

***In vitro* Propagation of Gladiolus Plantlets and Cormels**

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Abstract: Gladiolus is a potential cut flower grown throughout the world for its elegant attractive spikes of different hues and good keeping quality. Commercially it is propagated by vegetative mean by corms and cormels. However the multiplication rate of corms and cormels is slow and the conventional methods are insufficient to meet the demand of planting material (corms and cormels). *In vitro* techniques are applicable for the propagation of corm producing species. These techniques are adopted at commercial level in order to fulfill supply gap of huge demand. A number of *in vitro* protocols have been developed for regeneration of gladiolus plantlets by using various explants sources of the plant. However, literature is rather scanty on *in vitro* cormel formation and acclimatization of *in vitro* propagules.

Key words: Cormel propagation • *In vitro* • Gladiolus

INTRODUCTION

Gladiolus is cultivated for its elegant and attractive spikes of different hues throughout the world [1]. The major gladiolus producing countries are the United States (Florida and California), Holland, Italy, France, Poland, Bulgaria, Brazil, India, Australia and Israel. It is the best selling bulb of the United States with an estimated annual sale of more than 370 million corms [2]. Gladiolus is a member of the Iris family having short life cycle of 110-120 days and requires temperature regime between 10-25°C for optimal growth. Commercially, gladiolus is propagated by natural multiplication of new corms and cormels [3, 4, 5] whereas seed propagation is only used to evolve new and improved varieties by hybridization. However, Memon *et al.* [6] reported few improved conventional techniques including divisions of the corms and leaf/flower clippings for enhancement of corm and cormel production in gladiolus. Usually, the bulbous plants are perpetuated by using their underground storage organs such as rhizomes of tuberose, corms of gladiolus and bulbs of lilies. Although there are other methods which are applied to these underground storage organs such as chipping, scooping, scaling and scoring, these are not suitable for the propagation of the corms of gladiolus because predominantly bulb is a fleshy leaf scales and a corm is a compressed solid thickened stem with distinct nodes and

internodes [3]. Very few improved conventional techniques were reported for the multiplication of corms and cormels including cutting of the corms and leaf clippings by the Memon *et al.* [6]. Additionally, commercial production of corms and cormels is affected by *Fusarium* corm rot during storage. Besides, one mother corm normally produces about 25 cormels each season [1]. These cormels require three to four seasons to attain standard size of flowering spike and daughter corms. The dormancy of the corms and cormels is another problem in this regard [7]. Thus its commercial cultivation is limited by low rate of multiplication and does not fulfill the local demand of planting material which eventually affects the final cost of corms. Therefore novel cultivars need to be rapidly mass multiplied by using modern *in vitro* technologies in order to fulfill the supply gap of huge demand of our local market which is of course not possible through conventional methods.

Mass propagation of corms and cormels through modern technologies such as tissue culture techniques have adopted at commercial level. Advanced countries are using highly sophisticated modern technologies for the commercial production of desired varieties in order to compete in the international markets. This technology makes also possible to produce disease free and true to type planting material. However, in many developing countries the establishment cost of facilities and unit production cost of *in vitro* propagated plants is high and

often the return on investment is not in proportion to the potential economic advantages of the technology [8, 9]. Hence, it is emphasized that all plants should not be commercially produced from tissue culture. This technology works in a way when tissue culture methods are superior to conventional propagation, produce pathogen free plants in huge quantities, compete with conventional method and used for cloning.

In vitro techniques are useful for the propagation of corm producing species. As most of the hybrid cultivars of gladiolus have a very low rate of multiplication. *In vitro* propagation is an alternative to the conventional methods; it increases multiplication rates [10, 11, 12] and also generates material free from viruses and other pathogens [13, 14].

Since the pioneering efforts, a lot of data were generated and a number of papers have been published on different aspects of *in vitro* studies of gladiolus with a greater emphasis on micropropagation. However, literature is rather scanty on *in vitro* cormel formation. *In vitro* propagation of gladiolus plantlets and cormels is possible to achieve through direct or indirect (callogenesis) mode of regeneration. A consolidated account of tissue culture studies on gladiolus is dealt with in the present review.

Direct Mode of Regeneration: *In vitro* direct mode of regeneration is a faster method for mass cloning of bulbous plants. However, the rate of regeneration is mainly depends on a number of factors, including the type of the explant, composition of the culture medium, type and concentration of plant growth regulators and genotype of the plant material.

