***IN VITRO* REGENERATION OF GLADIOLUS (*Gladiolus* sp.)**

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SHER-E-BANGLA NAGAR, DHAKA -1207

JAN-JUNE, 2021

IN VITRO REGENERATION OF GLADIOLUS (*Gladiolus* sp.)

BY

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REGISTRATION NO: 14-5857

A Thesis
Submitted to the Faculty of Agriculture,
Sher-e-Bangla Agricultural University, Dhaka,
in partial fulfilment of the requirements
for the degree of

MASTER OF SCIENCE

IN

BIOTCHNOLOGY

SEMESTER: JAN-JUNE, 2021

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This is to certify that the thesis entitled ***IN VITRO RAPID REGENERATION OF GLADIOLUS (Gladiolus sp.)*** *submitted to the Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka-1207, in partial fulfillment of the requirements for the degree of* ***MASTER OF SCIENCE IN BIOTECHNOLOGY,*** *embodies the result of a piece of bonafide research work carried out by* ***DRISTY BAIDYA,*** ***Registration No. 14-5857****, under my supervision and guidance. No part of this thesis has been submitted for any other degree or diploma.*

I further certify that any help or sources of information as has been availed of during the course of this work has been duly acknowledged & style of the thesis have been approved and recommended for submission.

Dated: December,2021
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ACKNOWLEDGEMENTS

All praises are due to Almighty God, who created everything in the universe, empower me to complete the research work and the thesis for the degree of Master of Science in Biotechnology.

I pay my deepest gratitude to my research supervisor **Fahima Khatun, Assistant Professor,** Department of Biotechnology, Sher-e-Bangla Agricultural University, Dhaka-1207, Bangladesh, for her Patience, motivation, enthusiasm, immense knowledge, practical co-operation, stimulating counsel, distinguishable scholastic guidance and sincere advice throughout the research work, giving valuable suggestion, guidance, fruitful discussion and timely instruction. This work would not have been possible without her guidance, support and encouragement. Under her guidance I successfully overcome many difficulties and learn a lot. I can't forget her hard work.

I also feel pleasure to extend my heartiest respect, deepest gratitude and cordial thanks to my respectable Co-Supervisor **Dr. Md. Ekramul Hoque, Professor** , Department of Biotechnology, Sher-e-Bangla Agricultural University, Dhaka-1207, Bangladesh, for his valuable teaching, inspiration and encouragement during the whole course of study. I am also grateful to all the teachers Department of Biotechnology, Sher-e-Bangla Agricultural University, Dhaka-1207, Bangladesh for their inspiration and helpful advice throughout the research work.

I express my sincere respect to chairman, **Dr. Mohammad Nazrul Isalm, Asociate Professor,** Department of Biotechnology, Sher-e-Bangla Agricultural University, Dhaka-1207, Bangladesh for their valuable advice, encouragement, proper assistance and support during the period of research works.

I also wish to acknowledge my indebtedness to staff of the Department of Biotechnology for their co-operation in the implementation of research works.

Finally, I would like to thanks my parents, **Pankaj Baidya** and **Maya Roy** who had also sacrificed their happiness for me and rendered financial support and encouragement throughout my academic career. They all always with me. The research work would not have been possible without them. I will be always indebted to them and will be try to give a better volume in future.

JAN- JUNE , 2020 **The Author**
Dhaka, Bangladesh

IN VITRO REGENERATION OF *Gladiolus* sp.

BY

DRISTY BAIDYA

ABSTRACT

The present research was carried out in Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka -1207 from the period of July 2020 to June 2021 for in vitro rapid regeneration of Gladiolus. The healthy, disease free cormel of 1.5 cm- 2.0 cm were used as explants, was sterilized by 0.1% Bavistin followed by 0.1% HgCl2 mixing with few drops Tween-20. The explant were inoculated in MS media supplemented with different combination of Benzyl Adenene (1.0, 2.0, 3.0, 4.0, 5.0 mg/L) and Naphthalene Acetic Acid (0.5, 1.0, 1.5, 2.0 mg/L). The minimum days (11.00) to shoot (73.80%) induction with the highest percent of shoots induction were achieved in 4.0 mg/L BA. The highest number of shoot (1.60, 2.20, 2.40) and maximum shoot length (0.74 cm, 1.18 cm and 1.32 cm) were also observed in 4.0 mg/L BA and at 21, 35 and 45 Days After Induction (DAI) respectively. The combine effect of auxins and cytokinins were also investigated where 4.0 mg/L BA + 0.5 mg/L NAA performed the best incase highest frequency of shoot induction (74.80%) with minimum days (10.60) and gave the maximum shoot number (1.80, 2.60, 2.80) and shoot length (1.40 cm, 2.32cm, 2.82 cm) at 21, 35 and 42 DAI respectively. Moreover, the treatment 4.0 mg/L BA + 2.0 mg/L NAA showed the highest frequency (79.80%) of root induction with minimum days (8.40) and gave the maximum number (4.20, 6.00, 7.20) of root with optimum root length (1.02 cm, 1.26 cm, 1.34 cm) at 21, 35 and 42 DAI. In shaded condition the survival rate of plantlet was 83.33% and in open atmospheric condition the survival rate was 76%. Overall, a reliable and feasible protocol had been developed for in vitro multiplication of Gladiolus which can be a useful tool for tissue culture of Gladiolus

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LIST OF ACRONYMS

AEZ	=	Agro-Ecological Zone
BARI	=	Bangladesh Agricultural Research Institute
BBS	=	Bangladesh Bureau of Statistics
LAI	=	Leaf area index
ppm	=	Parts per million
et al.	=	And others
N	=	Nitrogen
TSP	=	Triple Super Phosphate
MP	=	Muriate of Potash
RCBD	=	Randomized complete block design
DAS	=	Days after sowing
ha^{-1}	=	Per hectare
G	=	gram (s)
Kg	=	Kilogram
µg	=	Micro gram
SAU	=	Sher-e-Bangla Agricultural University
SRDI	=	Soil Resources and Development Institute
HI	=	Harvest Index
No.	=	Number
Wt.	=	Weight
LSD	=	Least Significant Difference
^0C	=	Degree Celsius
mm	=	millimeter
Max	=	Maximum
Min	=	Minimum
%	=	Percent
cv.	=	Cultivar
NPK	=	Nitrogen, Phosphorus and Potassium
CV%	=	Percentage of coefficient of variance
Hr	=	Hour
T	=	Ton
viz.	=	Videlicet (namely)

CHAPTER I

INTRODUCTION

Gladiolus (*Gladiolus grandiflora* L.), belonging to the family Iridaceae, is one of the most commercially grown, economically important and widespread flowering plants and known as the queen of bulbous flowers (Azimi and Banijamali, 2019). The name "gladiolus" comes from the Latin "gladius" and means "sword" because the shape of the leaf resembles a sword. Because of this, they are sometimes referred to as "sword lilies". Gladioli is native to and around Africa, particularly South Africa, but some species also originate from the Mediterranean and surrounding areas of Europe (Singh *et al*., 2021).

The genus Gladiolus has 266 species all over the world. Gladiolus is exceptionally well known as a cut blossom, assessed 8th position in world trade (Solanky *et al.,*2019). Gladiolus could be a genus of perpetual bulbous blooming plants. These appealing, perpetual herbs are semi-hardy in mild climates. They develop from adjusted, symmetrical corms, that are encompassed in a few layers of brownish, sinewy tunics. Gladiolus are accessible in a wide assortment of colors counting white, green, cream, yellow, buff, orange, salmon, pink, ruddy, lavender, purple, blue and terra cotta. The cut plant will appreciate the vase life for least 6 days. Like most blooms, gladiolus carries a particular class and exceptionally much in request amid the month of November. Because of its florets' many colors and forms, gladiolus cultivars are classified according to floret size, basic color, and depth of color. The size of the floret can be determined by measuring the diameter of the lowest floret without flattening its petals. Gladiolus spikes are most popular in flower arrangements and for preparing attractive bouquet (Mishra *et al.,* 2006).

Gladiolus has a short life cycle of about 110 to 120 days and requires a temperature regime of 10 to 25°C (Memon, 2012). Gladiolus plants are classified into two types. They come in two varieties: summer flowering and spring flowering. The summer variety is more common and is widely used in the production of cut flowers (Azimi, 2017). Gladiolus has been cultivated all over the world since the late 16th century. The United States (Florida and California) are the leading producers of gladiolus, followed by

Holland, Italy, the Netherlands, France, Portugal, Poland, Bulgaria, Belgium, Brazil, Australia, Israel, and India (Memon, 2012).

Gladiolus is a popular flower use in landscaping and religious ceremonies. For aesthetic and decorative purposes gladiolus is mostly grown in pots (Solanky *et al*., 2019*).* There are some medicinal health benefits of dried bulb which is ground into a powder. Gladiolus flower bulb powder may help in curing common cold, relieve from diarrhea, alleviate constipation. Gladiolus flower bulb powder may reduce pain during menstruation in women, help in curing fungal infections, relieving ulcers, curing meningitis (gardenplants.comparespecies.com).

Gladiolus is a potential cut flower in the Bangladeshi economy and due to its popularity and demand in the country, it has become one of the first commercial targets for large-scale production. In the late 1980s, only a few shops in Dhaka city sold fresh cut flowers. Fresh cut flowers are now sold in the majority of Bangladesh's cities, districts, and even upazilla towns. In Bangladesh, gladiolus production is highest in Dhaka, Jessore, Kalakair, and Sreepur of Gazipur (Rakibuzzaman *et al.,* 2018). As a cut flower it has a great economic value and is widely used for decoration (Tazuddin S.M., 2020).

Gladiolus propagated by seeds or corm formation or by cormel differentiation. Although, seeds are successful means for the propagation of gladiolus but seed-raised plants may not produce true-to-type population and one mother corm normally produces about 25 cormels each season. With the enlargement of leaves and spikes, new corm forms at the base of the leaves and when the union of daughter corm and parent, stolon's grow out, terminating in cormels which are used for asexual propagation (Bhat *et al*., 2018). Moreover, for blooming it takes four seasons (Memon, 2012). The asexual propagation by corm and cormel differentiation is another conventional method of multiplication but it may transmit several viral, fungal and bacterial diseases e.g., *Fusarium* corm rot, bacterial leaf rot, Botrytis blight, etc., which cause a great loss (Aftab *et al*., 2008).

Breeders all over the world are very interested in breeding gladiolus, and each year a large number of cultivars are developed with the goal of producing novel colors, being resistant to biotic and abiotic stresses. However, conventional breeding takes about 10-14

years to produce a cultivar. Corms and cormels are used for traditional propagation, but their multiplication rate is slow, which accounts for the long duration of cultivar production. A gladiolus plant can produce 1-2 corms and 5-50 cormels under natural conditions, depending on the genotype. As a result, it takes nearly 8-10 years to produce the required amount of planting material on a commercial scale for a new variety. Furthermore, many abiotic and biotic stresses have negative consequences. Fusarium rot and a high percentage of corm spoilage also have a significant impact on commercial corm and cormel production (Memon, 2012).

