Studies on in vitro morphogenesis and cormlet formation of *Gladiolus hybridus* Hort.

A

Dissertation Report

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Under the Supervision of

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CERTIFICATE

This is to certify that the dissertation "**Studies on in vitro morphogenesis and cormlet formation of *Gladiolus hybridus* Hort.**" submitted by Miss. Shweta Bisht (Roll no. 301701027) in the partial fulfilment of the requirement for the award of the degree of Masters of Science in Biotechnology, Thapar Institute of Engineering and Technology, Patiala. She has fulfilled all the requirements in completing this work under my supervision and guidance.

The matter embodied in this thesis has not been submitted in part or full to any other institute or university for the award of any degree or diploma.

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DECLARATION

I, hereby declare that the work which is being presented in the dissertation entitled **"Studies on in vitro morphogenesis and cormlet formation of *Gladiolus hybridus* Hort."**, in partial fulfilment of the requirement for the award of the degree of Masters of Science in Biotechnology, Thapar Institute of Engineering and Technology (TIET), Patiala, Punjab, India. This is an authentic record of my own work during a period of six months under the supervision of Dr. Anil Kumar, Associate Professor, Department of Biotechnology, TIET. The matter embodied in this thesis has not been submitted in part or full to any other institute or university for the award of any other degree.

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Date: 26-August-2019

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LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

Abbreviation	*Full form*
%	Percent
℃	degree Celsius
et al	et alia alii (Latin) = and other people
µ mol ms^{-2} s^{1}	micromoles per square meter per second
µM	Micromolar
µl	Microliter
ANOVA	Analysis of variance
BA	6-benzyl adenine
CCC	Chloro Choline Chloride
2,4-D	2,4 Dichlorophenoxy acetic acid
EDTA	Ethylenediaminetetraacetic acid
et al	et alia alii (Latin) = and other people
g	Gram
IAA	Indole 3-acetic acid
IBA	Indole 3-butyric acid
Kn	Kinetin
mg/l	mili gram(s) per litre
min	Minutes
ml/l	milli litre/litre
mM	millimolar
MS	Murashige and Skoog medium (1962)

NAA	α-naphthaleneacetic acid
PGRs	Plant Growth Regulators
sec	Seconds
v/v	Volume by volume
w/v	Weight by volume

ABSTRACT

Gladiolus hybridus Hort. holds a paramount position in floriculture market because of its magnificent spikes in myriads of colours. Conventional methods of propagation are not much result yielding because of slow multiplication rate and due to pathogen attacks. In order to meet the huge demand of *Gladiolus,* the present study was undertaken to develop an efficient protocol for in vitro propagation of *Gladiolus*.

The cultivar of *G. hybridus* was multiplied on basal MS medium containing 3 % (w/v) sucrose. The effect of auxins and cytokinins was observed on shoot proliferation of *G. hybridus* using various combinations and concentrations of 6-benzylaminopurine (BA) and α-naphthaleneacetic acid (NAA). Out of which maximum shoot proliferation was recorded on MS medium containing 2.5 µM each of NAA and BA. Also, the organogenesis potential from leaf segments has been demonstrated in present study using auxins such as NAA, Indole-3-acetic acid (IAA), 2,4-Dichlorophenoxyacetic acid (2,4-D). Maximum shoot organogenesis was observed on MS medium containing 5 µM 2,4-D.

However, with increase in sucrose concentration shoot proliferation, rooting, cormlet formation increased significantly. Maximum shoot proliferation, rooting and cormlet formation was observed when 8 % (w/v) sucrose was used. Further, heat shock (45 ^{0} C) for 1 hour after 15 days of incubation stimulated shoot growth and rooting with maximum growth on medium containing 6 % (w/v) sucrose. Alongwith, the addition of plant growth inhibitors in basal MS medium containing 8 % (w/v) sucrose had a significant effect on growth and cormlet formation in *G. hybridus.* Maximum cormlet (3.86) per shoot clump were observed on medium containing 20 µM Chloro Choline Chloride.

1. INTRODUCTION

Gladiolus is one of the commercially important floriculture crop known for their beauty and economical value (Kumar et al. 1999; Ascough et al. 2009). It is also known as queen of bulbous flowers (Nagraju et al. 2002). *Gladiolus* holds third most important position in terms of area and production among all cut flowers (Kumar et al. 2018). *Gladiolus* belong to family Iridaceae (ornamental family) (Ascough et al. 2009) and have short life cycle of 110-120 days (Memon et al. 2012). It is placed in a major group of angiosperms due to its glorious flower and attractive spikes. Broadly, the cultivars (varieties) of *Gladiolus* are classified into two categories i.e. *big flowered* and *small flowered*. Some of the common cultivars are 'Friendship', 'Cherry blossom', 'Mayur', 'Black jack', 'Red canna', and 'Butterfly' (https://www.agrifarming.in/tag/gladiolus-project-report).

About 180 species of *Gladiolus* are known till date, among them *Gladiolus hybridus* Hort. holds an important position as in addition to ornamental value, it also have great medicinal benefits (Tripathi et al. 2017). Some of the therapeutic uses of *Gladiolus* bulb includes-

- curing of fungal infections and common cold
- providing relief from constipation
- curing of stomach and mouth ulcers
- reducing abdominal pain during mensuration in women
- curing meningitis (https://www.agrifarming.in/tag/gladiolus-project-report)

Gladiolus hybridus is a herbaceous plant that bears underground storage stem known as corm. An intact corm gives rise to spikes bearing sword shaped flowers. Due to its attractive floral arrangement and sword like shape of flower, it is commonly known as 'sword lily'. Spikes of *Gladiolus* are big and are arranged on one side. Colour of flowers generally ranges from reddish

to pink, or light purple to white or with contrasting markings (Memon et al. 2012; Tripathi et al. 2017).

Gladiolus is mainly grown in Europeon and American states, among them Germany, Holland, USA [(supplies 370 million corms per year) (Memon et al. 2012)] are major *Gladiolus* growing countries (Kumar et al. 2018). In India, more than 11660 hector (ha) area is covered for the cultivation of *Gladiolus* with an aim of producing 106 crore of cut flowers per year (Kadam et al. 2014; Kumar et al. 2018). Various states of India that cultivates *Gladiolus* are shown in Figure 1. About 150 ha area is covered in Punjab for the cultivation of *Gladiolus* sp. (NHB 2014). Growing season of *Gladiolus* range from October to March in plain areas and from June to September in hilly areas.

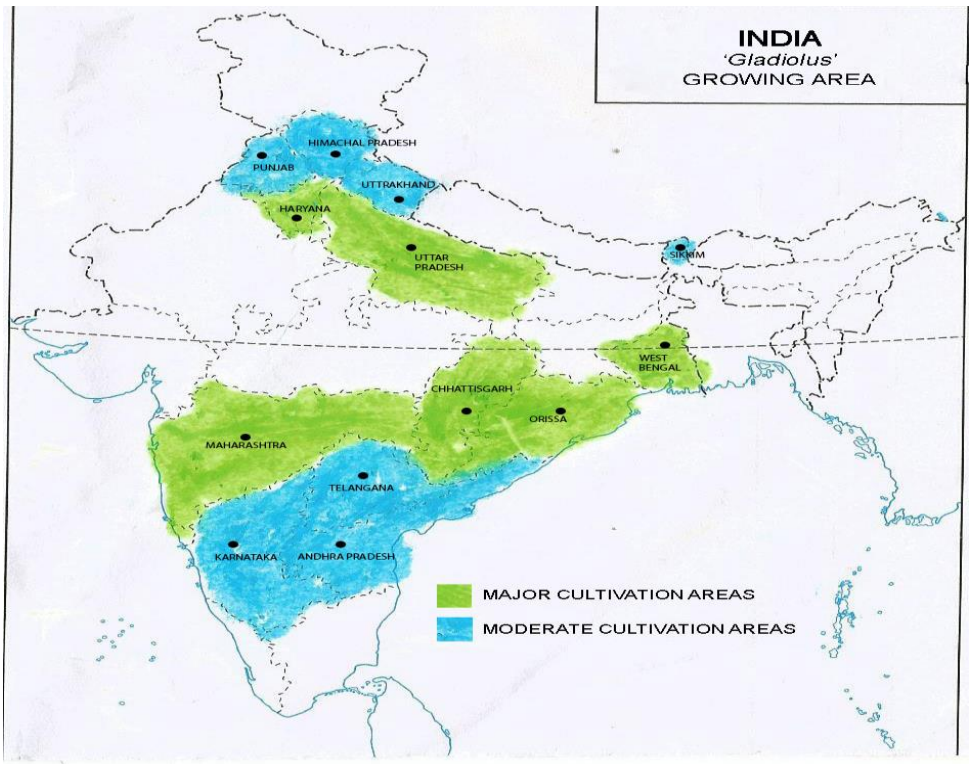

Figure 1 Cultivation of *Gladiolus* in different states of India. Major and moderate cultivators of *Gladiolus* are highlighted in green and blue colour respectively (Kadam et al. 2014; Kumar et al. 2018).