Choice of Explant: The choice of appropriate explant is critical in tissue culture [15]. The specific differences in the regeneration potential of different organs and explants have various explanations. In gladiolus, the most popular tissue source has been corms [16] or inflorescence explants [17]. Various explant types such as shoot tips or cormel sprouts [18-23] axillary buds [7, 24-27], basal leaf tissues [28], meristem [29], explants from inflorescence stalk [18, 30, 31, 32, 17, 22], cormel and cormel segments [33, 32, 34, 29, 22] have also been reported for *in vitro* direct shoot regeneration of gladiolus.

Different responses from different explants sources and even from the same explant were reported by a number of researchers. Memon *et al.* [22] used different explants at different stages/sizes including nodal cultures

from different developmental stages of flower spike, various sizes of whole cormels and cormel sprouts obtained at different intervals in cultivar *White Friendship* of gladiolus. Memon *et al.* [22] found heading stage the best explant for efficient shoot regeneration on MS medium supplemented with BAP (4 mg L^{-1}) from nodal cultures taken at various developmental stages of flower spike as compared to one bud or three bud opened stage in *White Friendship* variety of gladiolus. They also used different sizes of whole cormels and reported the best size of 0.6 g cormel for efficient shoot regeneration on the same MS medium as compared to 0.2 and 0.4 g sized cormels. Babu and Chawla [34] used corm slices and observed the better shoot induction from top slice of the cormels as compared to medium and bottom slices of the cormels. They also observed an average of 90% response for shooting from *in vitro* bisected shoot tips having no base of the cormel when inoculated on the medium containing kinetin ($18.6 \text{ } \mu\text{M}$) in the cut-side down orientation. Aftab *et al.* [29] used cormel and meristem explants and recorded cormel as a better source for shoot multiplication as compared to meristem. Nhut *et al.* [16] depicted highest shoot formation from basal plates (30 mm diameter) as compared to shoot tip (5 mm in length), longitudinal corm section (20 mm in width) and daughter corm (15-20 mm diameter) in the medium containing BA ($4.5 \text{ } \mu\text{M}$). Ahmad [26] achieved maximum number of shoots from nodal explants rather than terminal and axillary buds of cormel. Similar findings were observed by Grewal [31]. They found better response from nodal segments as compared to internodal, rachis and floret segments.

Composition of Culture Medium: The response of *in vitro* cultures for efficient direct shoot regeneration varies according to the strength of the MS medium and combination of the plant growth regulators. In gladiolus, multiple shoot regeneration was generally found on full strength MS medium [22, 23]. However successful multiple shoot induction was also observed in half strength MS medium when containing BAP (0.75 mg L^{-1}) by Begum [24]. Aftab [29] also observed multiple shoot induction response (up to 90%) on both full as well as half strength MS basal medium. Half strength of MS salts supplemented with 3-9% sucrose in order to produce multiple shoots was also reported by Ahmad [26]. High sucrose concentrations (8, 10 or 12%) significantly increased shoot production in *G. hybridus* cultures.

Optimum Concentration of Plant Growth Regulators:

The two most important plant growth regulators - cytokinins and auxins have been reported for *in vitro* regeneration and proliferation of shoots in gladiolus. In the class of cytokinins, beneficial effect of Benzyl aminopurine (BAP) or Benzyladenine (BA) over other cytokinins have been reported [35, 36, 22, 23]. Regarding auxins, naphthalene acetic acid (NAA) has also been found beneficial for the establishment of bud and meristem tip cultures [30, 19, 37]. Usually high levels of cytokinins and low levels of auxins are recommended for *in vitro* shoot proliferation. Whereas, at lower levels of cytokinins or in its absence, shoot elongation occurs [26, 38, 35] so organ initiation depend on the manipulation of auxin and cytokinins levels in the media. The optimum concentration of plant growth regulators for shoot proliferation varies from cultivar to cultivar [39, 35, 40]. Priyakumari [7] used intact cormels of cultivar "*Peach Blossom*" on MS medium supplemented with different levels of BA and Kinetin (1, 2, 4 mg L⁻¹ each) alone and in combination with NAA (0.1 and 0.5 mg L⁻¹) and IAA (1 and 2 mg L⁻¹). The highest rate of multiple shoots (33.7) was found in response to BA (4 mg L⁻¹) supplemented with NAA (0.5 mg L⁻¹). BA at low concentrations (1 or 2 mg L⁻¹) was however, suitable for further shoot multiplication. Memon *et al.* [22] and Memon *et al.* [23] also reported efficient shoot regeneration from different explants sources at BAP 4 mg L⁻¹. Prasad [28] studied comparative performance of three different culture systems viz; semi-solid agar (0.8% w/v), liquid media supported with membrane raft (MR) and duroplast foam (DF) in order to produce *in vitro* shoot regeneration from basal parts of leaves in *Gladiolus hybridus* Hort. cv. *Wedding Bouquet*. They used MS basal salt mixture along with different concentrations of NAA (0, 0.2, 0.5 and 1.0 mg L⁻¹) and BAP (0, 1, 2 and 4 mg L⁻¹) either alone or in combination. NAA and BAP alone didn't exhibit any response in all the three culture systems. Whereas a combination of NAA (1 mg L⁻¹) and BAP (2 mg L⁻¹) induced maximum (33.15) shoots/cluster in MR system followed by DF (24.74) with NAA (0.5 mg L⁻¹) and BAP (4.0 mg L⁻¹) and AS (21.34) with NAA (0.2 mg L⁻¹) and BAP (2.0 mg L⁻¹). Shoot multiplication was rapid in AS and MR systems as compared to DF system. BAP 2 to 4 mg L⁻¹ along with GA₃ was optimal for culture establishment of gladiolus cvs. '*Eurovision*' and '*Wine and Roses*' as reported by Pathania *et al.* [41]. Beura and Singh [42] demonstrated a higher rate of bud proliferation in gladiolus on MS medium supplemented