Moreover, plant tissue culture provides an excellent opportunity to produce large quantities of disease-free, true-to-type healthy stock in a relatively short period of time. By using various hormones and different types of in vitro methods, the time lapse for 8-10 years can be reduced to 2 years, allowing for the propagation of unique genotypes with low propagation rates. Most of the reports of gladiolus tissue culture indicated that Murashige and Skoog's (1962) medium supplemented with auxins and cytokinins is ideal for shoot initiation, multiplication and rooting (Lilien-Kipnis & Kochba, 1987). There are also some novel genotypes that have unique spike colors but cannot produce a sufficient number of corms or cormels, such as Pusa Urmil, that can be grown in tissue culture to produce a large number of disease-free planting materials. Furthermore, many *in vitro* methods, such as mutagenesis, protoplasmic fusion, or DNA uptake, are used to recover unique variants. If transgenic cell selection is effective, callus regeneration is preferable to direct regeneration for transformation. In the presence of IBA the growth of shoots was strongly inhibited as reported by Ruffoni *et al.*, (2012). Shoot regeneration was promoted using NAA, BA only, and their combinations. BAP is mostly used by the researcher because it is much more active then naturally forming cytokinis (Waring and Phillips, 1981). The root initiation was higher IN Murashige and Skoog formulation compared to the Linsmaired and skoog media for gladiolus (Wilfret, 1971). For the purpose of mass multiplication of desired genotypes and gene conversation (Pragya., 2012).

In vitro multiplication of Gladiolus sp. was accomplished by using axillary buds, cormels, shoot tips, and inflorescence axes. Furthermore, with the help of various hormones and growth regulators, successful *in vitro* corm formation organogenesis and

somatic embryogenesis have been achieved. There are numerous hormones that regenerate shoots and roots. Auxin is a plant hormone that promotes the development of adventitious roots. Indole acetic acid (IAA), a naturally occurring auxin, is found in plants and is involved in nearly every aspect of plant growth and development. Commercially available synthetic forms of auxin include Indolebutyric acid (IBA) and napthaleneacetic acid (NAA), which are used for root regeneration (Cerveny & Gibson, 2005). Cytokinesis, Gibberellins, and Abscisic acid (ABA) are other common plant hormones; Among them 6-Benzylaminopurine, also known as BAP or BA, is a first-generation synthetic hormone which is commonly used for shoot regeneration.

Gladiolus is a potential cut flower in the Bangladeshi economy, and due to its popularity and demand in the country, it has become one of the first commercial targets for large-scale production. To maximize gladiolus production effective steps should be taken to meet grower demand and enable them to enter international trade. The efficient *in vitro* protocols for the maximization of propagules, producing multiple shoots by using cormels and meristem as explants, and economical and effective ways of propagation in order to meet the future demands of growers and consumers should be developed. There has been very little data since the pioneering efforts (Memon, 2012). Based on above mentioned context and prospects, the present investigation, therefore, has been carried out with the following objectives :

- To establish an effective *in vitro* regeneration protocol of gladiolus genotypes.
- To assess the hormonal effect for *in vitro* response.
- To identify the best hormonal effect for *in vitro* regeneration of *Gladiolus* sp.

CHAPTER II

REVIEW OF LITERATURE

.

Plant tissue culture is the foundation of plant biotechnology, and it includes micro-propagation, induction of soma clones, somatic hybridization, cryopreservation, and transgenic plant regeneration. Plant tissue culture is a technique that allows any plant part to be cultured on a sterile nutrient medium under controlled temperature and light conditions in order to grow. There have been some studies on this plant in Bangladesh. The Department of Dhaka University, micro-propagation (Oscar) on gladiolus cultivar. Gladiolus research is also being conducted in India and other countries

2.1 Types of explant

Different sizes of the same explants have different regenerative capacity even they are cultured on the same nutrient medium. Memon *et al.* (2013) studied different stages/sizes of various explants including nodal cultures from different stages of flower spike, whole flower buds only at sleeping stage, whole cormels of different sizes, cormel sprouts of different sizes and cormel slices of the cormel were explored and optimized for efficient shoot regeneration in three commercial grown varieties of the gladiolus. Different plant growth regulators including benzyl aminopurine (BAP), kinetin (KIN) and naphthalene acetic acid (NAA) alone or in combination with each other were used for each explant in order to find out the possibility of increasing rate of shoot induction and regeneration. Out of five explants, cormel medium size sprouts (12 days old) were evaluated the best explant in White friendship.

Memon *et al.* (2010) conducted an experiment using different explant types and media supplemented with various growth regulators. The explants *viz,* nodal cultures from different stages of flower spike, whole cormels of various size, cormel sprouts taken at

different time intervals and cormel segments of White friendship were cultured. The heading stage of nodal cultures, large sized whole cormels (0.6 g), 12 days old cormel sprouts and top segments of cormels were evaluated the best stage/size from each explant type for efficient shoot regeneration on MS medium containing BAP (4 mg L(-1)). The best responded stage/size from each explant type was further explored for rooting and cormel production. The better response for rooting was observed from the shoots of cormel sprouts on MS medium supplemented with IBA (2 mg L(-1)) and sucrose (3%) as compared to other explants.

Ferdousi *et al.* (2018) investigated *in vitro* shoot multiplication and callogenesis in Gladiolus and for this purpose, cormel, meristem and leaf explants were chosen. Different concentrations of BAP i. e., 0.5, 1.0, 1.5, 2.0 or 2.5 mgL-1 (2.22, 4.44, 6.66, 8.88 or 11.10 μM) were supplemented to the MS medium. Both cormel and meristem explants exhibited maximum multiplication on MS medium containing 1mgL-1 BAP. Cormel proved to be a better source of explants for shoot multiplication as compared to meristem.

Nhut *et al.* (2004) examined an experiment with four explant sources (shoot-tip, longitudinal corm section, basal plate and daughter corm) to test the capacity of uniform shoot formation. Six vigorous and uniform shoots could be derived from longitudinal corm explants on MS agar medium with 2.2M BA and 30 g l−1 sucrose after 15 days and were most stable and proliferative. Separate plantlet and corm formation sometimes could be achieved when shoots were cultured on MS-agar medium with IBA (from 1.0 to 2.5M) at phytosynthetic photon flux (PPF) of 30 or 40mol m−2 s−1, respectively and at 15–20 or 25 ◦C, respectively.

Datta, (2020) conducted an *in vitro* regeneration and transformation studies on two cultivars of gladiolus. Cormels of 1.0 to 1.5 cm diameter cut into 2–3 mm thick slices of top, middle and bottom, and in *vitro* derived shoot tips were used as explants on MS medium containing 18.6 μM kinetin. Amongst the cormel slices, the top slice gave better shoot induction response of 89% having average of 2.4 shoots per explant over both cultivars. Shoot tips without attached cormel base and inoculated in the cut-side down

orientation showed an average of 90% shooting response. *In vitro* derived shoot tips were used as explants for transformation.

2.2 Sterilization of Explant

Sanyat & Ranvir (1999) conducted an experiment in vitro propagation of gladiolus cv. American Beauty, which revealed that cormel tips are excellent explants for culture establishment in MS medium when surface sterilized with 0,1% HgCl2 for 10 min than leaf and inflorescence segments.

In vitro culture aseptic condition is always related. So, *in vitro* culture sterilization is a prerequisite. Many types of chemicals are used for this, viz, calcium hypochlorite (Bini & Bellini, 1973; Monim, 1974), Sodium hypochlorite for surface sterilization of explants (Jones & Hopgood, 1979), 5% $CaClO_4$ for 10 minutes (Ziv, 1979) and rinsed 3 times in sterile water to ensure complete aseptic buds.

Some reports also found where the use of $HgCl_2$ for surface sterilization of mature plant (Nekrosova, 1964; Bennette & McComb, 1982; Roy, Rahman, & Datta, 1987). Sometimes plant organs were sterilized in various concentration of clorok solution containing 20 drops Tween-20 per liter (Kamo, Chen, & Lawson, 1990).

2.3 Media

Cultures can be initiated and maintained on medium containing Knop's (Knop, 1965) macronutrients and Murashige and Skoog (1962, MS) micronutrients and organic components, or MS medium supplemented with 2.2-4.4 µM 6-benzyladenine (BA), 0.5-2.5 µM indole- 3-butyric acid (IBA) and 0.3 µM gibberellic acid (GA3) at 23-25°C during the light period, and 17°C in the dark period; the quantum irradiance is 46 µmol m2 s -1 for a 16 h photo-period (Sowik *et al.*, 2001). For successful micropropagation and cormel development, the chemical composition of the growing medium is the most important factor. Most of the reports of gladiolus tissue culture indicated that Murashige and Skoog's (1962) medium supplemented with auxins and cytokinins is ideal for shoot initiation, multiplication and rooting (Lilien-Kipnis & Kochba, 1987). Explant can be initiated from *in vitro* culture on media containing AgNO3 (10-20 mg/l). GA3 (28.9-57.7

µM) increased the efficiency of $AgNO_3$ significantly. Agar (0.6-0.8%, w/v) is the most commonly used gelling agent for *in vitro* culture on semi-solid medium. The agar/galactomannan mixture in the proportion of 0.3/0.3 (w/v) in MS medium exhibited better performance and enhanced shoot proliferation compared to medium containing agar (0.6%, w/v) only. Cultures were maintained at 23°C under a photosynthetic photon flux density (PPFD) of 30 µmol m-2 s -1 from 'warm-white' fluorescent lamps and 16 h photoperiod. The use of light-emitting diodes, or LEDs, in particular 70% red and 30% blue at 60 µmol m-2 s -1 re- sulted in greatest shoot proliferation, plantlet and total fresh weight when three-leaved explants of cv. Also the addition of growth retardants and increased sucrose concentration improved cormel development (Ziv, 1989, 1990; Steinitz et al., 1991). Either using IBA or 2iP with different efficiency level, cormels can be developed depending on the genotype; it is clear that in the presence of the cytokinin 2 iP either corms or shoots can develop from mother plant but in the presence of IBA the growth of shoots was strongly inhibited as reported by Ruffoni *et al.*, (2012).