As the cultivation of any crop is dependent upon many factors such as soil quality, weather, photoperiod etc. Thus, the optimum conditions for growth and cultivation of *Gladiolus* are listed below-

a) Climate – *Gladiolus* grow well in subtropical and temperate climatic conditions. For the cultivation of this crop, mid climate i.e. neither too hot nor too cold is considered an ideal climate (http://agritech.tnau.ac.in/horticulture/horti_flower%20crops_gladioli.html).
b) Temperature – Around 25-30 ℃ is considered as an ideal temperature for growth of *Gladiolus.* At time of planting soil temperature should not be less than 10 ℃ as frost injury can harm the plant (Memon et al. 2012).
c) Sunlight – *Gladiolus* need 12-16 hour of sunlight. Frost conditions should be avoided as *Gladiolus* is sensitive to frost conditions. (http://agricoop.nic.in/sites/default/files/Gladiolus%20%281%29.pdf).
d) Soil type – *Gladiolus* grows well in deep, well drained, rich in nutrient and organic matter soil but it can also grow in light sandy to clay loom soil (Tripathi et al. 2017).
e) Soil pH – Soil should be slightly acidic (pH-5.7-5.95) as most of the nutrients become available to plant. pH of the soil should not exceed more than 7 as higher pH level supress cormel formation (https://www.agrifarming.in/tag/gladiolus-project-report).
f) Nutritional requirement – High quality flowers along with good yield is ensured by providing proper manures and fertilizers to the field. It has been reported that around 20 to 25 tons of farm yard manure (cow dung) should be provided during land preparation for production of top quality flowers. (https://www.agrifarming.in/tag/gladiolus-project-report).
g) Irrigation – Cultivation of *Gladiolus* needs regular watering (twice a week) to keep roots wet depending upon weather conditions. Irrigation frequency also depends on

type of soil. Over irrigation should be avoided. However, water stress should be avoided specially when spikes are emerging (https://www.agrifarming.in/tag/gladiolus-project-report).

h) Mulching – For controlling weed population and conserving moisture, mulching is important. Mulching is usually done between and across the rows. Fresh manure, chopped straw, husk, dried grass, saw dust can be used for effective mulching (https://www.agrifarming.in/tag/gladiolus-project-report).

Conventionally, *Gladiolus* is propagated through seeds, corm or cormels. Though seed germination is considered as an effective means of propagation, but mostly true phenotype is not produced due to cross pollination and recombination among the plants (Kumar et al. 1999; Hussain et al. 2001; Tripathi et al. 2017). Slow multiplication rate and seed dormancy is also a major concern (Priyakumari et al. 2005) and old conventional methods are not much effective (Kumar et al. 1999; Kumar et al. 2018). Also, the propagation through traditional methods is also affected by various fungal attacks such as *Fusarium oxysporum, Botrytis rot* during the storage (Tripathi et al. 2017). Further, commercial cultivation of *Gladiolus* is highly limited by low multiplication rate and does not fulfil the local demand which eventually affects the cost of corms (Memon et al. 2012; Tripathi et al. 2017). Therefore, to fulfill the growing demand, cultivars of *Gladiolus* need to be multiplied rapidly using in vitro techniques (Kumar et al. 1999; Memon et al. 2012; Kumar et al. 2018).

In *Gladiolus* many studies have demonstrated successfully on in vitro propagation (Dantu et al. 1987, 1995; Kumar et al. 1996, 1999; Ziv et al. 2000; Kumar et al. 2002). The plant cells are totipotent in nature potential and can develop into differentiated organs when cultured on formulized medium. In *Gladiolus*, in vitro propagation has been studied using various explants such as root segments and shoot buds (Ziv et al. 1989; Misra et al. 1999; Pathania et al. 2001; Priyakumari et al. 2005; Roy et al. 2006; Emek et al. 2007; Kumar et al. 2018), shoot tip

(Hussain et al. 2001), cormels (Nagraju et al. 1995; Sen et al. 1995; Choudhary et al. 2010; Budiarto 2012; Memon et al. 2012), auxillary buds (Begum et al. 1995; Boovanno et al. 2000), inflorescence axes (ziv et al. 2000). Studies have shown that success of in vitro propagation is dependent on various factors such as choice of explant, genotype of explant, medium composition, cultural conditions, plant growth regulators (PGRs) (such as auxins, cytokinin, gibberellins, abscisic acid or ethylene). Apart from the PGRs, the effect of different growth retardants such as paclobutrazol (Kumar et al. 2010; Memon et al. 2012), Chloro Choline chloride (CCC) (Dantu et al. 1995; Kumar et al. 2010) and daminozide (Memon et al. 2012) on morphogenesis and cormlet formation in *Gladiolus* has also been studied. In addition to these factors, sucrose concentration, heat shock treatment are known to play an important role in organogenesis and in vitro propagation of plants (Altschuler et al. 1992; Kumar et al. 1999, 2002; Dharmasena et al. 2011). But these kind of studies are very limited. As organogenesis and in vitro propagation can have an important impact on the plant multiplication and conservation. Thus, on the basis of gaps in study, present work is classified into following objectives-

1. To study the various factors affecting in vitro morphogenesis in *Gladiolus hybridus*
2. To study factors affecting in vitro cormlet formation in *Gladiolus hybridus*

2. LITERATURE REVIEW

Gladiolus hybridus Hort. is a bulbous, ornamental plant conventionally propagated through seed or cormlet. But these traditional methods are not considered as an effective means of propagation due to cross pollination and recombination among the plants (Hussain et al. 2001; Tripathi et al. 2017) and attacks from fungal species such as *Fusarium oxysporum*, *Botrytis rot* (Tripathi et al. 2017). Further, the cormel production is very low as one corm produces only 20-25 cormlels per year (Memon et al. 2012). Thus, commercial production of *Gladiolus* is highly limited and there is a strong need to design a well efficient in vitro propagation protocol to improve quality and quantity of *Gladiolus.*

In recent years, considerable attention has been drawn towards biotechnological approaches for genetic improvement of flower crops. Different parts of plant such as roots, leaves, cormlet, have been used for in vitro propagation of *Gladiolus* (Budiarto 2012). Various studies reporting different aspects of *Gladiolus* in vitro propagation are discussed below-

Dantu et al. (1992) reported that modified Murashige and skoog (1962) (MS) medium containing half strength NH_4NO_3, $NaH_2PO_4.2H_2O$ (300 mg/l) instead of KH_2PO_4 and omitted KI, to be a better medium for shoot multiplication.

In a successive study, Dantu et al. (1995) reported that the addition of 6-benzylaminopurine (BA) (0.5 mg/l) in MS medium can lead to maximum shoot elongation. Further, maximum shoots (96 %) formed corms of size 19.6 mm and weight 27.67 mg, on MS medium containing 6 % (w/v) sucrose concentration. No corms were observed on medium containing Gibberellic acid (GA), BA, Chloro Choline Chloride (CCC), Abscisic acid or activated charcoal. Corms formed in vitro when planted in soil, showed 100 % germination when treated for 8 weeks at 5 °C.

