv. Rose Supreme.

Sl. No.	Storage period (days)	Survival of encapsulated callus, shoot buds and SEs					
		Storage at 26 ± 2 °C			Storage at 5 ° ± 1° C		
		Callus	Shoot bud	Somatic embryo	Callus	Shoot bud	Somatic embryo
1.	0	20.00 (20.00)	20.00 (20.00)	20.00 (20.00)	20.00 (20.00)	20.00 (20.00)	20.00 (20.00)
2.	3	11.00 (5.50)	16.83 (10.00)	9.67 (3.50)	19.67 (10.33)	19.67 (14.67)	16.33 (5.50)
3.	6	9.67 (1.83)	12.67 (7.33)	6.83 (1.67)	17.50 (6.50)	19.00 (11.33)	13.50 (3.83)
4.	10	7.00 (0.33)	9.83 (3.50)	5.33 (0.50)	16.17 (2.50)	18.17 (6.83)	11.17 (1.17)
5.	15	4.33 (0.00)	6.50 (1.33)	1.17 (0.00)	10.33 (0.83)	15.67 (3.83)	8.17 (0.00)
6.	30	0.00 (0.00)	3.00 (0.00)	0.00 (0.00)	4.67 (0.00)	10.50 (0.00)	3.83 (0.00)
CD(0.05 P)		1.21 (0.64)	1.24 (0.83)	0.66 (0.60)	0.92 (1.03)	0.85 (0.89)	1.00 (5.38)
SEm(±)		0.86 (0.45)	0.88 (0.58)	0.47 (0.42)	0.65 (0.73)	0.60 (0.63)	0.70 (3.80)

Encapsulation gel : Sodium alginate (4%). Complexing agent : Calcium chloride (100mM).

No. of capsules/replication : 20. No. of replications : 6

Storage conditions : Temperature as per treatments. Light/ dark period : continuous dark.

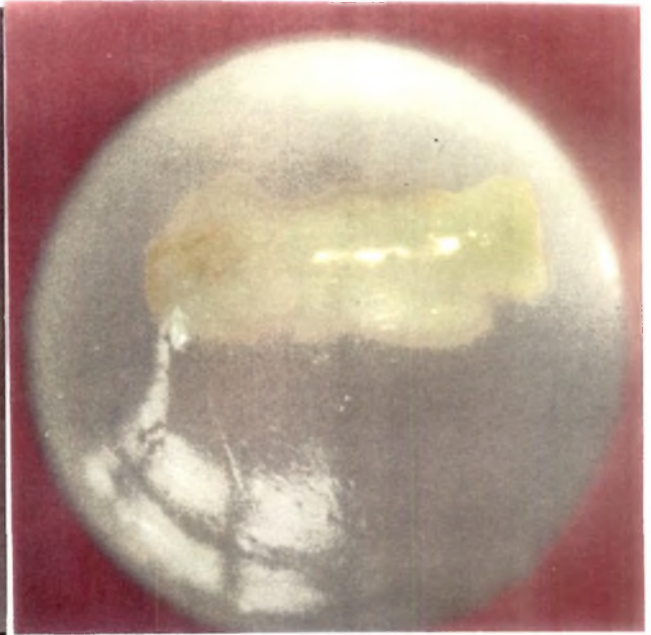
Figures in parenthesis : Survival of callus /shoot buds /SEs without encapsulation.

Plate 14. Encapsulated somatic embryos, adventitious shoot buds and callus.

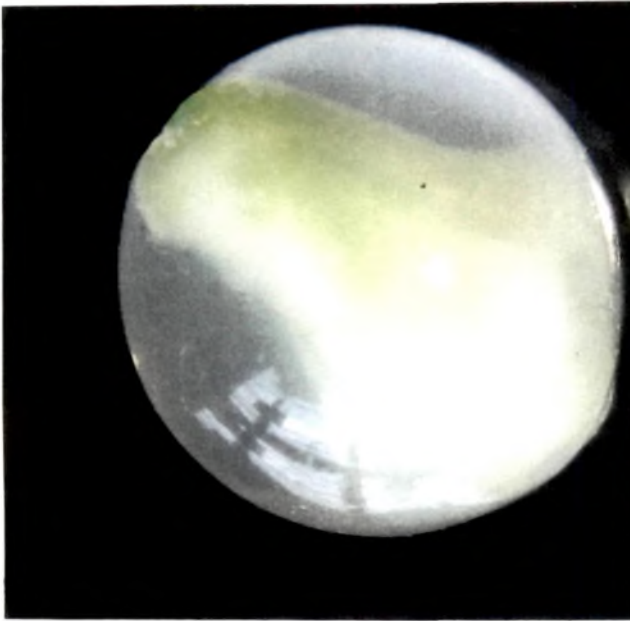
- A . Cluster of encapsulated
 - a. Somatic embryos
 - b. Adventitious shoot buds
 - c. Callus
- B . Close view of encapsulated somatic embryo
- C . Close view of encapsulated adventitious shoot bud
- D . Close view of encapsulated callus bit
- E . Stage of somatic embryo isolated and used for synthetic seed production



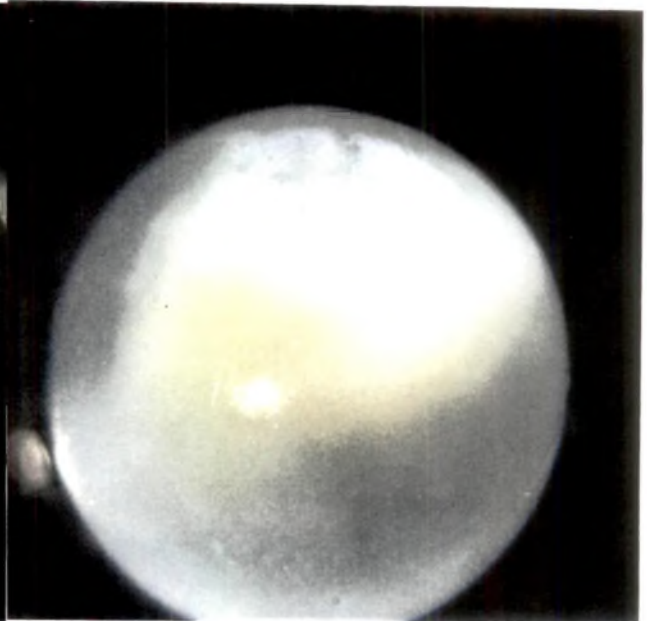
A



B



C



D



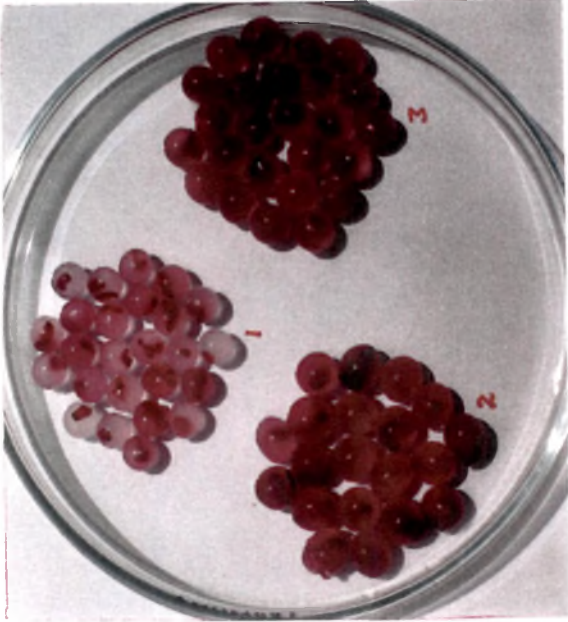
E

2. Effect of different additives on contamination, survival and germination of encapsulated somatic embryos in *Gladiolus* cv. Rose Supreme.

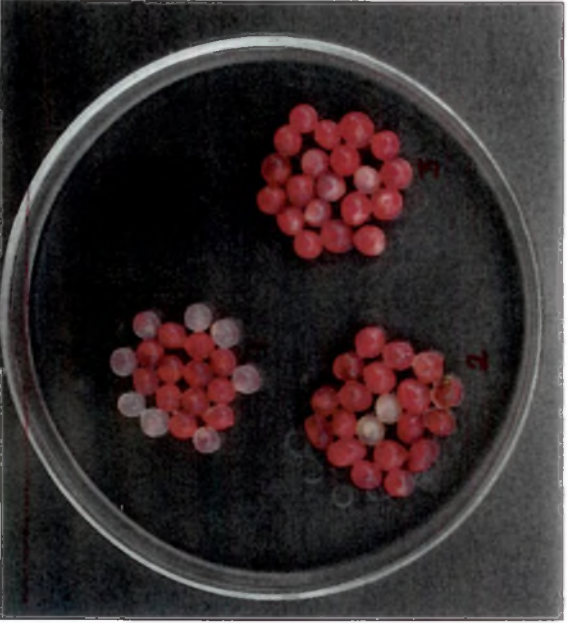
The treatments imposed in this experiment are shown in Table 15. The kind of additives incorporated in the encapsulating gel brought about marked differences with respect to contamination, survival and germination of encapsulated somatic embryos. In control, without any additives and without the sterilization of encapsulating gel and complexing agents, higher percent of capsules (57.1) was contaminated with only bacteria, 35.7 per cent with both fungi and bacteria and the remaining 7.1 percent with only fungi. In contrast to this, encapsulation with 0.1% carbendazim showed better protection effect. The bacterial contamination although recorded in 57.4 percent of the capsules, there was drastic reduction in fungal contamination and restricted to only 3.6 per cent. The remaining 39 per cent of the capsules were free from any contaminations. In the treatment involving 0.1% streptomycin, 42.8 percent of the capsules was contaminated with fungi and 57.2 per cent remained uncontaminated. The contamination level receded drastically when both carbendazim and streptomycin were incorporated, wherein nearly 94.82 percent of the capsules remained healthy without any kind of contaminations. However, in all the above treatments, when encapsulated embryos were plated on germination medium, they turned brown and died eventually. Contrary to this, in the treatment involving no additives but with sterilization of both encapsulating gel and complexing agents, not only all the capsules remained healthy, but 23.9 per cent of the encapsulated embryos remained viable upto 10 days. Nevertheless, they also failed to germinate. The last treatment, involving no additives but incorporation of half strength MS nutrients and sterilization of both encapsulating gel and complexing

Plate 15. Survival of encapsulated somatic embryos, adventitious shoot buds and callus.

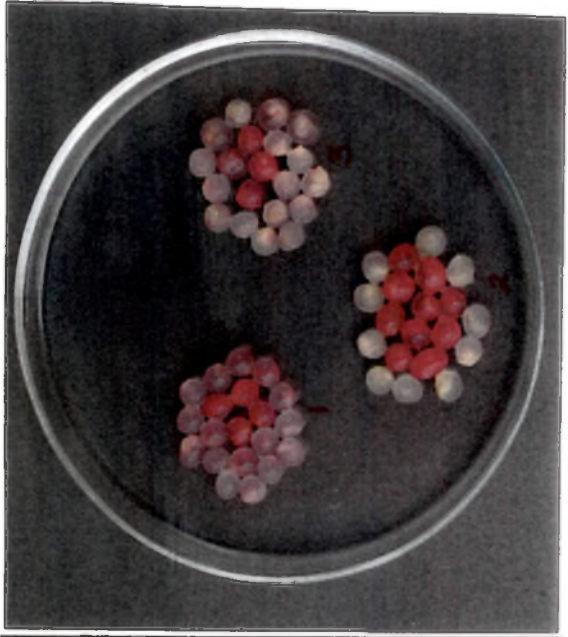
- A . Survival immediately after encapsulation
 - B . Survival after 10 days of storage at $5 \pm 1^{\circ}\text{C}$
 - C . Survival after 30 days of storage at $5 \pm 1^{\circ}\text{C}$
 - D . Close view of viable somatic embryos
 - E . Close view of viable callus
-
- 1 . Encapsulated somatic embryos
 - 2. Encapsulated adventitious shoot buds
 - 3. Encapsulated callus bits



