IN VITRO PROPAGATION AND VARIABILITY INDUCTION BY MUTAGEN IN GERBERA (*Gerbera jamesonii* Bolus.)

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REGISTRATION NO. 09-03597

A Thesis

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

SEMESTER: January-June, 2014

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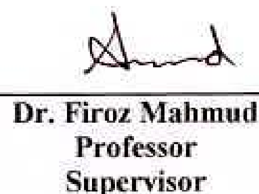
CERTIFICATE

This is to certify that thesis entitled, **"*IN VITRO* PROPAGATION AND VARIABILITY INDUCTION BY MUTAGEN IN GERBERA"** 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 **MST. SHAMIRA SULTANA**, Registration NO. 09-03597 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, as has been availed of during the course of this investigation has duly been acknowledged.



Dated: June, 2014
Place: Dhaka, Bangladesh

Dr. Firoz Mahmud
Professor
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Dedicated
to
My Beloved Parents

ACRONYMS

Abbreviations	Full Name
$^{\circ}C$	Degree Celsius
%	Percentage
IN	1 Normal
2, 4-D	2, 4-Dichlorophenoxyacetic acid
BAP	Benzyl Amino Purine
cm	Centimeter
CRD	Completely Randomized Design
Conc	Concentration
DAI	Days After Inoculation
DW	Distilled Water
EMS	Ethyl Methane Sulphonate
et al.	And others
etc.	Etcetra
Fig.	Figure
GA_3	Gibberelic Acetic Acid
gm	Gram
gm/L	Gram per litre
$HgCl_2$	Mercuric Chloride
IAA	Indole-3-Acetic Acid
i.e	id test (That is)
LSD	Least Significant Difference
mg	Milligram(s)
mg/L	Milligram per litre
ml	Milliliter
max.	Maximum
min.	Minimum
MS	Murashige and Skoog

Abbreviations	Full Name
μM	Micro mole
NAA	Naphthalene Acetic Acid
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
No.	Number
NS	Non Significant
ppm	Parts per million
pH	Negative logarithm of hydrogen ion concentration ($-\log[H^+]$)
PGRs	Plant Growth Regulators
SAU	Sher-e-Bangla Agricultural University
Sci	Science
SE	Standard Error
Soln.	Solution
Viz.	Namely

ACKNOWLEDGEMENT

First of all the author express her gratitude to the merciful and almighty Allah without whose grace he would have never been able to pursue her higher studies and to complete this thesis for the degree of MS in Genetics and Plant Breeding.

The author expresses her indebtedness, gratitude and appreciation to her reverend teacher and Research Supervisor **Dr. Firoz Mahmud, Professor**, Department of Genetics and Plant Breeding, Sher-e-Bangla Agricultural University, Dhaka for his scholastic supervision, valuable advices, constant inspiration, radical investigation and constructive criticism in all phases of this study and preparing the manuscript.

The author also expresses her heartfelt gratitude and profound regards to her respected teacher and Research Co-Supervisor **Dr. Md. Shahidur Rashid Bhuiyan, Professor**, Department of Genetics and Plant Breeding, Sher-e-Bangla Agricultural University, Dhaka for his kind co-operation and creative suggestions for the completion of the research work and preparation of this thesis.

The author pays her deepest respect to Professor **Dr. Md. Sarowar Hossain, Chairman**, Department of Genetics and Plant Breeding, Sher-e-Bangla Agricultural University, Dhaka for his cooperation and inspiration throughout the research work.

Special thanks and indebtedness are also due to all the respective teachers **Dr. Naheed Zeba, Dr. Mohammad Saiful Islam, Dr. Md.**

Abdur Rahim of the Department of Genetics and Plant Breeding, Sher-e-Bangla Agricultural University, Dhaka for their valuable teaching and cooperation throughout the period of the study.

The author expresses her gratitude to Ministry of Science and Technology, Government of the People's Republic of Bangladesh for awarding the National Science and Technology (NST) Fellowship and facilitating the author in the experimental procedure of this thesis.

The author also likes to give thanks to all of her friends of their support in her study period in SAU, Dhaka. Finally, the author found no words to thank her parents and family members for their love and continuous support for bringing her dream to proper shape. They were constant sources of inspiration, zeal and enthusiasm in the critical moment of her studies.

Dated: June, 2014
Place: SAU, Dhaka

The Author

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IN VITRO PROPAGATION AND VARIABILITY INDUCTION BY MUTAGEN IN GERBERA (*Gerbera jamesonii* Bolus.)