Kumar et al. (1999) established cultures of *G. hybridus* cultivars namely 'Bright Eye', 'Aldebaran', 'Her Majesty' using cormel segment or intact cormlet on MS medium containing various concentrations of PGRs. Callus was initiated from cut ends of cormel segments when cultured on medium containing 2,4-Dichlorophenoxyacetic acid (2,4-D) and BA. Shoot differentiation was maximum when cultured on medium containing 1.0 µM BA and 10.0 µM α-naphthaleneacetic acid (NAA). After one year, when callus was transferred to MS medium containing BA only, it failed to differentiate into somatic embryos. The effect of different sucrose concentrations on calli growth was also investigated. In this study, the effect of heat shock (50 ℃ for 1 hour) treatment on embryogenesis was also investigated. Medium containing high sucrose concentration (>174 mM) induced somatic embryos whereas low sucrose concentration (<116 mM) induced shoot bud differentiation. Embryos converted into plantlets (100 %) when transferred to MS basal medium.

Khan et al. (2000) found that the potential of micropropagation through leaves can be enhanced by the use of PGRs. The leaf explants induced callus on MS medium variously supplemented with concentration of Indole-3-butyric acid (IBA), BA, NAA, Indole-3-acetic acid (IAA). Maximum callogenesis was observed on MS medium containing 31.08 µM BA, 5.37 µM NAA, 9.84 µM IBA, 5.71 µM IAA. The regeneration of pseudo embryogenic bodies from callus was also observed in later stages.

Kumar et al. (2002) reported the best initiation of callus on MS medium containing 5.0 µM each of BA and 2,4-D. After one year, callus cultures were transferred to MS medium containing various concentrations of sucrose (58-290 mM) and were subjected to heat shock treatment (50 ℃ for 1 hour). It was observed that medium containing high sucrose (>174 mM) induced somatic embryogenesis and medium containing low sucrose (<116 mM) induced shoot bud differentiation. Later on, it was found that when high sucrose, over and above 58 mM replaced with mannitol, resulted in shoot bud differentiation. Thus, it was reported that

the presence of high sucrose more than normal cannot be only attributed to its role as an osmotic agent alone. Further, addition of putrescine into medium containing high sucrose and low sucrose medium leads to somatic embryogenesis and shoot bud differentiation respectively. Maximum initiation of somatic embryos were noticed in cultures containing medium gelled agar than phytagel. Somatic embryos converted into plantlets when cultured on basal MS medium. After acclimatization, survival rate was found to be 70 %.

Nhut et al. (2004) used longitudinal corm sections, shoot tips, daughter corms, basal plate for rapid mass propagation and shoot regeneration using continuous shake culture system. Maximum of six uniform and vigorous shoots (proliferative and most stable) were obtained after 15 days of culture on MS medium containing 2.2 µM BA. New shoots were cultured on same liquid medium (50 ml) for continuous mass propagation. Corm formation and separated plantlet formation achieved on MS medium containing IBA.

Aftab et al. (2005) reported the use of meristem, cormel, leaf for in vitro multiplication and callogenesis in *Gladiolus*. Maximum multiplication and callogenesis was observed in both meristems and cormel explant on MS medium containing 4.44 µM BA. Maximum of 16 shoots per cormel and 10 shoots per meristem were regenerated after 8 weeks of culture initiation. In comparison with meristem, cormel explants proved to be a better source of shoot regeneration. Further, maximum rooting was achieved on half strength MS medium containing 9.84 µM IBA and 2.69 µM NAA.

Priyakumari et al. (2005) reported that the addition of 17.76 µM BA in MS medium can lead to maximum shoot proliferation. Further, addition of 8.88 µM BA in MS medium can lead to longest (7 cm) and earliest (7 days) roots.

Prasad et al. (2006) established multiple shoot clusters on semi solid agar medium (AS) supported with Duroplast foam (DF) and membrane raft (MR) along with different

concentration of NAA and BA. Maximum regeneration (33.15 shoots per cluster) was observed on membrane support in liquid medium containing 5.37 µM NAA and 8.88 µM BA. High frequency of shoot regeneration (33.46 % and 25.37 %) in MR system was observed as compared to DF and AS systems. Early and maximum elongation of shoots was observed by same authors in DF in comparison to AS and MR.

Roy et al. (2006) reported that incorporation of NAA and BA in MS medium resulted in initiation of adventitious shoot buds via direct organogenesis. Maximum number of adventitious shoot buds (10-15) were observed on MS medium containing 2.0 µM BA and 9.0 µM NAA. Maximum micro corms were observed on MS medium containing 179 mM sucrose and 20 µM NAA.

Emek et al. (2007) used somatic embryogenesis (by fusing the leaves) for in vitro micropropagation for *Gladiolus anatolicus* (Boiss.). Maximum number of shoots (11.00±0.38) were recorded on MS medium containing 8.88 µM BA. Further, callus initiation was observed from basal and middle region of leaf explant on MS medium containing BA and NAA.

Choudhary et al. (2010) used corm slices to enhance the micropropagation of *G. hybridus*. Calli proliferation and multiplication was observed on MS medium containing NAA and BA. Maximum callus proliferation and multiplication was observed on MS medium containing 10.74 µM NAA and 4.44 µM BA. Further, callus regeneration was observed from basal region of corm slices in three weeks on callus induction medium. Maximum shoot proliferation was observed when one gram of callus (fresh weight) was transferred to MS medium containing 4.44 µM BA.

Kumar et al. (2011) studied number of various factor affecting in vitro cormlet formation of *G. hybridus*. Increase in fresh cormlet weight was recorded with increase in sucrose concentration (174-348 mM) in MS medium. Maximum number of corms (122) were recorded on MS

medium containing 232 mM sucrose. Further, in cultivar 'Her Majesty' maximum corms (200) with higher mass (386 mg/cormlet) were observed in 1000 ml flask. Maximum cormlet formation (19.3) and maximum weight (131.0 mg) was recorded on MS medium containing putrescine (100 µM) and Aminoguanidine (100 µM). Decline in cormlet formation and average mass was recorded on medium containing only Aminoguanidine (100 µM). The significant increase in sprouting was recorded from in vitro produce cormlets in comparison to conventionally produces cormlets under field conditions.

Memon et al. (2010) used whole cormels, cormel segments, cormels sprouted at different time intervals and nodal cultures from different stages of flower spike for optimizing *in vitro* propagation of *G. hybridus* cultivar friendship. Explants were cultured in completely randomized manner with factorial arrangement on MS medium containing various concentrations PGRs. Shoots from the cormel sprout produced better rooting response on MS medium containing 9.84 µM IBA in comparison to the other explants. Cormel sprout produced maximum cormel production on MS containing 4.92 µM IBA and 7 % (w/v) sucrose followed by IBA (4.92 µM)+KIN (4.65 µM). In vitro grading of cormels exhibited the highest corm formation (diameter = 2.8-3.2 mm) from sprouted cormels in comparison to the other explants.

Budiarto (2012) cultured cormlets of three cultivars of *G. hybridus* namely 'Kafia', 'Nabila', 'Clara' in hormone free media in order to break dormancy and to promote plant establishment. After plantlets establishment, they were transferred to MS medium containing NAA (2.69 µM) and BA (4.44- 17.76 µM). It was reported that in hormone free media, dormancy breakage varied from cultivar to cultivar. Optimum shoot formation and elongation depended on the level of BA and also varied from cultivar to cultivar.

Memon et al. (2012) used cormel sprout, whole cormels (0.6 g) for direct mode of organogenesis for regenerating cormels in *G. hybridus* cultivar 'White Friendship' and 'Peter

Pears'. Maximum number of shoots (22.07) were recorded in cultivar 'White Friendship'. Nearly same number of roots were obtained in *G. hybridus* cultivar 'White Friendship' (22.67) and 'Peters Pears' (19.60) when cultured on MS medium containing 9.84 µM IBA. Production of cormels was not affected on MS medium containing 4.92 µM IBA and 7 % (w/v) sucrose concentration.