Sucrose plays an important role for cormel formation in Gladiolus *in vitro* (Dantu and Bhojwani, 1987; Arora et al., 1996; Sinha and Roy, 2002; Memon et al., 2009b). It also has useful effect on multiplication of shoots (Kumar *et al*., 1999; De Bruyn and Ferreira, 1992), somatic embryogenesis (Loiseau *et al*., 1995) and rooting response of microshoots (Rahman *et al*., 1992; Romano *et al*., 1995). The higher growth of tuberous organs needs a relatively high (> 50 g/l) concentration of sucrose in the medium (Mares *et al*., 1985; Dantu and Memon *et al.*, 1705 Bhojwani, 1987; Nagaraju *et al*., 2002). Higher concentration (6 or 10%) of sucrose helps the formation of large corms (Dantu and Bhojwani, 1987). Hussain *et al.*, (1995) reported that a high concentration of sucrose (5%) with triadimefon resulted in 11 fold increase in size of *in vitro* corms in Cv. Friendship.

2.4 Effect of Growth Regulators

2.4.1 Shoot Multiplication

Aftab *et al.* (2008) investigated *In vitro* shoot multiplication and callogenesis in Gladiolus and for this purpose, cormel, meristem and leaf explants were chosen. Different concentrations of BAP i. e., 0.5, 1.0, 1.5, 2.0 or 2.5 mgL-1 (2.22, 4.44, 6.66, 8.88 or 11.10 µM) were supplemented to the MS medium. Both cormel and meristem explants exhibited maximum multiplication on MS medium containing 1mg/L BAP. Rooting of *in vitro* grown shoots was achieved on medium supplemented with 0.5mg/L (2.69 µM) NAA or 2mg/L (9.84 µM) IBA. Rooting was recorded in one month old cormel-derived callus cultures when NAA was used at either 0.3 or 0.4 mg/L concentration (1.61 or 2.15 µM). Sporadic shoot regeneration was noted from cormel explant-derived callus cultures in MS medium containing 2 mg/L (9.05 µM) 2, 4-D and 1.0 mg/L (4.44 µM) BAP.

Emek And Erdağ, (2007) experimented *in vitro* propagation method for *Gladiolus anatolicus* (Boiss.) Stapf (Iridaceae) using corm sections. Murashige & Skoog medium supplemented with different concentrations of NAA and without growth regulators were used. The highest rate of callus formation (75 %) was occured in MS medium suplrmrnted with 8.5 mg l-1 NAA. Shoot regeneration was promoted using NAA, BA only, and their combinations. The highest number of shoots per explant was obtained in MS medium containing 0.2 mg l-1 BA and 2 mg l-1 NAA (4.7 shoots per explant). Corm formation was observed in the medium with 0.1 mg l-1 BA. Rooting was obtained in the same medium, but rooting rates in shoots were very low (% 20).

Multiple shoot clusters of gladiolus were established (Prasad & Gupta, 2006) in semi-solid agar (AS) and liquid media appended with membrane raft (MR) and Duroplast foam (DF) support matrix. A difference in optimum combination of α-napthaleneacetic acid (NAA)/6-benzylaminopurine (BAP) for shoot multiplication was recorded with culture systems. Maximum regeneration of 33.15 shoots/cluster was obtained with 1 mg l^{-1} NAA and 2 mg l^{-1} BAP in liquid medium with membrane support. Shoot multiplication was observed rapid in AS and MR systems (relative differentiation rate of 5.7% per 7 days of

incubation) compared to DF system (relative differentiation rate of 4.6% per 7 days of incubation). Shoots in DF system showed faster elongation than those in AS and MR systems.

In Axillary shoot proliferation a number of cytokinin compounds are used such as BAP, Kinetin and 2iP. For specific plant species specific cytokinin is good but BAP is mostly used by the researcher because it is much more active then naturally forming cytokinis (Waring and Phillips, 1981). Dantu and Bhojwani (1995) reported that shoot culture of gladiolus initiated from axillary buds, showed best elongation on MS media containing 0.5 mg/ liter IBA in light.

In study of cultivars 'Beauls spot', 'Jo wagenaar', 'Vink's Glory' and 'Wild Rose' were cultivated on basal MS medium supplemented with BA or Kinetin each at 4-8 mg/L and solidified with 0.2% Gelrite. More shoots were produce with kinetin whereas BA produces more calluses. Higher number of shoots was gained with kinetin at 4 mg/liter (Rao *et al*., 1991). Zakustskaya and Murin (1990) outlined that higher number of shoots gained on media with BA and NAA at 0.3 and 0.5 mg/L respectively when corm buds were halves as explant.

Shoot culture of cultivar Friendship, Her Majesty and American Beauty were initiated from axillary buds from the cold stoned corms. Shoots multiplication for over 2 years at the rate of 3 fold every 2 weeks or 5 fold every 4 weeks on MS media with BA (0.5 mg/L). When the BA was omitted or the reduction to 0.1-0.2 mg/L, shoots elongation was observed (Dantu & Bhojwani, 1987). Jager *et al.*, (1998) reported that from the hypocotyl section MS medium containing 100 mg/L myoinositol, 3% sucrose and 1 mg/L NAA and BA respectively, Solidified with 0.8% Agar, Multiple shoots were occurred. Rapid Multiplication of shoots was initiated from pedicel, thalamus, nodal segment, young shoot tips of plants. The best performance was observed in MS medium containing 2.0 mg/L BAP, 3.0 mg/L BAP and 1.0 mg/L BAP + 0.5 mg/L NAA. Multiple shoot was observed best in half MS medium supplemented with 0.75 mg/L BAP (Begum and Hadiuzzaman, 1995)

2.4.2 Root Multiplication

Bettaieb *et al.* (2006) conducted an experiment to set up a new bulb formation and micropropagation of gladiolus *in vitro*, trials were undertaken using two ornemental gladiolus cultivars 'Peter pears' and 'White friend ship'. *In vitro* initiation and multiplication were observed for the two cultivars on Murashige and Skoog medium containing 2 mg/L of BA and 0.5 mg/L of AIB. *In vitro* root development and bulb formation have been occurred.

In most cases of *in vitro* regenerated shoots fails to produce roots at the root proliferation period. So that, separate root induction phase is important. For root initiation, most of the plant species required growth regulators. Most common growth regulators are Auxins, Napthaleneacetic Acid (NAA), Indole-3-butyric Acid (IBA), and Indole-3-acetic Acid (IAA), which are used for rooting. Morel and Muller (1946) reported that mineral salt medium containing NAA (0.05 mg/l) and IBA (1.0 mg/l) with sucrose (16g/l) is essential for adventitious root formation on the lamina and petioles of African violet. Kashem (1992) reported that 100% rooting was observed in several gladioli within 15-20 days when 1.0 mg/l IBA were used in half MS medium.

The root initiation was higher IN Murashige and Skoog formulation compared to the Linsmaired and skoog media for gladiolus (Wilfret, 1971). The presence of AC in the rooting medium has a beneficial effect on shoot growth as well as root length (Babu and Chawla, 2000).

2.5. *In Vitro* Regeneration

Memon (2012) investigated *in vitro* direct mode of regeneration is a faster method for mass cloning of bulbous plants. The rate of regeneration is mainly depends on a number of factors, including the type of the explants, composition of the culture medium, type and concentration of plant growth regulators and genotype of the plant material.

Various explants such as nodal buds (Memon *et al*., 2010; Grewal *et al.*, 1990; Arora *et al.*, 1996), cormel tips (Arora *et al.*, 1996), inflorescence stalk (Ziv *et al.*, 1970), axillary buds of corm (Dantu and Bhojwani, 1987; Ahmad *et al.*, 2000; Begum and

Haddiuzaman, 1995) and slices of cormel sprouts (Sinha and Roy, 2002) had been noted to be used for *in vitro* cormel production in gladiolus with the application of different growth regulators and sucrose in the medium.

The ultimate goal of *in vitro* propagation of gladiolus is the mass production of cormels (Steinitz et al., 1991; Dantu and Bhojwani, 1995; Sen and Sen, 1995; Al-Juboory *et al*., 1997; Nagaraju *et al*., 2002). The *in vitro* developed cormels can be easily stored and sown like seeds in plantation season (Wang and Hu, 1982; Ziv and Lilien-Kipnis, 1990). The transplantation difficulties also reduce during acclimatization (Ziv, 1979; Sengupta *et al*., 1984).

2.6. Acclimatization of *in vitro* propagules

It is a very critical step for successful propagation to acclimatization of *in vitro* propagules to the *ex vitro* environment. In gladiolus, successful acclimatization can be achieved by taking *in vitro* regenerates at three different stages: (i) When *in vitro* regenerated have optimum shoot/root ratio but no cormel formation; (ii) Before dormancy of the cormels but after cormel formation; (iii) When cormels goes under dormant stage and plant shoot dries up. Generally, the first option is more practiced. *In vitro* regenerated shoots are planted into rooting medium and then put it into high humidity environment with low irradiance and temperature for acclimatization. It is necessary because (i) *in vitro* plantlets are not autotrophic (McCartan *et al.*, 2004); (ii) poor formation of leaf cuticle; and (iii); weaken stomatal functioning (Preece and Sutter, 1991; Hazarika, 2006). *In vitro* grown plants also have less photosynthetic efficiency and vascular connection between the shoots and roots. This unnatural morphology, anatomy and physiology of *in vitro* plantlets (Pospisilova *et al.*, 1992, 1997; BuddendorfJoosten and Woltering, 1994; Desjardins, 1995) create difficult for the plantlets to survive *ex vitro*. In gladiolus, there are some reports of transplanting *in vitro* grown plants either from direct or indirect regeneration. No proper protocol has yet been developed for acclimatization technique in gladiolus. Ziv (1979) transferred *in vitro* raised propagules on Halfstrength MS medium containing a reduced sucrose concentration (1.5%), 0.4 mg/l thiamine, 0.5 mg/l NAA and 0.3% activated charcoal, and gown under a higher light intensity than used for maintaining the micro-propagated plants. Ziv (1991) also outlined

that the addition of paclobutrazol to the medium resulted in the development of cormels with 100% survival following transfer to the greenhouse, whereas 58% was noticed without paclobutrazol. Priyakumari and Sheela (2005) reported successful acclimatization of the gladiolus plantlets planted in 2:1 of sand and soil in plastic pots. Earlier Jager *et al.*, (1998) also reported similar results.

In *Lilium speciosum* Thunb var. *glorioso ides* Baker, 98% survival rate of rooted plantlets was observed in 35 cavity growing trays under mist condition for first four weeks (Chang *et al.*, 2000). Hannweg *et al.*, (1996) also observed more or less same results in *Bowiea volubilis*. They transplanted *in vitro* regenerated plantlets in sterilized soil (Memon *et al.*, 1707) and washed coarse river sand under three different conditions: (i) Covered tightly for 7 days to get high relative humidity; (ii) Used loose covering for 2-3 weeks to have medium relative humidity; (iii) Plantlets uncovered and mist sprayed all over it twice daily. From the mist sprayed plantlets, maximum survival rate (90.9%) as compared to other conditions was found.

