IN VITRO PROPAGATION OF GLADIOLUS (*Gladiolus grandiflorus*)

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IN VITRO PROPAGATION OF GLADIOLUS (*Gladiolus grandiflorus*)

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CERTIFICATE

This is to certify that thesis entitled, " ***In vitro propagation ofgladiolus (Gladiolus grandiflorus).****" submitted to the Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of* ***MASTER OF SCIENCE IN GENETICS AND PLANT BREEDING****, embodies the result of a piece of bona fide research work carried out by* ***JANCY FELOMENA BARLLA****, Registration No. 15-06904 under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.*

I further certify that such help or source of information, availed during the course of this investigation has duly been acknowledged.

Dated: December, 2016
Place: Dhaka, Bangladesh

(Prof. Dr. Firoz Mahmud)
Supervisor

DEDICATED
TO
MY BELOVED PARENTS

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SAU, Dhaka.

IN VITRO PROPAGATION OF GLADIOLUS (*Gladiolus grandiflorus*)[1]

ABSTRACT

BY

JANCY FELOMENA BARLLA[2]

Gladiolus (*Gladiolus grandiflorus*) being a potential ornamental cut flower is cultivated throughout the world for its attractive spikes. The present study was intended to establish a protocol for *in vitro* plant regeneration of two gladiolus varieties. This research was conducted in tissue culture laboratory of Genetics and Plant Breeding Department, Sher-e-Bangla Agricultural University, Dhaka. The cormels of each variety as explants and media supplemented with various plant growth regulators (BAP, BA, NAA and IBA) of different concentrations were explored in *In vitro* propagation techniques. The effect of different combination and concentration of plant growth regulators on *in vitro* callus induction and plant regeneration was studied. The maximum days (7.58 days) required to callus initiation was noticed in T_7 (2.0 mg/L BAP+ 1.0 mg /L NAA) in yellow variety (V_1) and the minimum days (4.06 days) to callus initiation was found in white variety(V_2) with T_9 (1.5 mg/L BAP+ 1.5 mg/L NAA). The highest size of callus (0.93, 1.67 and 2.80 cm at 14, 28 and 42 DAI, respectively) found in the white variety (V_2) was observed in T_9 (1.5 mg/L BAP + 1.5 mg /L NAA) and the minimum size of callus (0.50, 0.92 and 1.52 cm at 14, 28 and 42 DAI) was found in yellow variety (V_1) in T_7 (2.0 mg/L BAP+ 1.0 mg/L NAA). The highest fresh callus weight (0.95. 1.67 and 3.11 g at 14, 28 and 42 DAI, respectively) was recorded in white variety (V_2) in response to T_9 (1.5 mg/L BAP+1.5 mg/L NAA) and the lowest fresh callus weight i.e. 0.45 and 2.08 g at 14 and 28 DAI respectively in T_7 (2.0 mg/L BAP+1.0 mg/L NAA) and 1.00g in T_8(1.0 mg/L BAP+1.5 mg/L NAA) was recorded in yellow variety (V_1). The minimum days (6.61 days) to shoot initiation was found in T_4 (3 mg/L BA) in white variety (V_2) and the maximum days (25.20 days) to shoot initiation was found in T_7 (2.0 mg/L BA+ 0.5 mg/L NAA)in yellow variety (V_1). T_4 (3.0 mg/L BA) showed the highest number of shoots(7.47, 13.20 and 17.87 at 14, 28 and 42 DAI, respectively) in white variety (V_2). White variety (V_2) produced the longest shoot i.e. (3.86, 5.67 and 8.11 cm at 14, 28 and 42 DAI, respectively) in T_4 (3 mg/L BA) and yellow variety (V_1) produced the smallest shoots (0.00, 0.50 and 0.80 cm at 14, 28 and 42 DAI, respectively) in T_8 (3.0 mg/L BA+ 0.5 mg/L NAA). The maximum days (23.20 days) to root initiation, was found in yellow variety with T_7 (2.0 mg/L NAA) and the minimum days (6.12 days) to root initiation in white variety (V_2) at T_3 (1.5 mg/L IBA). The maximum no. of roots i.e. 2.56, 4.94 and 8.33 was found in white variety with T_4 (2.0 mg/L IBA) at 14, 28 and 42 DAI, respectively. White variety produced the longest root (2.45, 4.38 and 6.15 cm at 14, 28 and 42 DAI, respectively) at T_4 (2.0mg/L IBA). The survival rate of the plantlets of white variety (80%) was greater than yellow variety (66.67%) under *ex vitro* condition.

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SOME COMMONLY USED ABBEVIATIONS

FULL WORD	ABBREVIATIONS
Degree celsius	^{0}c
Percentage	%
1 Normal	1N
Benzyl adenine	BA
Benzyl amino purine	BAP
Centimeter	cm
Completely Randomized Design	CRD
Concentration	Conc.
Days after inoculation	DAI
Distillled water	DW
Etcetera	*et al.*
Gram	g
Gram per litre	g/L
Indole-3-butyric acid	IBA
id test (That is)	i.e.
Least Significant Difference	LSD
Milligram	mg
Milligram per litre	Mg/L
Milliliter	ml
Murashige and Skoog	MS
Naphthalene acetic acid	NAA
Sodium Chloride	NaCl
Sodium Hydroxide	NaOH
Number	No.
Negative logarithm of hydrogen ion concentration (-log[H^+])	pH
Plant Growth Regulators	PGRs
Sher-e –Bangla Agriultural University	SAU
Science	Sci
Namely	Viz.

CHAPTER I

INTRODUCTION

Gladiolus (*Gladiolus grandiflorus)* is a cut flower which belongs to the family Iridaceae having 1500 species. It is a perennial herb with an underground corm. The name gladiolus came from the Latin word ‘gladiolus’ means a sword. As the leaves of gladiolus look like sword and for this reason, it is also called ‘sword lily’.

Gladiolus has an attractive inflorescence. The flowers are variable in color with attractive shades of creamy white, pink, yellow, salmon, purple apricot, red scarlet, crimson or combination of two or more shades. Commercial and professional growers use it in various ways. It has aesthetic value for its beautiful appearance in a garden. For its long vase life, it has economic value as a cut flower.

Commercially, gladiolus is propagated by two types of vegetative organs, namely corms and cormels (Pfeiffer, 1931; Halevy, 1986; Bose *et al.*, 2003). However, non-availability of a large quantity of propagules is one of the major obstacles in commercial cultivation (Singh and Dohare, 1994). Only one daughter corm and few cormels are produced from a large corm. Besides,due to very slow rate of multiplication, most of the hybrid cultivars of gladiolus take many years before the cultivar can be released. Incidence of Fusarium corm rot (caused by *Fusarium oxysporum* f. gladioli, viral and some other diseases) caused to a great extent of commercial losses in gladiolus production (Roy *et al.*, 2006; Sinha and Roy, 2002).

In vitro propagation through tissue culture is an effective method for large scale rapid multiplication of plants (Rao and Lee, 1982; Mott, 1981; Bajaj, 1986). A small plant tissue can produce million of homogenous plants within a year (Dantu and Bhojwani, 1987). It provides scope for elimination of diseases and developing a new cultivar (Debergh and Read, 1990).

There are many reports on *in vitro* regeneration of gladiolus. Ziv *et al* (1970) probably first attempted to grow gladiolus *in vitro*. Simonsen and Hildebrandt (1971) were probably the pioneers in regenerating *in vitro* plants of gladiolus from cormel culture. Commercially, gladiolus is important as ornamental plant for its contribution to the florists industry and horticulture. In many South East Asian countries (such as India, Singapur and Thailand etc.), gladiolus industry is an important source of income. More than two million plants in a year from a single bud are produced in many exporting countries through tissue culture technique. Moreover, many of the commercial laboratories have production capabilities more than two million *in vitro* plantlets from a single bud per week (Hartman, 1988). So, there is a great scope of exporting millions of plantlets from Bangladesh propagated by tissue culture techniques which can bring foreign exchange.

Some cultivars are very gorgeous in color but they are shy in large number production of cormels that means they produce only one or two cormels from one mother corm which is a major constrain for commercial production. This problem may be solved by regenerating plantlets through *in vitro* propagation. Therefore, the present investigation was conducted to establish an effective *in vitro* propagation technique in two gladiolus cultivars with the following objectives:

1. To study optimum conditions for *in vitro* regeneration of callus, shoot and root and
2. To study the effect of plant growth regulators on callus, root and shoot growth.

CHAPTER II

REVIEW OF LITERATURE

Gladiolus has recently introduced as ornamental plant in Bangladesh. Mainly it is used as cut-flower, which attain good price in big city. Gladiolus spikes are most popularly used in flower arrangements. So, some study had been done in Bangladesh on this plant. First work on the micro-propagation on cultivar of Gladiolus (Oscar) was done in the Tissue Culture Laboratory of the Department of Botany, University of Dhaka.

Gladiolus(Iridaceae family), propagates by corms and cormels. In breeding programmes,hybrids of gladiolus are obtained by seeds. In recent years, *in vitro* propagation has become a standard and valuable tool for rapid clonal regeneration of several plant species (Murashige, 1974). The credit for the first effort to grow gladiolus clonal propagation should probably goes to Ziv *et al.* (1970). Available literature and relevant information on gladiolus and other plants were reviewed under the following heads:

2.1 Sterilizatioin of explants

Aseptic condition is always required for*in vitro* culture. So, the first steps *in vitro* culture is sterilization of explants. Different kinds of chemicals were used in vitro propagation, viz. sodium hypochlorite (Bini and Belini,1973;; Quoirin *et al.*, 1977), calcium hypochlorite for surface sterilization of explants.

Ziv (1979)treated explants with 5% $CaClO_4$ for 10 minutes and washed 3 times in sterile water to ensure complete septic condition of buds. There were many reports on surface sterilization of explant by using $HgCl_2$from mature plants (Nekrosova, 1964; Bennett and McComb, 1982; Roy *et al.*, 1987, Kamo *et al.* (1990) reported that sterilization plant organs was done by various concentrations of a Clorox solution with 20 drops Tween -20 per litre.

2.2 Types of explant

Various stages/sizes of any explant might have different regenerative capacity and this is much depended upon the type, concentration and combination of plant growth regulators.

An efficient *in vitro* protocol for mass production of plantlets of gladiolus cv. White friendship was developed using different explant types and media supplemented with various growth regulators (Memon *et al.*, 2013). 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 and arranged in completely randomized design with factorial arrangements. The heading stage of nodal cultures, large sized whole cormels (0.6 g), 12 days old cormel sprouts and top segments of cormels were found as the best stage/size from each explant type for efficient shoot regeneration on MS medium supplemented with BAP (4 mg/L).

Various explant types such as segments of inflorescence stalk (Ziv *et al.*, 1970, Kumar *et al*., 1999), cormel tips (Simonsen and Hildebrandt, 1971; Goo *et al.*, 2003), cormel slices or basal leaf regions (Kamo, 1994; Kamo, 1995; Kumar *et al*., 1999; Emek and Erdag, 2007; Remotti and Loffler, 1995; Aftab *et al*., 2008), axillary buds (Lilien-Kipnis and Kochba, 1987; Boonvanno and Kanchanapoom, 2000) and slices of cormel sprouts (Sinha and Roy, 2002) have been reported for callogenesis at various levels of different plant growth regulators. The shoot tip of the cormel was considered the best source for callus initiation (Simonsen and Hildebrandt, 1971; Goo *et al.*, 2003).

2.3 Effect of growth regulators forcallus initiation

The callus initiation and regeneration from any explant source is mainly based oncultivated varieties, concentrations and combinations of plant growth regulators used inthe culture media (Kamo, 1994, 1995).

The hormonal requirement for callus initiation and subsequent plant regenerationfrom the monocotyledonous bulbous crops in the floral industry were largely unknown(Kamo, 1994). Various results reported that the best callusing occurs in

gladiolus in thepresence of NAA (Goo *et al.*, 2003; Emek and Erdag, 2007) or 2,4-D (Kumar *et al.*, 1999and Remotti and Loffler, 1995) alone or in combination with BAP (Aftab *et al.*, 2008).

The variations in concentration of plant hormones may vary from hormone tohormone and genotype to genotype even for the same explant as reported by Aftab *et al.*(2008). They used cormel slices (2-3 mm thick) and leaf sections (1 to 1.5 cm2) for callusinduction on MS basal media supplemented with NAA or 2,4-D alone or in combinationwith BAP and found positive response for callus induction from slices of cormel atlower concentrations of 2,4-D (2 mg/L) supplemented with BAP (1 mg/L). They alsoreported callus induction from the same explant on MS basal medium containing NAA (3or 4 mg/L). Kim *et al.* (1988) observed the best callus formation from cormel explantson a medium containing 2,4-D (10 mg/L) in about 40 days. Kamo (1995) achieved thebest callus induction from cormel segments at higher levels of NAA (10 mg/L) or MSmedium with 2,4-D (0.5 mg/L) or dicamba (2.0 mg/L). Kumar *et al.* (1999) inducedbetter callus formation from cormel segments of gladiolus on MS basal media containingBAP (1.1 mg/L) and 2,4-D (1.1 to 2.2 mg/L) from '*Her Majesty'* and'*Aldebaran'* culivars. In the same way Remotti and Loffler (1995) reported thebest callus induction from cormel slices on a medium containing 2,4-D (2 mg/L) incultivar *Peter Pears*. They also reported that middle part of the cormel had thehighest competence for callus initiation. Emek and Erdag (2007) optimized NAA at the rate of 8.5 mg/L for maximum callus production from longitudinal corm slices in*Gladiolus anatolicus*. Cormel segments responded better for callus induction ascompared to inflorescences axes (Kumar *et al.*, 1999) and leaf explant (Aftab *et al.*,2008). It is clear from the above findings that NAA or 2,4-D alone or in combination hadmajor role in initiation of callus from cormels of any genotype.

Goo *et al.* (2003) also observed 100% callus formationratio from cormel tip of *Topaz* on MS medium supplemented with NAA (1.0 mg/L).