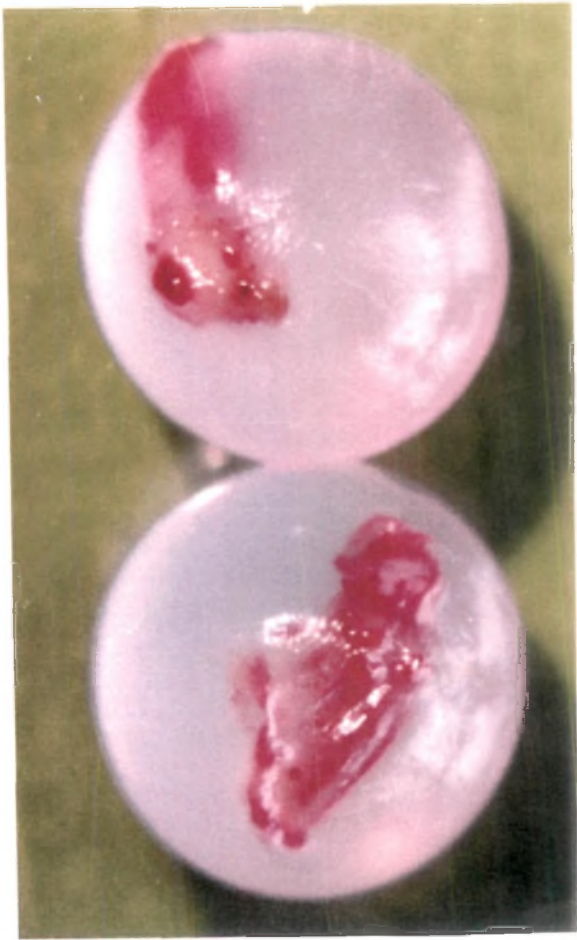
A



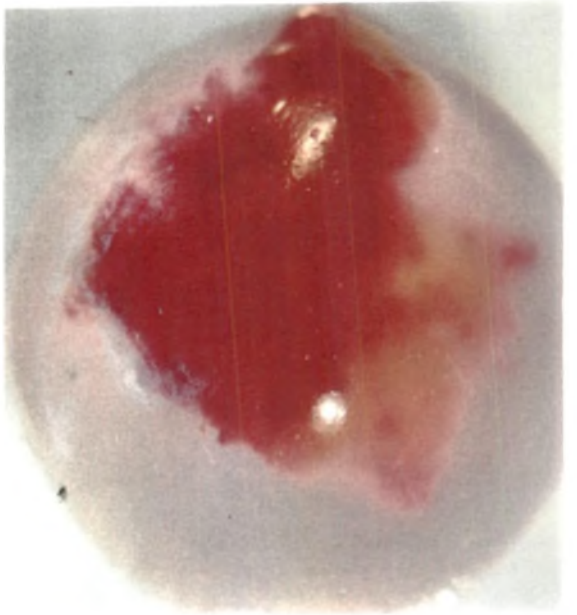
B



C



D



E

Plate 15

Table 15. Effect of different additives on contaminations, survival and germination of encapsulated SEs in *Gladiolus* cv. Rose Supreme.

Sl.No	Treatment	Capsules per plate (Nos)	Capsules contaminated with fungus + bacteria (Nos)	Capsules contaminated with only fungus (Nos)	Capsules contaminated with only bacteria (Nos)	Embryos survived (Nos)	Embryos germinated (Nos)
1.	Control : Encapsulating SEs with sodium alginate, complexed with CaCl ₂ both nonsterilised.	7	2.50 (35.7)	0.50 (7.1)	4.00 (57.1)	0.00 (0.0)	0.00 (0.0)
2.	Addition of 0.1% carbendazim to sodium alginate, complexed with CaCl ₂ both nonsterilized.	9	0.00 (0.0)	0.33 (3.6)	5.17 (57.4)	0.00 (0.0)	0.00 (0.0)
3.	Addition of 0.1% streptomycin to sodium alginate, complexed with CaCl ₂ both nonsterilized.	7	0.00 (0.0)	3.00 (42.8)	0.00 (0.0)	0.00 (0.0)	0.00 (0.0)
4.	Addition of 0.1% carbendazim and 0.1% streptomycin to sodium alginate, complexed with CaCl ₂ both nonsterilized	16	0.00 (0.0)	0.83 (5.18)	0.00 (0.0)	0.00 (0.0)	0.00 (0.0)
5.	Encapsulating SEs with sodium alginate gel, complexed with CaCl ₂ both sterilized.	16	0.00 (0.0)	0.00 (0.0)	0.00 (0.0)	3.83 (23.9)	0.00 (0.0)
6.	Encapsulating SEs with sodium alginate containing half MS basal nutrients complexed with CaCl ₂ both sterilized.	18	0.00 (0.0)	0.00 (0.0)	0.00 (0.0)	0.33 (57.33)	3.50 (19.4)
	CD (0.05 P)		0.48	0.81	0.54	1.20	0.48
	SEm (+)		0.34	0.57	0.38	0.85	0.34

Encapsulation gel : Sodium alginate (4%). Complexing agent : CaCl₂ (100mM).

Germination medium : Half strength MS basal. pH : 5.8 No. of replications : 6 Culture conditions : Temperature : 26 ± 2 °C.

Light /dark period : 16/8Hrs. Figures in parenthesis : Percentage to the no. of capsules per plate.

Observations : No. of capsules contaminated with fungus and bacteria on 10 th day after plating and germination on 15 th day after plating on germination medium.

agent, not only helped in higher survival (57.33 percent) but also resulted in germination of fewer encapsulated embryos (19.4 per cent). The results are presented in Table 15 and illustrated in Plates 16 and 17.

3. Effect of hardening treatments on survival of hydrogel encapsulated somatic embryos in *Gladiolus* cv. Rose Supreme.

The somatic embryos were encapsulated after subjecting them to various hardening treatments (Table 16) and stored at 26 ± 2 °C for a period of 30 days under dark. The assessment of the viability of the SEs immediately after encapsulation and after 5, 10 and 30 days of storage, showed varied responses among the treatments. While the addition of 1 μ M ABA to the development media improved the viability of the encapsulated embryos, the desiccation treatment brought about negative response. The magnitude of response in other treatments was highly variable, and inconclusive.

The somatic embryos obtained from control and ABA (1 μ M) treatments survived 100 per cent when tested for their viability immediately after encapsulation. But somatic embryos from desiccation, prechilling and desiccation combined with prechilling treatments failed to retain full viability after encapsulation. The adverse effect was more apparent in desiccation treatment as only 76.6 percent of the encapsulated somatic embryos survived. The addition of 1.0 μ M ABA ensured higher survival of encapsulated embryos upto 30 days of storage (12.2 percent), in contrast to 7.8 percent under ABA combined with chilling. The encapsulated somatic embryos from other treatments did not maintain higher viability for long periods. Due to desiccation treatment, the viability was reduced from 76.6 to 3.3 per

Plate 16. Contamination, survival and germination of encapsulated somatic embryos.

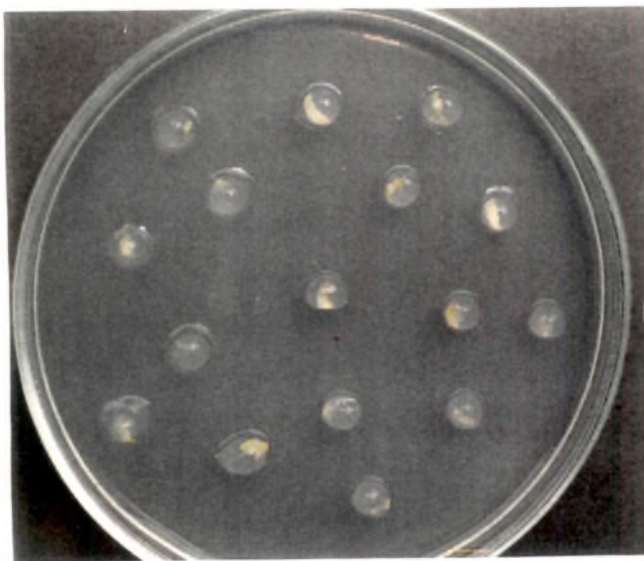
- A1. Encapsulated SEs contaminated with both fungi and bacteria without any additives
- A2. Encapsulated SEs contaminated with only bacteria with the addition of carbendazim (0.1 %)
- A3. Encapsulated SEs contaminated with only fungus with the addition of streptomycin (0.1 %)
- B . Encapsulated SEs free from contamination with sterilization of both encapsulating gel and complexing agent.
- C . Germination of encapsulated embryos with the incorporation of half MS nutrients and sterilization of encapsulating and complexing agents.



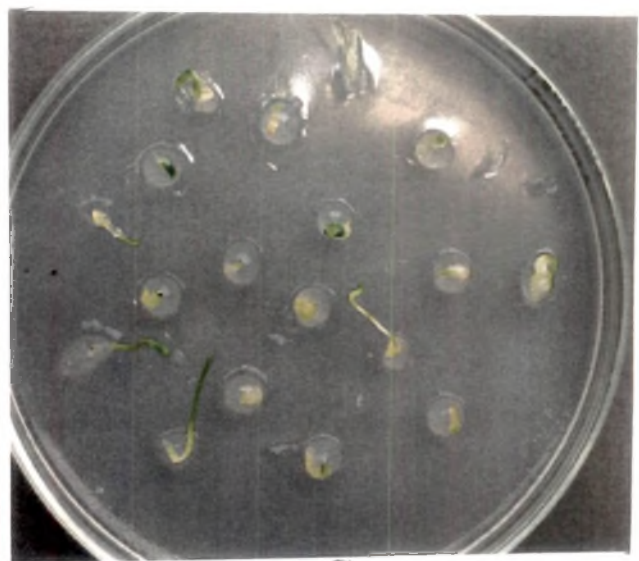
A1

A2

A3



B



C

Plate 16

Plate 17. Stages of germination of encapsulated somatic embryos.

- A . Initiation of germination
- B . Emergence of plumule
- C . Plantlet development



A



B



C

Plate 17

cent and due to chilling treatment, it reduced from 88.8 to 18.9 per cent, after 15 days of storage. The survival of encapsulated somatic embryos was comparatively better under ABA with chilling treatment (65.5, 33.3 per cent after 5 and 15 days of storage, respectively) over control and chilling alone. Although the SEs subjected to prechilling could not survive after 30 days of storage, their viability was better after 5 and 15 days of storage (48.9 and 18.9 percent, respectively) when compared with the control; wherein the survival was only 37.8 and 8.9 percent at the corresponding storage periods. The results are presented in Table 16 and illustrated in Plate 18.

Table 16. Effect of hardening treatments on survival of hydrogel encapsulated SEs in *Gladiolus* cv. Rose Supreme.

Sl.No.	Treatment	Survival of encapsulated embryo (Nos)					
		Storage period (days)					
		0	5	15	30		
1.	Encapsulation of SEs immediately after harvest from liquid maturation medium	15.00 (100)	5.67 (37.8)	1.33 (8.9)	0.00 (0.0)		
2.	Encapsulation of SEs after subjecting to desiccation on laminar airflow cabinet for 1 hour.	11.50 (76.6)	3.17 (21.3)	0.50 (3.3)	0.00 (0.0)		
3.	Addition of ABA 1 μ M to liquid development medium 6 days earlier to embryo harvest and immediate encapsulation.	15.00 (100)	11.00 (73.3)	6.50 (43.3)	1.83 (12.2)		
4.	SEs subjected to chilling @ 5 °C for 3 days before encapsulation.	13.33 (88.8)	7.33 (48.9)	2.83 (18.9)	0.00 (0.0)		
5.	Addition of ABA 1 μ M to the liquid maturation medium 6 days before harvest and subjecting SEs to chilling @ 5 °C for 3 days before encapsulation.	13.50 (90.0)	9.83 (65.5)	5.00 (33.3)	1.17 (7.8)		
	CD (0.05 P)	0.69	1.31	0.79	0.57		
	Sem($\pm$)	0.48	0.93	0.56	0.40		

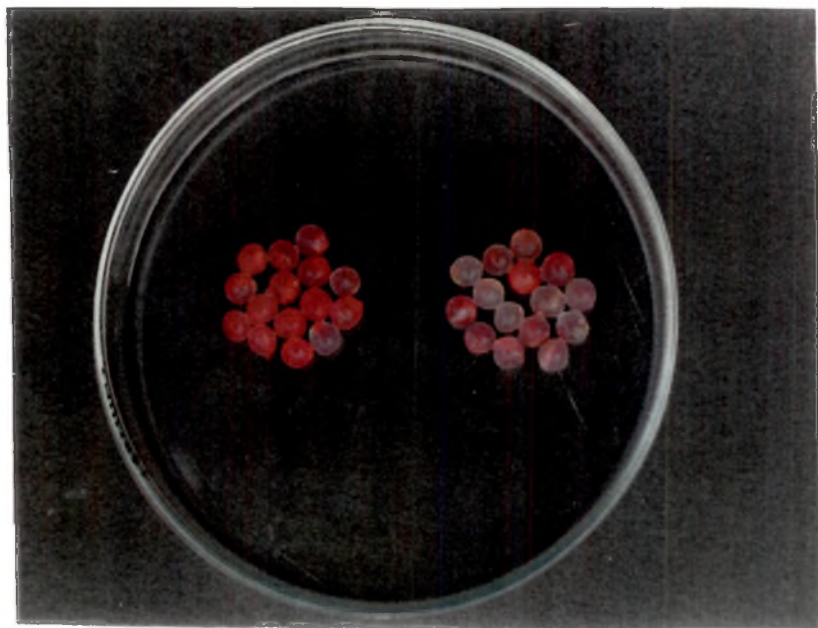
Encapsulation gel : Sodium alginate (4%). Complexing agent : 100mM.

No. of replications : 6. Storage conditions : Temperature : 26 $\pm$ 2°C. Light /dark period : continuous dark.

No. of capsules per replication : 15 . Figures in parenthesis : Percentage survival.