Tripathi et al. (2017) reported the initiation of callus on MS medium supplemented with 8.88 µM BA and 2.69 µM NAA. Maximum shoot proliferation efficiency was observed on MS medium containing BA (8.88-13.32 µM) in combination with NAA (2.69 µM). Maximum rooting efficiency was obtained on medium containing IBA (2.46 µM) and kinetin (2.32 µM). Maximum root length was observed on MS containing IBA (2.46 µM) and sucrose (15.0 g/l). After the acclimatization of the plantlets, normal phenotype was observed.

Kumar et al. (2018) reported in vitro regeneration from shoot buds of *G. hybridus* cultivar 'White Prosperity'. Multiple shoots were obtained on MS medium and B5 medium (Gamborg medium) supplemented with various concentrations of BA. Shoot number varied from 0.6 to 2.3 per explant on MS medium and 1.3 to 3.0 per explant on B5 medium. Followed by incubation of 30 days, length of in vitro produced shoots varied from 1.1 to 2.9 cm on MS medium and 2.2 cm to 3.8 cm on B5 media. In vitro raised plants were found to true to type.

In addition to medium composition, and above mentioned PGRs (Khan et al. 2000; Kumar et al. 2011), role of polyamines (Kumar et al. 2013), phenols, heat shock treatment (Kumar et al. 1999, 2002, 2011) has also been reported but such kind of studies are very limited in *Gladiolus hybridus*. In the present study, the effect of PGRs, sucrose concentration, heat shock treatment, and various plant growth inhibitors such as paclobutrazol, CCC, daminozide on morphogenesis and cormlet formation was studied.

3. MATERIALS AND METHODS

3.1 Plant material and culture conditions

Cultures of *Gladiolus hybridus* Hort. cultivar 'Friendship Pink' were maintained at plant tissue laboratory at TIFAC- CORE, Thapar Institute of Engineering and Technology (TIET), Patiala. Cultures were multiplied and maintained on Murashige and skoog (1962) (MS) medium containing 58 mM (3 % w/v) sucrose and 0.7 % agar (w/v) (pH 5.8; autoclaved for 15 minute at 121 ℃) (Annexure 1) through regular subculture cycle of 21 days. Cultures were incubated at 25 ± 1 ℃ under white fluorescent light (60 µmol $m^{-2}s^{-1}$) and photoperiod regime of 16 hour light and 8 hour dark cycle.

3.2 Chemicals and Glassware

All glassware were purchased from Borosil Glass Works Ltd. (Mumbai, India). Plant tissue culture experiments were carried out in 300 ml glass bottles purchased from Kasablanka Corporation (Mumbai, India). Experiment involving heat shock treatment were carried out in 250 ml erlynmeyer's flask (Borosil). All the PGRs and tissue culture grade chemicals were purchased from Hi-media Laboratories Pvt. Ltd. (Mumbai, India)

3.3 Effect of PGRs on shoot organogenesis of *G. hybridus*

To evaluate the effect of PGRs on shoot multiplication and proliferation, shoot clumps were cultured on MS medium containing 2 % (w/v) sucrose and variously supplemented with BA (0-5 µM) and NAA (0-5 µM).

To study the effect of PGRs on shoot organogenesis, leaves of *G. hybridus* were excised. Leaves were cultured with adaxial side on MS medium containing 3 % sucrose (w/v), 2 % (w/v) activated charcoal and variously supplemented with IAA (0-35 µM), NAA (0-35 µM), 2,4-D (0-35 µM).

The cultures were incubated at culture conditions till observations were made.

3.4 Effect of sucrose and heat shock on morphogenesis of *G. hybridus*

To evaluate the effect of sucrose concentration on morphogenesis of *G. hybridus*, shoot clumps were cultured on MS medium supplemented with different sucrose concentration i.e. 2 -10 % (w/v). The cumulative effect of sucrose concentration and heat shock was also evaluated by subjecting by shoot clumps growing for 15 days on MS medium variously supplemented with sucrose concentration i.e. 2 -10 % (w/v) to heat shock (HS) treatment at 45 ℃ for 1 hour. After the treatment cultures were immediately shifted to 25 ℃. Cultures were incubated at culture conditions till observations were made.

3.5 Effect of plant growth inhibitors such as paclobutrazol, daminozide, Chloro Choline Chloride (CCC) on morphogenesis and corm formation in *G. hybridus*

To evaluate the effect of paclobutrazol, daminozide, CCC on morphogenesis and cormlet formation, shoot clumps were cultured on MS medium containing 8 % (w/v) sucrose and variously supplemented with concentrations of paclobutrazol (0-20 µM), daminozide (0-20 µM), CCC (0-20 µM). Cultures were incubated at culture conditions till observations were made.

3.6 Statistical analysis

Each experiment was performed with three replicates, and data were analysed using one way analysis of variance using Graphpad prism 5.1 software. Means were compared using Newman keuls test at $P \leq 0.05$.

4. RESULTS

The shoot cultures of *Gladiolus hybridus*. Hort. cultivar 'Friendship Pink' maintained on MS medium were used as a source of explants to study the effect of plant growth regulators (PGRs), sucrose concentration, heat shock treatment, and various growth inhibitors on in vitro propagation, morphogenesis and cormlet formation of the plant.

4.1 The effect of PGRs on shoot proliferation of *G. hybridus*

Shoot length and number of shoots per explant varied significantly with the addition of various combinations and concentrations (0-5 µM) of BA and NAA in basal MS medium containing 2 % (w/v) sucrose. Although 100 % shoot proliferation was recorded in all the combinations, but shoot length of 7.33 cm was recorded on medium containing 2.5 µM each of BA and NAA. However, minimum shoot length (1.83 cm) was recorded on medium containing 5 µM BA and 1 µM NAA. Similarly, maximum (9) number of shoots were recorded on medium containing 1 µM NAA whereas, minimum (1.67) number of shoots were recorded on medium containing 5 µM BA and 1 µM NAA (Table 1).

Table 1. The effect of plant growth regulators on shoot proliferation, shoot length and number of shoots in the cultures vessel of *G. hybridus* on MS medium containing 2 % (w/v) sucrose along with various concentrations and combinations of BA and NAA.

PGR concentration (µM)		Shoot proliferation (%)	Shoot length (cm)	Number of shoots
BA	**NAA**			
0	0	100^{a}±0.00	7.16^{a}±0.61	6.66^{abc}±0.33
1	0	100^{a}±0.00	4.00^{abcd}±0.57	3.67^{bcd}±0.33
2.5	0	100^{a}±0.00	5.83^{abc}±0.44	7.67^{a}±0.33
5	0	100^{a}±0.00	4.66^{abcd}±0.44	3.33^{bcd}±0.33
0	1	100^{a}±0.00	6.00^{abc}±0.500	9.00^{a}±0.57
1	1	100^{a}±0.00	5.00^{abcd}±0.57	6.00^{abc}±1.52
2.5	1	100^{a} ±0.00	4.83^{abcd}±0.80	5.67^{abc}±0.33
5	1	100^{a}±0.00	1.83^{d}±0.601	1.67^{d}±0.33
0	2.5	100^{a}±0.00	3.83^{abcd}±0.726	3.66^{bcd}±0.33
1	2.5	100^{a}±0.00	6.83^{ab}±0.44	5.67^{abc}±0.33
2.5	2.5	100^{a}±0.00	7.33^{a}±0.601	5.33^{abcd}±0.33
5	2.5	100^{a}±0.00	3.33^{bcd} ±0.601	3.33^{bcd}±0.88
0	5	100^{a}±0.00	2.50^{cd}±0.289	2.66^{cd}±0.33
1	5	100^{a}±0.00	4.00^{abcd}±0.57	7.33^{ab}±1.45
2.5	5	100^{a}±0.00	5.33^{abcd}±0.60	7.00^{abcd}±0.57
5	5	100^{a}±0.00	5.00^{abcd}±0.76	5.33^{abcd}±1.45

Data were recorded after 6 weeks of culture and analysed by one way ANOVA. Means were compared using Newman keuls test $P \leq 0.05$. Values followed by same lowercase letters (within columns) were found to be non-significant at $P \leq 0.05$.