2.4 Effect of growth regulators for shoot induction

Regeneration through callogenesis is mainly based on quality of the callus, age ofthe obtained callus and its regeneration potential. In cultivar *Gladiolus primulinas* Baker,Sinha and Roy (2002) regenerated shoots from approximately one gram of the callus onMS media supplemented with 0.5-2.0 mg/L KIN or/and 0.5-2.0 mg/L BAP. Themaximum number of shoots (28) regenerated on MS medium with BAP (2.0 mg/L)from one month old callus and in the fifth month no regeneration of shoots was found onany medium. On the other side this is not the case with direct regeneration of *in vitro*cultures. The rate of regeneration depends on the combination of growth regulators usedin the medium and on the cultivar. Cytokinins play major role in this regard. Earlier,Dantu and Bhojwani (1987) and De Bruyn and Ferreira (1992) had reported thebeneficial effect of BAP over other cytokinins for shoot multiplication and regeneration.BAP and KIN used and its optimal concentration depend on the cultivar. In cultivar*Gladiolus grandzilorus* "pink" more shoots (6.1 per explant) from basal plates wererecorded on medium containing BAP 1 mg/L. In cultivar *Eurovision* 5-8shoots obtained per sub culture on a medium containing KIN (2 mg/L) (Ziv, 1979),whereas 11-15 shoots produced on medium having KIN (1 mg/L) for a series ofcultivars. Dantu and Bhojwani (1995) reported maximum number of shoots from axillarybuds of gladiolus on MS medium containing BAP (0.5 mg/L). Kamo (1995) culturedcormel slices on MS medium supplemented with BAP (1.0 mg/L) regenerated plantsfrom all six cultivars. Remotti and Loffler (1995) regenerated plants from yellowcompact cells of all genotypes on media containing zeatin and BA in variousconcentrations.

The use of NAA alone or in combination with BAP or KIN has also been foundbeneficial for the establishment of bud and meristem tip cultures (Ziv, 1979; Lilien-kipnisand Kachba, 1987; Boonvanno and Kanchanapoom, 2000). Emek and Erdag (2007) gotmore number of shoots per explant (4.71) at lower levels of BA (0.2 mg/L) and higherlevels of NAA (2 mg/L) in *Gladiolus anatolicus.* Kumar *et al.* (1999) noted shootdifferentiation from callus cultures at lower concentration of BA (0.2 mg/L)supplemented with higher concentration of NAA (1.9 mg/L) in three cultivars viz. HerMajesty, Aldebaran and Bright Eye. Boonvanno andKanchanapoom (2000) cultured axillary buds on MS medium supplemented with KIN(0.5 mg/L) or NAA (0.4 mg/L) to produced multiple shoots initiated from axillarybuds of the corms.

Callus induced from buds in the presence of sole NAA (0.4 mg/L)and multiple shoots were developed from the callus on MS medium containing BA (1 mgL-1).

A number of cytokinin compounds were used in axillary shoot proliferation. Those mostly used are BAP, Kinetin and 2ip. A specific cytokinin was sometimes better for a specific species of plant but BAP was widely used by many workers. BAP is much more active than naturally occuringcytokinins. But in gladiolus some worker used different cytokinin in single or with auxin for shoot proliferation. Dantu and Bhojwani (1995) reported that shoot cultures of Gladiolus cv. Friendship, initiated from axillary buds, exhibited best elongation on liquid MS medium containing 0.5 mg/l BA litre in light.

In a study cormel shoot tips of cultivars Beaty spot, Jo Wagenaar, Vink's Glory and wild Rose were cultured on basal MS medium supplemented with BA or Kinetin each at 4-8 mg/litre and solidified with 0.2% Gelrite. More calli was produced with BA, whereas kinetin produced more shoots. The highest number of shoots was obtained with Kinetin at 4 mg/litre (Rao *et al.*, 1991). Zakutskaya and Murin (1990) reported that the highest frequency of shoots was obtained on medium with BA and NAA atrepetitively 0.3 and 0.5 mg/litre when corm buds were halves as explant. Lilien and Kochba (1987) noted that for repeated proliferation of six new miniature hybrids of Gladiolus. MS medium was supplemented with low level of NAA and BA or Kinetin at varying concentrations.

Shoot cultures of cultivars Friendship, Her Majesty and American Beauty were initiated from axillary buds excised from cold stoned corms. Shoots multiplied for over 2 years at the rate of 3 fold every 2 weeks or 5 fold every 4 weeks on MS medium + BA (0.5 mg/ l). Shoots elongated only when BAwas omitted from the medium or when its level was reduced to 0.1 to 0.2 mg/l reported by Dantu and Bhojwani (1987). Jager *et al.* (1998) reported that multiple shoots were occurred from the hypocotyl sections on MS medium containing 100 mg/l myoinositol, 3% sucrose and 1 mg/l NAA and BA respectively, solidified with 0.8% ager. Multiple shoots occurred from the hypocotyls sections. Good rates of proliferation were also achieved for the six miniature hybrids at lower levels of cytokinin reported for large-flowered by Hussey (1978), Ziv (1979), and Logan and Zettler (1985).

Rapid multiplication of shoot was initiated from pedicel, thalamus, nodal segment, young shoot tip of mature or nursery plants. The best performance was in MS medium with 2.0 mg/l BAP, 3.0 mg/l BAP and 1.0 mg/l BAP+ 0.5 mg/l NAA. Multiplication rate was also considerable when MS solid medium was supplemented with similar concentration of cytokinin (1.0 mg/l BAP) and (1.0 mg /l NAA). The highest response was found in cv. White Goddess (100%) and lowest in cv. Friendship (40%) reported by Kashem (1992). Begum and Hadiuzzaman (1995) stated that multiple shoot induction was the best in half MS medium supplemented with 0.75 mg/l BAP. Grewal *et al.* (1995) reported that nodal segments regenerated single shoot per explant upon culturing on MS medium supplemented with 1.0 mg/l BAP, whereas,those cultured on MS + 5.0mg/l BAP medium developed 14-20 shoots primordial within 4 weeks.

2.5 Effect of growth regulators for rooting

In most cases, *in vitro* regenerated shoots fail to produce roots at the shoot proliferation phase. Therefore, a separate root induction phase is essential. Most of the plant species require single or combination, one or more growth regulators for root induction. The most common auxins, napthaleneacetic acid (NAA), Indole-3-butyric acid (IBA) and Indole-3-acetic acid (IAA) are mainly used for rooting. Sometimes one auxin appeared much better than that of another for a specific species of plant. In African violet, Start and Cummings (1976) found that a second medium higher in auxins essential for rooting.

Better rooting plays major role in acclimatization. The poor survival rate of transplanted plants could have been the result of poorly developed roots (Ziv *et al.*, 1970). Thus a major change in the pre-transplant stage is the shift to conditions that favor root initiation and shoot elongation.

Various factors such as low concentrations or completely elimination ofcytokinins and high concentrations of auxins (Logan and Zettler, 1985; Lilien-Kipnis and Kochba, 1987; Ziv, 1979), low concentrations of inorganic salts (Sriskandarajal and Mullins, 1981), addition of sucrose (Kumar *et al.*, 1999), addition of activated charcoal (Lilien-Kipnis and Kochba, 1987) and addition of vermiculite (Logan and

Zettler, 1985) instead of agar in the medium have been reported for better rooting response in in vitro cultures. Rooting in simple basal MS medium without plant growth regulators was also observed by Goo *et al.* (2003).

The role of various auxins (NAA, IBA and IAA) has been reported for initiation and regeneration of roots in various cultivars of gladiolus and other bulbous plants such as Snowdrop bulblets (Tipirdamaz, 2003) and Narcissus (Hosaki and Asahira, 1980; Chow *et al.*, 1992). In gladiolus, better rooting response was reported in the presence of NAA and IBA. Root length, number and its morphology was greatly affected with the increasing levels of NAA (Lilien-Kipnis and Kochba, 1987). Ahmad *et al.* (2000) also observed better rooting response on MS medium supplemented with NAA (0.5 mg/L) or IBA (3.9 mg/L) but the best survival rate was found in the medium containing NAA in White prosperity. IBA (2 mg/L) was also observed beneficial in response to rooting in *Golden Wave* as reported by Sinha and Roy (2002). Similar reports were observed by Priyakumari and Sheela (2005). They found earlier rooting (within 7 days) and longer roots (5 cm) on MS medium supplemented with IBA (2 mg/L) in cultivar Peach Blossom. However more number of roots (24) was produced by NAA (1 mg/L) in the same cultivar. Hussain *et al.* (1994) observed extensive growth of roots on MS medium supplemented with IBA (2 mg/L) in cultivar White Friendship. Cytokinins usually induce shoots but contrarily Emek and Erdag (2007) observed rooting on MS medium with BA (0.1 mg/L) in *Gladiolus anatolicus*but rooting percentage (20%) was low. Moreover, no rooting was observed with NAA (0.5 or 2 mg/L).

Aftab *et al.* (2008) found 100% rooting response in gladiolus on half strength MS medium supplemented with IBA (2 mg/L) after 5 days of inoculation. Sinha and Roy (2002) reported use of half strength MS medium supplemented with IBA (2 mgL-1) with sucrose (6%) promote roots in 100% cultures within two weeks.

Morel and Muller (1946) reported that mineral salt medium containing NAA and IBA at the rate of 0.05-1.0 mg/l with 16 g/l sucrose is essential for advedtitious root formation on the petioles and lamina of African violet. In chrysanthemum, the preferredauxin for root induction was IAA (Jain and Chaturvedi, 1993; Hoque and Fatema, 1995). In onion, strong tendency to root formation was found when plantlets

cultured on media containing low concentrations of hormones BAP 0.5-1.0 mg/l and NAA 0.03 mg/l (Hussey, 1978).

Kashem (1992) reported that 100% rooting was observed of several gladiolus cultivars within 15-20 days when 1.0 mg/l IBA were used in ½ MS medium. Dantu and Bhojwani (1987) used BM and MB+BAP(0.1, 0.2 mg/l) and 33% shoots were rooted. Wilfret (1971) studied that root production was higher in Gladiolus in the Murashige and Skoog formulation as compared to the Linsmaier and Skoog media. Kamo *et al.* (1990) found that the regenerated plantlets of Gladiolus formed roots whentransferred to an MS basal salt medium lacking hormones. Begum and. Fridborg and Eriksson (1975) reported that AC in the rooting medium has a beneficial effect on shoot growth as well as root length.

2.6 *Ex-vitro* establishment of plantlets

Tissue culture technique will be success when *in vitro* cultured plantlets will successfully establish in *ex- vitro* condition. Establishment of plantlets in the pot or in the field depends upon the development of suitable conditions to harden theplantlets rapidly. In the developed countries many potting mixtures are available which are specially formulated and contain no soil for cent percent survivability of *in vitro* plantlets. But these type of mixture were not available in Bangladesh. So, for *ex-vitro*establishment soil media must be appropriate. For good establishment of *in vitro* plantlets potting media must be porous, light and have a good water holding capacity. However, the nature or type of substrate used for transplanting *in vitro* plantlets to*ex-vitro* environment varies from species to species.

A mixture of peat: sand: loam (1:1:1v/v) covered with a layer of peat and sand than perlite or vermiculite mixture was better for good growth of carnation plantlets Stone (1963). Kashem (1992) reported that rate of survivability or adapted of transplanted plantlets was 75-80% when gladiolus plantlets were transferred into sterile compositions of loam and compost in a ratio of 1:1.

CHAPTER III

MATERIALS AND METHODS

The experiment was carried outduring 2015-2016 in the laboratory of Department of Genetics and Plant Breeding, Sher-e-Bangla Agricultural University. The materials and methods used to conduct the experiment and record the observations during the course of this experiment were presented in this chapter.

3.1 Experimental material

The experimental materials were the cormels of the following varieties of gladiolus

1. White variety (plate 1)
2. Yellow variety (plate 1)

3.1.1 Sources of plant material

All of these materials were collected from my supervisor Prof. Dr. Firoz Mahmud. Disease free materials were used.

3.1.2 Culture media

The degree of accomplishment in any technology employing cell, tissue and organ culture is related with some major factors. A noteworthy factor is the choice of nutritional components and growth regulators. Both for shoot regeneration and rooting of multiplied shoots, MS medium (Murashigeand Skoog, 1962) was used with different vitamins and hormonal supplemented. The composition of the MS medium has been presented in appendix I. Hormones were added independently to different media accordingto the requirements. For the preparation of the media, stock solutions were prepared at the beginning and stored at 4 $(\pm 1)^0$C. The relevant media were prepared from the stock solution.

(a)

(b)

Plate 1. Varieties of gladiolus used in the study: (a) white variety; (b) yellow variety

3.1.2.1 Preparation of the stock solution

The first step in the preparation of the medium was the preparation of the stock solution of the various constituents of the medium. As different media constituents were required in different concentrations, separate stock solution for the macronutrient, micronutrient, irons, vitamins, amino acids and growth regulators etc. were prepared for ready use.

3.1.2.1.1Stock solution of macronutrients (Soln. A)

Stock solution of macronutrients was prepared with 10 times the final strength of the medium in 1000 ml of distilled water (DW). Ten times the weight of the salts required for one litre of medium were weighted accurately and dissolved thoroughly in 750 ml of distilled water and final volume was made up to 1 litre by further addition of DW. The stock solution was filtered through a Whatman no. 1 filter paper to remove all the solid contaminants and solid particles like cellulose dust, cotton etc. The stock solution was poured into a clean plastic container and stored in refrigerator at 4(±1) ^{0}C for ready use.

3.1.2.1.2 Stock solution of micronutrients (Soln. B)

Stock solution of micronutrients was made up to 100 folds (100x) the final strength of the medium in 1000 ml DW as described earlier for stock solution A. The stock solution was filtered, labled and stored in a refrigerator at 4(±1)^{0}C for ready use.

3.1.2.1.3 Stock solution of iron (Soln. C)

It was made up to 100 folds (Soln. C) the final strength of the medium in 1000 ml of DW. Here two constituents, $FeSO_4$ and Na-EDTA were dissolved in 750 ml DW in a beaker by heating on a hitter cum magnetic stirrer. Then the volume was made up to 1000 ml by further addition of DW. Finally the stock solution was filtered and stored by wrapping with aluminium foils in a refrigerator at 4(±1)^{0}c for later use.

3.1.2.1.4 Stock solution of vitamins and amino acids (Soln. D)

The following vitamins and amino acids were used for the preparation of MS medium:

- Pyridoxine HCL(Vitamins B_6)
- Thiamine HCL (Vitamins B_1)
- Myoinositol (inositol)
- Glysine
- Nicotinic acid (Vitamins B_3)

Each of the above vitamins and amino acids except myoinositol were taken at 100 folds (100x) of their final strength in a measuring cylinder and dissolved in 400 ml of DW. Then the final volume was made up to 1000 ml by further addition of distilled water. Finally the stock solution filtered and stored in a refrigerator at a $4(\pm1)^0$C for later use. But the myoinositol was made separately 100 folds (100x) the final strength of the medium in 1000 ml of DW. This stock solution was also filtered and stored in a refrigerator at $4(\pm1)^0$C.