with high concentration of BAP (4 mg L⁻¹). Same results were observed by De Bruyn *et al.* [36]. They reported *in vitro* multiple shoot induction with BAP as single growth regulator. Direct shoot regeneration was observed from segments of cormels and inflorescence axes cultured on MS basal medium containing 5.0 µM each of BA and 2,4-D, besides callus formation [32]. Ahmad *et al.* [26] cultured axillary buds, cormel tips and nodal buds on MS basal medium supplemented with BA or KIN alone and BA with IBA for shoot production in gladiolus. They observed maximum number of shoots on MS basal medium supplemented with BAP (18 µM) and IBA (36 µM). Nodal buds were found better explant for efficient shoot regeneration as compared to other types of the explants. Grewal *et al.* [31] obtained single shoot per explant from nodal segments in cultivars viz. *Mayur*, *Sylvia*, *Spic* and *Span* on MS medium supplemented with BAP (1 mg L⁻¹) and developed 14-20 shoot primordia within 4 weeks from these developed shoots when these were cultured on MS medium containing BAP (5 mg L⁻¹). These clump sections of shoot primordia were successfully multiplied on the same medium.

From the works presented under this section, it is evident that direct shoot induction and proliferation is mainly dependent on the use of cytokinins especially BA (1-5 mg L⁻¹) alone or in combination with low levels of auxins. Among auxins, NAA (0.5-1 mg L⁻¹) is the most commonly used plant growth regulator. Although, IBA or 2,4-D along with BA is also reported in few reports for successful regeneration and proliferation of shoots.

Regarding genotype a thorough study is required for screening and selection of genotypes in gladiolus for productive *in vitro* regeneration of plantlets. It is not necessary that *in vitro* optimized protocol of one genotype is applicable for another even in the same species.

Indirect Mode of Regeneration (Through Callogenesis)

Choice of Explant: The bulbous ornamental plants are monocotyledons and among this group, callus is generally initiated from meristematic tissue such as embryos, basal meristems or shoot tips. However, in gladiolus, callus initiation is also reported from various explants sources such as segments of inflorescence stalk [18, 32], cormel tips [43, 44], cormel slices or basal leaf regions [45, 46, 32, 47, 48, 29], axillary buds [37, 27] and slices of cormel sprouts [1]. The shoot tip of the cormel was considered the best source for callus initiation [43, 44].

The callus initiation and regeneration from any explant source is mainly based on cultivated varieties, concentrations and combinations of plant growth regulators used in the culture media [45, 46]. The first published report on tissue culture of gladiolus was by Ziv *et al.*, (1970) who used inflorescence discs to induce callus [15] followed by Simonsen [43], who used stem tip of cormel to induce callus. A series of experiments by Hussey (1975, 1976, 39) showed that corms and inflorescence explants were superior to leaves or ovaries for callus production. Flower stalk as explants produced more callus compared to bracts, denuded flowers or inflorescence segments (Bajaj *et al.*, 1983). Segments of inflorescence stalk [32], cormel tips [44], cormel slices or basal leaf regions [45, 46, 32, 47, 48, 29], axillary buds [37, 27] and slices of cormel sprouts [1] have also been reported for successful regeneration of callus.