ABSTRACT

BY

MST. SHAMIRA SULTANA

The study was undertaken for establishing a protocol for *in vitro* callus induction, plant regeneration and mutagen sensitivity of two gerbera varieties. This research was held in the tissue culture laboratory of Genetics and Plant Breeding Department, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka. The effect of different combination and concentration of NAA and BAP on *in vitro* callus induction and plant regeneration was studied. The concentrations for BAP were 1.0 mg/L, 1.5 mg/L and 2.0mg/L. The concentration for combined NAA and BAP were1.0 mg/L NAA+1.0 mg/L BAP, 1.0mg/L NAA+2.0 mg/LBAP, 1.0 mg/L NAA+4.0 mg/L BAP, 1.5 mg/L NAA+1.0 mg/L BAP and 1.50 mg/L NAA+1.5 mg/L BAP. The Red variety took minimum days (4.00 days) for callus initiation and the largest size (2.77 cm) of callus were found with the same treatment of 1.50 mg/L NAA+1.5 mg/L BAP. The highest weight (3.12 gm) of callus was noticed in the variety Red with the treatment1.0 mg/L NAA+1.0 mg/L BAP at 42 DAI. In case of shoot initiation, variety Red took minimum time (5.60 days) followed by variety Yellow (8.74 days). The variety Red produced the maximum number of shoots/plantlet (16.80 shoots/plantlet) and the longest shoot (8.60 cm) followed by Yellow (13.29 shoots/plantlet and 2.63 cm) at 42 DAI. The Red variety also produced maximum number of leaves/plantlet (22.40 leaves) with the treatment 2.0mg/L BAP within 42 DAI. The variety Yellow took minimum days (9.30 days) to root initiation followed by Red (11.20 days). When variety and treatment took under considerations, the treatment 1.50 mg/L NAA+1.5 mg/L BAP with variety Red produced the maximum (14.70 roots/plantlet). The longest roots (7.50 cm) were found in the Red variety with the treatment of 1.5 mg/L NAA+1.0 mg/L BAP at 42 DAI. The investigation on induction of mutation *in vitro* was aimed for understanding the effect of chemical mutagens on the survival percentage of shoot and abnormalities of leaf. The survival percentage and the number of shoots produced from the treated shoot tips of gerbera decreased with different concentrations of the EMS used. Minimum percentage of survival shoot tips (39.59%) was found in the yellow variety with the concentrations 500 μM EMS. Yellow variety has shown maximum 60.41% mortality and Red variety shown 69.87% abnormal leaf with the same concentration 500 μM EMS. The regenerated plantlets were transferred successfully in earthen pot and subsequently to soil. The survival rate of the plantlets of Red variety was greater than Yellow variety under *ex vitro* conditions.

Chapter I
Introduction

CHAPTER I

INTRODUCTION

Gerbera (*Gerbera jamesonii* Bolus) is one of the foremost cut flowers and ranks among the top ten cut flowers of the world (Parthasarathy and Nagaraju, 1999). The flowers are hardy and stand the rigours of transportation and a long keeping quality fetches a good market price. It is an important commercial flower grown throughout the world. The long vase life, ability to rehydrate after long transportation makes it extremely popular as a cut flower. Gerbera flowers are now-a-days indispensable items for floral arrangements and decorations for marriages and other ceremonial functions.

The augmentation of floriculture industry has taken long strides world-wide especially in the developing countries as a result of outsourcing which is due to low cost of maintenance including low labor cost (Jain, 2006). For that reason gerbera has gained popularity in the past few years in many countries of the world and it is in great demand in the floral industry as cut flower as well as potted plant due to its beauty, color, long vase life (Kanwar and Kumar, 2008). The market value of gerbera is high in the US, Japan, Germany and in the UK. In Europe, the Netherlands and in Asia Indonesia as well as Malaysia are the dominant gerbera suppliers world-wide. In Bangladesh, public attraction is gradually increasing particularly in urban areas about gerbera as ornamental and home decorative plants for its gorgeous colors and size. However, the supply of gerbera plantlets is not adequate to fulfill the local demand. It may be mentioned here that Bangladesh has a favorable climatic condition and is capable of producing a wide array of gerberas of international standard (http://www.mdgbangla.org). In light of global demand, gerbera could be a major source of foreign exchange as one of the non-traditional export items in Bangladesh. In this aspect, mass commercial production of gerbera could be acted as a new aspect in Bangladesh economy. However, using conventional propagation system it is simply not possible to execute the demand of the export market. Though, it can be propagated both by sexual and asexual methods, seed propagation is not always satisfactory due to a great deal of variation (Schiva, 1975). The commercial cultivars are propagated through vegetative means so as to keep up uniformity, genetic purity and quality flower production (Peper *et al.,* 1971). Among the vegetative means, multiplication through division of clumps is the most common method used for several decades. Its