3.1.2.1.5 Stock solution of growth regulators (Soln. E)

In addition to the nutrients, it is generally necessary to add growth regulators (hormone) such as auxin and cytokinin to the medium to support good growth of tissues and organs. The following growth regulators were used in the present investigation, which were dissolved in appropriate solvent as shown against each of them.

Growth regulators (Solute)	**Solubility**
Auxins	
NAA (α-Napthalene acetic acid)	96% Ethanol
IBA(Indole-3-butyric acid	96% Ethanol
Cytokinins	
BA(Benzyladenine)	1N NaOH
BAP (Banzyl amino purine	1N NaOH

To prepare a stock solution of growth regulator, 10 mg of the growth regulators was taken on a clean watch glass then dissolved in 1 litre measuring cylinder and the volume was made up to 100 ml with distilled water. The solution was then poured into a clean glass container and stored at $4(\pm1)^0$C and used for a maximum period of two months.

3.1.2.1.6 Preparation of other stock solutions

3.1.2.1.6.1 Preparation of 1N NaOH

1. 1.40 g NaOH pellets was weighted
2. Then those pellets were put in dry 1L volumetric flask
3. 900 ml distilled water was added slowly and stirred until dissolved
4. The flask in a thermostat at 20 c and maintain for 1 hour
5. Distilled water was added up to the 1 L-mark mix the closed bottle.

3.1.2.1.6.2 Preparation of 70% Ethanol

1. In a 100 ml measuring cylinder 70 ml 99.9% ethanol was poured.
2. Double distilled water was poured up to the level of 100 ml
3. Stored the solution in a sterilized glass bottle use
4. This solution was made fresh each time before use.

3.1.2.1.7 MS media preparation

To prepare one litre of MS medium, the following steps were followed:

1. 500 ml double distilled water was taken into 1 litre beaker.
2. 100 ml of stock solution of macro-nutrients, 10 ml of stock solution of micro-nutrient, 10 ml of stock solution Na-EDTA and 10 ml of stocksolution of vitamins and growth regulators were added in this 500 ml double distilled water.
3. 30 mg sucrose was dissolved in this solution with the help of magnetic stirrer.
4. Different concentrations of hormonal supplements as required were added either in single or in different combination to this solution and were mixed thoroughly.

5. Each hormonal stock solution contained in 100 ml of solution. Later different combinations of these two plant growth regulators were used for callus, shoot and root induction. The whole mixture was then made up to 1 litre with further addition of double distilled water.

3.1.2.1.8 pH of the medium

pH of the medium was adjusted to 5.8 by pH meter with the addition of 1N NaOH or 0.1 N HClwhichever was necessary.

3.1.2.1.9 Agar

The media was gelled with 8 g/L agar and the whole mixture was gently heated on microwave oven at 250^0C for 8-10 minutes.

3.2 Sterilization

3.2.1 Sterilization of culture media

Fixed volume of medium was dispensed into vial. The vials were plugged with cork and marked with different codes with the help of permanent glass marker to indicate specific hormonal supplement. Then the vials were autoclaved at 15 psi pressure at 121^0C for 20 minutes. The medium was then transferred into the culture room and cooled at 24^0C temperature before used. Marking is also necessary.

3.2.2Sterilization of glassware and instruments

Glassware, culture vessels, beakers, petridishes, pipettes, slides, plastic caps, other instruments such as forceps, needles, scissors, spatula, surgical blades, brush, cotton, instrument stand and aluminium foil were sterilized in an autoclave at a temperature of 121^0C for 20 minutes at 15 psi pressure. Before this, all types of glassware instrument were washed with distilled water.

3.2.3 Sterilization of culture room and transfer area

In the beginning the culture room was sprayed with formaldehyde and then the room was kept closed for one day. Then the room was cleaned through gently washing the floors walls and rakes with a detergent. This is followed by careful wiping them with 70% ethnol. This process sterilization of culture room was repeated at regular

intervals. The transfer area also cleaned with detergent and also sterilized twice in a month by 70% ethanol. Laminar air flow cabinetwas usually sterilized by switching on the cabinet. The ultra violet Ray kills the microbes inside the laminar airflow. It switches on 30 minutes before working inempty condition and for 20 minutes with all the instruments. The working surface was wiping with 70% ethanol, 30 minutes before starting the transfer work.

3.2.4 Precaution of ensure aseptic condition

All inoculation and aseptic manipulation were carried out under laminar airflow cabinet. The cabinet was usually switched on with ultra violet light half an hour before use and wiped 70% ethanol to reduce the chances of contamination. The instruments like scalpels, forceps, needles, surgical blades, scissors, pipettes, slides, plastics caps, spatula, brush, cotton etc. were pre-sterilized by autoclaved and subsequent sterilization were done by dipping 70% ethanol and wearing of hand gloves. It is also necessary to wear apron and mask to avoid contamination rate. Other required materials like distilled water, culture vessels, beakers, glass plates and petridishes etc. were sterilized in an autoclave following method of media sterilization. The cork of vials was flamed before open. Aseptic conditions were followed during each and each and every operation to avoid the contamination of cultures.

3.3 Micropropagation protocol

The required numbers of plantlets were obtained following the micropropagation protocol developed in the tissue culture laboratory of Department of Genetics and Plant Breeding, Sher-e-Bangla Agricultural University. The details of protocol are described below:

3.3.1 Preparation of explants

The cormels of gladiolus were used as explant material. The outer ray florets were trimmed carefully and the calyx was retained.

3.3.2 Surface sterilization

The explants were washed in running tap water. Two drops of Tween-20 was added and the capitulums were washed with distilled water. The capitulums prepared in this manner were then treated with 0.1 per cent mercuric chloride for 10 minutes with constant shaking in laminar air flow chamber. Later, they were washed four times with sterile double distilled water to remove traces of mercuric chloride.

3.3.3 Initiation of culture

The prepared explants were transferred to Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with different concentrations of NAA, IBA, BA and BAP to promote callus, shoot and root induction. A pictorial view of inoculation of explant in MS medium with different concentrations of PGRs for callus initiation and culture in lab condition are presented in Plate 2.

3.3.4 Culture condition

The inoculated cultures were inoculed in the culture room at a temperature of 25 ± 2^0C with a photoperiod of 16 hours light was maintained by the illumination from white florescent tube light and light intensity was 2000 lux for the growth and development of the cultures.

3.4.1 Subculture

3.4.1.1Maintenance of calli

Callus initiated after 5-7 days of explant inoculation in the medium. The developed calli were also kept under 16 hour photoperiod at 25 ± 2^0C. The vials were checked daily to note the response and the developed of contamination.

3.4.1.2Maintenance of proliferating shoots

Initial sub-culturing was done when the explants had produced some shoots. For sub-culturing, the entire samples of *in vitro* shoots were cut into small pieces so that each piece would contain about one shoot. Each piece was inoculated into a similar fresh medium. The cultures were sub-cultured 25-30 days after inoculation in MS medium.

(a) **(b)**

Plate 2. Initiation of culture: (a) Inoculation of explant (cormel) in MS medium with different concentrations of PGRs for callus initiation; (b) Cultured in lab condition

3.5 *In vitro* rooting of shoots

Single shoots obtained were separated. The stem was cut from callus region using a sterile scalpel and such shoots were transferred to MS medium added with 3 percent sucrose, 0.8 per cent agar and supplemented with NAA and BAP. The cultures were incubated for a period 25 days. The rooted plantlets thus obtained were used for the experiment.

3.6 Hardening

The rooted plantlets were transferred to coir pith medium in net pots after washing off adhering agar. The plantlets were first kept in moist conditions for 15 days. Thenthese were planted in a potting mixture of 1:1:1 proportion of soil, sand and compost filled in earthen pot and kept in favourable conditions.

3.7 Experimental factors

The experimental consisted of two factors

1. Variety
2. Different concentration of Auxin(NAA& IBA) and cytokinin (BAP& BA)

3.7.1 Factor A

Experimental materials are two popular varieties of gladiolus

1. Yellow variety (V_1)
2. White variety(V_2)

3.7.2 Factor B

Different concentration of plant growth regulators for callus initiation

1) T_1=control
2) T_2=1.0 mg/L BAP
3) T_3=1.5 mg/L BAP
4) T_4=2.0 mg/L BAP
5) T_5=1.0 mg/L BAP + 1.0 mg/L NAA
6) T_6=1.5 mg/L BAP + 1.0 mg/L NAA
7) T_7=2.0 mg/L BAP + 1.0 mg/L NAA
8) T_8=1.0 mg/L BAP + 1.5 mg/L NAA
9) T_9=1.5 mg/L BAP + 1.5 mg/L NAA
10) T_{10}= 2.0 mg/L BAP+ 1.5 mg/L NAA

Different concentration of plant growth regulators for shoot induction

1) T_1=control
2) T_2=1.0 mg/L BA
3) T_3=2.0 mg/L BA
4) T_4=3.0 mg/L BA
5) T_5=4.0 mg/L BA
6) T_6= 1.0 mg/L BA+ 0.5 mg/L NAA
7) T_7= 2.0 mg/L BA+ 0.5 mg/L NAA
8) T_8= 3.0 mg/L BA+ 0.5 mg/L NAA
9) T_9= 4.0 mg/L BA+ 0.5 mg/L NAA

Different concentration of plant growth regulators for root induction

1) T_1=control
2) T_2= 1.0 mg/L IBA
3) T_3= 1.5 mg/L IBA
4) T_4= 2.0 mg/L IBA
5) T_5= 1.0 mg/L NAA
6) T_6= 1.5 mg/L NAA
7) T_7= 2.0 mg/L NAA

3.8 Experimental details

3.8.1 Experiment I, II and III: To study the effect of hormone on *in vitro* regeneration of callus, shoot and root

3.8.1.1 Experimental material

Healthy gladiolus plantlets of cultivars, white and yellow cormels were used as experimental material.

3.8.1.2 Recording of experimental data

3.8.1.2.1 Sampling procedure

Five plantlets each selected randomly from the 3 replications of each of the treatment combinations were tagged to record observations on the various parameters. Destructive sampling was done to record the observations on the root system.

3.8.1.2.2 *In vitro* callus induction

3.8.1.2.2.1 Days to callus induction

Generally callus induction started after few days of explants incubation. Days to callus induction were recorded until callus was not induced fromthe explants. The mean value of the data provided the days to callus induction.

3.8.1.2.2.2 Size of callus

It was measured with a plastic scale set under test tube from base to apex of callus. Size of the callus was recorded at 14, 28 and 42 days after inoculation (DAI) of callus. Callus length was measured horizontally and breath was measured vertically. The formula (Thadavong *et al.*, 2002)used for estimating of size of callus is given below:

$$\text{Size of callus} = \frac{\text{breath} + \text{length}}{2}$$

3.8.1.2.2.3 Fresh weight of callus

Callus weight was recorded at 14, 28 and 42 days in grams after inoculation of explants with the help of electrical digital balance inside the laminar airflow with proper precaution. After that the callus was place in its previous place.

3.8.1.3.3 *In vitro* plants regeneration

To investigate the effect of different hormonal treatment of this experiment, the following parameters were recorded.

3.8.1.3.3.1 Days to shoot initiation

Shoot initiation started after 14-28 days of incubation of explants. The mean value of the data provided the days required for shoot initiation.

3.8.1.3.3.2 Number of shoot/ plantlet

the number of shoot proliferated was recorded at 14, 28 and 42 days after inoculation (DAI) and the number shoots/ explants was counted and mean was recorded.

The mean value was calculated using the following formula:

$X = \sum X_i / n$

Where,

X= mean of shoots/plants

$\sum$= Summation

X_i= Number of observations/ replication

N = number of observations/ replication

3.8.1.3.3.3Length of shoots/plantlet

The length of the plantlet was recorded by measuring the length from the base to the tip of the leaf. It was expressed in cm. it was measured by using a plastic scale in laminar airflow cabinet at an interval of 14, 28 and 42 days after inoculation(DAI). The mean value of the data provided the number of leaves per plantlets.

3.8.1.3.3.4Days to root initiation

Root formation was initiated within 21-28 days, but in many cases within one week. The mean value of the data provided the days to root initiation.

3.8.1.3.3.5 Number of roots per plantlet

The number of roots per plantlet was recorded at an interval 14, 28 and 42 days after inoculation of explant inoculation and it was recorded and mean was calculated.

3.8.1.3.3.3 Length of roots per plantlet

The length of roots/plantlet was determined by using a plastic scale inside the laminar airflow cabinet by plotting the plant in a petridish. Length of root in cm was recordedat an interval of 14, 28 and 42 days after inoculation (DAI) of explants inoculation and it was recorded and mean was calculated.

3.8.1.3.4 Subculture of *in vitro* plantlet

- Number of shoots/plantlet
- Length of shoot/plantlet
- Number of roots/plantlet
- Length of roots/plantlet

3.8.2 Acclimatization of *in vitro* plantlets

3.8.2.1 Planting material

Healthy and uniform tissue cultured gladiolus plantlets of white and yellow variety were used as experimental material.

3.8.2.2 Hardening media

Potting mixture (sand:soil:compost=1:1:1) was used as the hardening medium. The hardening media was sterilized uniformly with Formaldehyde 4 per cent.

3.8.2.3 Transfer of plantlet from culture vessel to soil

During *in vitro* acclimatization and the establishments into the soil, data were collected for the following parameter.

3.8.2.4 Survival rate of plantlets

The survival rate of established plants was calculated based on the number of plants in the cubicals and the number of plants finally survived. The survival rate of plantlets established was calculated by using the following formula:

Survival rate(%) of plant= $\frac{\text{number of established plants}}{\text{total number of plantlets}} \times 100$

3.9 Statistical analysis

The data for the characters under present study were statistically analyzes where applicable. The experiment was conducted in growth chamber and arranged in Completely Randomized Design (CRD).Data were analyzed using MSTAT-C software. The analysis of variance was performed and differences among the means were compared by the Least Significant Difference test at 5% level of significance.