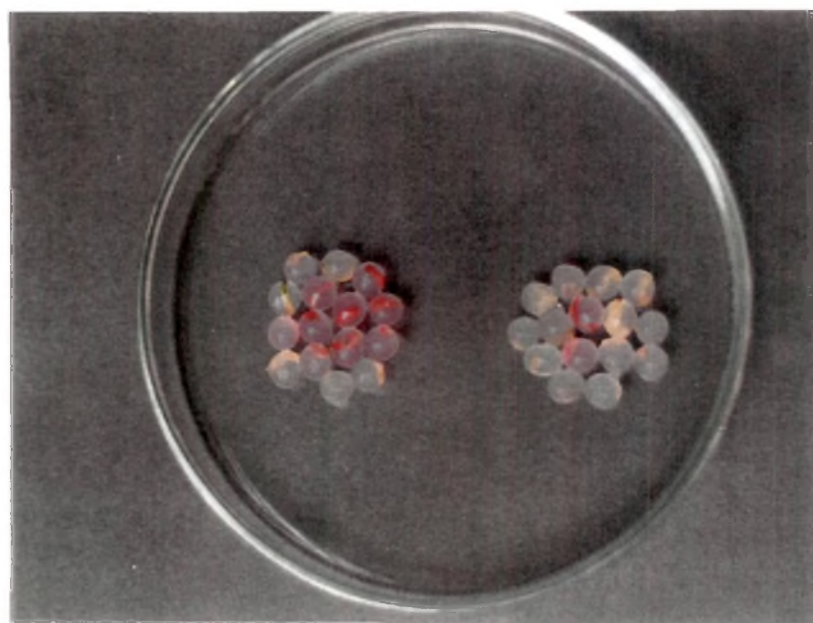
Plate 18. Survival of encapsulated somatic embryos with and without ABA (1 μ m) treatment.

- A1. Survival of encapsulated somatic embryos treated with ABA (1 μ m) after 5 days of storage
- A2. Survival of encapsulated somatic embryos (untreated) after 5 days of storage
- B1. Survival of encapsulated somatic embryos treated with ABA (1 μ m) after 15 days of storage
- B2. Survival of encapsulated (untreated) somatic embryos after 15 days of storage.



A1

A2



B1

B2

Plate 18

DISCUSSION

V. DISCUSSION

The results of the studies on somatic embryogenesis and production of synthetic seed in *Gladiolus* cultivars are discussed in this chapter in the light of established facts and available literature.

Response of explants to growth regulators *in vitro*

In *Gladiolus* cultivars namely, Priscilla, Victor Borge and Rose Supreme, shoot buds excised from the corm sections with pieces of basal corm tissue attached, were cultured on MS medium containing no growth regulators as well as with different levels of cytokinins. It was to test whether the endogenous growth regulators, if any in the explants, would be sufficient to induce multiple sprouting *in vitro* or required to be supplied exogenously into the growth medium. The explants of all the three cultivars showed positive response in the presence of growth regulators, BAP and kinetin at different concentrations in the medium. This was in contrast to minimum response obtained on medium that contains no growth regulators. Between BAP and kinetin, the response evinced by kinetin was more striking and the number of sprouts obtained was maximum on medium containing kinetin at 4.0 mg/l. This suggested that the endogenous growth regulators were either absent or their levels were inadequate in inducing multiple sprouts in all the three cultivars. Though BAP in a number of studies (Rumynin *et al.*, 1990 and De Bruyn and Ferreira, 1992) reportedly favoured multiple shoot production in *Gladiolus*, more positive performance of kinetin in the present investigation may be attributed to the differences in cultivars. However, Rao *et al.* (1991) reported that the shoot tips

of *Gladiolus* cultivars, namely Beauty Spot, Jo Wagenaar, Vink's Glory and Wild Rose, on MS medium supplemented with 4 mg/l kinetin resulted in production of highest number of shoots than with BAP at the same levels. The present observations are in agreement with the above reports. Among the cultivars, Rose Supreme produced highest number of sprouts per explant (7.67) followed by Priscilla and Victor Borge. These gradients can be attributed to varietal characters as the cultivar Rose Supreme was more vigorous with highest shoot and corm multiplication ratio of 1:2.60 in contrast to 1:2.15 of Priscilla and 1:1.50 of Victor Borge in the field.

The morphogenic response in cytokinins media was restricted to formation of multiple sprouts and no event of dedifferentiation at any level of BAP or kinetin was noticed in the first experiment. It was also evident from the reports that high level of auxins alone or in combination with the low level of cytokinins promote abundant cell proliferation with the formation of callus (Flick *et al.*, 1983). The most commonly used auxins for callus initiation in bulbous ornamentals are 2,4-D and NAA (Sheridan, 1968 and Hussey, 1977 in *Liliums* ; Ziv and Halevy, 1972 in *Gladiolus* and Hussey, 1977 in *Freesia*). Therefore, 2,4-D alone as well as with different combinations of BAP were tested to induce callus in the cultivars of *Gladiolus* selected for the study. Interestingly, there was no cell proliferation, which would have led to the disorganization of the existing structure and development of callus. On the contrary, there was mere enlargement of explants in their sizes even 30 days after inoculation. Enlargement of explants was more conspicuous on media containing higher levels of 2,4-D (6 or 8 mg/l) with BAP (0.2 or 0.5 mg/l). However, when the enlarged explants were recultured on the media constituents similar to those of the primary culture, there was callus development even on medium

containing the lowest level of 2,4-D (2.0 mg/l) with BAP (0.2 mg/l) in all the cultivars. The results are in contrary to the earlier reports (Kim and Lee, 1993) that the callus formation was observed to be directly on primary cultures, but with higher concentration of 2,4-D (10 mg/l). These observations suggested that either the volume of the culture medium or the level of growth stimulating substances were inadequate for the explant to undergo dedifferentiation in the primary culture. However, the response of enlarged explants to produce rapid cell proliferation and development of unorganised tissue, the callus, even under low levels of 2,4-D (2.0mg/l) with BAP (0.2 mg/l), may be attributed to the conditioning of explants and increase in the level of endogenous auxins that might have been imbibed during the primary culture.

It is well established that 2,4-D, a powerful and potent auxin, generally used for callus induction in several crop species. However, Ziv *et al.* (1970) and Kamo *et al.* (1990) were successful in inducing better callus in *Gladiolus* cultivars on NAA medium. These revelations lend support to believe that NAA could be used to promote callus development. Similar to 2,4-D experiment, there was no callus formation here also in the primary culture. The young meristem explants enlarged themselves and the degree of enlargement was quite positive at higher levels of NAA. While the cultivars Priscilla and Victor Borge responded better at both 6 and 8 mg/l NAA combined with 0.5 mg/l BAP, the cultivar Rose Supreme responded well at the same levels of NAA even without BAP. Interestingly, unlike in 2,4-D experiment, the enlarged explants required little higher levels of NAA (4.0 mg/l NAA with 0.2 mg/l BAP in Priscilla and Victor Borge and 6.0 mg/l NAA with 0.2 mg/l BAP in Rose Supreme) to support maximum development of callus in the second stage. The

callus developed on NAA medium was light creamy, compact and non-friable in contrast to soft, friable and light yellow calluses of 2,4-D medium. The growth of calluses was also marginally higher on NAA medium in all the cultivars. Hence, the auxin NAA, which is milder than 2,4-D was preferred for callus initiation under the present investigations. The results are in tune with the reports of Ziv *et al.* (1970), Kim *et al.* (1989), and Kamo *et al.* (1990).

Growth and maintenance of callus cultures

It is a common phenomenon and an established hypothesis that auxin, especially 2,4-D, is required to induce embryogenic determination in a proportion of the cells in callus or suspension cultures. Studies by Smith and Street (1974), Mc William *et al.* (1974), Sharp *et al.* (1980), Gorst *et al.* (1987) and George (1993) suggested that, callus, without any obvious sign of embryogenesis, should be maintained on medium containing 2,4-D as the source of auxin, if the callus is meant for inducing somatic embryogenesis. Therefore, to work out the critical balance between auxin and cytokinins, a callus growth study was taken up involving 2,4-D as the source of auxin and BAP and kinetin as cytokinins in order to maintain the induced callus in large quantities required for subsequent studies involving somatic embryogenesis.

The positive influence of 2,4-D in causing cell expansion was evident from the fact that the weight of the calluses increased with increase in the concentration of 2,4-D from 0.5 to 1.0 mg/l. However, when the level of 2,4-D was increased to 2.0 mg/l, there was not only repression in growth, but also the longevity of the tissue was also reduced.

Though, the promotive effect of BAP and kinetin in the presence of auxin (2,4-D) was subtle considering the numerical differences, the response obtained due to incorporation of BAP was better compared to kinetin. The growth of tissue was not only maximum but remained viable for long on medium containing 0.2 mg/l BAP and 1.0 mg/l 2,4-D in contrast to the corresponding concentrations of kinetin and 2,4-D in all the cultivars. Hence, it was felt pertinent that the tissues be maintained on medium containing 2,4-D (1.0 mg/l) and BAP (0.2 mg/l) for subsequent experiments. Similar views were advanced earlier by Gu and Gao, (1987) in *Narcissus tazetta*; Priyadarshi and Sen (1992) in *Lilium longiflorum* and Mizuguchi *et al.* (1994) in *Lilium japonicum*, but with different sources and levels of auxins and cytokinins. Contrary to these, Kim and Lee (1993) employed 0.5 and 0.1 to 1 mg/l of 2,4-D for maintenance of callus in several *Gladiolus* cultivars during the first and second subcultures, respectively, without the presence of BAP or kinetin. Probably, this may be the reason for the failure in maintenance of uniform callus, except in the cv. Topaz, although subculturing was possible in other cultivars viz. Fire Brand, True Love and Spic and Span.

Among the three cultivars tested in field, Victor Borge was a fast growing and short duration cultivar with least shoot and corm multiplication ratio, whereas Rose Supreme was a long duration cultivar with highest shoot and corm multiplication ratio and Priscilla coming in between. In light of these observations, it was thought that the study on callus growth patterns *in vitro* would reveal intriguing results, hence the experiment was scheduled. Accumulation of fresh and dryweights over a period of 40 days showed a definite growth pattern, depicting a typical sigmoid curve with

initial lag phase, middle exponential growth phase, followed by stationary and senescence phases in all the three cultivars. However, stationary and senescence phases started much earlier in Victor Borge, respectively, on 25th and 35th days after inoculation, in contrast to 35th and 45th days in Priscilla and Rose Supreme. Yet, there was a difference in the total accumulation of fresh and dryweights between Priscilla and Rose Supreme, as Rose Supreme showed higher accumulations than Priscilla. These differences in the growth pattern of calluses typify the cultivar characters. Kim and Lee (1993) obtained similar callus growth curves for *Gladiolus* cultivars namely Fire Brand, Topaz, True Love and Spic and Span with a two week lag phase and a stationary phase after 6 weeks.

Several published evidences favour the view that the incorporation of additional nitrogen sources into the growth medium would be helpful in promoting callus growth. Evans *et al.* (1976) found that soybean leaf callus grew more rapidly when ammonium nitrate was replaced with ammonium sulphate in the medium. Halesh (1995) reported that the incorporation of additional nitrogen in both inorganic form (urea) and organic form (casein hydrolysate), resulted in enhanced callus growth in *Gladiolus*. However, Bindu (1997) recorded positive influence on callus initiation and inhibitory effect on callus growth when ammonium sulphate was supplied as an additional source of nitrogen in *Syngonium podophyllum*. Therefore, different sources of nitrogen at different levels were incorporated into the media to test their role on callus growth in *Gladiolus* cultivars.

Incorporation of ammonium sulphate as an additional nitrogen source showed inhibitory effect on callus growth and there was no influence on morphogenesis. As

the concentration of ammonium sulphate increased, there was inhibition in the growth of callus either supplied alone or in combination with other supplements. However, the addition of nitrogen in the form of ammonium nitrate alone was superior to ammonium sulphate, casein hydrolysate and their combinations even with ammonium nitrate. This clearly suggests that a balance between the reduced form of nitrogen and nitrate form of nitrogen was more effective for the present cultivars to promote better growth and maintenance of healthy callus for longer periods. It was also observed that the organic form of nitrogen (casein hydrolysate) was in no way helpful to the extent of ammonium nitrate, which also indicated the fact that the inorganic form of nitrogen was more efficiently reduced and utilized than the organic form of nitrogen by these cultivars. The reduced growth of callus on media containing ammonium sulphate may be attributed to the deleterious effect of reduced nitrogen supplied in excess to the required balance with nitrate nitrogen. The promotive effect of casein hydrolysate and urea on callus growth in *Gladiolus* cultivars, namely Local Yellow and Black Jack (Halesh, 1995), suggested the cultivar differences in the capacity to uptake organic nitrogen under specific culture conditions.

The fact that the incorporation of additional nitrogen into the growth medium is likely to alter the balance between NO_3 and NH_4 ions and that the callus grown on such media is understood to have a bearing on the process of somatic embryogenesis (Schenk and Hildebrandt, 1972 and Walker and Sato, 1981), supplementation of any additional nitrogen other than the regular MS constituents to maintain callus meant for somatic embryogenesis, therefore, was avoided.