4.2 The effect of auxins on shoot organogenesis of *G. hybridus*

Leaf segments (about 1 cm) of *G. hybridus* were excised and adaxially cultured on basal MS medium containing 2 % (w/v) activated charcoal and various concentrations (0-35 µM) of auxins i.e. NAA, IAA, 2,4-D. Maximum regeneration frequency (9 %) was observed on MS medium containing 10 µM NAA with average 0.66 number of shoots per explant were

recorded. The average shoot length of regenerated shoot was found to be 0.9 cm on the same medium. In the absence of NAA, 6.67 % regeneration frequency was recorded with an average shoot length of 0.11 cm. The regeneration frequency increased from 6.67 % to 10.33 % when 2.5 µM IAA was incorporated in MS medium with no change in number of shoots and length of regenerated shoots. Maximum regeneration (13 %) was recorded on MS medium containing 5 µM 2,4-D and average length of regenerated shoot was found to be 0.12 cm on the same medium. However, no regeneration was recorded on medium containing higher concentrations of auxins (Figure 4.1 and Table 2) .

Figure 4.1 Leaf explants showing direct organogenesis in presence of auxins. **A.** Basal MS medium **B.** 2.5 µM Indole-3-acetic acid (IAA) **C**. 5 µM 2,4-Dichlorophenoxyacetic acid (2,4-D) **D**. 10 µM α-naphthaleneacetic acid (NAA).

Table 2. The effect of auxins on shoot organogenesis in the cultures vessel of *G. hybridus* on basal MS medium containing various concentrations (0-35 µM) of NAA, IAA and 2,4-D.

PGR	Concentration (µM)	Regeneration (%)	Number of shoots per explant	Shoot length (cm)
NAA	0	6.67^{ab}±1.66	1.00^{a}±0.00	0.11^{a}±0.017
	2.5	0.00^{b}±0.00	0.00^{a}±0.00	0.00^{d}±0.00
	5	0.00^{b} ±0.00	0.00^{c}±0.00	0.00^{d}±0.00
	10	8.67^{a}±4.66	0.66^{b}±0.33	0.90^{a}±0.051
	15	0.00^{b}±0.00	0.00^{c}±0.00	0.00^{d}±0.00
	25	0.00^{b}±0.00	0.00^{c}±0.00	0.00^{d}±0.00
	35	0.00^{b}±0.00	0.00^{c}±0.00	0.00^{d}±0.00
IAA	2.5	10.33^{a}±3.8	1.00^{a} ±0.00	0.11^{b}±0.017
	5	0.00^{b}±0.00	0.00^{c}±0.00	0.00^{d}±0.00
	10	0.00^{b}±0.00	0.00^{c}±0.00	0.00^{d}±0.00
	15	0.00^{b}±0.00	0.00^{c}±0.00	0.00^{d}±0.00
	25	0.00^{b}±0.00	0.00^{c}±0.00	0.00^{d}±0.00
	35	0.00^{b}±0.00	0.00^{c}±0.00	0.00^{d}±0.00
2, 4-D	2.5	0.00^{b}±0.00	0.00^{c}±0.00	0.00^{d}±0.00
	5	11.67^{a}±1.67	1.00^{a}±0.00	0.12^{b}±0.25
	10	0.00^{b}±0.00	0.00^{c}±0.00	0.00^{d}±0.00
	15	0.00^{b}±0.00	0.00^{c}±0.00	0.00^{d}±0.00
	25	0.00^{b}±0.00	0.00^{c}±0.00	0.00^{d}±0.00
	35	0.00^{b}±0.00	0.00^{c}±0.00	0.00^{d}±0.00

Data were recorded after 15 weeks of culture and analysed by one way ANOVA. Means were compared using Newman keuls test $P \leq 0.05$. Values followed by same lowercase letters (within columns) were found to be non-significant at $P \leq 0.05$.

4.3 The effect of sucrose concentration on shoot proliferation and rooting of microshoots of *G. hybridus*

The shoot clumps of *G. hybridus* were cultured on MS medium containing different concentrations of sucrose [2 -10 % (w/v)] to examine effect on shoot multiplication, root formation and cormlet production. A positive correlation was observed between sucrose concentration and shoot growth. Although, shoot proliferation and multiplication was observed on all the medium combinations but the shoot length was found to be significantly affected by varying sucrose concentrations (Figure 4.2 and 4.3).

A continuous increase in shoot length was observed from 6.16 to 9.16 cm with increase in sucrose concentration from 2 to 8 % (w/v). However, further on medium containing more than 8 % (w/v) sucrose, decrease in shoot length was recorded. It was also noteworthy vegetative growth of the shoot clumps was retarded on medium containing 10 % (w/v) sucrose concentration (Figure 4.2 and 4.3).

In addition to shoot multiplication, sucrose concentration was also found to have an important impact on root formation in microshoots of *G. hybridus*. Root formation was initiated on all the medium combinations but number of roots per shoot clump were found to be directly affected by the sucrose concentration (Figure 4.2 and 4.3).

The number of roots per explant were found to increase from 2.33 to 17.5 with increasing sucrose concentration. Maximum number of roots per shoot clump was found on the MS medium containing 8 % (w/v) sucrose. Further increasing the sucrose concentration resulted in a significant decrease of number of roots per explant (Figure 4.2 and 4.3).

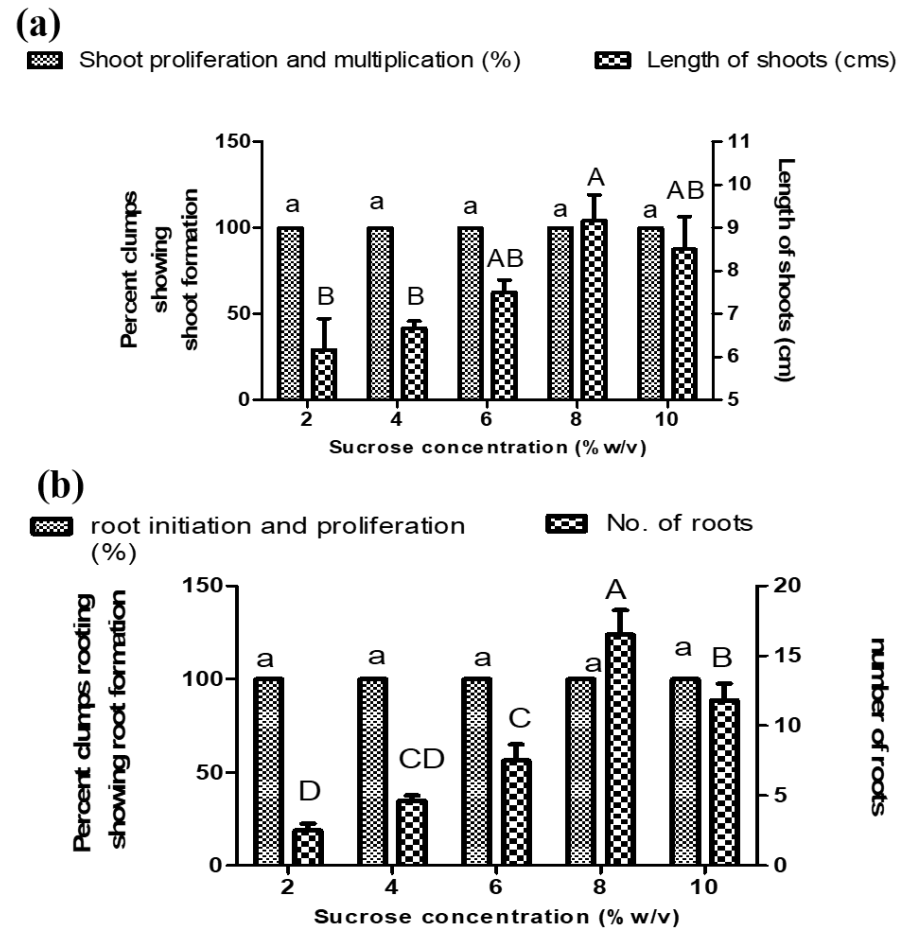

Figure 4.2 Changes in shoot multiplication rate and rooting of *G. hybridus* on medium containing various concentration [2-10 % (w/v)] of sucrose. Data were recorded after 8 weeks of culture and analysed by one way ANOVA. Means were compared by using Newman keuls test at P≤0.05. Values followed by same lowercase letters (between percent shoot proliferation) and uppercase letters (between shoot length) were found to be non-significant at P≤0.05.