CHAPTER IV

RESULTS AND DISCUSSION

The results of the investigation on the studies on *in vitro* regeneration by using PGRs and hardening of gladiolus plantlets conducted during 2015-2016 in the laboratory of Department of Genetics and Plant Breeding, Sher-e-Bangla Agricultural University were presented below:

4.1 Experiment I. Callus induction of two gladiolus varieties supplemented with different concentrations of plant growth regulators

This section of research was carried out for callus induction and subsequentregeneration from explants i.e.cormel of two gladiolus varieties *viz.*yellow and white variety. MS media supplemented with BAP alone or with NAA was used for initial callus induction. Callus initiation in cormel was observed as swelling of the explant following initiation from edges of the explant. The time to callus initiation, size of callus and fresh weight of callus varied with varieties and the concentration of plant growth regulators.

4.1.2 Days to callus initiation

Statistical analysis revealed significant differences in varieties, PGR combinations and their interaction for callus induction from cormel (Appendix II). However the time to callus initiation was observed dependent upon the variety grown and type/concentration of the PGR used and combinations of variety and PGRs (Appendix II).

The varietal comparison showedthat yellow variety (V_1)took more time (3.96 days) for callus initiation as compared to white variety(V_2) (2.77 days) (Table 1). Callus induction is genotype dependent (Kamo and Joung, 2007; Remotti and Loffler, 1995). In our study, white variety performed better than yellow variety (Table 1).

Table 1. Effect of different varieties on days to callus initiation, size of callus (cm) and fresh weight of callus (g) at different days after inoculation

Variety	Days to callus initiation	Size of callus (cm)			Fresh weight of callus (g)		
		14 DAI	28 DAI	42 DAI	14 DAI	28 DAI	42 DAI
V_1	3.96 a	0.39 b	0.78 b	1.31 b	0.39 b	0.81 b	1.53 b
V_2	2.77 b	0.48 a	0.96 a	1.59 a	0.46 a	0.92 a	1.67 a
$LSD_{(0.05)}$	0.139	0.016	0.047	0.040	0.017	0.020	0.073
CV (%)	7.91	6.82	10.38	5.15	6.47	4.28	8.88

V_1=yellow variety, V_2=white variety, T_1=control, T_2=1.0 mg/L BAP, T_3=1.5 mg/L BAP, T_4=2.0 mg/L BAP,T_5 = 1.0 mg/L BAP + 1.0 mg/L NAA, T_6=1.5 mg/L BAP + 1.0 mg/L NAA, T_7=2.0 mg/L BAP + 1.0 mg/L NAA, T_8=1.0 mg/L BAP + 1.5 mg/L NAA, T_9=1.5 mg/L BAP + 1.5 mg/L NAA, T_{10}= 2.0 mg/L BAP+ 1.5 mg/L NAA

In a column, means having similar letter(s) are statistically identical and those having different letter(s) differ significantly as per 0.05 level of probability

No callus initiation was observed in T_1 (control), T_2 (1.0 mg/L BAP), T_3 (1.5 mg/L BAP)and T_4 (2.0 mg/L BAP) (Table 2). The earlier mean callus induction was in T_9 (1.5 mg/L BAP+1.5 mg/L NAA) which was statistically similar to T_5 (1.0 mg/L BAP+1.0 mg/L NAA) and took 4.96 to 5.03 days to induce callus, respectively (Table 2).

In case of interaction between varieties and PGRs,in treatment T_1 (control), T_2 (1.0mg/L BAP), T_3 (1.5 mg/L BAP) and T_4(2.0 mg/L BAP),no callus was observed in any variety (Table 3).The maximum days (7.58 days) required to callus induction was noticed in the treatmentT_7(2.0 mg/L BAP+ 1.0 mg /L NAA) in yellow variety(V_1) and the minimum days (4.06 days) to callus induction was found in white variety(V_2) with T_9 (1.5 mg/L BAP+ 1.5 mg/L NAA) (Table 3).

From the above result it was observed that BAP with different concentrations produced no callus, but when BAP was supplemented with different concentrations of NAA, gradually callus initiation was observed (Table 2). Generally 2, 4-D and NAA are considered best auxins for callus initiation (Boonvanno and Kanchanpoom, 2000). Our results confirmed that the presence of NAA in the medium is must for callus induction and produced regenerable callus from cormel .

Our results are in harmony with Aftab *et al.* (2008) who reported callus initiation in cormel slices on MS medium supplemented with NAA (1 to 3mg/L). In contrast, callus formation was recorded from longitudinal sections of cormels at higher levels of NAA (8.5 mg/L) as reported by Emek and Erdag (2007).Our results also werenot in harmony with Kamo (1995) who reported quite a high concentration of NAA (10 mgL-1) for callus induction from cormel explants in gladiolus.

4.1.2 Size of callus

In case of varieties, significant differences had been found(Appendix II). White variety(V_2) produced the highest size of callus (0.48, 0.96 and 1.59 cm at 14, 28 and 42 DAI, respectively) than yellow variety(V_2) (0.39, 0.78 and 1.31 cm at 14, 28 and 42 DAI, respectively) (Table 1).

Table 2. Effect of different hormones on days to callus initiation, size of callus and fresh weight of callus at different days after inoculation

Treatment	Days to callus initiation	Size of callus (cm)			Fresh weight of callus (g)		
		14 DAI	28 DAI	42 DAI	14 DAI	28 DAI	42 DAI
T_1	0.00 e	0.00 e	0.00 e	0.00 e	0.00 f	0.00 g	0.00 f
T_2	0.00 e	0.00 e	0.00 e	0.00 e	0.00 f	0.00 g	0.00 f
T_3	0.00 e	0.00 e	0.00 e	0.00 e	0.00 f	0.00 g	0.00 f
T_4	0.00 e	0.00 e	0.00 e	0.00 e	0.00 f	0.00 g	0.00 f
T_5	5.03 d	0.77 b	1.59 a	2.62 a	0.84 b	1.58 b	2.96 ab
T_6	5.79 bc	0.71 c	1.31 c	2.38bc	0.74 c	1.50 c	2.64 cd
T_7	6.48 a	0.63 d	1.24 d	2. 06d	0.48 e	1.19 f	2.09 e
T_8	5.53 c	0.74 bc	1.55 a	2.47 b	0.75 c	1.27 e	2.80 bc
T_9	4.96 d	0.86 a	1.61 a	2.65 a	0.90 a	1.64 a	3.04 a
T_{10}	5.88 b	0.64 d	1.44 b	2.36 c	0.55 d	1.46 d	2.50 d
$LSD_{(0.05)}$	0.310	0.036	0.104	0.090	0.037	0.040	0.165
CV (%)	7.91	6.82	10.38	5.15	6.47	4.28	8.88

V_1=yellow variety, V_2=white variety, T_1=control, T_2=1.0 mg/L BAP, T_3=1.5 mg/L BAP, T_4=2.0 mg/L BAP,T_5 = 1.0 mg/L BAP + 1.0 mg/L NAA, T_6=1.5 mg/L BAP + 1.0 mg/L NAA, T_7=2.0 mg/L BAP + 1.0 mg/L NAA, T_8=1.0 mg/L BAP + 1.5 mg/L NAA, T_9=1.5 mg/L BAP + 1.5 mg/L NAA, T_{10}= 2.0 mg/L BAP+ 1.5 mg/L NAA

In a column, means having similar letter(s) are statistically identical and those having different letter(s) differ significantly as per 0.05 level of probability

Table 3. Interaction effect of different varieties and different hormones on days to callus initiation, size of callus and fresh weight of callus at different days after inoculation

Treatment	Days to callus initiation	Size of callus (cm)			Fresh weight of callus (g)		
		14 DAI	28 DAI	42 DAI	14 DAI	28 DAI	42 DAI
V_1 T_1	0.00 i	0.00 h	0.00 e	0.00 h	0.00 h	0.00h	0.00 g
V_1 T_2	0.00 i	0.00 h	0.00 e	0.00 h	0.00 h	0.00 h	0.00 g
V_1 T_3	0.00 i	0.00 h	0.00 e	0.00 h	0.00 h	0.00 h	0.00 g
V_1 T_4	0.00 i	0.00 h	0.00 e	0.00 h	0.00 h	0.00 h	0.00 g
V_1 T_5	5.52 de	0.77bcd	1.58 ab	2.60cd	0.87 b	1.62ab	2.98 a
V_1 T_6	7.31 ab	0.59 f	0.97 d	2.02 f	0.55 ef	1.36 e	2.21 ef
V_1 T_7	7.58 a	0.50 g	0.92 d	1.52 g	0.45 g	1.06 f	2.08 f
V_1 T_8	6.43 c	0.69 e	1.44 bc	2.30 e	0.70 d	1.00 g	2.70 bc
V_1 T_9	5.86 d	0.78bcd	1.54abc	2.50 d	0.85 bc	1.60 b	2.96 a
V_1 T_{10}	6.92 b	0.55 fg	1.42 c	2.20 e	0.50 fg	1.44 d	2.40 de
V_2 T_1	0.00 i	0.00 h	0.00 e	0.00 h	0.00 h	0.00 h	0.00 g
V_2 T_2	0.00 i	0.00 h	0.00 e	0.00 h	0.00 h	0.00 h	0.00 g
V_2 T_3	0.00 i	0.00 h	0.00 e	0.00 h	0.00 h	0.00 h	0.00 g
V_2 T_4	0.00 i	0.00 h	0.00 e	0.00 h	0.00 h	0.00 h	0.00 g
V_2 T_5	4.54 fg	0.77bcd	1.61 a	2.63bc	0.80 c	1.54 c	2.94a
V_2 T_6	4.28 gh	0.82 b	1.65 a	2.74ab	0.93 a	1.64ab	3.07 a
V_2 T_7	5.37 e	0.76 cd	1.55abc	2.60cd	0.50 fg	1.33 e	2.10 f
V_2 T_8	4.62 fg	0.79 bc	1.66 a	2.64bc	0.80 c	1.54 c	2.90 ab
V_2 T_9	4.06 h	0.93 a	1.67 a	2.80 a	0.95 a	1.67 a	3.11a
V_2 T_{10}	4.83 f	0.73 de	1.45 bc	2.52cd	0.60 e	1.47 d	2.60 cd
LSD $_{(0.05)}$	0.439	0.052	0.1476	0.1278	0.053	0.050	0.2334
CV (%)	7.91	6.82	10.38	5.15	6.47	4.28	8.88

V_1=yellow variety, V_2=white variety, T_1=control, T_2=1.0 mg/L BAP, T_3=1.5 mg/L BAP, T_4=2.0 mg/L BAP, T_5= 1.0 mg/L BAP + 1.0 mg/L NAA, T_6 = 1.5 mg/L BAP + 1.0 mg/L NAA, T_7=2.0 mg/L BAP + 1.0 mg/L NAA, T_8=1.0 mg/L BAP + 1.5 mg/L NAA, T_9=1.5 mg/L BAP + 1.5 mg/L NAA, T_{10}= 2.0 mg/L BAP+ 1.5 mg/L NAA

In a column, means having similar letter(s) are statistically identical and those having different letter(s) differ significantly as per 0.05 level of probability

Significant differences have been found among the treatments (Appendix II). Treatment T_9 (1.5 mg/L BAP+ 1.5 mg/L NAA) significantly produced the highest size of callus (0.86, 1.61 and 2.65 cm, respectively) and treatment T_7 (2.0 mg/L BAP+ 1.0 mg/L NAA) produced the lowest size of callus (0.63, 1.24 and 2.06 cm at 14, 28 and 42 DAI, respectively) (Table 2).

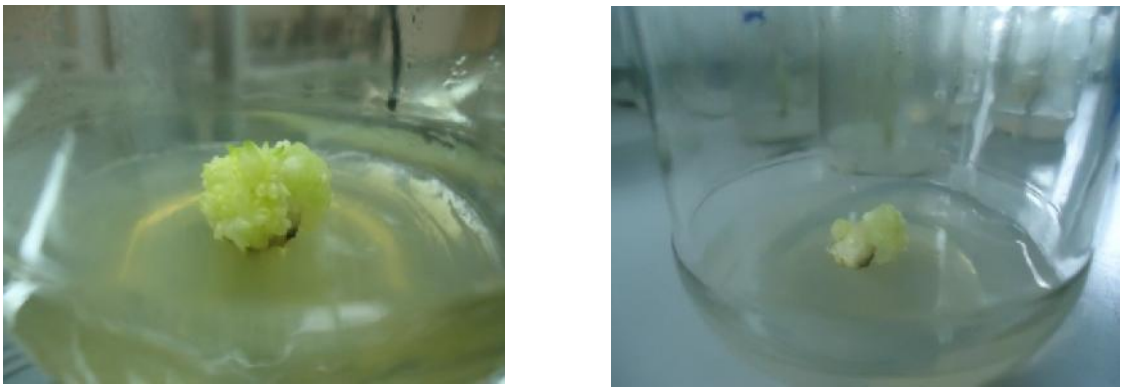
The interaction effect of varieties and different concentrations of PGRs showed significant variation on size of callus at different DAI (Appendix II). The highest size of callus (0.93, 1.67 and 2.80 cm at 14, 28 and 42 DAI, respectively) found in the white variety (V_2) was observed at T_9 (1.5 mg/L BAP + 1.5 mg /L NAA) (Table 3) (Plate 3) and the minimum size of callus (0.50, 0.92 and 1.52 cm at 14, 28 and 42 DAI) was found in yellow variety(V_1) at T_7 (2.0 mg/L BAP+ 1.0 mg/L NAA) (Table 3).A pictorial view of the highest size of callus 42 DAI on MS media supplemented with T_9 (1.5 mg/l BAP + 1.5 mg/l NAA) in white variety and the lowest size of callus 42 DAI on MS media supplemented with T_9 (1.5 mg/L BAP + 1.5 mg/LNAA) in yellow variety is presented in Plate 3.

Aftab *et al.,* 2008 showed that callus initiation from slices of cormels was possible when NAA was used at a concentration of 1 to 3 mg/L. The highest mean callus induction of 72.50% was observed on MS medium supplemented with NAA at 4 mg/L (Memon *et al.*, 2014). On the other hand, Kabir *et al.* (2014) showed that 90% explants showed potential callus on medium containing MS ± 7.5 mg/L NAA within the culture period of 90 days.

4.1.3 Fresh weight of callus

Significant differences were recorded for fresh weight of callus among varieties, treatments and their interactions (Appendix III).

White variety (V_2) significantly got the higher mean fresh weight (0.46, 0.92 and 1.67 g at 14, 28 and 42 DAI, respectively) of callus than yellow variety (V_1) (0.39, 0.81 and 1.53 g at 14, 28 and 42 DAI, respectively) (Table 1).