The period of transplantation from *in vitro* to *in vivo* usually needs some weeks of acclimatization with gradual lowering in air humidity (Preece and Sutter, 1991; Bolar *et al.*, 1998). To minimize the losses during the hardening process of *in vitro* grown plants, it is better to induce shoots to form storage organs such as cormels in gladiolus and bulbs of lilies. These underground storage organs are generally tough and can be planted or stored when desired. However, the survival of *in vitro* plantlets with cormels/bulblets is usually based on the size of the cormels (Naik and Nayak, 2005).

Cold treatment is required by gladiolus for a period of four weeks at a temperature range of 2 to 5°C as reported by Hussey (1977). He also outlined that dormancy can also be broken when *in vitro* produced cormels are sub-cultured on a medium containing BA. A period of 4 to 8 weeks at 0 to 5°C was required to break dormancy in bulblets (Bacchetta *et al.*, 2003).

CHAPTER III
MATERIALS AND METHODS

3.1 Time and location of the experiment

The present research was carried out in Biotechnology Laboratory in the Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207 from the period of July 2020 to June 2021.

3.2 Experimental materials

3.2.1 Source of material

The planting materials of Gladiolus (*Gladiolus* sp.) were collected from Horticulture farm of Sher-e-Bangla Agricultural University.

Plate 1. Source material of Gladiolus

3.2.2 Explant

Corm was grown in horticultural field of Sher-e-Bangla Agricultural University. After establishment of cormels, the healthy, disease free 0.5 cm – 1.0 cm sized cormels were used as explants for the study of *in vitro* regeneration (Plate 2).

Plate 2. Cormels of Gladiolus used as explant

3.2.3 Instruments

Metal instruments *viz*., forceps, scalpels, needless, spatulas and aluminum foils were sterilized in an autoclave at a temperature of 121^0C for 30 minutes at 1.06 kg/cm^2 (15 PSI) pressure.

3.2.4 Glass ware

The Borosil glassware was used for all the experiments. Oven dried (250^0C) Erlesn meyer flasks, culture bottles, flat bottom flasks, pipettes, Petri dishes, beaker and measuring cylinders (25 ml, 50 ml, 100 ml, 500 ml and 1000 ml) were used for media preparation**.** The glassware's were first rinsed with the liquid detergent (Trix) and washed thoroughly with tap water, until the detergent was removed completely. Finally, they were rinsed with distilled water and sterilized in oven at 160-180^0C for 3-4 hours.

3.2.5 Culture medium

Presence of plant growth regulators plays a significant role in a successful regeneration of any plant species. Media for tissue culture should contain all major and minor elements,

vitamins as well as growth regulators which are essential for normal plant growth. Explants were inoculated into media composed of basal MS (Murashige and Skoog, 1962) medium supplemented with the plant growth regulators. Composition of MS media have been shown in appendix I. Hormones were added separately to different media according to the requirements. To do so, stock solutions of hormones were prepared ahead of media preparation and stored at 4^{0}C temperature.

Treatments

1. BA (0.0, 1.00, 2.00, 3.00, 4.00 and 5.00 mg/L) were used for shoot proliferation.
2. NAA (0.0, 0.50, 1.00, 1.50 and 2.00 mg/L) in combination with, BA (0, 1.00, 2.00, 3.00, 4.00 and 5.00 mg/L) were used for shoot and root formation.

3.3 The preparation of hormonal stock solutions

To prepare these hormonal supplements, they were dissolved in proper solvent as shown against each of them below.

Hormones (Solute)	Solvents used
BA	1 N NaOH
NAA	96% ethyl alcohol

In present experiment, the stock solution of hormones was prepared by following procedure. 100 mg of powder hormone was placed in a small beaker and then dissolved in 10 ml of 96% ethyl alcohol or 1 (N) NaOH solvent. Finally, the volume was made upto 100 ml by the addition of sterile distilled water using a measuring cylinder. The prepared hormone solution was then labeled and stored at 4 ± 1^{0}C for use upto two month. (Growth regulators were purchased from Sigma, USA).

3.4 The preparation of culture media

To prepare 1000 ml of culture media the following steps were followed:

Step-1. Seven hundred ml of sterile distilled water was poured into 1000 ml beaker.

Step-2. Five gm of MS media and 30 gm of sucrose was added and gently stirrered to dissolve these ingredients completely with the help of a hot plate magnetic stirrer.

Step-3. Different concentrations of hormonal supplements were added to the so lution either in single or in combinations as required and mixed well.

Step-4. The volume was made up to 1000 ml with addition of sterile distilled water.

Step-5. The pH was adjusted at 5.8.

Step-6. Finally, 8 gm agar was added to the mixture and heated for 10 minutes in an electric oven for melting of agar.

3.5 Steam heat sterilization of media (Autoclaving)

For sterilization 30 mL culture medium was poured in 200 ml culture bottles and then autoclaving was done at a temperature of 121^0C for 15 minutes at 1.06 kg/cm^2 (15 PSI) pressure. After autoclaving the media were stored in at 25±2 °C for several hours to make it ready for inoculation with explants.

3.6 Preparation of explants

Gladiolus cormels prior to surface sterilization were washed with tap water and detergent followed by 4-5 times washing with distilled water. The cormels of gladiolus have an outer brown protective covering that was removed before surface sterilization. The surface sterilization of explants was accomplished by dipping cormels in 0.1% Bavistin for 1 hour. Subsequently the explants were transferred to laminar airflow cabinet and kept in a 250 ml sterilized beaker. The beaker with explants was constantly shaken during sterilization. They were treated with 70% ethanol for 1 minutes and rinsed with sterilized distilled water. After the explants were immersed in 0.1% $HgCl_2$ within a beaker and added 3-4 drops of Tween-20 for about 4-5 minutes with constant shaking in clockwise and anticlockwise direction. Then the explants were washed 3-4 times with autoclaved distilled water to make the material free from chemical and ready for inoculation in culture media.

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3.7 Inoculation of culture

The sterilized explants were inoculated carefully following proper sterilization process within laminar airflow cabinet. Prior to use, the surface of the laminar flow bench was swabbed down with 70 % ethyl alcohol and the interior sprayed with the same alcohol. All glassware, instruments and media were steam-sterilized in an autoclave. During the course of the work, instruments in use were placed in a beaker containing 70 % ethanol and were flamed repeatedly using a spirit burner. The worker's hands and forearms were washed thoroughly with soap and water and repeatedly sprayed with 70% alcohol during the period of work. The mouth of all culture vials were flamed before and after positioning of the explant on the medium.

Plate 3. Inoculation of explants in the MS culture medium

For inoculation, explants were transferred to large sterile glass petri dish or glass plate with the help of sterile forceps under strict aseptic conditions. Here the explants were further trimmed with sterile scalpel blade to make suitable size. Explants which are 0.6 g weight can be transferred to culture bottles either whole or tip of the cormel which contain the meristematic tissues or buds. After cutting, the cormel with 0.5 to 1.0 cm in size containing bud was inoculated in MS medium (Plate 3). After vertically inoculating the explants in culture bottle, the mouth of bottle was quickly flamed and capped tightly. After proper labeling with media code, date of inoculation etc. the bottles were transferred to growth room.

3.8 Incubation

The bottles were kept to the culture racks and allowed to grow in controlled condition (plate 4). The cultures were maintained at 14 hours photoperiod, 21±2 ^{0}C temperature and light intensity 3000-3500 lux (25 W white bulbs). White fluorescent lamps were used for growth of the culture. The photoperiod was generally 14 hours light and 10 hours dark having 70% relative humidity (RH).

Plate 4. Incubation of explants in controlled condition

3.9 Shoot proliferation

The explants were cultured on MS nutrient medium supplemented with different concentration of BA alone or in combination of NAA. Percentage of explants showing shoot proliferation, days for shoot induction, number shoots per explants and average length of shoots were considered as parameter for evaluating this experiment. After successful shoot proliferation, subculture was done with newly form shoots. Shoots ware excised in aseptic condition with help of sterile scalpel blade and sterile forceps and transferred to new MS media which was supplemented with same concentration of growth hormones in order to increase budding frequency. The observations on development pattern of shoots were made throughout the entire culture period. Data recording was started after 21, 35 and 42 days from inoculation.

3.10 Rooting of multiple shoots

Newly formed shoots with adequate length were excised individually from the culture vial and transferred to rooting media. Different concentration of combination of NAA and BA were used with MS media. The observations on development pattern of roots were made throughout the entire culture period. Data were recorded from 21, 35 and 42 days after inoculation.

3.11 Acclimatization

Acclimatization, also known as "hardening is a process by which *in vitro* propagated plants are made to adapt to an *in vivo* environment.

Step-1: After 42 days of culture on rooting media, the plantlets were taken out from culture vial with forceps with utmost care in order to prevent any damage to newly formed roots and dipped in gentle warm water to remove any traces of solidified agar media for acclimatization. Plastic pots (6.5 × 3 inches) were kept ready filled with garden soil and compost in the proportion of 1:1 respectively. Immediately after removing solidified agar media from newly formed roots, the plantlets were then transplanted in to the pots with special care.

Step-2: After planting, the plantlets were thoroughly watered and were kept at 21±2°C with light intensity varied from 3000–5000 lux. The photoperiod was generally 14 hours light and 10 hours dark and 70% RH for 7 days with consecutive irrigation.

Step-3: Then the plants were shifted to shade house with less humidity and indirect sunlight. The tops of the pots were covered with transparent plastic sheet and grew at room temperature and 70% RH for 14 days with periodic irrigation (2 days intervals).

Step-4: After 3 weeks, the plants were transferred to the soil following depotting and potting into different pot having bigger pot size. The plants were watered periodically and upper layer of the soil mulched occasionally whenever necessary.

3.12 Data recording

The observations on development pattern of shoots and roots were made throughout the entire culture period. Five replicates each of them containing 4 bottles (single shoot per culture bottle) were used per treatment. Data were recorded after 21, 35 and 42 days of culture, starting from day of inoculation on culture media in case of shoot proliferation. In event of root formation, it was done every day starting from 7 days to 42 days of culture. The following observations were recorded in cases of shoot and root formation under *in vitro* condition.

1. Days to shoot induction
2. Percentage of shoot induction
3. No. of shoots per explants
4. Length of shoot (cm)
5. Days to root induction
6. Percentage of root induction
7. No. of roots per explants
8. Length of root (cm)

3.12.1 Calculation of days to shoots and roots induction

Days to shoots and roots induction was calculated by counting the days from explants inoculation to the first induction of shoots/roots.