Regeneration response of callus

Callus cultures are widely used for regeneration in tissue culture, either through organogenesis or embryogenesis. Callus, which is generally thought to be undifferentiated tissue, contain diversity of cell types within a callus clump, but often there are no sharp boundaries between embryogenic and non embryogenic regions. However, several published reports suggest that in most tissue culture systems, only certain cell types may be multiplied due to media - dependent preferential growth of embryogenic and non embryogenic callus (Smith and Street, 1974; Williams and Maheswaran, 1986 and Kreuger *et al.*, 1995). Therefore, in order to test the morphogenic potential in callus cultures of *Gladiolus*, two experiments were conducted with different combinations and concentrations of auxin and cytokinins. The first experiment was to study the influence of BAP and NAA combinations and the second one with kinetin and NAA combinations under the present set of culture conditions. In both the experiments, the process of differentiation and regeneration occurred in 3 distinct stages. In stage I, within 30 days after inoculation, the undifferentiated callus cells, destined to produce somatic embryos were differentiated into proembrogenic masses (PEMs). The PEMs were identified by the presence of dense cytoplasm and thick cell walls and were transformed into somatic embryos after 45 days of inoculation (Stage II). The non embryogenic tissues remained as mere mass of cells and gave rise to adventitious shoots under the influence of specific hormonal concentrations. This transformation was observed in stage III, after 60 days of inoculation.

Between the two combinations of cytokinin with auxins, the combination of BAP and NAA was more preferred than kinetin and NAA to induce the events occurring in somatic embryogenesis. In the medium containing the BAP and NAA at 1:0.5 ratio, the degree of differentiation of callus cells into proembryogenic masses was highest after 30 days of inoculation. Similarly at the same hormonal combination and concentrations, maximum number of proembryogenic masses was transformed into somatic embryos in all the cultivars. Among the cultivars, the highest transformation of proembryogenic masses into somatic embryos was observed in Victor Borge, followed by Rose Supreme and Priscilla. Thus, the combination of BAP and NAA at 1.0 and 0.5 mg/l, respectively, appeared to be optimum for inducing somatic embryogeny, than the other combinations of BAP and NAA and all the combinations of kinetin and NAA. These findings are in conformity with the established facts that the callus, subcultured and maintained on 2,4-D medium, would elicit somatic embryogenesis when transferred to media containing a progressively reduced concentrations of auxin (De Vries *et al.*, 1988 and Merkle, *et al.*, 1990). However, Mc William *et al.* (1974) and Zimmerman (1993) reported that in carrot, proembryos were induced when the cells were shifted from 2,4-D medium to auxin free medium. These reports can be attributed to the differences in species and the culture conditions.

The development of adventitious shoots after 60 days of incubation was predominantly on medium containing cytokinins. In both the experiments, the number of adventitious shoots produced was significantly higher unlike somatic embryos, which suggest the predominance of adventitious shoots in drawing their

requirements from culture medium. Yet, the numerical differences in development of adventitious shoots and somatic embryos, reflect the role of growth regulators also. Between, BAP and kinetin, kinetin at 0.5 mg/l in Priscilla and 1.0 mg/l in Victor Borge and BAP at 2.0 mg/l in Rose Supreme supported regeneration of adventitious shoots to a maximum extent. Gu and Gao (1987) obtained regeneration of adventitious shoots from callus cultures of *Narcissus tazetta* on MS medium containing 1.0 mg/l BAP or 0.1 to 0.5 mg/l NAA. In *Gladiolus*, Ziv *et al.* (1970) reported the regeneration of adventitious shoots at 0.5 mg/l kinetin or 0.25 to 1.0 mg/l BAP. The present observations are in tune with the above reports. However, the difference in concentrations and sources of cytokinin may be attributed to the species and cultivar differences.

Somatic embryogenesis in callus cultures

The phenomenon of somatic embryogenesis has been exploited to regenerate whole plants of agronomically and horticulturally important species, since the discovery of this process for the first time by Steward *et al.* (1958) in callus cultures of carrot. Several published reports (Razdan, 1993; Zimmerman, 1993 and Kreuger, *et al.*, 1995) confirm the utility of this process for studying early events regulating the plant embryogenesis, in addition to its advantage in large scale production of propagules, repetitively, with low labour inputs by combining somatic embryogenesis with mechanized automated culture systems. Somatic embryogenesis *in vitro*, unlike the normal course of zygotic embryonic development, which is very closely controlled in the environment of the ovule and the embryo sac, is more responsive to external conditions (Steward and Krikorian, 1971 and Steward

et al., 1975). Therefore, somatic embryogenesis under regulated environment, medium and growth regulator influences may have to be developed into precision. This is required not only to amplify the process of somatic embryogenesis, but also to ensure that the somatic embryoids resemble more closely with that of zygotic embryoids in their morphology (Krikorian and Smith, 1992). On the strength of these assumptions, a series of experiments were conducted to optimize the process of somatic embryogenesis in *Gladiolus* cultivars.

On morphogenetic medium (MS + 1.0 mg/l NAA + 0.5 mg/l BAP), although the degree of response in differentiation of callus cultures into proembryogenic masses was high, there was no further transformation of proembryogenic masses into somatic embryos to the desired level even after 45 days. This led to the doubt that the continued presence of auxin (1.0 mg/l NAA) in the morphogenic medium may be inhibiting the process of transformation of PEMs into somatic embryos (De Vries *et al.*, 1988). Therefore, the proembryogenic masses were transferred to medium containing different concentrations of BAP and kinetins, but devoid of any auxins. The observations were encouraging as there was better transformation of PEMs into somatic embryos on media containing lower concentrations of both BAP and kinetin. Yet, kinetin was more effective than BAP in both Priscilla and Victor Borge, with highest recovery of somatic embryos emerging on medium containing kinetin at 0.2 mg/l. That the conversion of somatic embryos into plantlets was also highest on this medium with maximum recovery of 68 and 58 per cents in Victor Borge and Priscilla, respectively, suggests further the supremacy of kinetin at 0.2 mg/l in promoting embryo maturation and plantlet conversion. However, the elongation of plantlets recorded in terms of their length was better on medium

containing 0.5 mg/l kinetin. The plantlets produced on low concentrations (0.2 mg/l) of BAP and kinetin initiated roots in the same medium. Here again, plantlets produced on kinetin medium showed better root development. The present observations, however, are not in tune with the earlier reports in *Gladiolus*. Stefaniak (1994) used MS medium free from hormones for embryo maturation and plantlet development, whereas Jain *et al.* (1996) obtained embryo germination and plantlet formation on MS medium containing 1.0 mg/l kinetin and 0.1 mg/l NAA. These reports lend support to the concept that the media components and culture conditions used in the present context were restricting the transformation of proembryogenic masses into somatic embryos on the morphogenic medium itself. Thus, the findings suggest that somatic embryos in the present cultivars of *Gladiolus* required a period of maturation on medium containing the low concentrations of cytokinin, preferably kinetin at 0.2 mg/l for acquiring vigorous conversion and plantlet production. These observations are compatible with the studies of Barwale *et al.* (1986) in soybean, Wang *et al.* (1990) in *Freesia refracta*, Laublin *et al.* (1991) in *Iris* and Bach (1992) in *Freesia hybrida*.

The incubation of proembryogenic masses at lower concentrations of BAP and kinetin for a period of 20 days was given higher transformation rate suggested to conduct another experiment with further manipulations in the hormonal levels and duration of incubation. Here, the proembryogenic masses incubated under different concentrations of BAP and kinetin for a period of just 10 days were transferred to half strength MS medium containing no growth regulators. The observations on the process of development of somatic embryos and plantlets revealed that, like in the previous experiment, highest conversion of somatic embryos came from the

development medium containing 0.2 mg/l kinetin in Priscilla . But in case of Victor Borge, better transformation was noticed at higher levels of kinetin and BAP (1.0 mg/l). However, in both the cases, the period of incubation in embryo development medium was reduced from 20 to 10 days. In addition, there was reduction in the use of nutrients and hormones as better development of somatic embryos and plantlets occurred even on half strength MS medium without growth regulators. Stefaniak (1994) in *Gladiolus* and Gonsalves *et al.* (1995) in squash cultivars transferred somatic embryos to MS medium free from hormones for their maturation and plantlet development, but with full strength MS, unlike the half strength MS of the present study. This difference may be accounted to the change of species and cultivars.

The effective use of liquid medium for triggering higher rates of morphogenesis was advanced in orchids (Wimber, 1965), cocoa (Orchard *et al.*, 1979) and *Hyoscyamus mutius* (Grewal *et al.*, 1979). In cocoa, the development of zygotic embryos in liquid culture was more typical to that of *in vivo*, compared to embryos cultured on a semi solid medium (Pence *et al.*, 1980). In *Tylophora indica*, although somatic embryos were formed efficiently in liquid medium, they failed to develop further unless they were transferred onto an agar gelled medium (Rao and Narayanaswami, 1972). On the strength of these evidences, *Gladiolus* calluses grown on MS agar medium containing 1.0 mg/l 2,4-D and 0.2 mg/l BAP were transferred to liquid and agar embryo induction media both supplemented with 0.5 mg/l BAP and 1.0 mg/l NAA. The suspected proembryogenic clusters from both these media when transferred periodically at 5 days interval to gelled development medium containing 0.2 mg/l kinetin resulted in higher development of both somatic embryos and plantlets in general. Yet, the response evinced by the culture

incubated in liquid induction medium was far more superior. This was probably because of a greater surface area of the explant had in contact with liquid medium, allowing a more efficient uptake of its ingredients. In addition, toxic metabolites that might have accumulated in the vicinity of the tissue were also more effectively dispersed in liquid medium. Further examination of the results revealed that the process of embryo induction increased substantially with increase in the period of incubation in induction medium with the production of maximum number of embryos and plantlets from the culture incubated for 30 days. However, embryo induction was initiated only after 15 days in agar medium unlike in liquid medium, wherein it was visible even after 5 days in Victor Borge and 10 days in Priscilla. This also strengthens the view that liquid medium was more efficient than agar medium. That, even the number of roots developed per culture was also high and fell in line with the production of somatic embryos and plantlets, further points out the greater efficiency of liquid medium. These results are in agreement with the observations of Rao and Narayanaswami (1972) and Pence *et al.* (1980).

The differential results obtained in liquid and agar media were often effectively combined to obtain the most efficient micropropagation technique. By successive use of liquid and agar media, it was made possible to derive higher multiplication rates than any single medium. Simmonds and Cumming (1976) found that callus of *Lilium* hybrids could be multiplied most rapidly on a liquid medium, but that for the maximum rate of plantlet production, it was necessary to transfer the tissue to solid medium. Therefore, subsequent to the testing of agar-agar and liquid-agar systems for embryo induction and development, another experiment involving liquid - liquid - agar system was conducted to improve further the number of somatic

embryos and their sizes. The proembryogenic masses induced in liquid medium for a period of 20 days not only resulted in production of more number of embryos (93 % and 26% higher, respectively, over agar - agar and liquid - agar systems) but also improved embryo sizes (60% and 40% in length and 71% and 48% in thickness, respectively, over agar-agar and liquid-agar systems). This might be due to better absorption of nutrients as more surface area of the explant was in contact with the medium, giving further proof of the positive role played by the liquid medium. While majorities of the somatic embryos were still attached to the parental tissue, few of them got separated due to agitation and started germinating on the same medium. Such germinating embryos were seen floating freely on liquid medium. When the separated embryos from this medium were transferred onto agar half strength MS medium free from growth regulators, they developed into healthy and vigorous plantlets. Similar observations have been made by Krikorian and Kann (1979) in daylilies and Pence *et al.* (1980) in cocoa, wherein they reported higher morphogenesis and shoot development in liquid medium than in the semisolid agar medium.