(a) (b)

Plate 3. The size of callus of different varieties of gladiolus: (a) The highest size of callus 42 DAI on MS media supplemented with T_9 (1.5 mg/l BAP + 1.5 mg/l NAA) in white variety; (b)The lowest size of callus 42 DAI on MS media supplemented with T_9 (1.5 mg/l BAP + 1.5 mg/l NAA) in yellow variety

Among the treatments, the highest mean fresh callus weight (0.90, 1.64 and 3.04 g at 14 DAI, 28 DAI and 42 DAI, respectively) was observed in treatment T_9 (1.5 mg/L BAP+1.5 mg/L NAA) and the lowest mean fresh callus weight (0.48, 1.19 and 2.09 g at 14, 28 and 42 DAI, respectively) was observed in treatment T_7 (2.0 mg/L BAP+1.0 mg/L NAA) (Table 2).

Regarding interaction, the highest fresh callus weight (0.95. 1.67 and 3.11 g at 14,28and 42 DAI, respectively) was recorded in white variety (V_2) in response to T_9 (1.5 mg/L BAP+1.5 mg/L NAA) and the lowest fresh callus weight (0.45, 1.06and 2.08 g at 14,28 and 42 DAI, respectively) was recorded in yellow variety (V_1) in response to T_7 (2.0 mg/L BAP+1.0 mg/L NAA) (Table 3).In contrast, Memon *et al.* (2014) showed that the highest fresh callus weight (0.59 g) was found in MS+0.5 mg/LBAPand 0.080 g fresh growth was found in MS+ 0.5 mg/L NAA.

4.2 Experiment II. Shoot initiation of two gladiolus varieties supplemented with different concentrations of plant growth regulators

The fresh calli was subdivided into equal clumps and cultured on shoot regeneration media. Green shoots were induced on calli and time for shoot initiation was recorded. Number of shoots and length of shoots were recorded at 14, 28 and 42 DAI for analysis the effect of varieties, PGRs and their interactions (Table 4, 5 and 6).

4.2.1 Days to shoot initiation

Statistical analysis showed that there was significant difference among the varieties (Appendix IV).The yellow variety (V_1) required more days (17.36 days) for shoot initiation compared to the white variety (V_2) (15.59 days) (Table 4).

In case of treatments, significant differences had been found among the treatments (Appendix IV). The minimum days (9.11 days) to shoot initiation was found in treatment T_4 (3.0 mg/L BA) and the maximum days (24.15 days) to shoot initiation was found treatment T_7(2.0 mg/L BA +0.5 mg/L NAA)(Table 5).

It is evident from the analysis of variance (Appendix IV) that both varieties and PGR combinations had independently and interactively significant differences for days to shoot initiation from cormel. The minimum days (6.61 days) to shoot initiation was found in treatment T_4(3 mg/L BA) in white variety(V_2) and the maximum days (25.20 days) to shoot initiation was found in treatment T_7 (2.0 mg/L BA+ 0.5 mg/L NAA) in yellow variety (V_1) (Table 6).

Dantu and Bhojwani (1987) reported BA as effective cytokinin for shoot multiplication in variety "Her Majesty". Memon *et al.* (2013) showed that the earlier mean shoot induction (17.50%) was observed on MS medium supplemented with BAP and KIN (2 mg/L) each in16.33 days.

4.2.2 Number of shoots per plantlet

Varieties depicted significant differences (Appendix IV) and white variety (V_2) yielded the highest number of shoots (2.50, 5.30 and 7.57 at 14, 28 and 42 DAI,

Table 4. Effect of different varieties on days to shoot initiation, no. of shoots per plantlet and length of shoots (cm) at different days after inoculation

variety	Days to shoot initiation	No. of shoots per plantlet			Length of shoots(cm)		
		14 DAI	28 DAI	42 DAI	14 DAI	28 DAI	42 DAI
V_1	17.36 a	0.80 b	2.52 b	3.99 b	0.67 b	2.13 b	2.81 b
V_2	15.59 b	2.50 a	5.30 a	7.57 a	1.15 a	2.66 a	3.92 a
$LSD_{(0.05)}$	0.29	0.18	0.28	0.35	0.03	0.07	0.27
CV (%)	3.26	13.76	13.09	11.10	5.72	5.55	14.61

V_1=yellow variety, V_2=white variety, T_1=control, T_2=1.0 mg/L BA, T_3=2.0 mg/L BA, T_4=3.0 mg/L BA, T_5= 4.0 mg/L BA, T_6=1.0 mg/L BA+ 0.5 mg/L NAA, T_7=2.0 mg/L BA+ 0.5 mg/L NAA, T_8=3.0 mg/L BA+ 0.5 mg/L NAA, T_9=4.0 mg/L BA+ 0.5 mg/L NAA
In a column, means having similar letter(s) are statistically identical and those having different letter(s) differ significantly as per 0.05 level of probability

Table 5. Effect of different hormones on days to shoot initiation, no. of shoots per plantlet and length of shoots (cm) at different days after inoculation

Treatment	Days to shoot initiation	No. of shoots per plantlet			Length of shoots(cm)		
		14 DAI	28 DAI	42 DAI	14 DAI	28 DAI	42 DAI
T_1	19.63 c	0.00 d	2.43 d	3.21 d	0.00 e	1.37 f	1.58 d
T_2	18.03 d	0.00 d	2.28 d	3.27 d	0.00 e	1.48 f	1.59 d
T_3	9.58 g	4.84 a	7.27 b	11.08 a	2.33 b	4.75 b	6.84 a
T_4	9.11 g	4.91 a	8.40 a	11.76 a	3.05 a	5.07 a	7.06 a
T_5	21.30 b	0.00 d	1.23 e	2.78 d	0.00 e	1.38 f	2.50 c
T_6	21.00 b	0.00 d	2.12 d	3.48 d	1.14 c	2.77 c	3.58 b
T_7	24.15 a	0.00 d	0.81 e	1.41 e	1.09 c	2.42 d	3.67 b
T_8	13.92 e	2.83 b	7.14 b	9.01 b	0.00 e	0.48 g	1.13 d
T_9	11.58 f	2.24 c	3.55 c	6.02 c	0.62 d	1.83 e	2.31 c
LSD $_{(0.05)}$	0.63	0.38	0.59	0.75	0.06	0.16	0.57
CV (%)	3.26	19.76	13.09	11.10	5.72	5.55	14.61

V_1=yellow variety, V_2=white variety, T_1=control, T_2=1.0 mg/L BA, T_3=2.0 mg/L BA, T_4=3.0 mg/L BA, T_5= 4.0 mg/L BA, $T_{6=}$1.0 mg/L BA+ 0.5 mg/L NAA, T_7=2.0 mg/L BA+ 0.5 mg/L NAA, T_8=3.0 mg/L BA+ 0.5 mg/L NAA, T_9=4.0 mg/L BA+ 0.5 mg/L NAA

In a column, means having similar letter(s) are statistically identical and those having different letter(s) differ significantly as per 0.05 level of probability

Table 6. Interaction effect of different varieties and different hormones on days to shoot initiation, no. of shoots per plantlet and length of shoots (cm) at different days after inoculation

Treatment	Days to shoot initiation	No. of shoots per plantlet			Length of shoots(cm)		
		14 DAI	28 DAI	42 DAI	14 DAI	28 DAI	42 DAI
V_1 T_1	20.30 d	0.00 f	2.21 hi	3.22 i	0.00 f	1.32 g	1.52fgh
V_1 T_2	20.60 d	0.00 f	1.57 ij	2.12 j	0.00 f	2.05 f	1.48 fgh
V_1 T_3	10.50 jk	2.80 d	4.20 de	7.07 e	2.56 b	4.87 b	7.04 b
V_1 T_4	11.60 i	2.34de	3.60 def	5.67 f	2.23 c	4.46 c	6.00 cd
V_1 T_5	21.50 c	0.00 f	1.30 j	3.43hi	0.00 f	1.34 g	2.75 e
V_1 T_6	16.80 g	0.00 f	3.45 efg	5.45fg	0.00 f	0.98 h	1.40 gh
V_1 T_7	25.2 a	0.00 f	0.76 j	1.38j	0.00 f	0.86 h	1.35 gh
V_1 T_8	17.8 f	0.00 f	2.87 fgh	4.02hi	0.00 f	0.50 i	0.80 h
V_1 T_9	11.97 i	2.02 e	2.76 fgh	3.56hi	1.23 e	2.76 e	2.95 e
V_2 T_1	18.97 e	0.00 f	2.65 gh	3.20 i	0.00 f	1.42 g	1.65 fg
V_2 T_2	15.47 h	0.00 f	2.98 fgh	4.42gh	0.00 f	0.92 h	1.70 fg
V_2 T_3	8.67 l	6.87 b	10.33 c	15.10b	2.10 d	4.62 c	6.65bc
V_2 T_4	6.61 m	7.47 a	13.20 a	17.87a	3.86 a	5.67 a	8.11 a
V_2 T_5	21.10 cd	0.00 f	1.16 j	2.12j	0.00 f	1.43 g	2.25 ef
V_2 T_6	25.20 a	0.00 f	0.78 j	1.51j	2.27 c	4.57 c	5.76 d
V_2 T_7	23.10 b	0.00 f	0.86 j	1.44j	2.19 c	3.98 d	6.00 cd
V_2 T_8	10.04 k	5.65 c	11.40 b	14.00c	0.00 f	0.45 i	1.46 fgh
V_2 T_9	11.20 ij	2.45de	4.34 d	8.48d	0.00 f	0.89 h	1.67 fg
$LSD_{(0.05)}$	0.89	0.53	0.84	1.06	0.09	0.22	0.81
CV (%)	3.26	19.76	13.09	11.10	5.72	5.55	14.61

V_1=yellow variety, V_2=white variety, T_1=control, T_2=1.0 mg/L BA, T_3=2.0 mg/L BA, T_4=3.0 mg/L BA, T_5= 4.0 mg/L BA, T_6=1.0 mg/L BA+ 0.5 mg/L NAA, T_7=2.0 mg/L BA+ 0.5 mg/L NAA, T_8=3.0 mg/L BA+ 0.5 mg/L NAA, T_9=4.0 mg/L BA+ 0.5 mg/L NAA

In a column, means having similar letter(s) are statistically identical and those having different letter(s) differ significantly as per 0.05 level of probability

respectively) followed by yellow variety($V_{1)}$ (0.8, 2.52 and 3.99 at 14, 28 and 42 DAI, respectively) from cormel (Table 4).

Treatment T_4(3.0 mg/L BA) exhibited themaximum numberof shoots (4.91, 8.40 and 11.76 at 14, 28 and 42 DAI, respectively) and treatment T_7(2.0 mg/L BA+0.5 mg/L NAA) produced the minimum number of shoots (0.00, 0.81 and 1.41 at 14, 28 and 42 DAI, respectively) (Table 5).

The interaction effect of varieties and PGRs showed that treatment T_4 (3.0 mg/L BA) showed the highest number of shoots(7.47, 13.20 and 17.87 at 14, 28 and 42 DAI, respectively) in white variety (V_2) and T_3 (2.0 mg/L BA) and the highest number of shoots(2.80, 4.20 and 7.07 at 14, 28 and 42 DAI, respectively) in yellow variety (V_1)(Tabe 6). A pictorial view of the maximum no. of shoots at 42 DAI on MS media supplemented with T_4 (3.0 mg/l BA) in white variety and the maximum no. of shoots at 42 DAI on MS media supplemented with T_3(2.0 mg/l BA) in white variety is presented inPlate 4.

The addition of cytokinins in the medium encourages bud/shoot regeneration as reported by Sinha and Roy (2002). Budiarto (2009) recommended BA concentrations of 2–3 mg/L for potential shoot induction in gladiolus. In the present study, maximum number of shoots were observed on medium containing BA (3mg/L). Boonvanno and Kanchanapoom (2000) observed multiple shoots from callus initiated from axillary buds on MS medium containing 1 mg/L BA. Shaheenuzzaman *et al.* (2011) recorded the highest percentage of shoot regeneration (91.66%) on MS basal medium supplemented with BAP (3 mg/L) and Kinetin (0.5 mg/L).Memon *et al.*, 2014 showed that the mean shoot induction (92.92%) and number of shoots (18.25) from callus of cormel slices increased as the concentration of BAP increased from 2 to 4 mg/L.

Different responses of shoot regeneration were recorded from different cultivars. However, higher dose requirement of BA was recognized as to be genotype dependent (Hussain *et al.*, 2001). They studied shoot regeneration of two varieties (white and pink) of gladiolus and reported maximum number of shoots from shoot tip explants at a much higher BAP concentration (3.0 mg/L and 4.0 mg/L respectively for

(a) **(b)**

Plate 4. The different number of shoots initiation from gladiolus varieties: (a) The maximum no. of shoots at 42 DAI in T_4 (3.0 mg/l BA) in white variety; (b) The maximum no. of shoots at 42 DAI on MS in T_3 (2.0 mg/l BA) in yellow variety

the two varieties).3.0 mg/L BAP was also found the best and needed least number of days for shooting. Maximum numbers of days (21.66 days) were required in the medium containing 6.0 mg/l BA(Shaheenuzzaman *et al.*, 2011).

Budiarto (2009) showed that plantlet of cv. Nabila produced highest shoot and leaf formations on the BA concentration of 2 mg/l, while cv. Kaifa and Clara showed their most productivity in 3 mg/l BA. However, in general, the number of shoot and leaves of plantlet decreased when higher BA was applied. These conditions indicated that BA concentrations ranged from 2 to 3 mg/l were the most favorable level of BA in MS medium + 0.5 mg/l NAA for shoot and leaf formations. Similar finding was reported by Emek & Erdag (2007) on the study of somatic embryogenesis induction from the leaf explants that higher BA concentration than 3 mg/l in almost all cases, reduced the vegetative organ development.

4.2.3 Length of shoots per plantlet

Length of shoots was also significantly influenced by varieties and different concentrations of NAA and BA (Appendix V).White variety (V_2) produced the highest length (1.15, 2.66 and 3.92 cm at 14, 28 and 42 DAI,respectively) of shoots than yellow variety (V_2) (0.67, 2.13 and 2.81 cm at 14, 28 and 42 DAI, respectively (Table 4).