3.12.2 Calculation of number of shoots per explant

Number of shoots per explants was calculated by using the following formula,

$$\text{Number of shoots per explants} = \frac{\text{Number of shoots per explants}}{\text{Number of observation}}$$

3.12.3 Calculation of number of roots per explant

Number of roots per explants was calculated by using the following formula,

$$\text{Number of roots per explants} = \frac{\text{Number of roots per explant}}{\text{Number of observation}}$$

3.12.4 Calculation of percent of shoot induction from culture

Number of shoot were recorded and the percentage of shoot induction was calculated as:

$$\text{Percent (\%) of shoot induction} = \frac{\text{Number of explant induced shoot}}{\text{Number of explant incubated}} \times 100$$

3.12.5 Calculation of percent of root induction from culture

Number of root were recorded and the percentage of root induction was calculated as:

$$\text{Percent (\%) of root induction} = \frac{\text{Number of explant induced root}}{\text{Number of explant incubated}} \times 100$$

3.12.6 Calculation of shoots length (cm)

Shoot length was measured in centimeter (cm) from the base to the top of the explants by a measuring scale. Then the mean was then calculated.

3.12.7 Calculation of root length

Root length was measured in centimeter (cm) from the base to the top of the explants by a measuring scale. Then the mean was then calculated.

3.13 Statistical analysis

The experiment was one factorial set up in a completely randomized design (CRD) with five replications per treatment. Data were statistically analyzed by analysis of variance (ANOVA) technique and differences among treatment means were compared by using Duncan's multiple range test (DMRT) at 5% probability level using Statistix 10 program.

CHAPTER IV

RESULTS AND DISCUSSION

Different investigations were made for regeneration of gladiolus ability. The results obtained from the experiment are described and discussed here and analysis of variance (ANOVA) are presented in Appendix II-X.

4.1 Sub-experiment 1. Effect of BA on shoot regeneration potentiality in Gladiolus.

The result of the effect of different concentrations of BA has been presented under following headings with Figure (1.1-1.2) and Table (1-3).

4.1.1 Days to shoot induction

Significant variations were found on days to shoot induction imposing doses of BA. The maximum days (23.20) to shoot induction were recorded in control treatment, followed by 18.80 days and 17.60 days at 1.0 mg/L and 2.0 mg/L BA respectively. On the other hand, minimum days (11.00) were required in 4.0 mg/L BA, followed by 12.00 days and 15.80 days at 5.0 mg/l and 3.0 mg/l BA respectively (Figure 1.1). Emek and Erdag (2007) reported cormel cultured on MS media containing 1 mg/l BA (3-4 days from culture initiation) at the base, followed by single shoot elongation after approximately 20-25 days of incubation.

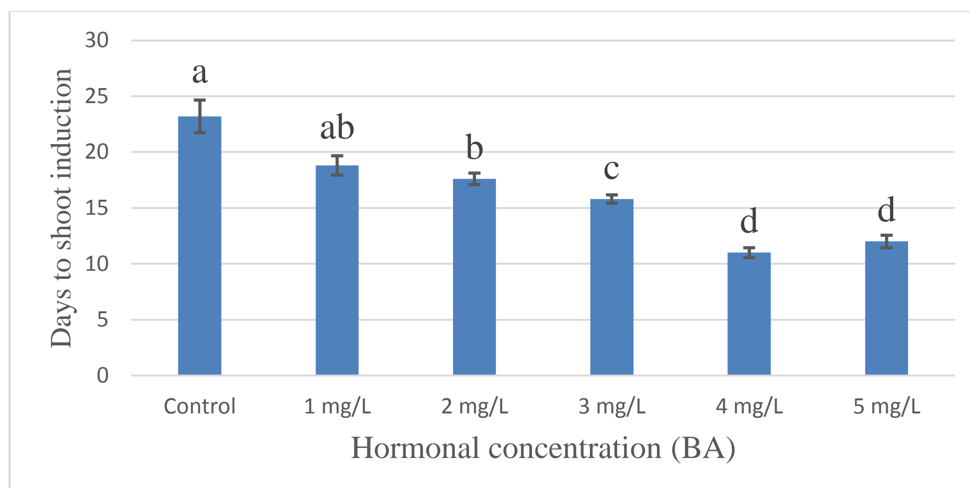

Figure 1.1 Effect of BA on days to shoot induction in Gladiolus

4.1.2 Percentage of response of explant

Different concentrations of BA showed significant variations on percentage of shoot induction. The treatment 4.0 mg/L BA had produced the highest frequency of shoot (73.80%) followed by 71.40% and 62.40% at 5.0 mg/L and 3.0 mg/L BA respectively. Whereas, the lowest percentage of shoot (45.00%) was observed in control treatment followed by 55.20% and 58.20% at 1.0 mg/L and 2.0 mg/L BA respectively (Figure 1.2). Mamun *et al.* (2014) reported that percentage of shoot formation from the callus of the explants was 20%, where the MS media contained (0.5 mg/l) BA.

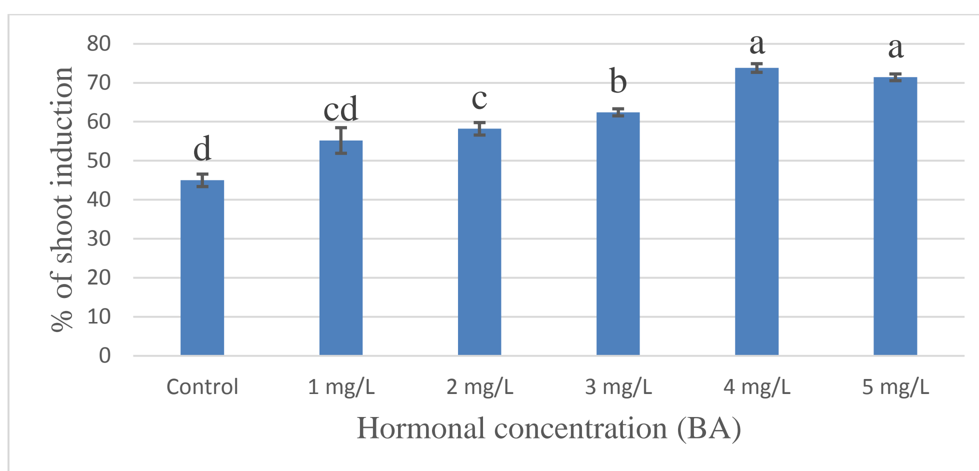

Figure 1.2 Effect of BA on percentage of shoot induction in Gladiolus

4.1.3 Number of shoot per explant

Significant variations were observed among different treatments of BA on the number of shoots per explant. It is observed that, the highest number of shoots (1.60, 2.20, 2.40) at 21, 35 and 42 DAI (Days after inoculation), respectively were observed in 4.0 mg/L BA was given (Table 2 and Plate 3). While, the control treatment showed the lowest number of shoot (1.00, 1.00 and 1.00) at 21, 35 and 42 DAI, respectively (Table 2). Emek and Erdag (2007) reported number of shoots on lateral bud cultured on MS media containing 2.0 mg/L BA was higher (11.00±0.38) than the media containing 0.5 mg/L BA, where number of shoots were (7.96±0.41). Budiarto (2009) outlined that among the gladiolus cultivars the number of shoots was varied on the 1.0-2.0 mg/L BA concentrations. Plantlet of cv. Nabila produced highest number of shoots on the 2.0 mg/L BA, while cv. Kaifa and Clara showed their most productivity in 3.0 mg/L BA. However, in general, the number of shoot decreased when higher concentration of BA was applied.

Table 1. Effect of different concentrations of BA on number of shoot at different Days after induction (DAI)

BA (mg/L)	Number of shoot per explant		
	21 DAI	35 DAI	42 DAI
0.0 (Control)	1.00b	1.00b	1.00b
1.0	1.20ab	1.20b	1.40b
2.0	1.20ab	1.40b	1.40b
3.0	1.00b	1.00b	1.20b
4.0	1.60a	2.20a	2.40a
5.0	1.00b	1.40b	1.40b
$LSD_{(0.05)}$	0.45	0.65	0.83
CV (%)	29.28	36.59	43.12

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, $LSD_{(0.05)}$=Least significant difference.

Plate 5. Effect of BA on number of shoot with 4.0 mg/L of BA at 35 DAI

4.1.4 Length of shoot (cm)

There was significant influence of different treatment of BA on the length of shoot. The highest length of shoot (0.74 cm, 1.18 cm and 1.32 cm) at 21, 35 and 42 DAI, respectively was found in 4.0 mg/L BA. The second highest length (0.66 cm, 1.02 cm and 1.20 cm) in 5.0 mg/L BA at 21, 35 and 42 DAI respectively (Table 2) was observed. While, the control treatment gave the lowest length (0.34 cm, 0.60 cm and 0.66 cm) of shoot at 21, 35 and 42 DAI, respectively (Table 2). Mamun *et al.* (2014) reported the length of shoot from explants was (2.5±0.30), with 0.5 mg/L BA.

Table 2. Effect of different concentrations of BA on length of shoot at different DAI

BA (mg/L)	Length of shoot (cm)		
	21 DAI	35 DAI	42 DAI
0.0(Control)	0.34b	0.60b	0.66b
1.0	0.54ab	0.86ab	1.02ab
2.0	0.68a	0.98a	1.04a
3.0	0.66a	0.98a	1.08a
4.0	0.74a	1.18a	1.32a
5.0	0.66a	1.02a	1.20a
LSD	0.24	0.33	0.38
CV (%)	30.41	26.90	27.60

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, $LSD_{(0.05)}$=Least significant difference

Plate 6. Effect of BA on shoot length at 35 DAI at 4.0 mg/L concentration of BA

4.2 Sub-experiment 2. Combined effect of BA and NAA on shoot and root regeneration potentiality in gladiolus

The results of the combined effect of different concentrations of BA and NAA have been presented under following headings with Table 4-9.

4.2.1 Days to shoot induction

Significance variations were observed for the combine used of BA+NAA on days to shoot initiation. The maximum days (23.60 days) to shoot induction was recorded in control condition, followed by 20.40 days, 18.60 days and 17.00 days at 1.0 mg/L BA + 0.5 mg/L NAA, 1.0 mg/L BA + 1.0 mg/L NAA and 1.0 mg/L BA + 1.5 mg/L NAA, respectively. Whereas, combination of 4.0 mg/L BA + 0.5 mg/L NAA showed the minimum 10.60 days for shoot induction followed by 11.80, 12.60 and 12.80 days at 4.0 mg/L BA + 2.0 mg/L NAA , 2.0 mg/L BA + 1.0 mg/L NAA and 1.0 mg/L BA + 2.0 mg/L NAA combine concentration respectively (Table 3). Kumar *et al.,* (1999) reported that medium containing 10.0 μM NAA and reduced level (1.0 μM) of BA, differentiated into pale green shoot buds within 15 days.