The plantlets obtained on half strength agar medium initiated roots and produced cormels on the same medium after 90 days. However, the rooted plantlets when transferred to MS agar medium containing 0.5 mg/l IBA and 6 per cent sucrose gave better sized cormels in all the cultivars. Selection of the above medium was based on the results of the experiment conducted separately to induce rooting and cormels *in vitro*, the results of which are not reported here. The fact that the *in vitro* rooted plantlets failed to establish properly even under green house conditions, suggests that embryonic plantlets may not be developed with proper vascular

connections between the shoot and the roots for better establishment. Therefore, *in vitro* produced cormels are recommended for field planting. The schematic representation of the protocol of somatic embryogenesis in *Gladiolus* cv. Victor Borge is shown in Fig. 3

The morphogenetic events in somatic embryogeny involve proembryogenic masses, globular, heart and torpedo stages (Street and Withers, 1974; Fujimura and Komamine, 1980 and Zimmerman, 1993). These stages are specific in their requirements for sequential development (Van Engelen and De Vries, 1992 and Domon *et al.*, 1995). During the somatic embryogenesis of *Daucus carota* a number of proteins are released into the suspension culture medium (De Vries *et al.*, 1988 and Hendricks and De Vries, 1995). A cell line impaired in somatic embryogenesis failed to release one or several of these proteins. Somatic embryogenesis in *Daucus carota* can be blocked by the glycosylation inhibitor, tunicamycin. However, blocked cultures could be completely rescued by addition of extracellular proteins from untreated embryo cultures (De Vries *et al.*, 1988). These published evidences prompted to test the role of cytoplasmic protein synthesis and time of synthesis on somatic embryogenesis in *Gladiolus* by incorporating cycloheximide, a protein synthesis inhibitor into the liquid induction medium. The absence of cycloheximide in the induction medium gave somatic embryos irrespective of the duration of incubation. Nevertheless, the number of somatic embryos and plantlets increased with the duration of incubation upto 25th day. Further examination of the morphogenic events in the induction medium revealed that the undifferentiated cell clusters in the beginning, were differentiated into globular stage characterized by larger group of cells not yet having a definite embryo shape. This was followed by a

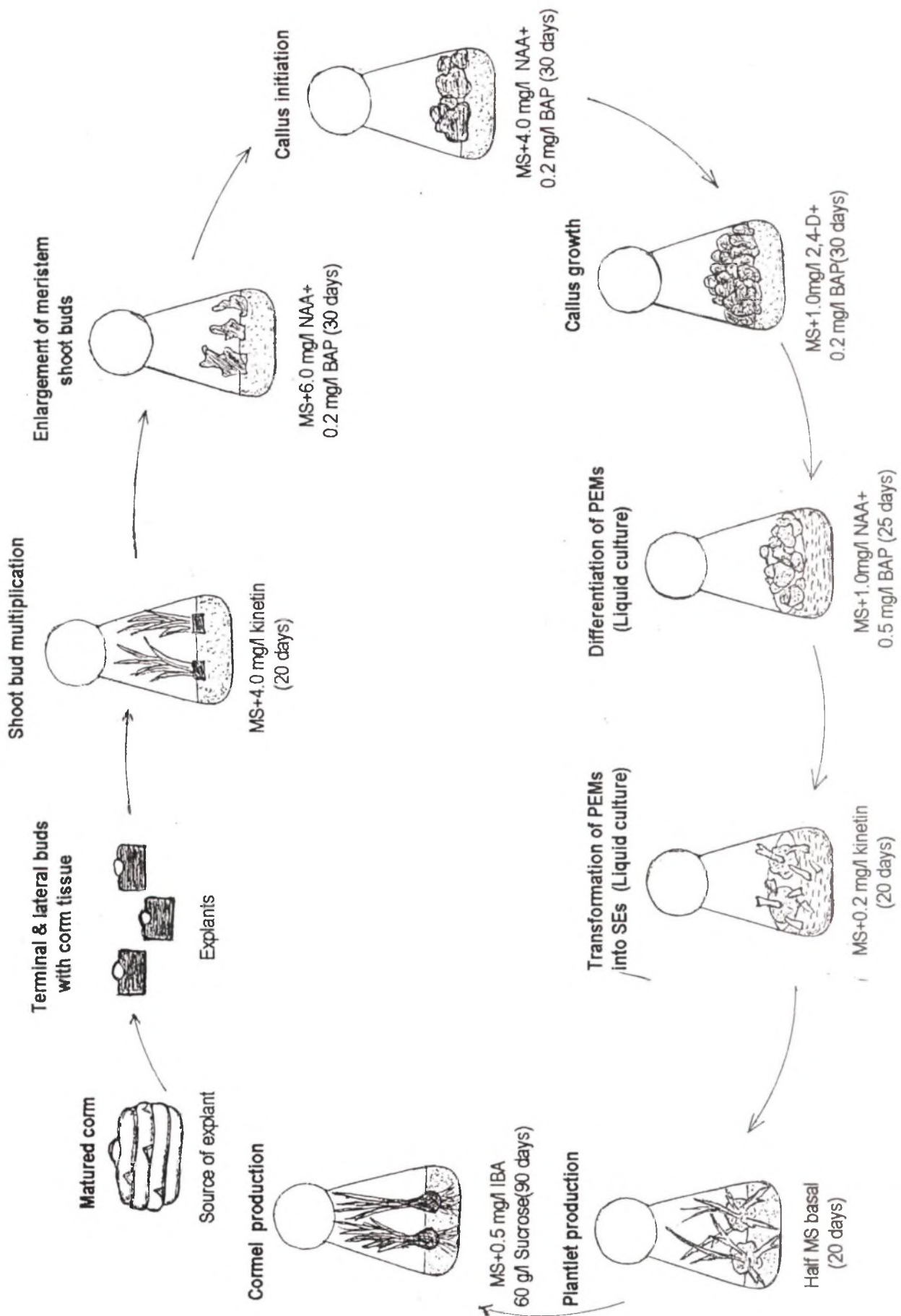


Fig 3. Schematic representation of the protocol of somatic embryogenesis in *Gladiolus* cv. Victor Borge

characteristic three lobed stage, called the heart stage. These two stages of development were seen between 7 and 10th days of incubation. The heart shaped embryos elongated themselves into torpedo stage and then to advance stage embryos. Both these two stages were intermixing and overlapping and were observed between 12 and 20 days after incubation.

That the incorporation of cycloheximide into the induction medium upto 15th day did not produce any somatic embryos or plantlets confirms the importance of cytoplasmic protein synthesis between the early globular to the late torpedo stages of embryo development. However, incorporation of cycloheximide in the later stages of embryo development was less effective as there was no influence of cycloheximide on the normal development of somatic embryos when added on the 30th day of incubation. Yet, the number of somatic embryos and plantlets produced per culture was substantially lower when compared with the production of somatic embryos and plantlets after 25th day of incubation in induction medium without cycloheximide. Somatic embryogenesis in carrot (Mc Cabe *et al.*, 1997) cell cultures involved the synthesis of certain signal molecules and release into the medium. These molecules were identified as arabinogalactan proteins that operate in conserved cell and stimulate somatic embryogenesis. In *Picea abies*, the secretion of specific extracellular proteins by somatic embryos was dependent on embryo morphology (Mo, *et al.*, 1996). In *Picea glauca*, different genes were active during the early and later stages of somatic embryogeny (Dong and Dunstan, 1996). These reports may be related to the present effect of cycloheximide that inhibited the cytoplasmic protein synthesis, affecting the normal process of somatic embryogenesis. The greater effect of cycloheximide in the early stages of somatic

embryogeny can also be attributed to the inhibitory effect on translocation of genes, active in the early stages.

Production of synthetic seeds and their viability

Somatic embryos produced *in vitro* can be grown directly into plants under controlled conditions. However, their versatility could be improved further if they are converted into synthetic seeds or artificial seeds by encapsulating them with synthetic seed coat and made them analogous to true seeds (Kitto and Janick, 1985a and Redenbaugh *et al.*, 1987). Embryos placed individually into the alginate beads would be advantageous for economical mass production of elite plants and facilitate easy handling and delivery. Most importantly, embryos in alginate beads can be stored for long without the loss of regeneration (Fujii *et al.*, 1987, Nouaille and Petiard, 1988 and Datta and Potrykus, 1989). In light of the above reports, three experiments were conducted in succession to test experimentally the prospects of synthetic seed production in *Gladiolus* cv. Rose Supreme.

The first experiment under the series was to test the viability of hydrated synthetic seeds produced by encapsulating the somatic embryos with sodium alginate gel through a simple process developed by Redenbaugh *et al.* (1987). Along with somatic embryos, callus bits and adventitious shoot buds obtained *in vitro* were also encapsulated and stored at two temperatures (26 ± 2 °C and 5 ± 1 °C), in darkness, together with non encapsulated counterparts for 30 days. The comparison of the values of survival in general revealed that there was a progressive decline in the viability of both encapsulated and naked embryos with increase in the storage period at both the temperatures. However, the viability was severely affected in

naked embryos stored at higher temperature, than the storage of encapsulated embryos at lower temperature. This could be seen from the fact that nearly 50 per cent of the encapsulated embryos survived even after 10 days of storage at 5°C in contrast to just 2.5 per cent of the naked embryos at 26°C. Within the storage temperature, encapsulated embryos survived for longer duration than the naked ones. Such trends were also observed for the adventitious shoot buds as well as for calluses, suggesting the role played by hydrogel encapsulation in restoring the viability for longer periods. This was probably due to the protection given by the synthetic seed coat against desiccation and other damages. Datta and Potrykus (1989) made similar observations in *Hordeum vulgare*. Further examination of the results indicated that the adventitious shoot buds either encapsulated or not, survived better than callus or somatic embryos at a given storage temperature, suggesting that the adventitious shoot buds were more tolerant to desiccation and other damages. Between the two storage temperatures, storage at 5°C was most preferable as more than 19% of the encapsulated embryos survived for as long as 30 days, in contrast to nil at 26 °C. Similar trends were also observed with callus and adventitious shoots. Better survival of somatic embryos, callus or adventitious shoot buds, either encapsulated or naked, was probably due to least desiccation that might have occurred under low temperature (5°C). Kitto and Janick (1985b) reported that carrot asexual embryos survived as long as 16 days at 4°C storage temperature compared to 4 days at 26°C. The present results are comparable with the above reports.

The synthetic seed coat used in encapsulation in addition to providing physical protection to the embryos shall also serve as synthetic endosperm containing carbon sources, nutrients, growth regulators, antibiotics, fungicides, etc., to assist germination and conversion without inducing undesirable variation (Redenbaugh, 1993a). These reports gave enough support to the view that the incorporation of different additives into the encapsulating gel would be beneficial in avoiding contamination, enhancing embryo survival and improving germination of *Gladiolus* somatic embryos within the hydrogel capsule. Therefore, an experiment involving incorporation of different additives into sodium alginate gel before encapsulation and manipulations in sterilization of encapsulating and complexing agents was conducted. The results of the experiment revealed that the addition of either fungicide (0.1 % carbendazim) or bacteriomycin (0.1% streptomycin) individually into the encapsulating gel, respectively, brought down the incidence of fungal and bacterial contaminations to a minimum level. Nevertheless, the incidences of fungi when bacteriomycin was added and bacteria when fungicide was added were as high as in control. But incorporation of both fungicide and bactericide into the alginate gel recedes the contamination level drastically and nearly 95 per cent of the capsules remained healthy when they were planted for germination. However, in all the above cases, the encapsulated embryos failed to germinate on germination medium. The embryos within the capsules turned brown initially and within 10 days turned to dark brown colour signaling their death. Probably this was due to the lethal effects of fungicide and bactericide on somatic embryos within the synthetic seed coat. In all these treatments including the control, both encapsulating gel and complexing agents were used without sterilization. Contrary to this, in treatment involving no fungicide or bactericide but sterilization of both encapsulating

gel and complexing agents before the act of encapsulation, not only did all the capsules remain healthy without any contamination, but nearly 24 per cent of the embryos within the capsule remained viable upto 10 days. Yet, there was no occurrence of germination and the embryos succumbed to death after 15 days. However, last treatment involving no additives but incorporation of half strength MS basal nutrients and sterilization of both encapsulating gel and complexing agents not only did help in survival of nearly 58 per cent of the encapsulated embryos upto 15 days but also accounted for germination of nearly 20 per cent of the embryos. The improvement in survival and germination of encapsulated embryos may be attributed to the deriving of nutrients from the synthetic endosperm that contained half strength MS nutrients as well as normal level of carbon source. These findings suggested that the incorporation of nutrients in the synthetic seed coat and aseptic production and handling of synthetic seeds may be beneficial for avoiding contamination, maintenance of viability as well as to improve germination of encapsulated embryos in *Gladiolus*. The results are in conformity with the reports of Redenbaugh *et al.* (1987) in celery, wherein they recommended the addition of nutrients and carbohydrates for better survival of encapsulated embryos.

Studies on the effect of hardening somatic embryos during initiation and development phases to prolong their storage life after encapsulations have been reported. The possibility of increasing the storage life of encapsulated embryos by exposing the embryo suspensions during induction phase to high inoculum density, higher sucrose or chilling treatments alone or in combination with abscisic acid as hardening agents, was explored in carrot with varied response (Kitto and Janick, 1985b). Ammirato (1988) reported a number of striking effects of abscisic acid

(ABA) when added at moderate concentrations to the population of proembryos as well as fully developed embryos. Maturation of somatic embryos on a medium containing ABA prior to encapsulation increased plant establishment (Fujii *et al.*, 1989). With these references in view, an experiment was carried out to test the effect of various hardening treatments on the survival of encapsulated embryos in *Gladiolus* cv. Rose Supreme. An examination of the values of survival of encapsulated embryos on 5, 15 and 30 days after storage revealed that the treatment of embryos with ABA at 0.1 μ M for 6 days prior to their harvest, or exposing the embryos to chilling treatment ($5 \pm 1^\circ\text{C}$) for 3 days ensured better survival of embryos than untreated control. Yet, the survival of encapsulated embryos treated with ABA was substantially high as 12 per cent of them were able to survive after 30 days of storage, compared to 7.8 per cent from the combined treatment of ABA and chilling. The influence of chilling treatment alone was reasonably better than control as 19 per cent of embryos exposed to this treatment survived, in contrast to 9 per cent of the control, after 15 days of storage.