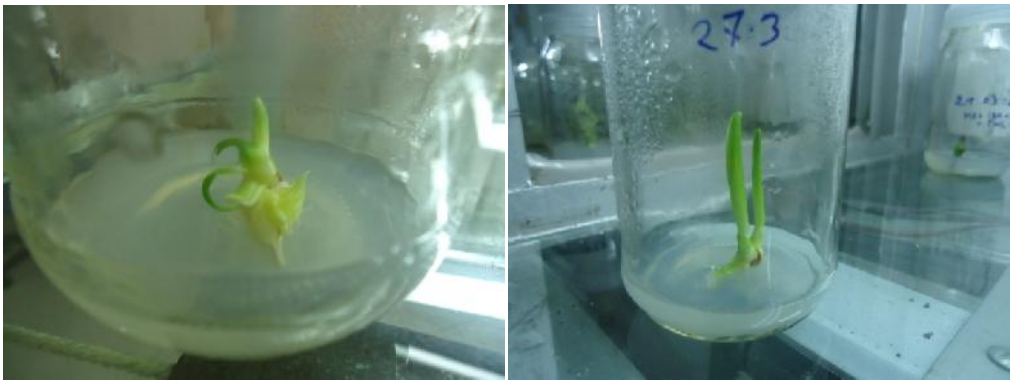
Treatment T_4 (3.0 mg/L BA) produced the highest length i. e. 3.05, 5.07 and 7.06 cm of shoot at 14, 28 and 42 DAI, respectively and treatment T_8 produced the lowest length of shoots (0.00, 0.48 and 1.13 cm at 14, 28 and42 DAI, respectively) (Table 5).

Theinteraction effect of varieties and PGRs showed thatwhite variety (V_2) produced the highest length of shoots (3.86, 5.67 and 8.11 cm at 14, 28 and 42 DAI, respectively) in T_4 (3 mg/L BA) (Plate 5) and the longest shoots (2.56, 4.87 and 7.04 cm at 14, 28 and 42 DAI, respectively) of yellow variety was found in T_3 (2.0 mg/L) (Plate 6) (Table 6)Yellow variety (V_1) produced the smallest shoots (0.00, 0.50 and

(a) **(b)**

(c)

Plate 5. The length of shoots of white varieties (*Gladiolus grandiflorus*) at different DAI: (a) The highest length of shoots at 14 DAI in T_4 (3mg/L BA); (b) The highest length of shoots at 28 DAI in T_4 (3mg/L BA); (c) The highest length of shoots in white variety at 42 DAI in T_4 (3mg/L BA)



(a) **(b)**

(c)

Plate 6. The length of shoots of yellow varieties (*Gladiolus grandiflorus*) at different DAI: (a) The highest length of shoots at 14 DAI in T_3 (2mg/L BA); (b) The highest length of shoots at 28 DAI in T_3 (2mg/L BA); (c) The highest length of shoots at 42 DAI in T_3 (2mg/L BA)

0.80 cm at 14, 28 and 42 DAI, respectively) in T_8 (3.0 mg/L BA+ 0.5 mg/L NAA) (Table 6)

Shaheenuzzaman *et al.*(2011) reported that 3.0 mg/L BA resulted the longest shoot (3.65 cm) and BA at 4.0 (mg/L) was reported obtained 2.5 cm length of shoot andthe shortest shoot was 0.79 cm was observed in medium without BA.

4.3 Experiment III.Root initiation of two gladiolus varieties supplemented with different concentrations of plant growth regulators

4.3.1 Days to root initiation

To achieve root proliferation from cormels derived plantlets of gladiolus varieties, MS medium supplemented with different concentration of NAA and IBA were used. Days to root initiation were recorded at 14, 28 and 42 DAI and the results have been presented in Table 7, 8 and 9 and AppendixVI.

Minimumdays (9.93 days) to root initiation were found in white variety (V_2) than yellow variety (13.58 days) (Table 7).

In case of treatment, T_4 treatment (2.0 mg/L IBA) required minimum days (10.00 days) for root initiationand the maximum days (15.37 days) to root initiation was required in T_7 (2.0 mg/L NAA) (Table 8).

The gladiolus varieties and different levels of PGRs showed significant interaction in relation to the days to root initiation.In Table 9, the maximum days (23.20 days) to root initiationwas found in yellow variety (V_1) with T_7 (2.0 mg/L NAA) and the minimum days (6.12 days) to root initiation in white variety (V_2) atT_4(2.0 mg/L IBA) (Table 9).

Efficient methods for developing roots are equally important for better plantlet establishment. In the present study, it was observed that roots were produced at each PGR combination except control (MS medium) in both varieties (Table 9). It means addition of IBA was a potent auxin for root formation.Priyakumari and Sheela (2005) exhibited that MS medium supplemented with IBA 2 mg/L recorded earliest rooting (7 days). Aftab *et al.* (2008) also found that rooting response for the shoots was 100% on half strength MS medium containing 2 mgL-1 IBA after 5 days of inoculation. Begum & Haddiuzaman (1995) also achieved the rooting of *in vitro* grown shoots using 0.5mgL-1 IBA in half strength MS basal medium.

Table 7. Effect of different varieties on days to root initiation, no. of roots per plantlet and length of roots (cm) at different days after inoculation

Variety	Days to root initiation	No. of roots per plantlet			Length of roots(cm)		
		14 DAI	28 DAI	42 DAI	14 DAI	28 DAI	42 DAI
V_1	13.58 a	0.35 b	2.23 b	3.86 b	0.48 b	1.55 b	2.48 b
V_2	9.93 b	0.98 a	2.74 a	4.68 a	0.77 a	2.14 a	3.27 a
$LSD_{(0.05)}$	0.29	0.02	0.15	0.03	0.10	0.19	0.38
CV (%)	3.92	4.37	9.78	1.18	26.42	16.56	21.24

V1=yellow variety, $V_{2=}$white variety,T_1=Control, T_2=1.0 mg/L IBA, T_3=1.5 mg/L IBA, T_4=2.0 mg/L IBA, T_5=1.0 mg/L NAA, T_6=1.5 mg/L NAA, T_7=2.0 mg/L NAA
In a column, means having similar letter(s) are statistically identical and those having different letter(s) differ significantly as per 0.05 level of probability

Table 8. Effect of different hormones on days to root initiation, no. of roots per plantlet and length of roots (cm) at different days after inoculation

Treatment	Days to root initiation	No. of roots per plantlet			Length of roots(cm)		
		14 DAI	28 DAI	42 DAI	14 DAI	28 DAI	42 DAI
T_1	0.00 g	0.00 f	0.00 e	0.00 f	0.00 d	0.00 f	0.00 d
T_2	18.97 a	0.00 f	1.94 d	4.05 d	0.00 d	1.72 d	2.65 b
T_3	10.66 e	0.62 d	3.96 b	5.94 b	0.58 c	2.09 c	3.25 b
T_4	10.00 f	1.94 a	4.37 a	7.68 a	2.09 a	3.96 a	5.33 a
T_5	13.30 d	0.72 c	2.76 c	4.84 c	0.67bc	1.52 d	2.69 b
T_6	14.00 c	0.57 e	2.54 c	3.71 e	0.83 b	2.91 b	4.80 a
T_7	15.37 b	0.85 b	1.83 d	3.68 e	0.19 d	0.70 e	1.42 c
LSD $_{(0.05)}$	0.54	0.04	0.28	0.06	0.19	0.36	0.72
CV (%)	3.92	4.37	9.78	1.18	26.42	16.56	21.24

V1=yellow variety, V_2=white variety,T_1=Control, T_2=1.0 mg/L IBA, T_3=1.5 mg/L IBA, T_4=2.0 mg/L IBA, T_5=1.0 mg/L NAA, T_6=1.5 mg/L NAA, T_7=2.0 mg/L NAA
In a column, means having similar letter(s) are statistically identical and those having different letter (s) differ significantly as per 0.05 level of probability

Table 9. Interaction effect of different varieties and different hormones on days to root initiation, no. of roots per plantlet and length of roots (cm) at different days after inoculation

Treatment	Days to root initiation	No. of roots/plantlet			Length of roots(cm)		
		14 DAI	28 DAI	42 DAI	14 DAI	28 DAI	42 DAI
V_1 T_1	0.00 j	0.00 g	0.00 g	0.00 l	0.00 e	0.00 f	0.00 f
V_1 T_2	20.60 b	0.00 g	1.32 e	3.26 i	0.00 e	0.98 e	1.30 de
V_1 T_3	15.20 b	0.00 g	3.23 c	6.21 c	0.00 e	2.57 c	4.00 b
V_1 T_4	11.50 c	1.32 d	3.80 b	7.02 b	1.74 b	3.53 b	4.50 b
V_1 T_5	16.60 c	0.00 g	3.03 c	5.08 e	0.00 e	0.76 e	1.76cde
V_1 T_6	8.00 gh	1.13 f	3.65 b	4.30 h	1.66 b	2.14 c	3.83 b
V_1 T_7	23.20 a	0.00 g	0.57 f	1.13 k	0.00 e	0.86 e	1.99 cd
V_2 T_1	0.00 j	0.00 g	0.00 g	0.00 l	0.00 e	0.00 f	0.00 f
V_2 T_2	17.33 c	0.00 g	2.56 d	4.83 f	0.00 e	2.45 c	4.00 b
V_2 T_3	6.12 i	1.23 e	4.68 a	5.67 d	1.17 c	1.62 d	2.50 c
V_2 T_4	8.50 g	2.56 a	4.94 a	8.33 a	2.45 a	4.38 a	6.15 a
V_2 T_5	9.99 f	1.43 c	2.48 d	4.60 g	1.34 c	2.28 c	3.63 b
V_2 T_6	20.00 b	0.00 g	1.42 e	3.12 j	0.00 e	3.68 b	5.77 a
V_2 T_7	7.54 h	1.70 b	3.08 c	6.23 c	0.38 d	0.54 e	0.85 ef
LSD $_{(0.05)}$	0.77	0.05	0.41	0.09	0.27	0.51	1.02
CV (%)	3.92	4.37	9.78	1.18	26.42	16.56	21.24

V_1=yellow variety, V_2=white variety,T_1=Control, T_2=1.0 mg/L IBA, T_3=1.5 mg/L IBA, T_4=2.0 mg/L IBA, T_5=1.0 mg/L NAA, T_6=1.5 mg/L NAA, T_7=2.0 mg/L NAA

In a column, means having similar letter(s) are statistically identical and those having different letter(s) differ significantly as per 0.05 level of probability

4.3.2 Number of roots per plantlet

The response of varieties on number of roots per plantlets at different DAI was found significant. The variety white produced more number (0.98, 2.74 and 4.68 at 14, 28 and 42 DAI, respectively) of roots per plantlets compared to yellow (0.35, 2.23 and 3.86 days at 14, 28 and 42 DAI, respectively) (Table 7). These results indicated that white variety (V_2) showed better performance inroot formation over yellow variety (0.35, 2.23 and 3.86 at 14, 28 and 42 DAI, respectively).

In case of treatments, T_4(2 mg/L IBA) treatment produced highest no. of roots i.e. 1.94, 4.37 and 7.68 at 14, 28 and 42 DAI, respectively (Table 8).

Different varieties and concentrations of NAA and IBA influenced the number of roots produced per plantlet. In Table 9, the maximum no. of roots i.e. 2.56, 4.94 and 8.33 was found in white variety (V_2) with T_4 (2.0 mg/l IBA) at 14, 28 and 42 DAI, respectively (Table 9). A pictorial view of maximum number of root initiation of white variety (V_2) is presented in Plate 7**.**Aftab *et al.* (2008) also found the highest number of roots (24) produced by the treatment having NAA 1 mg/L. MS medium supplemented with 2 mg/L IBA induced extensive root growth of *in vitro* raised shoots for cv. 'Friendship' (Hussain *et al.*, 1994).

4.3.3 Length of roots/plantlet

The response of varieties on the increase of root in length at 14, 28 and 42 DAI was found significantly different (Appendix VII). There was sharp increasing trend in root length at different DAI both the varieties but the increment of root length (0.77cm, 2.14 cm and 3.27 cm at 14, 28 and 42 DAI, respectively) of white variety was superior to yellow variety (V_1) (0.48, 1.55 and 2.48 cm at 14, 28 and 42 DAI, respectively)(Table 7). These results indicated that the white variety (V_2) was better than yellow variety (V_1) on the increment of root length.

A significant variation was found among the concentrations of NAA and IBA. T_4 (2.0 mg/L IBA) treatment produced highest roots i.e. 2.09. 3.96 and 5.33 cm, at 14, 28 and

Plate 7.The maximum no. of roots initiated at 42 DAI on MS medium supplemented with T_4 (2.0 mg/l IBA) in white variety

42 DAI, respectively) and T_7 (2.0 mg/L NAA) treatment produced shortest roots i.e. 0.19, 0.70 and 1.42 cm, at 14, 28 and 42 DAI, respectively (Table 8).

Root length was also influenced by varieties and different concentrations of IBA and NAA. In Table 9,white variety produced longest roots (2.45, 4.38 and 6.15 cm at 14, 28 and 42 DAI, respectively) at T_4 (2.0mg/L IBA) (Table 9). A pictorial view of maximum length of root initiation in white variety is presented in Plate 8.Kabir *et al.* (2014) observed the average root length of 5.5 ± 0.70 cm in MS medium supplemented with 2.0 mg/L IBA. The superiority of IBA for rooting over other auxins has also been reported by Grewal *et al.*(1995).

Plate 8. Themaximum length of roots initiated at 42 DAI on MS medium supplimented with T_4 (2.0 mg/L) IBA in white variety

4.4 Acclimatization and establishment of plantlets in soil

4.4.1*Ex-vitro* hardening of plantlets

Hardening requires time and intensive labour contributing to major portion of the production cost. The successful *ex-vitro* acclimatization of micro propagated plants determines the quality of the end product and in commercial production, the economic viability of the enterprise (Conner and Thomas, 1982).

When shoots or plantlets are transplanted from culture room to greenhouse conditions, they may dessicate or wilt rapidly and can die as a result of the change in environment, unless substantial precautions are taken to accommodate them. In commercial micro propagation, this step is often the limiting factor (Poole andconover, 1983) and at best, is challenging, labour intensive and costly (Debergh, 1988). Traditionally the acclimatization environment *ex vitro* is adjusted to accommodate transplants from culture, gradually weaning them towards ambient relative humidity and light levels. Transplants must undergo a period of acclimatization, more specifically, a period of transitional development in which both anatomical characteristics and physiological performance escape the influence of the *in vitro* conditions (Donnelly *et al.*, 1984). Tissue cultured plants aresusceptible to transplantation shocks leaing to high mortality during the final stage of micro propagation (Dhawan and Bhojwani, 1986). This is because the growth conditions inside the culture vials induce abnormal morphology and physiology of the plants Sutter, 1984). Understanding these abnormalities is a prerequisite to develop efficient transplantation protocols. After *ex vitro* transfer, these plantlets might easily be impaired by sudden changes in environmental conditions and so need a period of acclimatization to correct the abnormalities (Pospisilova *et al.*, 1999). Therefore, after *ex vitro* transplantation plants usually need some period of acclimatization with gradual lowering in air humidity (Bolar et at., 1998).acclimatization units have been developed with temperature, humidity, irradiance, CO_2 concentration and air flow rate controlled by computer (Hayashi *et al.*, 1988).