Table 3. Combined effect of different concentrations of BA and NAA on days to shoot induction and percentage of shoot

Treatments	Days to Shoot Induction	% of shoot initiation
0.0 mg/L + NAA 0.0 mg/L	23.60a	43.60e
BA 1.0 mg/L + NAA 0.5 mg/L	20.40ab	51.60de
BA 1.0 mg/L + NAA 1.0 mg/L	18.60a-c	55.60c-e
BA 1.0 mg/L + NAA 1.5 mg/L	17.00b-d	59.40b-d
BA 1.0 mg/L + NAA 2.0 mg/L	12.80c-e	69.40a-c
BA 2.0 mg/L + NAA 0.5 mg/L	13.60c-e	67.60a-c
BA 2.0 mg/L + NAA 1.0 mg/L	12.60c-e	70.00ab
BA 2.0 mg/L + NAA 1.5 mg/L	16.80b-d	60.00b-d
BA 2.0 mg/L + NAA 2.0 mg/L	14.20c-e	66.20a-c
BA 3.0 mg/L + NAA 0.5 mg/L	15.20b-e	63.60a-d
BA 3.0 mg/L + NAA 1.0 mg/L	15.40b-e	63.40a-d
BA 3.0 mg/L + NAA 1.5 mg/L	14.40b-e	65.60a-d
BA 3.0 mg/L + NAA 2.0 mg/L	13.80c-e	67.20a-c
BA 4.0 mg/L + NAA 0.5 mg/L	10.60e	74.80a
BA 4.0 mg/L + NAA 1.0 mg/L	16.40b-e	60.80a-d
BA 4.0 mg/L + NAA 1.5 mg/L	14.40b-e	65.80a-d
BA 4.0 mg/L + NAA 2.0 mg/L	11.80de	72.00ab
BA 5.0 mg/L + NAA 1.0 mg/L	15.00b-e	64.20a-d
BA 5.0 mg/L + NAA 0.5 mg/L	14.80b-e	64.80a-d
BA 5.0 mg/L + NAA 1.5 mg/L	14.20c-e	66.20a-c
BA 5.0 mg/L + NAA 2.0 mg/L	15.80b-e	62.20a-d
$LSD_{(0.05)}$	6.04	14.39
CV (%)	31.38	18.01

Figures in a column followed by different letter(s) differs significantly whereas figures havin g common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, $LSD_{(0.05)}$=Least significant difference.

Plate 7. The combine effect of BA and NAA on days to shoot initiation at 14 DAI

4.2.2 Percentage of shoot induction

Significant variation was observed for the combine treatment of BA and NAA on the percent of shoot initiation per explants. Maximum percentage (74.80%) of shoot induction was noticed in the treatment BA 4.0 mg/L + NAA 0.5 mg/L followed by 72.00%, 70.00%, and 69.40% at BA 4.0 mg/L + NAA 2.0 mg/L, BA 2.0 mg/L + NAA 1.0 mg/L and BA 1.0 mg/L + NAA 2.0 mg/L, respectively. Whereas, minimum percentage of shoot induction was observed 51.60% in 1.0mg/L BA + NAA 1.0 mg/L in control media followed by 55.60% and 59.40% at BA 1.0 mg/L + NAA 1.0 mg/L and BA 1.0 mg/L + NAA 1.5 mg/L respectively (Table 3). Sreekumar *et al.,* (2000) reported that 70% of shoot induction was observed in higher concentration of BA (4.4 µM) along with NAA (2.69 µM). however, isolated youngest nodes (2nd–3rd node from above) showed 96% percent of shooting in cultures.

4.2.3 Number of shoot per explant

There was significant influence of combined concentrations of BA and NAA on the number of shoot per explant at 21, 35 and 42 DAI. The treatment BA 4.0 mg/L + NAA 0.5 mg/L gave the highest number of shoot (1.80, 2.60 and 2.80) at 21, 35 and 42 DAI respectively. The second largest number of shoot (1.40, 1.60 and 2.00) in BA 3.0 mg/L + NAA 2.0 mg/L at 21, 35 and 42 DAI (Plate 8) followed by (1.40, 1.80 and 1.80), (1.40, 1.40 and 1.80) and (1.00, 1.00, 1.00, 1.00, 1.40 and 1.80) in 2.0 mg/L BA + 1.0 mg/L NAA, 1.0 mg/L BA + 2.0 mg/L NAA and 2.0 mg/L BA + 2.0 mg/L NAA concentration respectively at 21, 35 and 42 DAI. Whereas, the lowest number of shoot (0.80, 0.80 and

0.80) at 21, 35 and 42 DAI, respectively was found in (Table 4). Priyakumari and Sheela, (2005) reported that the highest number of shoot (4) was obtained for the treatment with 4 mg/L BA + 0.5 mg/L NAA. Kumar *et. al.* (1999) reported that using medium containing 10.0 µM NAA and 1.0 µM BA the maximum number of shoots were 200–250 per flask.

Table 4. Combined effect of BA and NAA on number of shoot at different DAI

Treatments	Number of shoot		
	21 DAI	**35 DAI**	**42 DAI**
BA 0.0 mg/L + NAA 0.0 mg/L	0.80c	0.80e	0.80e
BA 1.0 mg/L + NAA 0.5 mg/L	1.00bc	1.20c-e	1.20c-e
BA 1.0 mg/L + NAA 1.0 mg/L	1.00bc	1.00de	1.00de
BA 1.0 mg/L + NAA 1.5 mg/L	1.00bc	1.00de	1.00de
BA 1.0 mg/L + NAA 2.0 mg/L	1.40ab	1.40b-d	1.80bc
BA 2.0 mg/L + NAA 0.5 mg/L	1.20bc	1.40b-d	1.60b-d
BA 2.0 mg/L + NAA 1.0 mg/L	1.40ab	1.80b	1.80bc
BA 2.0 mg/L + NAA 1.5 mg/L	1.00bc	1.00de	1.00de
BA 2.0 mg/L + NAA 2.0 mg/L	1.00bc	1.40b-d	1.80bc
BA 3.0 mg/L + NAA 0.5 mg/L	1.00bc	1.20c-e	1.40b-e
BA 3.0 mg/L + NAA 1.0 mg/L	1.20bc	1.20c-e	1.20c-e
BA 3.0 mg/L + NAA 1.5 mg/L	1.00bc	1.20c-e	1.60b-d
BA 3.0 mg/L + NAA 2.0 mg/L	1.40ab	1.60bc	2.00b
BA 4.0 mg/L + NAA 0.5 mg/L	1.80a	2.60a	2.80a
BA 4.0 mg/L + NAA 1.0 mg/L	1.40ab	1.60bc	1.60b-d
BA 4.0 mg/L + NAA 1.5 mg/L	1.00bc	1.20c-e	1.40b-e
BA 4.0 mg/L + NAA 2.0 mg/L	1.00bc	1.40b-d	1.60b-d
BA 5.0 mg/L + NAA 1.0 mg/L	1.20bc	1.20c-e	1.60b-d
BA 5.0 mg/L + NAA 0.5 mg/L	1.00bc	1.00de	1.60b-d
BA 5.0 mg/L + NAA 1.5 mg/L	1.20bc	1.20c-e	1.60b-d
BA 5.0 mg/L + NAA 2.0 mg/L	1.40ab	1.40b-d	1.40b-e
$LSD_{(0.05)}$	0.45	0.56	0.61
CV (%)	30.86	33.78	31.90

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, $LSD_{(0.05)}$=Least significant difference.

4.2.4 Length of shoot (cm)

With combine concentrations of BA and NAA, significant influence was observed on the average length of shoot. The optimum length of shoot (1.40 cm, 2.32 cm and 2.82 cm) at

Plate 8. Maximum number of shoot observed at 42 DAI in the treatment of 4.0 mg/L BA + 0.5 mg/L NAA

21, 35 and 42 DAI respectively was noticed from the 4.0 mg/L BA+ 0.5 mg/L NAA (Plate 9). The second largest length was observed (1.14 cm, 1.40 cm and 2.46 cm) at 21, 35 and 42 DAI respectively in 2.0 mg/L BA + 1.0 mg/L NAA followed by (1.14 cm, 1.56 cm and 2.40 cm), (1.22 cm, 1.92 cm and 2.36 cm) and (0.94 cm, 1.74 cm and 2.18 cm) in 3.0 mg/L BA+ 2.0 mg/L NAA, 1.0 mg/L BA + 2.0 mg/L NAA and 5.0 mg/L BA + 2.0 mg/L NAA combine concentration respectively at 21, 35 and 42 DAI. Whereas, the minimum (0.62, 1.04 and 1.26) at 21, 35 and 42 in control treatment respectively (Table 6). Sreekumar *et al.,* (2000) reported that MS medium containing 2.22 µM BA and 1.07 µM NAA, showed 7-8 cm shoot length within 5 to 6 weeks. Priyakumari *et al.,* (2005) reported that to obtain ideal size of shoots (2.5 to 3.5 cm) for *in vitro*, lower concentrations of BA (1 and 2 mg L-1) in combination with NAA (0.1 and 0.5 mg L-1) are appropriate.

Plate 9. Maximum length of shoot observed at 42 DAI in the treatment 4.0 mg/L BA +0.5 mg/L NAA

Table 5. Combined effect of BA and NAA on length of shoot at different DAI

Treatments	Length of shoot (cm)		
	21 DAI	**35 DAI**	**42 DAI**
BA 0 mg/L + NAA 0 mg/L	0.62e	1.04g	1.26h
BA 1.0 mg/L + NAA 0.5 mg/L	0.82de	1.26fg	1.44gh
BA 1.0 mg/L + NAA 1.0 mg/L	0.90cd	1.36e-g	1.66f-h
BA 1.0 mg/L + NAA 1.5 mg/L	0.96b-d	1.50d-f	1.76e-h
BA 1.0 mg/L + NAA 2.0 mg/L	1.22ab	1.92a-d	2.36a-d
BA 2.0 mg/L + NAA 0.5 mg/L	0.86de	1.52c-f	1.88d-g
BA 2.0 mg/L + NAA 1.0 mg/L	1.14a-c	2.02ab	2.46ab
BA 2.0 mg/L + NAA 1.5 mg/L	0.96b-d	1.80b-e	2.00b-f
BA 2.0 mg/L + NAA 2.0 mg/L	1.06b-d	1.64b-f	2.02b-f
BA 3.0 mg/L + NAA 0.5 mg/L	1.08b-d	1.76b-e	2.06b-f
BA 3.0 mg/L + NAA 1.0 mg/L	0.98b-d	1.64b-f	1.96b-f
BA 3.0 mg/L + NAA 1.5 mg/L	0.86de	1.52c-f	1.82e-g
BA 3.0 mg/L + NAA 2.0 mg/L	1.14a-c	1.96a-c	2.40a-c
BA 4.0 mg/L + NAA 0.5 mg/L	1.40a	2.32a	2.82a
BA 4.0 mg/L + NAA 1.0 mg/L	0.96b-d	1.72b-e	2.00b-f
BA 4.0 mg/L + NAA 1.5 mg/L	0.94cd	1.52c-f	1.80e-g
BA 4.0 mg/L + NAA 2.0 mg/L	1.04b-d	1.7400b-e	2.08b-f
BA 5.0 mg/L + NAA 1.0 mg/L	1.16a-c	1.82b-d	2.06b-f
BA 5.0 mg/L + NAA 0.5 mg/L	0.90cd	1.50d-f	1.90c-g
BA 5.0 mg/L + NAA 1.5 mg/L	1.02b-d	1.70b-f	2.00b-f
BA 5.0 mg/L + NAA 2.0 mg/L	0.94cd	1.74b-e	2.18b-e
$LSD_{(0.05)}$	0.27	0.45	0.52
CV (%)	21.84	21.45	20.58

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, $LSD_{(0.05)}$=Least significant difference.