Increase in the endogenous level of ABA prevented precocious germination, suggesting the quiescent state of embryos. Quiescent embryos continue to synthesise proteins that are associated with embryonic growth (Crouch and Sussex, 1981). This may be the possible reason for better survival of embryos when they were exposed to ABA treatment in the present experiment. Further, reasonably good survival of embryos with chilling treatment may be linked to acquisition of tolerance by the protoplasm to dehydration due to reduction in intracellular water (Chen and Gusta, 1978) and increase in ABA levels (Eamus and Wilson, 1983). However, there was reduction in the per cent survival of encapsulated embryos

under the combined treatment of ABA and chilling in contrast to ABA alone. The possible explanation for the absence of additivity in the present study is that the treatment with ABA in combination with another hardening regime (chilling), could have resulted in supra optimal ABA levels. ABA at high concentrations inhibited the formation and growth of asexual embryos in carrot (Kamada and Harada, 1979). Similar reasons were quoted by Kitto and Janick (1985b), for not achieving the additivity in enhancing the storage life of encapsulated embryos in carrot. Further examination of the results revealed the negative response brought about by desiccation treatment as embryos subjected to this treatment survived far below the control. Exposure of naked embryos to laminar air flow current for a period of 1 hour reduced the survival of encapsulated embryos to 76 per cent compared to 100 per cent of the control even without further storage. This may be attributed to the desiccation damage caused to the embryos before encapsulation itself. The present finding can be related to the findings of Kitto and Janick (1985b) in carrot, and Datta and Potrykus (1989) in *Hordeum vulgare*, that non encapsulated asexual embryos when exposed to outside environment, undergo desiccation injury and lose their viability much earlier than the encapsulated embryos.

Schematic representation of the protocol for production of synthetic seeds in *Gladiolus* is shown in Fig. 4

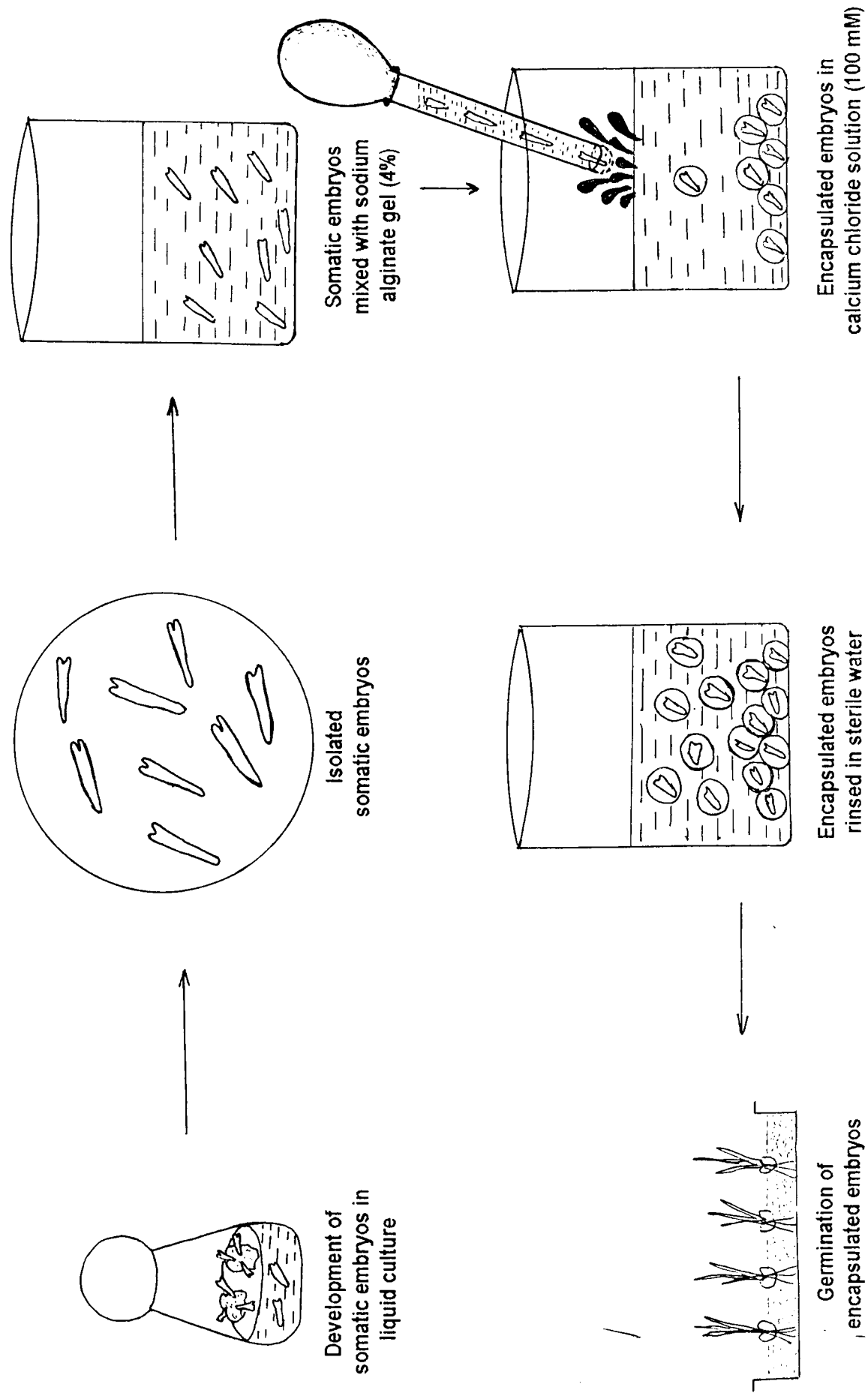


Fig 4 . Schematic representation of steps in synthetic seed production in *Gladiolus*.

SUMMARY

VI. SUMMARY

The present study, to develop a system for rapid clonal propagation via somatic embryogenesis and production of synthetic seeds as a means of embryo delivery, was carried out in *Gladiolus* cultivars viz. Priscilla, Victor Borge and Rose Supreme. The salient findings of the study are as follows:

Culturing of shoot buds excised from corm sections with pieces of basal tissue attached on MS medium containing different concentrations of BAP and kinetin resulted in bud bursting and production of multiple sprouts. However, in all the cultivars, the response was better on kinetin media with the maximum response coming from kinetin at 4.0 mg/l. Among the cultivars, the response was maximum in Rose Supreme, with the production of 7.67 sprouts per explant within 20 days of incubation followed by Priscilla (6.17) and Victor Borge(5.17).

Morphogenetic response in cytokinin media was restricted to the production of multiple shoots and there was no event of disorganization of the existing structure. Culturing of young meristems on media containing 2,4-D and NAA combined with BAP at different concentrations and combinations resulted in mere enlargement of explants and there was no sign of callus formation. However, culturing of these enlarged explants in the identical concentrations of auxin and cytokinins as in the primary culture, exhibited rapid cell proliferation and development of callus with maximum stimulus coming from NAA (4.0 mg/l) in combination with BAP (0.2 mg/l). NAA medium also supported qualitatively superior callus than the 2,4-D medium.

Attempts to optimize the concentrations and combinations of 2,4-D with BAP and kinetin in subculture medium in order to maintain the induced callus in large quantity, particularly in the embryogenic state showed better growth and longevity of tissues on medium containing 1.0 mg/l 2,4-D and 0.2 mg/l BAP.

The experiment, carried out to understand the callus growth patterns in the selected cultivars of *Gladiolus* exhibited early stationary and senescence phases in Victor Borge compared to Priscilla and Rose Supreme. Accumulation of total fresh and dry weights was maximum in Rose Supreme, followed by Priscilla and Victor Borge.

Incorporation of 100 mg/l ammonium nitrate into the maintenance medium, as an additional nitrogen source, promoted better growth and longevity of the tissues. However, ammonium sulphate brought about inhibitory effect on callus growth, indicating the deleterious effect of reduced nitrogen supplied in excess of nitrate nitrogen.

Regeneration of callus, either by embryogenesis or organogenesis, occurred in three stages on morphogenic medium containing different levels of BAP and kinetin in combination with NAA. After 30 days of incubation, undifferentiated callus cells, destined to form somatic embryos, first differentiated into proembryogenic masses, followed by transformation into somatic embryos after 45 days. The degree of differentiation and transformation was highest on medium containing 0.5 mg/l BAP and 1.0 mg/l NAA. However, somatic embryos failed to develop into plantlets on the same media constituents. The non embryogenic mass of cells after 60 days of

incubation gave luxurious adventitious shoot buds on medium containing cytokinin alone in the range of 0.5 to 2.0 mg/l depending upon the variety.

The process of transformation of somatic embryos and development of plantlets did not occur to the desired level on morphogenic medium. Therefore, investigations to optimize media constituents for higher transformation and development of somatic embryos showed encouraging results on medium containing low levels of cytokinins. The development medium supplemented with 0.2 mg/l kinetin attributed for higher development of both somatic embryos and plantlets, within 20 days in both Priscilla and Victor Borge.

The experiment involving pre incubation of proembryogenic masses for a short period of 10 days on medium containing different levels of BAP and kinetin, followed by transfer to half strength MS medium free from growth regulators did not improve either embryo development or plantlet recovery, substantially. However, there was a definite indication that the duration of the process of embryogenesis could be reduced by about 10 days.

Liquid medium was more effective in triggering not only higher rates of embryogenesis, but also inducing the process more quickly than the agar medium. The number of somatic embryos induced in liquid-agar system was higher by 52 and 35 per cents, respectively, in Priscilla and Victor Borge, compared to agar-agar system. However, the use of liquid-liquid-agar system, respectively, for embryo induction, development and plantlet production resulted in further improvement in somatic embryogenesis by production of 26 per cent higher embryos compared to

liquid-agar system. Further, the quality of embryos in terms of their length and thickness also improved substantially in liquid-liquid-agar system.

The embryonic plantlets initiated roots and produced cormels *in vitro* on MS agar medium containing 0.5 mg/l IBA and 6 per cent sucrose. The *in vitro* rooted plantlets however failed to establish properly even under green house conditions. Hence, *in vitro* produced cormels are recommended for field planting.

The study on morphogenetic events in somatic embryogenesis of *Gladiolus* revealed the development of globular to heartshaped embryos from the undifferentiated cell clusters between 7-10 days of incubation in embryo induction medium. The heart shaped embryos elongated themselves into torpedo stage and then to advanced stage embryos. These two stages were intermixing and overlapping and observed between 12 and 20 days after incubation.

The importance of cytoplasmic protein synthesis in different stages of embryo development was determined by incorporating cycloheximide (0.5%), a cytoplasmic protein synthesis inhibitor, into the embryo induction medium. The addition of cycloheximide at early stages of embryogenesis inhibited embryo development indicating the critical role of protein synthesis between early globular and late torpedo stages of embryo development. However, the influence of cycloheximide was only marginal during the later stages of embryo development.

The synthetic seeds were produced by encapsulating the somatic embryos with sodium alginate gel (4%) and complexing with calcium chloride (100mM). The encapsulated somatic embryos survived for longer duration than the naked embryos

at a given storage temperature. Storage at lower temperature (5°C) was more advantageous than the storage at higher temperature (26°C). Among somatic embryos, callus bits and adventitious shoots, whether naked or encapsulated, adventitious shoot buds survived for longer duration at a given temperature.

The somatic embryos encapsulated with sodium alginate and complexed with calcium chloride, both without sterilization, were contaminated with fungus and bacteria when they were plated for germination. Incorporation of carbendazim (0.1%) and streptomycin (0.1%) to the encapsulating gel or sterilization of both encapsulating and complexing agents gave good protection against both fungal and bacterial contaminations. However, there was no germination of the encapsulated embryos. Addition of half strength MS nutrients to encapsulating gel, followed by sterilization, gave not only good protection against contaminations, but also resulted in germination of nearly 20 per cent of the encapsulated embryos.

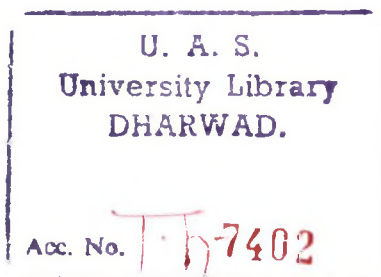
Study on the effect of hardening somatic embryos to prolong their storage life after encapsulation showed favourable effect with ABA treatment. Nearly 12 per cent of the embryos treated with ABA (0.1 μ M) for 6 days prior to their harvest survived for 30 days of storage compared to nil under control. However, combination of ABA with chilling did not ensure more than 7.8 per cent survival. Chilling treatment gave better results than the control. Desiccation treatment brought about negative impact on survival of encapsulated embryos.