4.4.3 Transplantation

Potting mixture (sand: soil: compost=1:1:1) was mixed properly and autoclaved one hour in 121^0c for 20 minutes. After cooling, the soil mixtures were taken into 10 cm pots for growing the pots *in vitro* condition. 30 plantlets were taken out from the vials without damaging any roots. Medium attached to the roots was gently washed out running tap water to prevent further microbial infection. The plantlets were then transplanted to pot containing potting mixture mentioned above. Immediately after transplantation the plants along with the pots were covered with moist polythene bag to prevent desiccation. To reduce sudden shock the pots were kept in favourable conditions for 7-15 days. The interior of the polythene bags was sprayed with distilled water at every 24 hours to maintain around the plantlets.

After 2-3 days, the polythene bags were gradually perforated to expose the plants to natural environment. The polythene bags were completely removed after 10-15 days when the plantlets, appeared to be self- sustainable. Finally after 15-20 days, they were transferred to the natural conditions for the adaptation to the soil (Plate 8). About 24 out of 30 plants (80.00%) survived in natural condition in case of white variety and 20 out of 30 plants (66.67%) of yellow variety survived in natural condition (Table 10).

Table 10. Survival rate of *in vitro* regenerated plantlets of two gladiolus varieties in earthen pot

variety	Number of transplanted plants	Number of plants survived	Survival rate(%)
White	30	24	80.00
Yellow	30	20	66.67

Plate 9. Transfer of plants in potting mixture (sand:soil:compost=1:1:1) for acclimatization

CHAPTER V

SUMMARY AND CONCLUSION

The present experiment was conducted at the tissue culture laboratory of Genetics and Plant Breeding Department, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka, during the period of 2015-2016 from cormels as explants of gladiolus white and yellow variety to establish *in vitro* callus induction and plant regeneration using different concentrations of plant growth regulators. The experiment was conducted at completely randomized Design (CRD) with 3 replications.

Cormels from source materials were used as experimental materials. Explants were sterilized by 0.1% $HgCl_2$ for 10 minutes. Explants were cultured on MS medium supplemented with different concentration of PGRs. To investigate the effect of different treatments of this experiment, the parameters were recorded on days to callus induction, size of callus, fresh weight of callus, days to shoot initiation, number of shoots /plantlets, length of shoots/plantlet, days to root initiation, number of roots /plantlets, length of roots /plantlets and survival rate of plants.

For callus regeneration, different concentration of BAP and NAA were used are,T_1(Control), T_2 (1.0 mg/L BAP), T_3(1.5 mg/L BAP), T_4(2.0 mg/L BAP), T_5(1.0 mg/L BAP + 1.0 mg/L NAA), T_6(1.5 mg/L BAP +1.0 mg/L NAA), T_7(2.0 mg/L BAP +1.0 mg/L NAA), T_8(1.0 mg/L BAP+1.5 mg/L NAA), T_9(1.5 mg/L BAP +1.5 mg/L NAA) and T_{10}(2.0 mg/L BAP+1.5 mg/L NAA).

Statistical analysis revealed significant differences in varieties, PGR combinations and their interaction for callus induction from cormel. The maximum days (7.58 days) required to callus induction was noticed in the treatment T_7 (2.0 mg/L BAP+ 1.0 mg /L NAA) in yellow variety (V_1) and the minimum days (4.06 days) to callus induction was found in white variety(V_2) with T_9 (1.5 mg/L BAP+ 1.5 mg/L NAA).

In case of varieties, significant differences have been found insize of callus. The highest size of callus (0.93, 1.67 and 2.80 cm at 14, 28 and 42 DAI, respectively)

found in the white variety (V_2) was observed at T_9 (1.5 mg/L BAP + 1.5 mg /L NAA) and the minimum size of callus (0.50, 0.92 and 1.52 cm at 14, 28 and 42 DAI) was found in yellow variety(V_1) at T_7 (2.0 mg/L BAP+ 1.0 mg/L NAA).

Significant differences were recorded for fresh weight of callus among varieties, treatments and their interactions.The highest fresh callus weight (0.95. 1.67 and 3.11 g at 14, 28 and 42 DAI, respectively) was recorded in white variety (V_2) in response to T_9 (1.5 mg/L BAP+1.5 mg/L NAA) and the lowest fresh callus weight (0.45, 1.06 and 2.08 g at 14, 28 and 42 DAI, respectively) was recorded in yellow variety (V_1) in response to T_7 (2.0 mg/L BAP+1.0 mg/L NAA).

For shoot regeneration, different concentration of BA and NAA were used are, T_1 (control), T_2 (1.0 mg/L BA), T_3 (2.0 mg/L BA), T_4 (3.0 mg/L BA), T_5 (4.0 mg/L BA), T_6 (1.0 mg/L BA+ 0.5 mg/L NAA), T_7 (2.0 mg/L BA+ 0.5 mg/L NAA), T_8 (3.0 mg/L BA+ 0.5 mg/L NAA), T_9 (4.0 mg/L BA+ 0.5 mg/L NAA).

It is evident from the analysis of variance that both varieties and PGR combinations had independently and interactively significant differences for days to shoot initiation from cormel. The minimum days (6.61 days) to shoot initiation was found in treatment T_4 (3 mg/L BA) in white variety(V_2) and the maximum days (25.20 days) to shoot initiation was found in treatment T_7 (2.0 mg/L BA+ 0.5 mg/L NAA)in yellow variety (V_1).

The interaction effect of varieties and PGRs showed that treatment T_4 (3.0 mg/L BA) produced the highest number of shoots(7.47, 13.20 and 17.87 at 14, 28 and 42 DAI, respectively) in white variety (V_2).

Length of shoots was also significantly influenced by varieties and different concentrations of NAA and BA. White variety (V_2) produced longest shoot i.e. (3.86, 5.67 and 8.11 cm at 14, 28 and 42 DAI, respectively) in T_4 (3 mg/L BA) and yellow variety (V_1) produced the smallest shoots (0.00, 0.50 and 0.80 cm at 14, 28 and 42 DAI, respectively) in T_8 (3.0 mg/L BA+ 0.5 mg/L NAA).

For root regeneration, different concentration of IBA and NAA were used are, T_1 (Control), T_2 (1.0 mg/L IBA), T_3 (1.5 mg/L IBA), T_4 (2.0 mg/L IBA), T_5 (1.0 mg/L NAA), T_6 (1.5 mg/L NAA), T_7 (2.0 mg/L NAA).

The gladiolus varieties and different levels of PGRs showed significant differencesto the days to root initiation. The maximum days (23.20 days) to root initiation, found in yellow variety was found with T_7 (2.0 mg/L NAA) and the minimum days (6.12 days) to root initiation in white variety (V_2) at T_3 (1.5 mg/l IBA).

Different varieties and concentrations of NAA and IBA significantly influenced the number of roots produced per plantlet. The maximum no. of roots i.e. 2.56, 4.94 and 8.33 was found in white variety with T_4 (2.0 mg/l IBA) at 14, 28 and 42 DAI, respectively.

Root length was also significantly influenced by varieties and different concentrations of IBA and NAA. The response of varieties showed that white variety produced longer root (2.45, 4.38 and 6.15 cm at 14, 28 and 42 DAI, respectively) at T_4 (2.0mg/L IBA). For acclimatization, plantlets were transplanted from culture media to earthen pot.

The protocol developed from the present study may be useful for large scale production of healthy and disease free planting material of gladiolus commercially. This protocol could be used for *in vitro* breeding program.

Conclusion

1) Treatment T_9 (1.5 mg/L BAP+ 1.5 mg/L NAA) was found best for early callus initiation, the highest size of callus and the highest fresh weight of callus.
2) Treatment T_4 (3.0 mg/L BA) was found best for early shoot initiation, the highest number of shoot and the highest length of shoot.
3) Treatment T_4 (2 mg/L IBA) was found for early root initiation, the highest number of root and the highest length of root.
4) Among the variety, the performance of white variety was better than yellow variety.

REFERENCES

Aftab, F., Alam, M. and Afrasiab, H. (2008). *In vitro* shoot multiplication and callus induction in *Gladiolushybridus. Hort. Pakistan J. Bot*. **40**(2):517-522.

Ahmad, T., Ahmad, M.S., Nasir, I.A. and Riazuddin, S.(2000). *In vitro* production of cormels in gladiolus. *Pakistan J. Biol. Sci.***3**(5):819-821.

Bajaj, Y.P.S., Sidhu, M.M.S., and Gill, A.P.S. (1986). Micropropagation of gladiolus. In: Bajaj, Y.P.S. (Ed.). Biotechnology in agriculture and forestry. High-Tech and Micropropagation IV Springer-Verlag, Berlin Heidelberg, **20**:135-143.

Bajaj, Y.P.S., Sidhu, M.M.S. and Gill, A.P.S. (1983). Some factors affecting the *in vitro* propagation gladiolus. *Hort. Sci.* **18**: 269-275.

Begum, S. and Hadiuzzaman, S. (1995). *In vitro* rapid shoot proliferation and corm development in *Gladiolus grandiflorus* cv. *Red. Plant Tiss. Cult.***5**(1):7-12.

Bose, T.K., Yadav, L.P., Pal, P., Das, P. and Parthasarathy, V.A. (2003). Commercial Flowers. Vol. II (2nd rev. ed.) NAYA UDYOG, Bidhan Sarani, Kolkata.

Bennette, I.J. and McComb, J.A. (1982). Propagation of Jarrah (*Eucalyptus marginata*) by organ and tissue culture. *Australian For. Res.***12**: 121-127.

Bini, G. and Bellini, F. (1973). Premeirs results de-la-technique del “imbyo culture applique a 1” amelioration genetique de preeneir. In: Gerestetter, R. (ed.). Genetique et amelioration de preuneir. *Acta Hort*. **48**: 51-88.

Bolar, J.P., Norelli, J., Aldwinckle, H. S. and Hanke, V. (1998). An efficient method for rooting and acclimatization of micropropagated apple culture. *Hort. Sci*.**37**:1241-1252.

Boonvanno, K. and Kanchanapoom, K. (2000). *In vitro* propagation of gladiolus. *Suranaree J. Sci. Tech.***7**:25-29.

Budiarto, K. (2009). *In vitro* regeneration of three gladiolus cultivars using cormel explants. *J. Ilm. Das.***10**(2): 109-113.

Chow, Y.N., Selby, C. and Harvey, B.M.R. (1992). Stimulation by sucrose of narcissus bulbil formation *in vitro. J. Hort. Sci.***67**:289-293.

Conner, A.J. and Thomas, M.B. (1982). Re-establishing plants from tissue culture –a review. *Proc. Inter. Prop*. *Coc*. **31**: 342-357.

Conouer, C.A. and Poole, R.T. (1984). Aclimatization of indoor foliage plants. *Hort. Rev.***6**: 120-154.

Dantu, P.K. and Bhojwani, S.S. (1987). *In vitro* propagation and corm formation in gladiolus. *Gart*. **52**: 90-93.

Dantu, P.K. and Bhojwani, S.S. (1995). *In vitro* corm formation and field evaluation of corm-derived plants of gladiolus. *Sci. Hort.***61**(1):115-129.

Debergh, P. (1988). Micropropagation of woody species state of the art on *in vitro* aspect.*Acta Hort.* **227**: 287-295.

Debergh, P.C. and Read, R.E. (1990). Micropropagation. In: Micropropagation: Technology and Application. Debergh, P.C. and R.H. Zimmerman, (Eds.). Kluwer Academic Publ. Dorclnecht. The Netherlands, pp.1-12.

De Bruyn, M.H. and Ferreira, D.I. (1992). *In vitro* corm production of *Gladiolus dalenii* and *G. tritis*. *Plant Cell Tiss. Org. Cult.***31**:123-128.

Donnelly, D.J. and Vidaver, W.E. (1984). Leaf Anatomy of red raspberry transferred from culture to soil. *J. American Soc. Hort. Sci*. **109**: 177-181.

Dhawan, V. and Bhojwani, S.S. (1987). Hardening *in vitro* and morpho-physiological changes in the leaves during acclimatization of micropropagated plants of *Leucaena leucocephela* (Lam) de wit. *Plant Sci*. **53**: 65-72.

Emek, Y. and Erdag, B. (2007). *In vitro* propagation of *Gladiolus anatolicus* (Boiss.) Stapf. *Pakistan J. Bot*. **39**(1):23-30.

Fridborg, G. and Eriksson, T. (1975). Effects of activated charcoal on growth and morphologenesis in cell cultures. *Physiol. Plant*. **34**: 306-308.

Goo, D.H., Joung, H.Y. and Kim, K.W. (2003). Differentiation of gladiolus plantlets from callus and subsequent flowering. *Acta Hort.***620**: 339-342.

Gosal, S.S. and Grewal, H.S. (1991). Tissue culture propagation: problems and potentials. In: Horticulture - new technologies and applications. Proc. International Seminar on New Frontiers in Horticulture. J. Prakash and RLM Pierikh (Eds.), Kluwer Publ. The Netherlands,pp. 197-200.

Grewal, M.S., Arora, J.S. and Gosal, S.S. (1995). Micro-propagation of Gladiolus through *in vitro* cormels production. *Plant Tiss. Cult.***5**(1): 27-33.

Halevy, A.H. (1986). The induction of contractile roots in *Gladiolus grandiflorus*. *Planta*. **167**: 94-100.

Hartman, R.D. (1988). The role of the micropropagation business in the invention and commercialization of plants. pp. 193-203. In: Mabry T.J. (ed.). Plant Biotechnology. Inc. Institute, Austin.

Hayashi, M., Nakayama, M. and Kozai, T. (1988). An application of the acclimatization unit for growth of carnation explants and rooting and acclimatization of the plants. *Acta. Hort.***230**:189-194.

Hoque, M.I. and Fatema, M. (1995). *In vitro* multiple shoot regeneration in *Chrysanthemum morifolium* . *Plant Tiss. Cult.***5(**2):153-162.

Hosaki, T. and Asahira, T. (1980). *In vitro* propagation of narcissus. *Hort. Sci.***15**:602-603.