4.2.5 Days to root induction

The significant variations were observed among different concentrations of BA and NAA on days to root induction at 5% level of significance in laboratory. The optimum days (16.80a days) to root induction was recorded in control treatment followed by 15.20, 14.40 and 14.40 days in BA 1.0 mg/L + NAA 0.5 mg/L, BA 1.0 mg/L + NAA 1.0 mg/L and BA 1.0 mg/L + NAA 1.5 mg/L combined media respectively. While, the minimum days (8.40 days) was required in BA 4.0 mg/L + NAA 2.0 mg/L concentration followed by 9.40, 10.20 and 10.80 days in BA 2.0 mg/L + NAA 2.0 mg/L, BA 5.0 mg/L + NAA 2.0 mg/L and BA 3.0 mg/L + NAA 2.0 mg/L combined treatment respectively (Table 7). (Sreekumar *et al.,* 2000) reported that the root segments required higher concentration of BA (4.4 µM) along with NAA (2.69 µM) and indicating induction period 15 days.

Table 6. Combined effect of different concentration of BA and NAA on days to root initiation and percent of root initiation

Treatment	DRI	% of Root initiation
BA 0 mg/L + NAA 0 mg/L	16.80a	60.00f
BA 1.0 mg/L + NAA 0.5 mg/L	15.20ab	63.80ef
BA 1.0 mg/L + NAA 1.0 mg/L	14.40a-c	65.80d-f
BA 1.0 mg/L + NAA 1.5 mg/L	14.40a-c	65.60d-f
BA 1.0 mg/L + NAA 2.0 mg/L	11.80b-e	71.80a-e
BA 2.0 mg/L + NAA 0.5 mg/L	13.40a-d	68.00b-f
BA 2.0 mg/L + NAA 1.0 mg/L	12.20b-e	71.00a-e
BA 2.0 mg/L + NAA 1.5 mg/L	12.60b-d	70.00a-e
BA 2.0 mg/L + NAA 2.0 mg/L	9.40de	77.80ab
BA 3.0 mg/L + NAA 0.5 mg/L	13.20a-d	68.40b-f
BA 3.0 mg/L + NAA 1.0 mg/L	12.40b-e	70.40a-e
BA 3.0 mg/L + NAA 1.5 mg/L	13.00a-d	69.20b-f
BA 3.0 mg/L + NAA 2.0 mg/L	10.80c-e	74.40a-d
BA 4.0 mg/L + NAA 0.5 mg/L	13.40a-d	67.80c-f
BA 4.0 mg/L + NAA 1.0 mg/L	12.20b-e	70.80a-e
BA 4.0 mg/L + NAA 1.5 mg/L	11.40b-e	73.00a-e
BA 4.0 mg/L + NAA 2.0 mg/L	8.40e	79.80a
BA 5.0 mg/L + NAA 1.0 mg/L	12.40b-e	70.60a-e
BA 5.0 mg/L + NAA 0.5 mg/L	12.80a-d	69.40b-f
BA 5.0 mg/L + NAA 1.5 mg/L	12.40b-e	70.40a-e
BA 5.0 mg/L + NAA 2.0 mg/L	10.20de	75.80a-c
LSD	4.15	9.84
CV (%)	26.36	11.15

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, $LSD_{(0.05)}$=Least significant difference.

Plate 10. The effect of BA and NAA on days to root induction

4.2.6 Percentage of root induction

Among different concentrations of BA and IBA on percent of explants significance variations were observed in percentage of root induction potentiality. The highest percentage (79.80%) of root induction was recorded with BA 4.0 mg/L + NAA 2.0 mg/L followed by 77.80%,75.80% and 74.40% in 2.0 mg/L BA + 2.0 mg/L NAA, 5.0 mg/L BA + 2.0 mg/L NAA and 3.0 mg/L BA + 2.0 mg/L NAA, respectively. Whereas, the lowest percentage (60.00%) of root induction was recorded in control condition followed by 63.80%, 65.80% and 65.60% in 1.0 mg/L BA + 0.5 mg/L NAA, 1.0 mg/L BA + 1.0 mg/L NAA and 1.0 mg/L BA + 1.5 mg/L NAA, respectively (Table 7). Emek and Erdağ (2007) reported that media containing 0.5 and 2 mg/L NAA to promote rooting, But transferring to media containing 0.1 mg/L BA, rooting rates were very low 20%.

4.2.7 Number of roots

There was significant variation on number of roots at different concentrations of BA and NAA on the number of root per explant. The treatment BA 4.0 mg/L + NAA 2.0 mg/L gave the highest number of root (4.20, 6.00 and 7.20) at 21, 35 and 42 DAI, respectively (Plate 11). The second highest number of roots (3.20, 5.00 and 6.00) in BA 3.0 mg/L +

NAA 2.0 mg/L followed by (2.80, 4.80 and 5.80), (3.00, 4.40 and 5.60) and (2.60, 4.00 and 5.40) in BA 2.0 mg/L + NAA 2.0 mg/L, BA 4.0 mg/L + NAA 1.5 mg/L and BA 1.0 mg/L + NAA 2.0 mg/L combine treatment respectively at 21, 35 and 42 DAI. Whereas, the lowest number of root (1.20g, 1.80 and 1.80) at 21, 35 and 42 DAI, respectively was found in control treatment (Table 8).

Plate 11. Maximum number of shoot observed at 42 DAI in the treatment BA 4.0 mg/L + NAA 2.0 mg/L

Table 7. Combined effect of BA and NAA on number of root at different DAI

Treatments	Number of root		
	21 DAI	**35 DAI**	**42 DAI**
BA 0 mg/L + NAA 0 mg/L	1.20g	1.80h	1.80h
BA 1.0 mg/L + NAA 0.5 mg/L	1.80e-g	2.00gh	2.60gh
BA 1.0 mg/L + NAA 1.0 mg/L	2.20c-f	3.00d-g	3.20f-h
BA 1.0 mg/L + NAA 1.5 mg/L	2.20c-f	3.40c-e	4.40c-f
BA 1.0 mg/L + NAA 2.0 mg/L	2.60b-e	4.00b-d	5.40b-e
BA 2.0 mg/L + NAA 0.5 mg/L	1.80e-g	2.20f-h	3.00f-h
BA 2.0 mg/L + NAA 1.0 mg/L	2.40b-f	3.20d-f	3.60fg
BA 2.0 mg/L + NAA 1.5 mg/L	2.00d-g	3.40c-e	4.20d-f
BA 2.0 mg/L + NAA 2.0 mg/L	2.80b-d	4.80b	5.80a-c
BA 3.0 mg/L + NAA 0.5 mg/L	2.00d-g	2.40e-h	3.00f-h
BA 3.0 mg/L + NAA 1.0 mg/L	2.20c-f	2.60e-h	3.20f-h
BA 3.0 mg/L + NAA 1.5 mg/L	2.00d-g	3.00d-g	4.00e-g
BA 3.0 mg/L + NAA 2.0 mg/L	3.20b	5.00ab	6.00ab
BA 4.0 mg/L + NAA 0.5 mg/L	1.80e-g	2.80e-h	3.00f-h
BA 4.0 mg/L + NAA 1.0 mg/L	2.20c-f	2.60e-h	3.40fg
BA 4.0 mg/L + NAA 1.5 mg/L	3.00bc	4.40bc	5.60b-d
BA 4.0 mg/L + NAA 2.0 mg/L	4.20a	6.00a	7.20a
BA 5.0 mg/L + NAA 1.0 mg/L	1.60fg	2.60e-h	3.20f-h
BA 5.0 mg/L + NAA 0.5 mg/L	2.00d-g	2.80e-h	3.60fg
BA 5.0 mg/L + NAA 1.5 mg/L	2.40b-f	2.80e-h	4.20d-f
BA 5.0 mg/L + NAA 2.0 mg/L	3.00bc	4.00b-d	5.20b-e
$LSD_{(0.05)}$	0.89	1.06	1.44
CV (%)	30.41	25.80	28.02

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, $LSD_{(0.05)}$=Least significant difference.

4.2.8 Length of root (cm)

There was 5% significant influence of different combined concentrations of BA and IBA on the length of root. The highest length of root (1.02 cm, 1.26 cm and 1.34 cm) was found in 4.0 mg/L BA + 2.0 mg/L NAA concentration at 21, 35 and 42 DAI, respectively (Plate 15). The second largest length (0.88 cm, 1.06 cm and 1.16 cm) at 21, 35 and 42 DAI respectively was found in 3.0 mg/L BA + 2.0 mg/L NAA followed by (0.82 cm, 0.92 cm, and 1.14 cm), (0.72 cm, 0.98 cm and 1.14 cm) and (0.74 cm, 0.94 cm and 1.04 cm) in 2.0 mg/L BA + 2.0 mg/L NAA, 1.0 mg/L BA + 2.0 mg/L NAA and 5.0 mg/L BA + 1.5 mg/L NAA respectively at 21, 35 and 42 DAI. Whereas, the lowest number of root (0.20, 0.32 and 0.36) at 21, 35 and 42 DAI, respectively was found in control treatment (Table 8).

Plate12. Maximum length of shoot observed at 42 at DAI in the treatment 4.0mg/L BA + NAA 2.0 mg/L.