commercial propagation through division of clumps and other conventional methods of propagation is slow and inadequate for the production of large number of uniform propagules (Aswath & Choudhary, 2001). For commercialization of this crop, planting material is required on large scales, which further needs the development of an easier, quicker and economically viable method of propagation. Micropropagation is the only viable alternative for large-scale multiplication of gerbera. This method is free of seasonal bonds and enables manifold multiplication of the selected plants. The other advantages are product uniformity, disease-free plants, easy exchange of germplasm and planting material (Murashige *et al.*,1974). The *in vitro* response in gerbera varies with cultivar, explants and composition of media. From many years gerbera is being propagated by direct or indirect organogenesis using various explants including stem tips, floral buds, leaf, capitulum etc. The plants are produced from explants of capitulum in red flower gerbera (Pierik *et al.*, 1975, Pierik *et al.*, 1982), leaves (Kumar *et al.*, 2004, Jerzy & Lubomski, 1991), floral buds (Mandal *et al.*, 2002), floral bracts (Maia *et al.*, 1983) and inflorescence (Schum & Busold, 1985). Shoot tip culture is by far the most common *in vitro* method for commercial multiplication as shoot tip commences the growth more rapidly and contain more number of axillary buds (Murashige *et al.*, 1974). The advantages of the capitulum method over shoot tip are the easier sterile isolation *in vitro*. It is also nondestructive method, as only inflorescences are used and no shoots are lost from the plant (Pierik *et al.*, 1982). The present investigation was undertaken to develop an efficient and viable protocol for commercial micropropagation of gerbera.

One of the disadvantages of traditional breeding is the limited gene pool of any single species (Kanwar and Kumar, 2008). Since the genetic variability within Gerbera genus is relatively limited, breeding potential for new flower colors and patterns as well as resistance to biotic and abiotic stresses are also limited (Aswath and Choudhary, 2002). Genetic variability is the first requirement for the improvement of any trait. The application of chemical mutagens as well as 'somaclonal variation from tissue-culture' is quite common in the creation of genetic variation (Puchooa and Sookun, 2003). The variability available to breeders comes from spontaneous or artificially induced mutations. Mutation is a permanent heritable change in the primary structure of the genetic material which consists of the total genome of a cell or plant. The combination of tissue culture with mutation induction

techniques may be an effective way for crop improvement (Gao *et al.*, 1992). Particularly in plants with reproductive sterility, this is the only alternative (Broertjes and Van Harten, 1988). Artificial mutation induction is carried out using physical and chemical mutagens which can increase the mutation frequency when compared to its spontaneous occurrence. Induced mutagenesis and mutant induction through the application of physical and chemical mutagens remain, despite the advent of new technologies for novel variety production, as an important part of ornamental and floricultural plant breeding (Jain and Spencer, 2006).

Once mutation has been induced and identified, there is a distinctive advantage in vegetatively propagated plants, in the sense that the created variation can be multiplied and preserved. In addition to the variants/mutants (cell lines and plants) obtained as a result of the application of a selective agent in the presence or absence of a mutagen, many variants have been obtained through the tissue-culture cycle itself. The term somaclonal variation was introduced by Larkin and Scowcroft (1981). The plant variants obtained from tissue culture is called somaclones. These somaclonal variants, which are dependent on the natural variation in a population of cells, may be genetic or epigenetic, and are usually observed in the regenerated plantlets (Larkin and Scowcroft, 1981). Many of the changes observed in plants regenerated *in vitro* have potential agricultural and horticultural significance. These include alterations in plant pigmentation, seed yield, plant vigour and size, leaf and flower morphology, essential oils, fruit solids, and disease tolerance or resistance (Karp, 1994). One of the most critical problems is the transfer of *in vitro* plantlets to *ex vitro* conditions. This often results in high mortality rates, since plantlets have limitations to resist transplant stress (Puthur *et al.*, 1998). Micropropagated plants have been continuously exposed to an unique micro environment that has been selected to provide minimal stress and nearly optimal conditions for plant multiplication. These conditions lead to a phenotype which is more fragile than greenhouse grown plants due to different anatomical, morphological and physiological factors. High mortality is often observed upon transfer to *ex vitro* conditions because the cultured plants have functionally impaired stomata and a poorly developed cuticle and root system (Wang *et al.*, 1993). After transfer to *ex vitro* conditions the plantlets have to correct the above mentioned abnormalities to

become adapted to a natural environment. An acclimatization process before transfer to the nursery is required to improve survival and growth of the plantlets (Schultz, 2001). Most of the work has been carried on plant regeneration by adventitious organogenesis from capitulum, shoot tip, leaf, petiole and other parts of the plant.