Hussain, S.C.T., Geetha, C.K., Rajeevan, P.K. and Valsalakumari, P.K. (1994). Plant regeneration from root derived callus in gladiolus. *J. Orna. Hort.***2**:46-50.

Hussain, I., Muhammad, A., Rashid, H. andQuraishi, A. (2001). *In vitro* multiplication of gladiolus (*Gladiolus crassifolius). Plant Tiss. Cult*. **11**:121-126.

Hussey, G. (1978). *In vitro* propagation of three onion *Allium cepa* by axillary and adventitious shoot proliferation. *Sci. Hort*. **9**(3): 227-236.

Hussey, G. (1978). *In vitro* propagation of some members of Liliaceae, Iridaceae and Amaryllidaceae . *Act. Hort.***8**: 303-309.

Jain, A.K., McAlister, B.G. and Vanstaden, J. (1998). *In vitro* culture of *Gladiolus carneus*. *South Africa J. Bot*. **64** (2): 146-147.

Jain, M. and Chaturvedi, H.C. (1993). *In vitro* mass multiplication of Chrysanthemum cinerarifolium, in proc., XVI Annual Meeting of Plant Tissue culture Association, India and Symp. on plant tissue culture and its biotechnological applications. Mar. 1-3, 1993.Vniv. Rajashtal, Jaipur, India, pp. 9-10.

Kabir, M.H., Mamun, A.N.K., Yesmin, F. and Subramaniam, S. (2014). *In vitro* propagation of *Gladiolus dalenii* from the callus through the culture of corm slices. *J. Phyto*. **6**: 40-45.

Kamo, K., Chen, J. and Lawson, R. (1990). The establishment of cell suspension cultures of gladiolus that regenerated plants *in vitro* cells. *Dev. Biol*. **26**: 425-430.

Kamo, K. (1994). Effect of phytohormones on plant regeneration from callus of gladiolus cultivar "Jenny Lee". *In vitro Cell Dev. Biol*.**30:**26-31.

Kamo, K. (1995). A cultivar comparison of plant regeneration from suspension cells, callus, and cormel slices of gladiolus. *In vitro Cell Dev. Biol. Plant*.**31**:113-115.

Kashem, A. (1992). *In vitro* Regenerative Performance of Seven Cultivars of *Gladiolus grandiflorus L*. M. Sc. In Botany thesis submitted to the Department of Botany, University of Dhaka. pp. 1-116.

Kim, K., Jung-Bun, C. and Kee-Young, K. (1988). Rapid multiplication of gladiolus plants through callus culture. *J. Korean Soc. Hort. Sci*.**29**:312-318.

Kamo, K. and Joung, Y.H. (200)7. Gladiolus. In: Pua, E.C. and M.R. Davey (ed) Biotechnology in agriculture and forestry (Vol. 61) Transgenic Crops VI. Springer-Verlag Berlin Heidelberg, pp. 289-298.

Kumar,A., Sood, A, Palni,L.M.S. and Gupta, A.K. (1999). *In vitro* propagation of *Gladiolus hybridus* hort.: Synergistic effect of heat shock and sucrose on morphogenesis. *Plant Cell Tiss. Org. Cult*.**57**:105-112.

Lilien-Kipnis, H. and Kochba, M. (1987). Mass propagation of new *Gladiolus* hybrids.*Act. Hort*. **212**: 631-637.

Logan, A.E. and Zettler, F.W. (1985). Rapid *in vitro* propagation of virus indexed gladiolus. *Act. Hort*. **164**: 169-180.

Memon, N., Jasakni, M.J., Qasim, M. and Sharif, N. (2014). Cormel formation in gladiolus through tissue culture. *Pakistan J. Agri. Sci*.**51**(2): 475-482.

Memon, N., Qasim, M., Jaskani, M.J., Khooharo, A.A., Hussain, Z. and Ahmad, I. (2013). Comparison of various explants on the basis of efficient shoot regeneration in gladiolus. *Pakistan J. Bot*. **45**(3): 877-885.

Memon, N., Qasim, M., Jaskani, M.J., Awan, F.S., Khan, A.I., Sadia, B. and Hussain, Z. (2012). Assessement of somaclonal variation in in vitro propagated cormels of gladiolus. *Pakistan J. Bot.***44**(2): 769-776.

Morel, G. and Muller, J.F. (1946). La culture *in vitro* de meristem apical de la pomme de terre. Compl. *R. Acad. Sci. Paris*. **258**: 5250-5252.

Mott, R.L. (1981). Trees. In: Conger B.V. (ed.) Cloning Agriculture Plants via *in vitro* Techniques CRC Press Inc.,Boca Raton, Florida. pp. 217-254.

Murashige, T. (1974). Plant propagation through tissue cultures. *Ann. Rev. Plant Physiol*. **25**: 135-165.

Nekrosova, T.V. (1964). The culture of isolated buds of fruit trees. *Sov. Plant Physiol.***11**: 107-113.

Pospilova, J., Ticha, I., Kadlecek, P., Haisel, D. and Plzakova, S. (1999). Acclimatization of micropropagated plants to *ex vitro* conditions. *Biol. Planta*. **42**(4): 481-497.

Pfeiffer, E.N. (1931). A morphological study of Gladiolus contr. Boyce. Tompson. *Ins. Plant Res*. **3**: 173-196.

Priyakumari, I. and Sheela, V.L. (2005). Micropropagation of gladiolus cv. 'Peach Blossom' through enhanced release of axillary buds. *J. Trop. Agric.***43**(1-2):47-50.

Quoirin, M., Lepoivre, P. and Boxus, P. (1977). Un premier bilan de 10 annees. On recherché Sur les culture de meristems et. A. la nultiplication *in vitro* d fruitie ligomux, C.R. Rech. 1976-1977. Stn. Cult, Fruit. Maracheres Gemblu, pp. 93-117.

Remotti, P.C. and Loffler, H.J.M. (1995). Callus induction and plant regeneration from gladiolus. *Plant Cell Tiss. Org. Cult.***42**(2):171-178.

Rao, A.N. and Lee, S.K. (1982). Importance of tissue cuture in tree propagation. In fujiwara, A. (ed.) Proceedings of 5th Internastional Congress on plant Tissue at Cell Culture. Maruzen Co., Tokyo, pp. 715-718.

Rao, T.M., Negi, S.S. and Swamy, R.D. (1991). Micropropagation of Gladiolus. *Indian J. Hort*. **48**(2): 171-176.

Roy, S.K., Rahman, S.K.L. and Datta, P.C. (1987). *In vitro* propagation of *Mitragyna parviflorakorth.Plant Cell Tiss. Org Cult*. **12**: 75-80.

Roy, S.K., Gangopadhyay, G., Bandyopadhyay, T., Modak, B.K., Datta, S. and Mukherjee, K.K. (2006). Enhancement of *in vitro* micro corm production in gladiolus using alternative matrix. *African J. Biotechnol*.**5**(12):1204-1209.

Shaheenuzzaman, M., Haque, M.S., Karim, M.M. and Noor, Z.U. (2011). *In vitro* shoot proliferation and development of micropropagation protocol from leaf disc of gladiolus. *J. Bangladesh Agril. Univ*.**9**(1): 21–26.

Simonsen, J. and Hildebrandt, A.C. (1971). *In vitro* growth and differentiation of Gladiolus in liquid shake cultures. *Plant cell Tiss. Org. Cul*. **26:** 63-70.

Singh, A.P. and Dohare, S.R. (1994). Maximization of corms and cormel production in gladiolus. In: Floriculture-Technology, trades and trends (Ed. J. Prakash & K.R. Bhandary), p.205-208. Oxford & IBH Pub.Co. Pvt. Ltd. India.

Sinha, P. and Roy, S.K. (2002). Plant regeneration through *in vitro* cormel formation from callus culture of *Gladiolus primulinus* Baker. *Plant Tiss. Cult.***12(**2):139-145.

Sriskandarajal, C. and Mullins, M.G. (1981). Micropropagation of granny smith apple: Factors affecting root formation *in vitro*. *J. Hort. Sci*. **56**:71-76.

Start, N.D. and Cumming, B.G. (1976). *In vitro* propagation of *Saintpaulia ionanthawendl. Hort. Sci*. **11**: 204-206.

Stone, O.M. (1963). Factors affecting of carnation plants from shoot apices. *Ann. Appl. Biol*. **52**: 199-209.

Sutter, E.G. (1984). Chemical compositions of epicuticular wax of cabbage plants grown *in vitro. Canadian J. Bot*. 62: 74-77.

Thadavong, S., Sripichitt, P., Wongyai, W. and Jumpuk, P. (2002). Callus induction and plant regeneration from mature embryos of glutinous rice (*Oryza sativaL.)* cultivar TDK1. *Kasetsart J. (Nat. Sci.)***36**:334-344.

Tipirdamaz, R. (2003). Rooting and acclimatization of *in vitro* micropropagated snowdrop (*Galanthus ikariae* Baker) bulblets. *Akdeniz Univ. Ziraat Fakultesi Dergisi.***16**(2):121-126.

Torabi-Giglou, M. and Hajieghrari, B. (2008). *In vitro* study on regeneration of *Gladiolus grandiflorus* corm calli as affected by plant growth regulators. *Pakistan. J. Biol. Sci.* **11**(8):1147-1151.

Wilfret, G.J. (1971). Shoot tip culture of Gladiolus: An evaluation of nutrient media for callus tissue development. *Proc. Fl. Stat. Hort. Soc*.**84**: 389-393.

Zakutskaya, E.S. and Murin, A.V. (1990). Use of tissue culture method in propagating gladiolus IZVestiya- Akademii- Nauk-SSR- Moldova. *Biol. Khim. Nauk*. **6**: 63.

Ziv, M. (1979). Transplanting gladiolus plants propagated *in vitro. Scientia Hort.* **11**:257-260.

Ziv, M., Halevy, A.H. and Shillo, R. (1970). Organs and Plantlets regeneration of Gldiolus through tissue culture. *Ann. Bot.***34**(136): 671-76.

APPENDICES

Appendix I. Concentration and composition used for the preparation of MS medium (Murashige and Skoog,1962).

Category	Compound	Weight mg1-1	Stock solution g 1-1
A	**Macronutrients**		10 X
	NH_4NO_3	1650	16.5
	KNO_3	1900	19.0
	$CaCl_2.2H_2O$	440	4.40
	$MgSO_4.7H_2O$	370	3.70
	KH_2PO_4	170	1.70
B	**Micronutrients**		1000 X
	KI	0.830	83.0
	H_3BO_3	6.20	620
	$MnSO_4.7H_2O$	16.90	1690
	$ZnSO_4.7H_2O$	8.60	860
	$Na_2\ MoO_4.2H_2O$	0.25	25
	$CuSO_4.5H_2O$	0.025	2.5
	$CaCl_2.6H_2O$	0.025	2.5
C	**Iron**		100 X
	Na-EDTA	37.3	373
	$FeSO_4.7H_2O$	27.8	278
D	**Vitamins**		1000
	Myo-inositol dehydrate	100	4
	Thiamine HCL	0.4	5
	Nicotinic acid	0.5	5
	Pyriduxin HCL	0.5	

Stock solutions were kept in refrigerator at 4^0C

Appendix II. Analysis of variance of days to callus initiation and size of callus.

Source of variance	Degrees of freedom (df)	Mean square of days to callus initiation	Mean square of size of callus (cm)		
			14 DAI	28 DAI	42 DAI
Factor A (Variety)	1	21.313**	0.127**	0.444**	1.168**
Factor B (Treatment)	9	51.435**	0.861**	3.468**	9.542**
AB (Variety × Treatment	9	1.950**	0.017**	0.105**	0.203**
error	40	0.071	0.001	0.008	0.006

**= Significant at 1% level of probability
*= Significant at 5% level of probability

Appendix III. Analysis of variance of days to fresh weight of callus.

Source of variance	Degrees of freedom (df)	Mean square of fresh weight of callus (g)		
		14 DAI	28 DAI	42 DAI
Factor A (Variety)	1	0.065**	0.184**	0.290**
Factor B (Treatment)	9	0.893**	3.413**	11.814**
AB (Variety × Treatment	9	0.023**	0.055**	0.108**
error	40	0.001	0.001	0.020

**= Significant at 1% level of probability
*= Significant at 5% level of probability

Appendix IV. Analysis of variance of days to shoot initiation and number of shoots per plant.

Source of variance	Degrees of freedom (df)	Mean square of days to shoot initiation	Mean square of number of shoots per plant.		
			14 DAI	28 DAI	42 DAI
Factor A (Variety)	1	42.206**	38.913**	104.028**	172.914**
Factor B (Treatment)	8	185.483**	27.056**	50.127**	90.610**
AB (Variety × Treatment	8	30.783**	9.196**	27.192**	45.795**
error	36	0.289	0.106	0.262	0.411

**= Significant at 1% level of probability
*= Significant at 5% level of probability

Appendix V. Analysis of variance length of shoots per plantlet.

Source of variance	Degrees of freedom (df)	Mean square of length of shoot		
		14 DAI	28 DAI	42 DAI
Factor A (Variety)	1	3.232**	3.856**	16.545**
Factor B (Treatment)	8	7.545**	14.780**	29.286**
AB (Variety × Treatment	8	2.286**	4.945**	6.860**
error	36	0.003	0.018	0.241

**= Significant at 1% level of probability
*= Significant at 5% level of probability

Appendix VI. Analysis of variance of days to root initiation and number of roots per plant.

Source of variance	Degrees of freedom (df)	Mean square of days to root initiation	Mean square of number of roots per plant.		
			14 DAI	28DAI	42DAI
Factor A (Variety)	1	140.581**	4.282**	2.716**	7.159**
Factor B (Treatment)	6	214.952**	2.559**	12.697**	33.653**
AB (Variety × Treatment	6	110.320**	1.602**	3.676**	6.833**
error	28	0.212	0.001	0.059	0.003

**= Significant at 1% level of probability
*= Significant at 5% level of probability

Appendix VII. Analysis of variance length of roots per plantlet.

Source of variance	Degrees of freedom (df)	Mean square of length of root per plantlet		
		14 DAI	28 DAI	42 DAI
Factor A (Variety)	1	0.809**	3.620**	6.529**
Factor B (Treatment)	6	3.176**	10.486**	20.314**
AB (Variety × Treatment	6	1.500**	1.539**	4.117**
error	28	0.027	0.093	0.374

**= Significant at 1% level of probability
*= Significant at 5% level of probability