Future line of work

In *Gladiolus* cultivars, viz. Priscilla, Victor Borge and Rose Supreme, an attempt was made to develop a system for rapid clonal propagation via somatic embryogenesis and production of synthetic seeds as a means of low cost embryo delivery system. Though the rate of regeneration of plantlets through callus mediated somatic embryogenesis and cormels from these *in vitro* was appreciable, there was a need to evaluate their performance in field, so as to adopt this method for commercial propagation. The fact that the *in vitro* rooted plantlets failed to establish properly even under green house conditions, it is reasonable to foresee a further refined process for hardening. The germination test conducted to regenerate plantlets from encapsulated somatic embryos was restricted to growth room conditions. Therefore, there is a need to investigate their regeneration capability under green house and field conditions.

REFERENCES

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VII. REFERENCES

- Ammirato, P.V., 1988, Role of ABA in regulation of somatic embryogenesis. Hort. Sci., **23(3)** : 520.
- Bach, A., 1992, Somatic embryogenesis from zygotic embryos and meristems of *Freesia hybrida*. Acta. Hort., **325** : 429-435.
- Barwale, U.B., Kerns, H.R. and Widholm, J.M., 1986, Plant regeneration from callus cultures of several soybean genotypes via. embryogenesis and organogenesis. Planta , **167** : 473 -481.
- Bewley, J.D. and Black, M., 1985, In :Seeds: Physiology of Development and Germination. Plenum press, New York.
- Bindu, B., 1997, Dedifferentiation and redifferentiation studies in *Syngonium podophyllum* L. M.Sc(Agri.) Thesis submitted to the University of Agricultural Sciences, Bangalore.
- Buchheim, J.A., Colburn, S.M. and Ranch, J.P., 1989, Maturation of soybean embryos and the transition to plantlet growth. Plant Physiol., **89** : 768 - 775.
- Carman, J.G., 1988, Improved somatic embryogenesis in wheat by partial stimulation of the *in-voulo* oxygen growth-regulator and desiccation environments. Planta, **175** : 417-424.
- Chee, P.P., 1990, High frequency somatic embryogenesis and recovery of fertile cucumber plants. Hort. Sci., **25(7)** : 792 - 793.
- * Chen. P. and Gusta. L.V., 1978, The role of water in cold hardiness of winter cereals. In : Plant Cold Hardiness and Freezing Stress : Mechanisms and Crop Implications (P.H. Li. and A. Sakai, eds.), pp.165 -174. Academic Press, New York.

- Chetia, S. and Devi, J., 1996, Regeneration of three orchid species from artificial seeds. In : Souvenir . Nat. Symp. Hort. Biotechnol., Hort. Soc. India and I I H R, Bangalore. pp.6.
- Cocking, E.C., 1960, A method for isolation of plant protoplasts and vacuoles. Nature, **187** : 927-929.
- Crouch, M.L. and Sussex, I.M., 1981, Development and storage protein synthesis in *Brassica napus* L. embryos *in vivo* and *in vitro*. Planta , **153** : 64-74.
- Datta, S.K. and Potrykus. I., 1989, Artificial seeds in barley: encapsulation of microspore - derived embryos. Theor. Appl. Genet. , **77** : 820 - 824.
- * Davies, D.R. and Griffiths, P., 1971, *In vitro* propagation of *Freesia* : Ann. Rep., PP.45. Jhon Innes Inst.
- * De Bruyn, M.H. and Ferreira, D.I., 1992, *In vitro* corm production of *Gladiolus dalenii*, and *G. tristis*. Plant Cell Tissue and Organ Culture, **31** : 123-128.
- De Vries, S.C., Booij, H., Meyerink, P., Huisman. G., Daytonwilde. H., Thomas, T. and Van Kammen, A., 1988, Acquisition of embryogenic potential in carrot cell-suspension cultures. Planta, **176** : 196 - 204.
- Domon, J.M., Dumas, B., Laine, E., Meyer, Y., Davis, A. and David, H., 1995, Three glycosylated polypeptides secreted by several embryogenic cell cultures of pine show highly specific serological affinity to antibodies directed against the wheat germin apoprotein monomer. Plant Physiol., **108** : 141 - 148.
- Dong., J. Z. and Dunstan, D.I., 1996, Expression of abundant mRNAs during somatic embryogenesis of white spruce (*Picea glauca*). Planta, **199(3)** : 459 -466
- Eamus, D. and Wilson, J.M., 1983, ABA levels and effects in chilled and hardened *phaseolus vulgaris*. J. Expt. Bot., **34** : 1000 - 1006.
- Evans. D.A., Sharp, W.R. and Flick, C.E., 1981, Growth and behavior of cell

cultures. Embryogenesis and organogenesis. In: Plant Tissue Culture: Methods and Applications in Agriculture (T.A. Thorpe, ed.), pp. 45 -113. Academic press, New York.

- * Evans, D.A., Sharp, W.R. and Paddock, E.F., 1976, Variation in callus proliferation and root morphogenesis in leaf tissue of *Glycine max* strain T 219. Phytomorph., **26** : 379 - 384.

- Flick, C.E, Evans, D.A. and Sharp, W.R., 1983, Organogenesis. In : Handbook of Plant Cell Culture. Vol.I. (D.A. Evans; W.R. Sharp; P.V. Ammirato and Y. Yamada, eds.), pp.63. Macmillan Publishing Co., New York.

- Frey, L., Saranga, Y. and Janick, J., 1992, Somatic embryogenesis in carnation. Hort. Sci., **27(1)** : 63-65.

- * Fujii, J.A., Slade, D.T. and Redenbaugh, K., 1989, Maturation and greenhouse planting of alfalfa artificial seeds. In vitro cell. Dev. Biol., **25** : 1179 -1182.

- * Fujii, J.A., Slade, D.T., Redenbaugh, K. and Walker, K.A., 1987, Artificial seeds for plant propagation. Trends Biotechnol., **5** : 335 -339.

- Fujimura, T. and Komamine, A., 1980, The serial observation of embryogenesis in a carrot cell-suspension culture. New Phytol., **86** : 213- 216

- * Gautheret, R.J., 1939, Sur La possibilite de realiser la culture indefinie des tissus tubercule de carotte. Compt. Rend. Acad. Sci., Paris. **208** : 118 -120

- * Geier, T., Kohlenbach, H.W. and Reuther, G., 1979, Klonale vermehrung von *Cyclamen persicum* durch Gewebekultur. Gartenbauwiss., **44** : 226 -237.

- George, E.F., 1993, Morphogenesis. In : Plant Propagation by Tissue Culture. Part 1. The Technology. pp. 25-36. Exegetics Ltd., Edington, Wilts, BA 13 4QG, England.

- Gonsalves,C., Xue, B. and Gonsalves, D., 1995, Somatic embryogenesis and regeneration from cotyledon explants of six squash cultivars. Hort. Sci., **30(6)** : 1295 - 1297.

- * Gorst, J., Overall, R.L. and Wernicke, W., 1987, Ionic currents traversing cell clusters from carrot suspension cultures reveal perpetuation of morphogenetic potential as distinct from induction of embryogenesis. Cell Differ., **21** : 101 -110.

- Gray, D.J., 1987, Synthetic seed technology for the mass cloning of crop plants: problems and prospectives. Hort. Sci., **22** : 795 - 814.

- Gray, D.J., 1990, Synthetic seed for clonal production of crop plants. In: Recent Advances in the Development and Germination of Seeds. (R.B. Taylorson, ed.) pp.29. Plenum press, New York.

- Grewal, S., Koul, S., Ahuja, A. and Atal, C.K., 1979, Hormonal control of growth, organogenesis and alkaloid production in *in vitro* cultures of *Hyoscyamus muticus* L. Indian J. Exp. Biol., **17** : 558 - 561.

- * Gu, H.S. and Gao, C.H., 1987, The effects of plant growth regulators on callus induction from bulb segments of *Narcissus tazetta*., maintenance and organogenesis in callus cultures. Acta. Hort. Sinica, **14 (1)** : 53- 56.

- * Guha, S. and Maheshwari, S.C., 1966, Cell division and differentiation of embryos in the pollen grains of *Datura in vitro*. Nature (London), **212** : 97-98.

- * Haberlandt, G., 1902, Culturversuche mit isolierten pflanzenzellen. Sitzungsber, Math. Naturwiss Kl. Kais Akad. Wiss., **111** : 69 - 92.

- Halesh, G.K., 1995, Studies on callus growth and morphogenesis in *Gladiolus gradiflorum* L. cultivars Local Yellow and Black Jack. M.Sc. (Agri.) Thesis submitted to the University of Agricultural Sciences, Bangalore.

- Halperin, W., 1966, Alternative morphogenetic events in cell suspensions. Am. J. Bot., **53** : 443 - 53.

- * Hendricks, T., and De Vries, S., 1995, The role of secreted proteins in carrot somatic embryogenesis. In: Current Issues in Plant Molecular and Cellular

Biology (M.Terzi, R. Cella., and A. Falavigna, eds.), pp.359-368. Dordrecht, Kluwer Academic Publishers.

- * Hicks, G.S., 1980, Patterns of organ development in plant tissue culture and the problem of organ determination. Bot. Rev., **46** : 1-23.

- Ho, W.J. and Vasil, I.K., 1983, Somatic embryogenesis in sugarcane (*Saccharum officinarum* L.): Growth and plant regeneration from embryogenic cell suspension cultures. Ann. Bot., **51** : 719 -726.

- * Huang, L.C. and Liu, D.M., 1989, Clonal multiplication of *Lycosis aurea* by tissue culture. Sci. Hort., **40 (2)** : 145 -152.

- Hussey, G., 1977, *In vitro* propagation of some members of the Liliaceae, Iridaceae and Amaryllidaceae. Acta Hort., **78** : 303 - 309.

- Hussey, G., 1978, The application of tissue culture to the vegetative propagation of plants. Sci. Prog., **65** : 185 - 208.

- * Hussey, G. and Wyvill. C., 1973, *In vitro* responses of bulbs and corms. Ann. Rep., Jhon Innes Institute. pp.64 -67.

- Jain, A., Yadav, R.C., Arora, N. and Chowdhury, J.B., 1996, Somatic embryogenesis from callus and suspension cultures of *Gladiolus*. In: *Souvenir*, Nat. Symp. Hort. Biotechnol., Hort. Soc. India and IIHR, Bangalore, pp.39.

- * Kamada, H. and Harada,H., 1979, Studies on the organogenesis in carrot tissue cultures. I. Effects of growth regulators on somatic embryogeneis and root formation. Z. Pflanzenphysiol, **91** : 225 - 266.

- * Kamo, K., Chen, J. and Lawson, R., 1990, The establishment of cell suspension cultures of *Gladiolus* that regenerate plants. In vitro cellular and Developmental Biol., **26 (4)** : 425 - 430.

- Kartha. K.K., Gamborg, O.L and Constabel, F., 1974, *In vitro* plant formation from stem explants of rape (*Brassica napus* cv. zephyr). Physiol. Plant ., **31** : 217 -220.
- * Kim, K.W., Choi, J.B., Kwon, K.Y., 1988, The rapid multiplication of *Gladiolus* plants by callus culture. J. Kor. Soc. Hort. Sci., **29(4)** : 312 - 318.
- * Kim, K.W., Kim, Y.S, Woo,C.K., Kim,O.H. and Kim,J.S., 1989, Adventitious bud formation from tulip callus. J. Kor. Soc. Hort. Sci., **7(1)** : 162 - 163.
- * Kim , K.W. and Lee, J.S., 1993, Differences in cv. on formation of the *Gladiolus* callus *in vitro*. J. Kor. Soc. Hort. Sci., **34(4)** : 301 - 307.
- Kitto, S.L. and Janick, J., 1985a, Production of synthetic seeds by encapsulating asexual embryos of carrot. J. Am. Soc. Hort. Sci., **110(2)** : 277-282.
- Kitto, S.L. and Janick, J., 1985b, Hardening treatments increase survival of synthetically coated asexual embryos of carrot. J. Am. Soc. Hort. Sci., **110(2)** : 283 - 285.
- * Konchak, I. and Rodeva, V., 1986, Effect of some growth regulators on *in vitro* production of regenerated *Lilium* plants. Genetikai Seleksiya., **19 (5)** : 394 - 395.
- Kreuger, M., Postma, E., Brouwer, Y and Van Holst. G.J., 1995, Somatic embryogenesis of *Cyclamen persicum* in liquid medium. Physiol. Plant., **94** : 605 -612.
- Krikorian, A.D. and Kann, R . P., 1979, Clonal propagation of daylilies. In: Plant cell and Tissue Culture. (W.R. Sharp., P.O. Larsen., E.F. Paddock, and Raghavan, eds.), PP.835 - 836. Ohino State University Press, Columbus.
- Krikorian, A.D. and Smith, D.L., 1992, Somatic embryogenesis in carrot (*Daucus Carota*). In: Plant Tissue Culture Manual. (K. Lindsey, ed.), pp.A9:1-32. Kluwer Academic Publishers, London.