Hormonal Requirement for Callus Initiation: The hormonal requirement for callus initiation and subsequent plant regeneration from the monocotyledonous bulbous crops in the floral industry are largely unknown [45]. Various results reported that the best callusing occurs in gladiolus in the presence of NAA [44, 47, 6], 2,4-D alone [32, 48] or in combination with NAA was also reported by [29]. Callus induction was also reported in the presence of BAP supplemented with 2,4-D [32, 29]. Media supplemented with either 1-10 mg L⁻¹ naphthalene acetic acid (NAA), 0.5-2.0 mg L⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D), 2 mg L⁻¹ dicamba or 1 mg L⁻¹ picloram have also been used for callus induction and maintenance [49].

The variations in concentration of plant hormones may vary from hormone to hormone and genotype to genotype even for the same explant as reported by Aftab [29]. They used cormel slices (2-3 mm thick) and leaf sections (1 to 1.5 cm²) for callus induction on MS basal media supplemented with NAA or 2,4-D alone or in combination with BAP. They found positive response for callus induction from slices of cormel at lower concentrations of 2,4-D (2 mg L⁻¹) supplemented with BAP (1 mg L⁻¹). They also reported callus induction from the same explant on MS basal medium containing NAA (3 or 4 mg L⁻¹). Kim [50] observed the best callus formation from cormel explants on a medium containing 2,4-D (10 ppm) in about 40 days. Kamo [46] achieved the best callus induction from cormel segments at higher levels of NAA (10 mg L⁻¹) or MS medium with 2,4-D (0.5 mg L⁻¹) or dicamba (2.0 mg L⁻¹). Kumar *et al.* [32] induced better callus formation from cormel segments of gladiolus on MS basal media containing BAP (5.0 µM)

and 2,4-D (5.0 to 10.0 µM) from 'Her Majesty and Aldebaran' cultivars. In the same way Remotti and Löffler [48] reported the best callus induction from cormel slices on a medium containing 2,4-D (9 mM) in cultivar *Peter Pears*. They also reported that middle part of the cormel had the highest competence for callus initiation. Emek and Erdag [47] optimized NAA at the rate of 8.5 mg L⁻¹ for maximum callus production from longitudinal corm slices in *Gladiolus anatolicus*. Cormel segments responded better for callus induction as compared to inflorescences axes [32] and leaf explant [29]. It is clear from the above findings that NAA or 2,4-D alone or in combination had major role in initiation of callus in different genotypes.

For most of the explants, callus initiation and regeneration was reported by using same type of growth regulators such as NAA, 2,4-D, BAP and KIN. When explants from inflorescence stalk (flower stalks, debudded flower, bract, perianth) and leaf segments cultured on MS basal medium containing NAA and KIN showed the best callus formation from the segments of flower stalks [51]. Callus formation from leaf sections was observed on MS basal medium supplemented with 2,4-D (3.0 mg L⁻¹) [29]. Seven days old sprouts of *in vitro* cultured cormels of *Gladiolus primulinus* cv. Golden wave initiated callus on MS medium supplemented with NAA (4 mg L⁻¹) [1]. Goo *et al.*, (2003) also observed 100% callus formation ratio from cormel tip of *Topaz* on MS medium supplemented with NAA (1.0 mg L⁻¹). Kasumi *et al.* [52] observed callus induction from all explants of ovaries on MS medium containing NAA (5 mg L⁻¹) and BAP (5 mg L⁻¹).

Kinetin (KIN) induced callus formation from explants of Inflorescence stem and the shoot tip on a medium containing 26.9 µM or 53.8 µM NAA and 2.3 µM KIN [18, Bajaj *et al.*, 1982]. Simonsen and Hildebrandt [43] achieved the best callus induction on agar as compared to liquid medium in the presence of 2.3 µM KIN. On the basis of their results they suggested the best callus growth at lower levels of KIN.

Callus induction was demonstrated as genotype dependent phenomenon and among the tested cultivars, 'Peter Pears' and 'White Prosperity' were found superior with respect to callus production on the media with either 2,4-dichlorophenoxyacetic acid or picloram [48].

Regeneration of *In vitro* Cultures: *In vitro* regeneration of plantlets has been successfully carried out from callus derived either from slices of cormel sprouts [1], cormel slices and basal leaf region [45, 47] cormel tips [43, 44]

and inflorescence stalk [18]. Additionally, direct organogenesis was also reported from cormel tips [43, 38], corm and corm slices [34, 16, 29], nodal segments [31, 38], axillary/lateral buds [37, 24, 25, 7] and basal plates [16]. However, cormel tips were found most suitable explant for culture establishment than leaf and inflorescence segments [53].