However, the *in vitro* propagation systems described by various scientists in the past need to be improved further in view of their reproducibility in respect to the conditions prevail in Bangladesh. The present investigation was undertaken with a view to developing an *in vitro* plant regeneration protocol for large scale propagation of selected Gerbera varieties cultivated in Bangladesh using suitable explants. Considering the above facts, investigations were carried out on inducing variability *in vitro* condition in gerbera with the following objectives:

- to study optimal conditions for *in vitro* regeneration of callus, shoot and root;
- to study the mutagen sensitivity of gerbera under *in vitro* condition; and
- to study the extent of variations in the micropropagated plantlets of gerbera.

Chapter II
Review of Literature

CHAPTER II

REVIEW OF LITERATURE

Gerbera (*Gerbera jamesonii* Bolus ex Hooker f.) has become one of the leading ornamental crops in the cut flower industry. It can be grown in both hills and plains and is also ideal for bed, border, pot and rock garden. In Bangladesh, it is fast catching up among the general circles of public. Since plant tissue culture can establish plants with uniform and predictable selected qualities (Murashige, 1976), this technique is used for mass propagation of high quality cut flowers of gerbera (Aswath and Choudhary, 2001). Today, floriculture industry is highly competitive and is always seeking novel characters to surprise the market. Thus the improvement for quality attributes such as flower colour, longevity and form, plant shape and the creation of novel variants are very important. Chemical and ionizing radiation mutagenesis (Jain and Spencer, 2006) have been routinely used to generate genetic variability for breeding research and genetic studies, especially in ornamental crops. Also through tissue culture cycles itself several variants are obtained called as somaclonal variants (Larkin and Scowcroft, 1981). Over the past few years, micropropagation techniques are being widely used as an important and advantageous tool for rapid propagation of several commercially important varieties of horticultural and forest tree species. Although, this technology has got several successful applications, low survival rates and poor growth while shifting these plantlets to field conditions are the most common problems which hinder its usage for commercial plant production practices.

2.1 Micropropagation of Gerbera

2.1.1 Establishment and multiplication

Flower producers have used the most advanced production, genetic breeding and commercialization techniques to meet this high quality standard. Nowadays, *in vitro* propagation of plants has offered producers enough high quality seedlings to meet this demand in a short period of time (Tombolato and Costa, 1998).

Gerbera is propagated by direct or indirect organogenesis using various explants including stem tips, floral buds, leaf, capitulum etc. The plants were produced from explants of capitulum in red flower gerbera (Pierik *et al.*, 1982), leaves (Hedtrich, 1979; Barbosa *et al.*, 1994, Aswath and Choudhary, 2004, Hasbullah *et al.*,

2008), shoot tip (Huang and Chu, 2007), floral buds (Akter *et al.*, 2012), (Posada *et al.*, 1999), floral bracts (Maia *et al.*, 1983), torus (Zhang, 2002) and inflorescence (Schum and Busold, 1985).

MS medium (Murashige and Skoog, 1962) was successfully used by many workers for callus formation as well as shoot regeneration (Pierik et al., 1982; Le *et al.*, 1999; Modh *et al.*, 2002; Aswath and Wazneen, 2004; Kumar and Kanwar, 2005, 2006).

The capitulum explants for propagation of gerbera were used by a number of workers (Pierik *et al.*, 1982; Modh *et al.*, 2002; Tyagi and Kothari, 2004; Ray *et al.*, 2005; Mohammed and Ozzambak, 2007). The advantages of the capitulum method over shoot tipare the easier sterile isolation *in vitro* and it is also non-destructive, only inflorescences are used and no shoots are lost from the plant (Pierik *et al.*, 1982).

Rahman *et al.*, (2014) showed that when the explants were cultured in higher concentration (5.0 mg/l) of BA produced shoots and 5.0 mg/l BA with 1.0 mg/l NAA was found to be the best for shoot proliferation of the three explants optimum response was obtained from flower buds.

Minerva G. et al., (2013) showed that direct shoot generation was accomplished from stem apices on MS medium supplemented with 1 mg/L 6-benzyladenine (BA) and 1 mg/L kinetin. Indirect shoot induction succeeded from callus differentiation has been achived on MS medium containing 2 mg/L 2,4-dichlorophenoxyacetic acid, 0.5 mg/L indole-3-acetic acid and 2 mg/L BA.