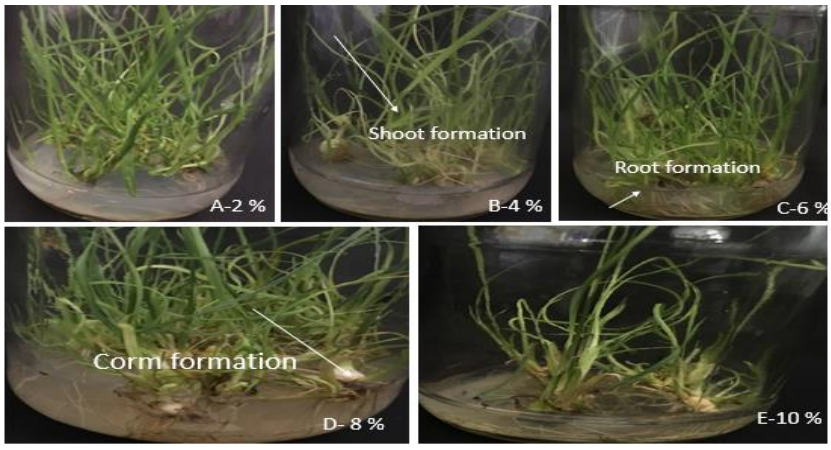

Figure 4.3 Effect of sucrose concentration [2-10 % (w/v)] on shoot and root growth in cultures of *G. hybridus* **A-E** Shoot multiplication and root formation in cultures of *G. hybridus* on MS medium supplemented with **A** 2 % **B** 4 % **C** 6 % **D** 8 % **E** 10 % sucrose**.**

4.4 The effect of sucrose concentration on cormlet fomation of *G. hybridus*

Higher sucrose concentration was found to influence the corm initiation and development. Cormlet formation was observed in cultures containing 8 % (w/v) and 10 % (w/v) sucrose concentration. Maximum number of cormlets (4.8) per explant were observed with average weight of 0.18 g at sucrose concentration 8 % (w/v). However, at higher sucrose concentration [10 % (w/v)], cormlets were observed to be (3.2) per explant with average weight of 0.08 g (Figure 4.4, Table 3).

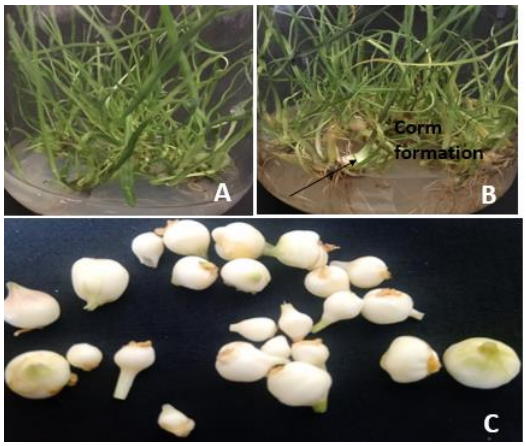

Figure 4.4 Effect of sucrose concentration on the corm formation in cultures of *G. hybridus* **A** No corm formation in cultures on MS medium containing 2 % (w/v) sucrose **B** Corm formation from the cultures of *G. hybridus* on MS medium containing 8 % (w/v) sucrose. **C** Harvested cormlets.

Table 3. Corm formation in the cultures of *G. hybridus* on basal MS medium containing various concentration [2 -10 % (w/v)] of sucrose.

Sucrose concentration [% (w/v)]	No. of corms per explant	Average weight of corms (g)
2	$0.00^{c}\pm0.00$	$0.00^{c}\pm0.00$
4	$0.00^{c}\pm0.00$	$0.00^{c}\pm0.00$
6	$0.00^{c}\pm0.00$	$0.00^{c}\pm0.00$
8	$4.80^{a}\pm0.11$	$0.18^{c}\pm0.00$
10	$3.20^{b}\pm0.30$	$0.08^{b}\pm0.00$

Data were recorded after 16 weeks of culture and analysed by one way ANOVA. Means were compared using Newman keuls test $P\leq0.05$. Values followed by same lowercase letters (within columns) were found to be non-significant at $P\leq0.05$.

4.5 The effect of sucrose concentration and Heat shock on shoot proliferation of *G. hybridus*

In order to evaluate the effect of heat shock on shoot proliferation in the cultures of *G. hybridus* growing on medium containing various concentrations of sucrose [2 -10 % (w/v)] were exposed to a heat shock at 45 ℃ for 1 hour after 15 days of culture. A significant increase in shoot proliferation was observed on all the medium combinations subjected to heat shock as compared to the cultures without heat shock (Figure 4.6, Table 4).

In case of heat shock, shoot length increased considerably from 9.16 to 14.50 cm as sucrose concentration increased from 2 to 6 % (w/v) in the medium. Thereafter, increase in sucrose concentration [>6 % (w/v)] resulted in decline of shoot length and shoot length of 7.5 cm was recorded on medium containing 10 % (w/v) sucrose. Moreover, maximum number of shoots per explant (14.50) were recorded on medium containing 6 % (w/v) sucrose, whereas minimum number of shoots per explant (3.23) were recorded on medium containing 10 % (w/v) sucrose (Figure 4.6, Table 4)

4.6 The effect of sucrose concentration and Heat shock on the rooting of microshoots of *G. hybridus*

Root formation (100 %) was observed after exposure to heat shock in all cultures of *G. hybridus*. The average root length increased from 3.53 to 10.66 cm with an increase in sucrose concentration form 2 to 6 % (w/v). However, average root length reduced to 3.03 cm when 10 % (w/v) sucrose was incorporated to the medium (Figure 4.6, Table 4). The average number of roots also increased from 9.6 to 27 as concentration of sucrose increased from 2 to 6 % (w/v) (Figure 4.6). On medium containing 10 % (w/v) sucrose, minimum number of roots (4) were recorded.

Table 4. Effect of heat shock on cultures of *G. hybridus* on basal MS medium containing various concentrations [2 -10 % (w/v)] of sucrose.

Sucrose concentration % (w/v)	Shoot proliferation (%)	No. of shoots	Shoot length (cm)	Root proliferation (%)	No. of Roots	Root length (cm)
2	100^{a}±0.00	7.37^{ab}±1.12	9.16^{b}±0.44	100^{a}±0.00	9.66^{bc}±1.85	3.53^{c}±0.13
4	100^{a}±0.00^{-}	10.86^{a} ±1.46	12.33^{a}±0.44	100^{a}±0.00	14.33^{b}±2.86	5.06^{bc}±0.13
6	100^{a}±0.00	12.33^{a}±1.22	14.50^{a}±0.28	100^{a}±0.00	27.00^{a}±1.52	10.66^{a}±0.38
8	100^{a}±0.00	6.77^{ab}±0.99	8.50^{b}±1.20	100^{a}±0.00	14.66^{b}±1.76	6.23^{b}±0.11
10	100^{a}±0.00	3.23^{b}±0.73	7.50^{b}±0.78	100^{a}±0.00	4.00^{c}±1.55	3.03^{c}±0.57

Data were recorded after 4 weeks of culture and analysed by one way ANOVA. Means were compared using Newman keuls test P≤0.05. Values followed by same lowercase letters (within columns) were found to be non-significant at P≤0.05.