Regeneration through callogenesis is mainly based on the quality of callus, its age and regeneration potential. In cultivar *Gladiolus primulinas* Baker, Sinha and Roy [1] regenerated shoots from approximately one gram of the callus on MS media supplemented with 0.5-2.0 mg L⁻¹ KIN or/and 0.5-2.0 mg L⁻¹ BAP. The maximum number of shoots [28] regenerated on MS medium with BAP (2.0 mg L⁻¹) from one month old callus and in the five month old callus, no regeneration of shoots was observed on any medium. On the other side this is not the case with direct regeneration of *in vitro* cultures. The rate of regeneration depends on the combination of growth regulators used in the medium and on the cultivar. Cytokinins play major role in this regard. Earlier, Dantu and Bhojwani [35] and De Bruyn and Ferreira [36] had reported the beneficial effect of BAP over other cytokinins for shoot multiplication and regeneration. when cytokinins are used their optimal concentration depend on the cultivar. In cultivar *Gladiolus grandzilorus* "pink" more shoots (6.1 per explant) were recorded from the basal plates on medium containing BAP 1 mg L⁻¹. In cultivar *Eurovision* 5-8 shoots obtained per sub culture on a medium containing KIN (2 mg L⁻¹) [30], whereas 11-15 shoots produced on medium having KIN (1 mg L⁻¹) for a series of cultivars. Dantu and Bhojwani [25] reported maximum number of shoots from axillary buds of gladiolus on MS medium containing BAP (0.5 mg L⁻¹). Kamo [46] cultured cormel slices on MS medium supplemented with BAP (1.0 mg L⁻¹) and regenerated plants from all six tested cultivars. Remotti and Löffler [48] regenerated plants from yellow compact cells of all genotypes on media containing zeatin and BA in various concentrations.

The use of NAA alone or in combination with BAP or KIN has also been found beneficial for the establishment of bud and meristem tip cultures [30, 37, 27]. Emek and Erdag [47] got more shoots per explant (4.71) at the combination of low level of BA (0.2 mg L⁻¹) and high level of NAA (2 mg L⁻¹) in *Gladiolus anatolicus*. The same observations were recorded by Kumar *et al.* [32]; they noted shoot differentiation from callus cultures at lower concentration of BA (0.2 mg L⁻¹) supplemented with higher concentration of NAA (1.9 mg L⁻¹) in three cultivars viz. *Her Majesty*, *Aldebaran* and *Bright Eye*.

Boonvanno and Kanchanapoom [27] cultured axillary buds on MS medium supplemented with KIN (0.5 mg L⁻¹) or NAA (0.4 mg L⁻¹) to produce multiple shoots initiated from axillary buds of the corms. Callus induced from buds in the presence of sole NAA (0.4 mg L⁻¹) and multiple shoots were developed from the callus on MS medium containing BA (1 mg L⁻¹).

It is very obvious from the cited reports here that in vitro direct or indirect regeneration of gladiolus is possible through different kind of explants, however regeneration potential depends on plant genotype and choice of growth regulators. KIN, BAP and NAA are the most important growth regulators which alone or in combination effect the regeneration of gladiolus plantlets.

Rooting of *In vitro* Regenerated Cultures: Better rooting plays major role in acclimatization. The poor survival rate of transplanted plants is usually the result of poorly developed roots [18]. Thus a major change in the pre-transplant stage is the change in culture environment to promote root initiation and shoot elongation.

Various factors such as low concentrations or completely elimination of cytokinins and high concentrations of auxins [19, 37, 30], low concentrations of inorganic salts [54], addition of sucrose [32], addition of activated charcoal [37] and addition of vermiculite [19] instead of agar in the medium is reported for better rooting response in *in vitro* cultures. Rooting in simple basal MS medium without plant growth regulators was also observed by Goo *et al.* [44].

The role of various auxins (NAA, IBA and IAA) has been reported for initiation and regeneration of roots in various cultivars of gladiolus and other bulbous plants such as *Snowdrop* bulblets [55] and *Narcissus* [56, 57]. In gladiolus, better rooting response was demonstrated in the presence of NAA and IBA. Root length, number and its morphology was greatly affected with the increasing levels of NAA [37]. Ahmad *et al.* [26] also observed better rooting response on MS medium supplemented with NAA (0.5 mg L⁻¹) or IBA (3.9 mg L⁻¹) but the best survival rate was found in the medium containing NAA in *White prosperity*. IBA (2 mg L⁻¹) was also observed beneficial in to induce rooting in *Golden Wave* as documented by Sinha and Roy [1]. Similar reports were published by Priyakumari and Sheela [7]. They found earlier rooting (within 7 days) and longer roots (5 cm) on MS medium supplemented with IBA (2 mg L⁻¹) in cultivar *Peach Blossom*. However more number of roots (24) was produced by NAA (1 mg L⁻¹) in the same cultivar. Hussain *et al.* [58] observed extensive growth of

roots on MS medium supplemented with IBA (2 mg L^{-1}) in cultivar *White Friendship*. Although cytokinins induce shoots Emek and Erdag [47] observed rooting (20%) on MS medium with BA (0.1 mg L^{-1}) in *Gladiolus anaticus* and no rooting was observed with NAA (0.5 or 2 mg L^{-1}). Memon *et al.*, (2010) found good rooting response from different explant sources by using IBA at 2 mg L^{-1} plus sucrose (3%) in *White Friendship* variety of gladiolus.