- Lankon, G., 1949, The topographical tetrazolium method for determining the germinating capacity of seeds. Plant Physiol., **24** : 389 – 94.
- Laublin, G., Saini, H.S. and Cappadocia, M., 1991, *In vitro* regeneration via somatic embryogenesis from root culture of some rhizomatous irises. Plant cell tissue and organ culture, **27** : 15 - 21.
- * Le, N.M., 1989, The production of adventitious buds by *in vitro* culture of explants of flowering stems of some actively growing tulips (*Tulipa gesneriana*). Sciences de la vie, **308 (14)** : 389 - 394.
- Lelien - Kipnis, H., Ziv, M., Kahany, S. and Azizbekov, N., 1992, Proliferation and regeneration of *Nerine* in liquid culture . Acta Hort. , **325** : 467 - 474.
- Linde, P.C.G. Van-Der., Blom-Barnhoorn, G.J., Aartrix, J. Van., 1986, Towards *in vitro* propagation of bulbous iris. Acta Hort., **177** : 569.
- * Linde, P.C.G. Van-Der., Hol, G.M., 1988, Tissue culture of iris propagation is much improved Bloembollencultuur, **99 (26)** : 36 - 37.
- Liu, L. and Burger, D.W., 1986, *In vitro* propagation of easter lily from pedicels. Hort. Sci., **21(6)** : 1437 -38.
- Loewenberg, J.R., 1969, *Cyclamen* callus culture. Can. J. Bot., **47** : 2065 - 2067.
- * Lutz, A., 1969, Etude des aptitudes morphogenetiques des cultures de tissu. Analyse par la methode des clones d' origine unicellulaire. Rev. Gen. Bot., **76** : 309 -359.
- Maheswaran, G. and Williams, E.G., 1985, Origin and development of somatic embryos formed directly on immature embryos of *Trifolium repens* *in vitro*. Ann. Bot. ; **56** : 619 - 30
- Maheswaran, G. and Williams, E.G., 1986, Direct secondary somatic embryogenesis from immature sexual embryos of *Trifolium repens* cultured *in vitro*. Ann. Bot., **57** : 109 -117.

- Mc Cabe, P.F., Valentine, T.A., Forsberg, L.S. and Pennel, R.I., 1997, Soluble signals from cells identified at the cell wall establish a developmental pathway in carrot. Plant Cell, **9 (12)** : 2225 - 2241.
- Mc William, A.A., Smith, S.M. and Street, H.E., 1974, The origin and development of embryoids in suspension cultures of carrot (*Daucus carota*) Ann. Bot., **38** : 243 -50.
- Merkle, S.A., Parrot, W.A. and Williams, E.G., 1990, Applications of somatic embryogenesis and embryo cloning. In: Plant Tissue Culture Applications and Limitations (S.S . Bhojwani, ed.), pp.67 -101. Elsevier Science Publishers, B.V. Amsterdam.
- Miller, C.O., Skoog, F., Saltz, M and Strong, F.M., 1955, Kinetin, a cell division factor from deoxyribonucleic acid. J. Am. Chem. Soc., **77** : 1329.
- * Mizuguchi, S., Ohkawa, M., and Ikekawa, T. 1994, Effect of naphthaleneacetic acid and benzyladenine on the growth of white callus and formation of bulblet from callus induced from mother scale of *Lilium japonicum*. J. Jap. Soc. Hort. Sci., **63 (1)** : 131 - 137.
- Mo, L.H., Egertsdotter, U. and Arnold, S.V. 1996, Secretion of specific extracellular proteins by somatic embryos of *Picea abies* is dependent on embryo morphology. Ann. Bot., **77** : 143 -152.
- * Morel, G. 1960, Producing Virus - free cymbidium. Am. Orchid Soc. Bull., **29** : 495 - 497.
- * Morel, G.M. and Martin, C., 1952, Guérison de dahlias atteints d'une maladie à virus. C. R. Acad. Sci., Paris. **235** : 1324 - 1325.
- Muir, W.H., Hildebrandt, A.C. and Riker, A.J., 1954, Plant tissue culture produced from single isolated plant cells. Sci., **119** : 877 - 75.
- Murashige, T., 1974, Plant Propagation through tissue culture. Ann. Rev. Plant Physiol., **25** : 135 - 165.

- Murshige, T., 1978, The impact of plant tissue culture on agriculture. In : Frontiers of Plant Tissue Culture. (T.A. Thorpe, ed.), pp. 15 - 26. University of Calgary, Canada.
- Murashige, T. and Skoog, F., 1962, A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiol., **15** : 473 - 97.
- * Nitsch, C., 1974, Pollen culture a new technique for mass production of haploid and homozygous plants. In : Haploids in Higher Plants: Advances and Potential, (K.J.Kash, ed.), PP.123 -35.
- * Nobecourt, P.,1939, Sur la perennite et l' angmentation de volume des cultures de tissus vegetaux. Compte rendu de la societe de biologic., **130** : 1270 -71.
- * Nouaille, C. and Petiard, V., 1988, Semences artificielles: Reves et Realites. Biofutur, **67** : 33 -38.
- Orchard, J. E., Collin, H. A. and Hardwick, K., 1979, Culture of shoot apices of *Theobroma cacao*. Physiol. Plant., **47** : 207 -210.
- Panase, V.G. and Sukhatme, P.V., 1978, Statistical Methods for Agricultural Workers. PP. 347. I C A R, New Delhi, 3rd Ed.
- * Pence, V.C., Hasegawa, P.M. and Janick.J., 1980, Initiation and development of asexual embryos of *Theobroma cacao in vitro*. Z. pflanzenphysiol, **98** : 1 - 14.
- Priyadarshi, S. and Sen.S., 1992, A revised scheme for mass propagation of easterlily. Plant Cell Tissue Organ Cult., **30** : 193-197.
- Raghavan, L.J., 1976, Adventive embryogenesis: Induction of diploid embryoids. In: Experimental Embryogenesis in Vascular Plants. pp.349-81. Academic Press, London.

- Rao, P.S., Handro, W. and Harada, H., 1973, Hormonal control of differentiation of shoots, roots and embryos in leaf and stem cultures of *Petunia inflata* and *P. hybrida*. Physiol. Plant., **28** : 458 -463.
- Rao , P.S. and Narayanaswami, S., 1972, Morphogenetic investigations in callus cultures to *Tylophora india*. Physiol. Plant., **27** : 271 - 276.
- Rao, T.M., Negi, S.S. and Swamy, R.D., 1991 , Micropropagation of *Gladiolus*. Indian J. Hort., **48(2)** : 171-176.
- Razdan, M.K., 1993, Somatic embryogenesis. In: An Introduction to Plant Tissue Culture., pp:87-100. Oxford and IBH Pub.Co. Pvt. Ltd., ND, Calcutta.
- Redenbaugh, K., 1990, Application of artificial seed to tropical crops. Hort. Sci., **25(3)** : 251.
- Redenbaugh, K., 1993a, Introduction. In: Synseeds. (K. Redenbaugh, ed.) pp.3 -10. CRC Press, London.
- Redenbaugh, K., 1993b, Hydrogel encapsulated synseed. In : Synseeds. (K. Redenbaugh, ed.), pp.199. CRC Press, London.
- * Redenbaugh, K., Paasch, B., Nichol, J., Kosler, M., Viss, P. and Walker, K., 1986, Somatic seeds: encapsulation of asexual embryos. Biotechnol., **4** : 797-801.
- Redenbaugh, K., Slade, D., Viss, P. and Fujii, J.A., 1987, Encapsulation of somatic embryos in synthetic seed coats. Hort. Sci., **22(5)** : 803 - 809.
- * Reinert, J., 1959, Über die kontrolle der morphogenese and die induktion von adventivaembryonen and genebed ulturen aus karotten. Planta, **53** : 318-33.
- * Rumynin, V.A., Agadzhanyan, I.V., Slyysanrenko, A.G. 1990, Mass clonal propagation of *Gladiolus* plants. Byulleten Glavnogo Botanicheskogo Sada, **155** : 68 - 72.

- Schenk, R.U. and Hildebrandt, A.C., 1972, Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can. J. Bot., **50** : 199 - 204.
- * Schleiden, M.J., 1838, Beitrage zur phytogenesis. Muller Arch. Anat. Wiss. Med., pp: 137 - 176.
- * Schwann, T., 1839, Mikroskopische untersuchungen uber die uberreinstimmung in der struktur und dem Wachstume der Tiere and Pflenzen leipzig. W. Englemann, Nr. 176, Oswalds Klassiker der Wissenschaften, 1910.
- Sharp, W.R., Evans, D.A. and Sondahl, M.R., 1982, Application of somatic embryogenesis to crop improvement. In: Plant Tissue Culture (A. Fujiwara, ed.), Proc. 5th Inter. Congr. Plant Tissue and Cell Culture, Japan. Japanese Association for Plant Tissue Culture.
- Sharp, W.R., Sondahl, M.R. Caldas, L.S. and Maraffa, S.B., 1980, The physiology of *in vitro* asexual embryogenesis. Hort. Rev., **2** : 268 -310.
- Sheridan, W.F., 1968, Tissue culture of the monocot *Lilium*. Planta, **82** : 189 - 192.
- * Simmonds, J.A. and Cumming, B.G., 1976, Propagation of *Lilium hybrids*. II. Production of plantlets from bulb scale callus cultures for increased propagation rates. Sci. Hort., **5** : 161 - 170.
- * Skoog, F. and Miller, C.O., 1957, Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. Symp. Soc. Exp. Biol., **11** : 118 - 131.
- Smith, D.L. and Krikorian, A.D. 1988, Production of somatic embryos from carrot tissues in hormone free medium. Plant Sci., **58** : 103 - 110.
- Smith, S.M. and Street, H.E., 1974, The decline of embryogenic potential as callus and suspension cultures of carrot are serially subcultured. Ann. Bot., **38** : 223 - 241.

- * Stefaniak, B., 1994, Somatic embryogenesis and plant regeneration of *Gladiolus*. Plant Cell Reports, **13 (7)** : 386 - 379.
- * Steward F.C., Israel, H.W., Mott, R.L. Wilson, H.J. and Krikorian A.D., 1975, Observations on growth and morphogenesis in cultured cells of carrot (*Daucus carota* L.). Philosophical Transactions of the Royal Society of London. B **273** : 33 - 53.
- * Steward, F.C. and Krikorian, A.D., 1971, Plants, chemicals and growth. Academic Press, New York.
- Steward, F.C., Mapel, M.O., and Smith, J., 1958, Growth and organized development of cultured cells. 1. Growth and division of freely suspended cells. Am. J. Bot., **45** : 693 - 703.
- Street, H.E. and Withers, L.A., 1974, The anatomy of embryogenesis in culture. In: Tissue Culture and Plant Science. (H.E. Street, ed.), pp.71-100. Proc. 3rd Inter. Congr. Plant Tissue and Cell Culture, University of Leicester, Leicester, U.K., Academic Press, London.
- Takayama, S., Amo, T. and Fukano, M., 1991, Rapid clonal propagation of *Hyacinthus orientalis* bulbs by shake culture. Sci. Hort., **45 (3 - 4)** : 315 -321.
- Tisserat, B., Esan, E.B. and Murashige, T., 1979, Somatic embryogenesis in angiosperms. Hort. Rev., **1** : 1- 78.
- Van Engelen, F.A. and De Vries, S.C., 1992, Extracellular proteins in plant embryogenesis. Trends Genet., **8** : 66 - 70.
- Vasil , V. and Hilderbrandt, A.C., 1965, Differentiation of tobacco plants from single isolated cells in micro cultures. Sci., **150** : 889 - 892.
- * Vasil, I.K. and Vasil, V., 1980, Clonal propagation. Inter. Rev. Cytology, Supplement IIA: 145 - 73.

- * Walker, K.A. and Sato, S.J., 1981, Morphogenesis in callus tissue of *Medicago sativa*: the role of ammonium ion in somatic embryogenesis. Plant Cell, Tissue and Organ Culture, **1** :109 - 121.
- Wang, L., Huang, B.C., He, M.Y. and Hao, S., 1990, Somatic embryogenesis and its hormonal regulation in tissue cultures of *Freesia refracta*. Ann. Bot., **68 (3)** : 271 - 276.
- White, P.R., 1934, Potentially unlimited growth of excised tomato root tips in a liquid medium Plant Physiol., **9** : 585 - 600.
- White, P.R. 1939, Potentially unlimited growth of excised plant callus in an artificial medium Am. J. Bot., **26** : 59 - 64.
- Williams, E.G., and Maheswaran, G., 1986, Somatic embryogenesis: Factors influencing co-ordinated behaviour of cells as an embryogenic group. Ann. Bot., **57** : 443 - 462.
- * Wimber, D.E., 1965, Additional observations on clonal multiplication of cymbidiums through culture of shoot meristems. Cymb. Soc. News, **20 (4)** : 7 - 10.
- Zimmerman, J.L., 1993, Somatic embryogenesis: A model for early development in higher plants. Plant Cell, **5** : 1411 - 1423.
- * Ziv, M. and Halevy, A.H., 1972., Organs and plantlets regeneration of *Gladiolus* through tissue culture Proc. Inter. Hort. Congr., **18 (1)** : 211 - 212.
- Ziv, M., Halevy, H. and Shilo, R., 1970, Organs and plantlets regeneration of *Gladiolus* through tissue culture. Ann. Bot., **34** : 671 - 676.
- * Original not seen.