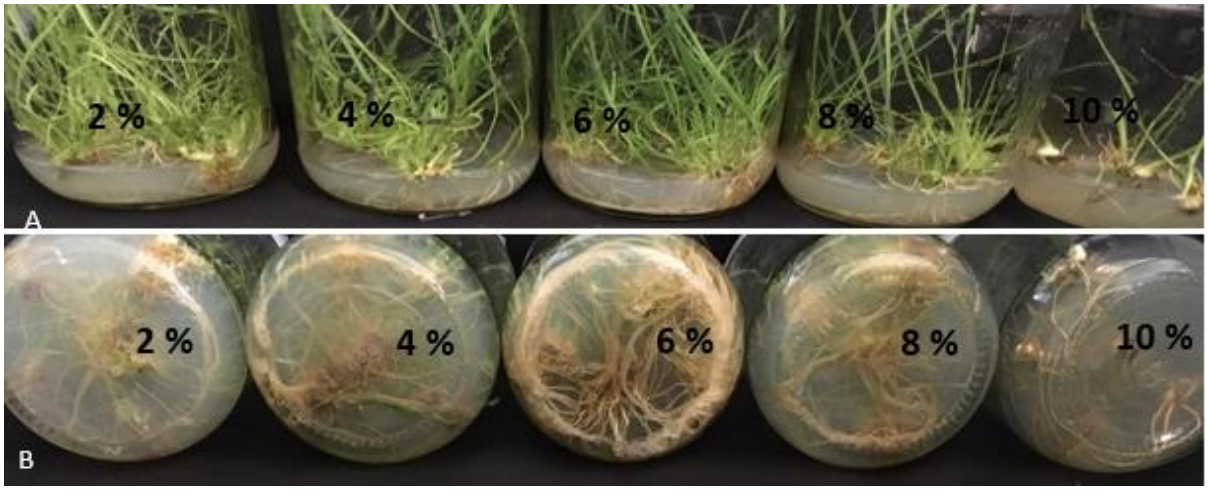

Figure 4.5 Effect of sucrose concentration [2-10% (w/v)] subjected to heat shock (45°C for 1 hour) on morphogenesis in cultures of *G. hybridus* **A** Shoot proliferation and multiplication after subjecting to heat shock in cultures on medium containing various concentrations (2-10 % w/v) of sucrose. **B** Root formation after subjecting to heat shock in cultures of *G. hybridus* on medium containing various concentrations [2-10 % (w/v)] of sucrose.

4.7 The effect of plant growth inhibitors on cormlet formation on *G. hybridus*

The shoot clumps of *G. hybridus* were cultured on basal MS medium containing 8 % (w/v) sucrose and various concentrations (0-20 µM) of different plant growth inhibitors such as paclobutrazol, Chloro Choline Chloride (CCC), daminozide to study their effect on growth and cormlet formation of *G. hybridus.* It was interesting to note that shoot proliferation and multiplication decreased with the increasing concentration of paclobutrazol whereas, 100 % shoot proliferation and multiplication was observed in the cultures of *G. hybridus* on medium containing various concentrations of CCC and daminozide (Figure 4.7)

In the presence of paclobutrazol (10 µM), thick green leaves were observed along with formation of organogenic calli on medium containing 5 µM, 15 µM and 20 µM paclobutrazol (Figure 4.7). Maximum number of cormlets (1.9) per shoot clump were observed on medium containing 10 µM paclobutrazol (Figure 4.7, Table 5).

However, CCC and daminozide had a positive effect on shoot proliferation and multiplication present at different concentrations. It was also observed that proliferated shoots appeared to be much thicker and greener on medium containing 20 µM CCC or 10 µM daminozide when compared to control medium (Figure 4.7). Maximum (3.86) and minimum (1.4) number of cormlet per clump were observed on medium containing 20 µM and 5 µM of CCC respectively. Similarly, in case of daminozide, maximum (3.3) and minimum (2.6) number of cormlet per clump were observed on medium containing 10 µM and 15 µM of daminozide respectively (Figure 4.7, Table 5).

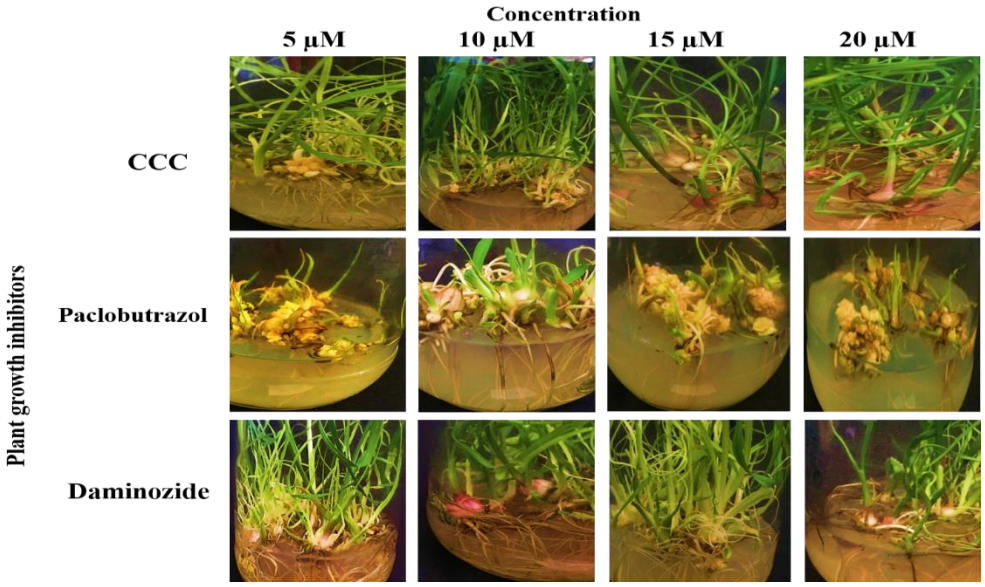

Figure 4.6 Effect of various concentrations (5-20 µM) of plant growth inhibitors such as Chloro Choline Chloride, paclobutrazol and daminozide on morphogenesis and cormlet formation in cultures of *G. hybridus*.

Table 5. The effect of various concentration (0-20 µM) of plant growth inhibitors such as Chloro Choline Chloride, paclobutrazol and daminozide cormlet formation in the cultures of *G. hybridus* on MS medium containing 8 % (w/v) sucrose concentration.

Plant growth inhibitors	Concentration (µM)	Average no. of cormlets per shoot clump
Chloro Choline Chloride	0	2.4^{abc}
	5	1.4^{cd}
	10	2.8^{abc}
	15	3.3^{ab}
	20	3.9^{a}
Paclobutrazol	5	0^{d}
	10	1.9^{bc}
	15	0.6^{d}
	20	0.3^{d}
Daminozide	5	2.9^{abc}
	10	3.3^{ab}
	15	2.6^{abc}
	20	2.9^{abc}

Data were recorded after 16 weeks of culture and analysed by one way ANOVA. Values followed by same lowercase letters were found to be non-significant at $P \leq 0.05$.

5. DISCUSSSION

Gladiolus hybridus Hort. is economically important floriculture crop. However, it has lower propagation and yield due to attack of pathogens and slow multiplication rate. Thus, in present study various factors have been studied to enhance micropropagation potential of *G. hybridus*. A number of studies had been undertaken on micropropagation of *G. hybridus* (Aftab et al. 2005; Prasad et al. 2006; Priyakumari et al. 2005; Roy et al. 2006; Tripathi et al. 2017). Keeping the view, present study was undertaken to examine the role of the various factors (i.e. PGRs, sucrose concentration, heat shock, plant growth retardants) on morphogenesis and cormlet formation in *G. hybridus.* The role of these factors on shoot proliferation, cormlet formation and organogenesis had been earlier reported in other cultivars of *Gladiolus* (Dantu et al. 1994; Kumar et al. 1999; Kumar et al. 2002; Nhut et al. 2004; Memon et al. 2010; Kumar et al. 2018).