According to published reports besides auxins, use of activated charcoal and sucrose also promote rooting positively. Activated charcoal can have two effects in rooting formation; it can adsorption all organic compounds with the exception of sugars and block light supply to the nutrient medium creating similar conditions as soil do in nature. Liliën-Kipnis and Kochba [37] observed rooting in the presence of auxins and activated charcoal. They reported white branched roots and differentiation of roots in the presence of activated charcoal and observed no effect of the increasing levels of NAA on rooting.

Low concentrations of salts have also been reported for successful rooting in number of plants [54]. The species in which induction of shoot multiplication requires a full strength MS medium, reduction of the salt concentration to half or one-quarter ([1]) is found satisfactory for rooting. Aftab *et al.* [29] found 100% rooting response in gladiolus on half strength MS medium supplemented with IBA (2 mg L^{-1}) after 5 days of inoculation. Kumar *et al.* [32] reported that sucrose concentration without use of the plant growth regulators had a direct affect on both the quality of the roots and induction of rooting in *Her Majesty* and *Aldebaran*. The beneficial effect of sucrose on rooting was also reported as Sinha and Roy [1] used half strength MS medium supplemented with IBA (2 mg L^{-1}) and sucrose (6%) that promoted roots in 100% cultures within two weeks. Kumar *et al.* [32] replaced sucrose by equimolar concentrations of mannitol had no beneficial effect on the rooting in gladiolus.

Vermiculite is an alternative method to ensure good root system for easy transplanting to soil [19]. They used horticultural grade vermiculite instead of agar; the vermiculite was enriched with a half strength liquid MS medium, NAA and activated charcoal which resulted in extensive root development.

Heat shock is also useful to induce rooting in gladiolus [32]. They tested heat shock at temperature 35, 40, 45 and 50°C and found best rooting response at 50°C in cvs. *Her Majesty* and *Aldebaran*.

Regeneration of Cormels: The ultimate goal of the successful *in vitro* propagation of gladiolus is mass production of cormels in gladiolus [59, 25, 60, 61]. The *in vitro* raised cormels can be easily stored and sown like seeds in plantation season [62, 4]. This may also reduce the transplantation difficulties occurred during acclimatization [30, 643].

Various explants such as nodal buds [40, 38], cormel tips [38], inflorescence stalk [18], axillary buds of corm [35, 26, 24] and slices of cormel sprouts [1] have been utilized for *in vitro* cormel production in gladiolus on MS basal medium with different concentrations of growth hormones and sucrose.

Sucrose Requirement for Cormel Formation: Sucrose plays an important role for *in vitro* cormel formation in gladiolus [35, 38, 1]. It effect multiplication of shoots [32, 36], somatic embryogenesis [64] and rooting of microshoots [65, 66]. The increased growth of tuberous organs needs a relatively high ($> 50 \text{ g L}^{-1}$) concentration of sucrose in the medium [35, 61]. Roy *et al.* [67] compared agar-gelled medium with liquid medium supported with coir as the matrix at two different concentrations of sucrose (3 and 6%) using basal portion of innermost leaves as an explant. They obtained large number of microcorms in liquid medium at higher concentration (6%) of sucrose as compared to agar-gelled medium.

The addition of sucrose had a positive effect on cormel weight and number in gladiolus as reported by Nagaraju *et al.* [61]. Other works [30, 20, 68] showed that the sucrose is totally utilized for corm filling as indicated by weight. Nagaraju *et al.* [61] further reported that the presence of 12% sucrose in MS basal medium exhibited elongated leaves but small cormels. This suggests that higher concentration of sucrose limit growth in general due to osmotic effect of sugars. According to Ziv [30], the growth of these longer leaves need not be related to the synthesis of more food by photosynthesis for the development of cormels. This might be poor photosynthetic rate of *in vitro* cultures under low irradiance. Sinha and Roy [1] produced three categories of corms viz. small (5-10 mm), medium (10-15 mm) and large (16-22 mm) from rooted shoots cultured in half strength of MS supplemented with IBA (2 mg L^{-1}) and sucrose (6%). Better corm production also obtained on a medium supplemented with sucrose (6-9%) and cultured at 15°C [36]. They also reported that sucrose that replaced by mannitol had no beneficial effect on corm production. Goo and Kim [69] also reported *in vitro* cormel formation from the shoot base of gladiolus cv. *Topaz*; that was