Table 8. Combined effect of BA and NAA on length of root at different DAI

Treatments	Length of root (cm)		
	21 DAI	**35 DAI**	**42 DAI**
BA 0 mg/L + NAA 0 mg/L	0.20h	0.32k	0.36k
BA 1.0 mg/L + NAA 0.5 mg/L	0.28gh	0.40jk	0.50i-k
BA 1.0 mg/L + NAA 1.0 mg/L	0.36f-h	0.50i-k	0.60h-k
BA 1.0 mg/L + NAA 1.5 mg/L	0.60cd	0.80c-g	0.90c-g
BA 1.0 mg/L + NAA 2.0 mg/L	0.72bc	0.98b-d	1.14a-c
BA 2.0 mg/L + NAA 0.5 mg/L	0.36f-h	0.40jk	0.48jk
BA 2.0 mg/L + NAA 1.0 mg/L	0.40d-h	0.60f-j	0.66g-j
BA 2.0 mg/L + NAA 1.5 mg/L	0.60cd	0.76d-h	0.84d-h
BA 2.0 mg/L + NAA 2.0 mg/L	0.82ab	1.02bc	1.14a-c
BA 3.0 mg/L + NAA 0.5 mg/L	0.38e-h	0.58g-j	0.74f-i
BA 3.0 mg/L + NAA 1.0 mg/L	0.42d-g	0.60f-j	0.70f-j
BA 3.0 mg/L + NAA 1.5 mg/L	0.58c-e	0.82c-f	0.94b-f
BA 3.0 mg/L + NAA 2.0 mg/L	0.88ab	1.06ab	1.16ab
BA 4.0 mg/L + NAA 0.5 mg/L	0.30gh	0.50i-k	0.60h-k
BA 4.0 mg/L + NAA 1.0 mg/L	0.42d-g	0.66f-i	0.78e-h
BA 4.0 mg/L + NAA 1.5 mg/L	0.70bc	0.92b-e	1.02b-e
BA 4.0 mg/L + NAA 2.0 mg/L	1.02a	1.26a	1.34a
BA 5.0 mg/L + NAA 1.0 mg/L	0.40d-h	0.56h-j	0.66g-j
BA 5.0 mg/L + NAA 0.5 mg/L	0.54c-f	0.72e-i	0.82d-h
BA 5.0 mg/L + NAA 1.5 mg/L	0.74bc	0.94b-e	1.04b-d
BA 5.0 mg/L + NAA 2.0 mg/L	0.20h	0.32k	0.36k
$LSD_{(0.05)}$	0.20	0.24	0.25
CV (%)	30.13	26.35	24.19

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, $LSD_{(0.05)}$=Least significant difference.

4.3 Sub-experiment

4.3.1 *Ex vitro* acclimatization and establishment of plantlets on soil

After a satisfactory number of shoot and root development at 6-8 weeks of culture the individual plantlets were moved from vial carefully without any root damage. The roots were washed with running tap water for removing surplus media and were kept at 21±2°C with light intensity varied from 3000–5000 lux. The plantlets were then transplanted into small plastic pot prepared with a standard ratio of cow dung and soil and shifted them in a shade house with less humidity and indirect sunlight. The plantlets were sprayed occasionally with water for maintaining humidity. At first 30 plants were transplanted, 25 survived in shade condition and survival rate was 83.33%. Finally in open atmospheric condition 25 plants were transplanted 19 survived (Table 11) and survival rate was 76% (Table 12) (*Sreekumar et al.*, 2000) reported that after 5–10 weeks of field transplantation, the micropropagated plants produced 4–5 additional axillary shoots from the base of the existing shoot each of which grew in length and in turn produced 5–7 lateral branches. Successful performances of micropropagules were observed.So, analyzing the survival rate it can be said that acclimatization potentiality of gladiolus was satisfactory.

Table 09. Survival rate of *in vitro* regenerated plantlets of gladiolus

Acclimatization	**No. of plants transplanted**	**No. of plants survived**	**Percentage of survival rate**
In shade house with controlled atmosphere	30	25	83.33
In open atmospheric area	25	19	76

Plate 13. Hardening of gladiolus plantlet in shaded atmosphere

Plate 14. Hardening of gladiolus plantlet in open atmosphere

CHAPTER V

SUMMARY AND CONCLUSION

The experiment was conducted at the Biotechnology Laboratory in the Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207, during the period of July 2019 to June 2020 to investigate the effect of different concentrations of BA singly as well as BA combine with NAA on *in vitro* regeneration, shoot proliferation and root formation in Gladiolus (*Gladiolus spp.*). Healthy, disease free 1.5cm- 2.0cm sized cormels were used as explants for the study.

In experiment I. The effects of different concentrations BA (0, 1.00, 2.00, 3.00, 4.00 and 5.00 mg/L) were studied on shoot proliferation of gladiolus. The results of this experiment revealed that 4.00 mg/L BA performed as the best treatment for gladiolus, as it took lowest days (11.00) to produce percent shoot induction (73.80%). It also expressed that, the highest number of shoot (1.60, 2.20, 2.40) with the highest length of shoot (0.74 cm, 1.18 cm and 1.32 cm) were found at 21, 35 and 42 DAI respectively when the treatment BA 4.00 mg/L was given.

In experiment II. The effects of NAA (0, 0.50, 1.00, 1.50 and 2.00 mg/L) in combination with, BA (0, 1.00, 2.00, 3.00, 4.00 and 5.00 mg/L) were studied for shoot and root proliferation of gladiolus. The results of this experiment revealed that the combination of BA 4.0 mg/L + NAA 0.5 mg/L showed the minimum (10.60 days) with maximum frequency (74.800%) of shoot induction, also the same treatment gave the highest number of shoot (1.80, 2.60 and 2.80) with longest shoot (1.40 cm, 2.32 cm and 2.82 cm) at 21, 35 and 42 DAI respectively.

For root initiation it was observed that the treatment BA 4.0 mg/L + NAA 2.0 mg/L showing largest frequency (79.80%) of root induction with minimum days (8.40 days) and also in the same concentration highest length of root (1.02cm, 1.16cm, and 1.34cm) with the maximum number of root (4.20, 6.00 and 7.20) at 21, 35 and 42 DAI were observed.

From the above summary, the results of the present experiment indicated that gladiolus could be successfully micro propagated with 4.00 mg/L BA for rapid *in vitro* shoot regeneration and proliferation.

Whereas for the combine treatment 4.0 mg/L BA + 0.5 mg/L NAA perform best for shoot regeneration and proliferation and 4.0 mg/L BA + 2.0 NAA mg/L showed the best response for root regeneration and proliferation. The results showed that an efficient protocol has been developed for *in vitro* regeneration of Gladiolus which can be used as an important tool for molecular biology and improvement of breeding programme.

RECOMMENDATIONS

Based on the summary and conclusions following recommendations can be made:

i. For further study of different levels of concentrations of auxins and cytokinines should be performed to evaluate their influence on *in vitro* gladiolus proliferation.
ii. Other than cormels other explants like shoot tip, petiole, leaf and root portion could be practiced to reflect in the best method
iii. Researches could be conducted on different genotypes of gladiolus to check if there is any genotypic influence or not.
iv. For callus induction, 2,4-D or other callus induction hormone could be used individually or in combine dose for large number of shoot induction.
v. Influence of other factors (elicitors, antioxidants) such as ascorbic acid, activated charcoal should be considered.
vi. Further extension and continuation of the research to assess field performance should be carried out.

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APPENDICES

Appendix I. Composition of Duchefa Biochemic MS (Murashige and Skoog, 1962) medium including vitamins

Components	Concentrations (mg/L)	Concentrations (µM)
Micro Elements		
$CoCl_2.6H_2O$	0.025	0.11
$CuSO_4.5H_2O$	0.025	0.10
Fe Na EDTA	36.70	100.00
H_3BO_3	6.20	100.27
KI	0.83	5.00
$MnSO_4.H_2O$	16.90	100.00
$Na_2MoO_4.2H_2O$	0.25	1.03
$ZnSO_4.7H_2O$	8.60	29.91
Macro Elements		
$CaCl_2$	332.02	2.99
KH_2PO_4	170.00	1.25
KNO_3	1900.00	18.79
$MgSO_4$	180.54	1.50
$NH4NO_3$	1650.00	20.61
Vitamins		
Glycine	2.00	26.64
Myo-Inositol	100.00	554.94
Nicotinic acid	0.50	4.06
Pyridoxine HCl	0.50	2.43
Thiamine HCl	0.10	0.30

Appendix II. Analysis of variance (ANOVA) of effect of different concentration of BA on days to shoot initiation and percent of shoot initiation

Source of variance	d.f.	DSI	% of Shoot induction
Treatment	5	102.32*	571.44*
Error	24	26.57	150.28
Total	29		

Appendix III. Analysis of variance (ANOVA) of effect of different concentration of BA on number of shoot at different DAI

Source of variance	d.f.	Number of shoot per explant		
		21 DAI	35 DAI	42 DAI
Treatment	5	0.37*	0.99**	1.17*
Error	24	0.12	0.25	0.40
Total	29			

Appendix IV. Analysis of variance (ANOVA) of effect of different concentration of BA on length of shoot at different DAI

Source of variance	d.f.	Length of shoot (cm)		
		21 DAI	35 DAI	42 DAI
Treatment	5	0.10*	0.19*	0.25*
Error	24	0.03	0.06	0.08
Total	29			

Appendix V. Analysis of variance (ANOVA) of combined effect of different concentration of BA and NAA on days to shoot initiation, % of shoot initiation

Source of variance	d.f.	DSI	% of Shoot initiation
Treatment	20	42.45*	243.01*
Error	84	23.06	130.95
Total	104		

Appendix VI. Analysis of variance (ANOVA) of combined effect of different concentration of BA and NAA on number of shoot at different DAI

Source of variance	d.f.	Number of shoot per explant		
		21 DAI	35 DAI	42 DAI
Treatment	20	0.27**	0.71**	0.93**
Error	84	0.13	0.20	0.23
Total	104			

Appendix VII. Analysis of variance (ANOVA) of combined effect of different concentration of BA and NAA on length of shoot at different DAI

Source of variance	d.f.	Length of shoot (cm)		
		21 DAI	35 DAI	42 DAI
Treatment	20	0.13**	0.39**	0.59**
Error	84	0.05	0.13	0.17
Total	104			

Appendix VIII. Analysis of variance (ANOVA) of combined effect of different concentration of BA and NAA on days to root initiation and % of root initiation

Source of variance	d.f.	DRI	% of Root initiation
Treatment	20	18.00*	102.90*
Error	84	10.89	61.26
Total	104		

Appendix IX. Analysis of variance (ANOVA) of combined effect of different concentration of BA and NAA on number of root at different DAI

Source of variance	d.f.	Number of root per explant		
		21 DAI	35 DAI	42 DAI
Treatment	20	2.15**	5.75**	8.99**
Error	84	0.50	0.71	1.30
Total	104			

Appendix X. Analysis of variance (ANOVA) of combined effect of different concentration of BA and NAA on length of root at different DAI

Source of variance	d.f.	Length of root (cm)		
		21 DAI	35 DAI	42 DAI
Treatment	20	0.23**	0.30**	0.33**
Error	84	0.03	0.04	0.04
Total	104			