The effect of various combination and concentrations of auxin (NAA) and cytokinin (BA) on shoot multiplication and proliferation was studied during present investigation. Maximum shoot length was observed on medium containing 2.5 µM each of NAA and BA (Table 2). As reported earlier, auxin and cytokinins had significant effect on shoot multiplication of *Gladiolus* (Dantu et al. 1995; Kumar et al. 1999; Priyakumari et al. 2005).Thus, our results are in line with these reports.

In the present study, in vitro shoot organogenesis was achieved from leaf segments of *G. hybridus* on medium containing different concentrations (0-35 µM) of auxins such as NAA, IAA and 2,4-D (Figure 4.2, Table 3). In *Gladiolus* this kind of study is very limited. However, many reports had been already reported shoot organogenesis from various explants such as corm slice (Sinha et al. 2002), cormel tips (ziv et al. 2000) etc on medium containing cytokininis. Earlier some reports also highlighted shoot organogenesis in various plants with the incorporation of auxins (Pablo et al. 2001; Vanegas et al. 2002; Pragya et al. 2011).

In the present study, it was recorded that with increasing concentration of sucrose, shoot and root length increased significantly. Maximum shoot length (9.16 cm) was found on basal MS medium containing 8 % (w/v) sucrose (Figure 4.3, 4.4). Previously, it has been reported that the addition of high concentration of sucrose in the medium have positive effect on plant growth (Dantu et al. 1994; Kumar et al. 1999; Nagraju et al. 2002; Kumar et al. 2011) including shoot multiplication and proliferation (De bryun et al. 1992; Kumar et al. 1999), induction of rooting and increase of root length (Romano et al. 1995; Kumar et al. 1999) and induction of somatic embryogenesis from the callus of *Gladiolus* (Loiseau et al. 1995; Kumar et al. 2002). Sucrose is widely utilized as carbohydrate source and played a major role in growth and development of plant under *in vitro* conditions (Coleman et al. 2009; Winter and Huber 2007; Kumar et al. 2011).

Further, it was also reported that relatively higher concentration of sucrose is responsible for the growth of tuberous organs (Taeb et al. 1990; Kumar et al. 2010; Dantu et al. 1987; Nagraju et al. 2002; Dharmasena et al. 2011). In the present study, addition of higher concentration of sucrose [>10 % (w/v)] resulted in shoot growth inhibition and formation of small cormlets (Figure 4.5, Table 4), which may be due to osmotic effect of sugars (Nagraju et al. 2002). The results are in line with previous findings, where cormlet formation and increase in the weight of cormlet was directly correlated with increase of sucrose concentration (Ziv et al. 1979; Steinitz and Yahel 1982; Sutter 1986; Dantu et al. 1995; Dharmasena et al. 2011; Jala et al. 2013). Various other studies reported that maximum cormlet formation occurs on the medium containing high sucrose (Goo and Kim 1994; Dantu et al. 1995; Kumar et al. 1999; Kumar et al. 2002; Memon et al. 2012).

The present study also indicated that in addition to different concentrations of sucrose by heat shock is beneficial for shoot multiplication and induction of rooting in the cultures of *G. hybridus* (Figure 4.6). The changes in shoot formation can be due to alternation in endogenous

PGR level (Altschuler et al. 1992; Blakesley et al. 1995; Kumar et al. 1996, 1999). It is also reported that rooting played an important role in acclimatization of plants (Romano et al. 1995; Kumar et al. 1999; Memon et al. 2012). Heat shock induces accumulation of higher level of IAA and polyamines endogenously, which can be corelated to increase in rooting frequency (Kares et al. 1990; Altschuler et al. 1992; Blakesley et al. 1993; Kumar et al. 1996, 2002, 2013). The effect of heat shock had been earlier reported in various plants influencing the plant growth under culture conditions (Altschuler et al. 1992; Yang et al. 1992; Kumar et al. 1999, 2002, 2011).

The incorporation of various growth inhibitors such as CCC, paclobutrazol, daminozide to basal MS medium reported to influence shoot elongation, rooting and cormlet formation (Dantu et al. 1995; Kumar et al. 2002; Mansuroglu et al. 2009; Kumar et al. 2011; Memon et al. 2012). They are commonly known as inhibitors of Gibberellic acid (GA) biosynthesis (Mansuroglu et al. 2009; Kumar et al. 2011). It was reported that these growth inhibitors induces cormlet formation (Kumar et al. 2011). In present study, maximum number of cormlets were observed at 20 µM CCC and 10 µM each of paclobutrazol and daminozide. A direct correlation between shoot growth and cormlet formation had been reported earlier in many plant species including *Gladiolus* (Wang et al. 1985, 1987; Steintiz et al. 1989, 1991; Kumar et al. 2011; Memon et al. 2012).

Thus, from above mentioned results, it can be concluded that sucrose concentration, heat shock, PGRs, plant growth retardants significantly influenced the morphogenesis of *G. hybridus*.

6. CONCLUSION

Gladiolus hybridus Hort. is commercially important ornamental and floriculture crop. From the present study, it was concluded that PGRs ,sucrose concentration, heat shock, plant growth inhibitors played an important role in growth, morphogenesis and cormlet formation of *G. hybridus*.

1. The cultures of *G. hybridus* were multiplied on basal MS medium containing 3 % (w/v) sucrose.
2. Significant increase in shoot length was recorded on medium containing 2.5 µM each of NAA and BA.
3. Shoot regeneration was also recorded from the leaf segments of *G. hybridus* when cultured on MS containing either of 10 µM NAA, 2.5 µM IAA or 5 µM 2,4-D.
4. MS medium fortified with 8 % (w/v) sucrose was found to be optimum for shoot elongation, induction of rooting, and cormlet formation.
5. Cultures of *G. hybridus* when subjected to heat shock of 45 ºC for 1 hour after 15 days promoted maximum shoot growth and rooting on MS medium containing 6 % (w/v) sucrose.
6. The addition of paclobutrazol to the medium lead to inhibition of shoot, root and promoted cormlet formation. Maximum number of corms were observed at 10 µM paclobutrazol.
7. In presence of daminozide and CCC, shoots were observed to be thicker and greener as compared to control. However, Maximum number of corms were observed at 20 µM and 10 µM of CCC and daminozide respectively.

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Annexure I

Composition of Murashige and Skoog medium

Macronutrients (mg/l)

NH_4NO_3	1650
KNO_3	1900
$MgSO_4.7H_2O$	370
$CaCl_2.2H_2O$	440
KH_2PO_4	170

Micronutrients (mg/l)

$MnSO_4.H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Na_2Fe-EDTA	30

Vitamins (mg/l)

Thiamine HCl	0.1
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Glycine	2.0
Myo – inositol	100

Sucrose	30g/l
Agar-agar	7g/l

Annexure II

Stock solution preparation of Plant Growth Regulators

S.no	Growth Regulator	Amount (mg)	Solvent	Volume make up (100 ml)
1.	2.5µM BA	56.31	HCl	Distilled H_2O
2.	2.5µM NAA	46.5	KOH	Distilled H_2O
3.	2.5µM 2,4-D	55.01	KOH	Distilled H_2O
4.	2.5µM IAA	43.79	KOH	Distilled H_2O

Annexure III

Stock solution preparation of Plant Growth Inhibitors

S.no	Growth Inhibitors	Amount (mg)	Solvent	Volume make up (20 ml)
1.	5µM Chloro Choline Chloride	15.80	DMSO	Alcohol
2.	5µM Paclobutrazol	29.38	DMSO	Alcohol
3.	5µM Daminozide	16.01	DMSO	Alcohol

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