greatest (90%) with 9% sucrose. Dantu and Bhojwani [25] reported cormel formation from 96% of shoots on liquid MS medium supplemented with sucrose (6%). Kumar *et al.* [32] observed cormel formation on medium containing high sucrose concentration (> 6% and up to 12%). Memon *et al.* (2010) reported successful cormel formation on medium containing IBA (1 mg L^{-1}) with high levels of sucrose (7%) in variety *White Friendship* of gladiolus. They also reported cormel formation using same medium in variety *Traderhorn* and *Peter Pears* [23].

Use of Growth Retardants for Cormel Formation: Growth retardants such as chloromequat [70], paclobutrazol [71, 72], daminozide and ancymidol [73] play major role in *in vitro* cormel formation in gladiolus. Ziv [73] produced cormels by using bud explants propagated in agitated liquid medium and supplemented with growth retardants like daminozide, ancymidol and paclobutrazol. The regeneration of buds was proliferated without leaves and these buds developed into procorms and after sub-culture to a hardening agar solidified medium, formed cormels 8-10 mm in diameter. Supplementation of paclobutrazol and sucrose also found beneficial in the medium for *in vitro* cormel formation. Formation of bigger cormels was favored with paclobutrazol at 10 mg L^{-1} and sucrose (120 g L^{-1}) in MS medium [61].

Reduced *in vitro* growth due to the presence of growth retardant such as paclobutrazol was also reported by Nagarju *et al.* [61]. The absence of paclobutrazol resulted in elongated leaves and the formation of smaller cormels. Same responses were also reported by Ritchie *et al.* [74] in chrysanthemum. Coulston and Shearing [75] observed reduction in stem elongation due to paclobutrazol in several ornamental species due to the anti-gibberellin activity of paclobutrazol [76] and promoted corm formation [77, 78] when grown in media enriched with sucrose. Steinitz *et al.* [59] obtained shoots on agar solidified media, whereas corm regeneration was obtained in subsequent liquid cultures. They also reported that paclobutrazol at 10 mg L^{-1} and sucrose promoted corm formation in liquid media.

Role of Cytokinins in Cormel Formation: There are a number of reports on the role of cytokinins in *in vitro* stimulation of tuberization [79, 80, 81]. There is apparent ambiguity about their role in the regulation of gladiolus corm formation. Emek and Erdag [47] reported corm formation on MS basal media containing BA (0.1 mg L^{-1}). Kinetin also found inducing cormel formation on excised stolon tips [82]. Memon *et al.* [22] reported the role of

kinetin in cormel formation. BA adversely affects corm formation at the shoot base [78]. Ginzburg and Ziv [82] used four plant hormones viz. kinetin, gibberellin, abscisic acid and naphthalene acetic acid for cormel development in gladiolus. Kinetin induced cormel formation, whereas, other three had no effect on tuberization. While on other side Kumar *et al.* [83] reported corm formation on MS media without growth regulators.

According to published reports on cormel formation we can conclude that this process of tuberization can be boost up by optimizing concentration sucrose, growth retardants and concentration of cytokinins in the growth medium.

Acclimatization of *In vitro* Propagules: Acclimatization of *in vitro* propagated plantlets to the *ex vitro* environment is a critical step for successful propagation. It is necessary because i) *in vitro* plantlets are not autotrophic [84] ii) poor development of leaf cuticle and iii) impaired stomatal functioning [85, 86]. *In vitro* grown plants also have poor photosynthetic efficiency and vascular connection between the shoots and roots. This abnormal morphology, anatomy and physiology of *in vitro* plantlets [87, 88, 89, 90, 91] make difficult to survive the plantlets *ex vivo*. In gladiolus, there are very few but varied reports of transplanting *in vitro* grown plants either from direct or indirect regeneration. No optimized protocol has yet been developed for acclimatization process in gladiolus. Ziv *et al.* [18] reported poor survival of transplanted plantlets in gladiolus. In 1979, he faced difficulty in transplanting gladiolus plants of the cultivar *Eurovision* to the soil until the plants were cultured on half-strength MS medium supplemented with a reduced sucrose concentration (1.5%), 0.4 mg L^{-1} thiamine, 0.5 mg L^{-1} NAA and 0.3% activated charcoal and grown under a higher light intensity than used for maintaining the microporpagated plants. Ziv [92] also reported that the addition of paclobutrazol to the medium resulted in the formation of cormels with 100% survival following transfer to the greenhouse, whereas 58% was observed without paclobutrazol. Priyakumari and Sheela [7] reported successful acclimatization of the gladiolus plantlets planted in 2:1 of sand and soil in plastic pots. Earlier Jager *et al.* [93] also reported the similar results.

In *Lilium speciosum* Thunb. var. *gloriosoides* Baker, 98% survival rate of rooted plantlets was recorded in 35 cavity growing trays under mist condition for first four weeks [94]. Hannweg *et al.* [95] also found almost same results in *Bowiea volubilis*. They transplanted *in vitro*

regenerated plantlets in sterilized soil and washed coarse river sand under three different conditions i) covered tightly for seven days to achieve high relative humidity ii) used loose covering for two to three weeks to acquire medium relative humidity iii) plantlets uncovered and mist sprayed twice a daily. Mist sprayed plantlets gave maximum survival rate (90.9%) as compared to other conditions. This phase of transplantation from *in vitro* to *in vivo* usually needs some weeks of acclimatization with gradual lowering in air humidity [85, 96].

In gladiolus, a successful acclimatization is possible at three different stages of *in vitro* propagation; i) when *in vitro* regenerated plantlets have optimal shoot/root ratio but no cormel formation ii) after cormel formation and before their dormancy iii) when cormels go under dormant period and plant shoot dries up. Generally the first option is practiced more in which *in vitro* regenerated shoots are planted into rooting medium and then placed into high humidity environment with low irradiance and temperature for acclimatization. However, to reduce the losses occur during the hardening process of *in vitro* grown plants, it is better especially in bulbous plants to induce shoots to form storage organs such as cormels in gladiolus and bulbs of lilies. These underground storage organs are generally resilient and can be planted or stored when desired. The survival of *in vitro* plantlets with cormels/bulblets is usually based on the size of the cormels as reported by Naik and Nayak [97] in *Ornithogalum virens*; Slabbert and Niederwieser [98] in *Lachenalia*. Smaller bulbs (2-3 mm diameter) showed low survival as compared to large one (4-10 mm diameter) [97]. Paek and Murthy [99] reported 100% survival of *in vitro* rooted bulblets those had a diameter of more than 10 mm. Cormels usually undergo dormancy and thus do not sprout upon planting. To overcome dormancy and with the idea of producing plants year round, a cold treatment is followed to break the dormancy before the cormels are sown in the soil [100]. Gladiolus require cold treatment for a period of four weeks at a temperature range of 2-5°C [39]. He also reported that the dormancy can also be broken when *in vitro* produced cormels are subcultured on a medium containing BA. A period of 4-8 weeks at 0-5°C was required to break dormancy in bulblets [101]. Paek and Murthy [99] employed cold treatment for 5 weeks at 5°C in *Fritillaria thunbergii*.

Role of Corm Size in Acclimatization: Corm size plays major role in the acclimatization of the bulbous plants as poor survival rate was observed within five to seven days from bulblets having diameter smaller than 4 mm whereas

the survival rate of larger bulblets was high [95]. Paek and Murthy [99] planted *in vitro* regenerated bulblets of *Fritillaria thunbergii* of different sizes in equal ratio of peat moss, vermiculite and perlite. They recorded survival rate after five weeks of planting, it was 17.6% for bulblets having a diameter less than 5 mm whereas bulblets having a diameter 6-10 mm or more showed 86% to 100% survival. In an experiment Naik and Nayak [97] reported that bulblets of small size (2-3 mm diameter) showed a survival rate of 40-50%, whereas the larger bulblets (4-10 mm diameter) had a 70 to 80% survival rate. This is also supported by Slabbert and Niederwieser [98] in *Lachenalia*.

CONCLUSIONS

The broad expansion in the international trade of floriculture products is forcing researchers and growers to establish new methods for rapid multiplication of elite clones, production of disease-free plants, introduction of new exotic varieties or novel cultivars with desirable traits and improvement of regular varieties as a part of gladiolus improvement program. In our opinion micropropagation techniques can play a vital role in this regard. We have combined most of the published work on the micropropagation of gladiolus as reference for future studies in this paper. Additionally, most of the critically important factors affecting successful micropropagation of gladiolus are discussed here in detail. Although it is obvious from literature that there are many reproducible protocols for micropropagation of gladiolus, there are many challenges being faced by the tissue culture industry at present. These include research for cost effective protocols, applying automation for commercialization and optimizing microenvironment etc. Therefore, it is important to bring further improvements in the existing tissue culture protocols to go for commercialization and to maintain the clonal fidelity of elite clones.

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