Akter *et al.*, (2012) found that among the explants flower bud and flower stalk were suitable and superior for callus induction and subsequent regeneration of *in vitro* shoots when cultured on MS supplemented with 5.0 mg/l BAP and 1.0 mg/l NAA. They also found that highest numbers of multiple shoots were obtained when the flower bud derived callus was sub-cultured on MS supplemented with 2.0 mg/l BAP.

Shagufta Naz *et al.*, (2012) showed that maximum number of multiple shoots was obtained on MS medium containing 10 mg/L BAP. These multiple shoots increased in the number when addition of NAA and kinetin to BAP showed good shoot multiplication response.

The study of Hussein *et al.*, (2008) showed that the highest shoot regeneration frequency was 36.6 % which was obtained on induction medium containing 2 mg/l BAP and 0.25 mg/l ABA and shoot formation medium containing 2 mg/l of each of BAP and NAA. Regenerated shoots were successfully rooted on MS medium containing 40 μg/l NAA.

Chen *et al.*, (2006) used DKW (Driver, Kuniyuki, 1984) medium for tissue culture studies of gerbera stem nodes with buds.

Ray *et al.*, (2005) developed an efficient protocol for large scale propagation using young capitulum as explant on a medium containing 7 mg/dm 3 BA and 0.1 mg/dm 3IAA, which initiate multiple shoots (10 shoots per explant).

Tyagi and Kothari (2004) used capitular sections for rapid *in vitro* multiplication of shoots with 4 mg/dm 3 kinetin and 0.5 mg/dm 3 IAA. About 20–25 shoot buds were developed from the callus.

Xu *et al.*, (2002) and Kumar *et al.*, (2004) regenerated adventitious shoots from petiole and leaf pieces of *G. jamesonii* in a medium supplemented with different concentrations of auxins and cytokinins.

Proliferated mass of shoots was obtained from mature capitula with 0.1 mg/dm 3 IAA and 10 mg/ dm 3 BA in the medium (Modh *et al.*, 2002; Zhang, 2002).

Wang and Yu (2001) studied the tissue culture of floral receptacle on MS medium supplemented with 8 mg/dm^3 BA and 0.1 mg/dm^3 NAA. Mandal and Datta (2002) established organogenic callus cultures from immature flower buds on modified MS medium with IAA and BA. Kuan *et al.*, (2002) conducted an experiment to study the effect of carbon source and concentrations on the *in vitro* growth and maintenance of plantlets produced from young capitulum explants. The addition of lactose at 1% was effective in lowering the growth rate of explants up to five months and the growth vigour could be resumed after transferring the plantlets to culture medium with 1% sucrose.

Transfer of the shoots to the medium with 1mg/dm^3 BA led to the production of axillary buds at the rate of 10 new-formed shoots per initial explant (Le *et al.*, 1999).

Reynoird *et al.*, (1993) gave modified MS medium supplemented with 10µM BA and 2.5µM NAA for plant regeneration from *in vitro* leaf culture of several gerbera species. The morphogenetic potential varied with the developmental stage of the leaves and up to 90% of excised developing leaves formed 3–5 shoots per explant.

Jerzy and Lubomski (1991) examined the effects of preparation of leaf explants, composition of culture medium, the kind of *ex vitro* stock plant and low temperature pre-treatment on the formation of adventitious shoots in cv. Sardis. Adventitious shoot formation occurred at the base of leaf petiole with 0.5 mg/dm^3 IAA

Capitulum explants of the varieties Appel Bloesem, Marleen, Clementine and Pimpernel were cultured on modified MS media supplemented with 2 mg/dm^3 BA and 0.5 mg/dm^3 IAA, the only treatment to produce a large translucent callus in Appel Bloesem and Marleen (Arello *et al.*, 1991).

Napaskamon (1991) studied the effect of kinetin and NAA on induction and growth of callus from mature capitulum of local gerberas. The plantlets showed enhanced callus formation and growth with NAA whereas kinetin had no effect

Topoonyanont and Dillen (1988) cultured capitulum explants of orange, yellow, red and pink cultivars on half-strength MS basal medium supplemented with 5–15 mg/dm^3 BA. The orange cultivar produced eight shoots per explant with 5.0–7.7 mg/dm^3 BA. No shoot induction from pink cultivar was observed.

The medium containing 0.1 mg/dm^3 IAA and 1 or 2 mg/dm^3 BA gave the best shoot production (Laliberte *et al.*, 1985).

Sahavacharin (1985) used young capitulum of gerbera hybrids for rapid multiplication of shoots on MS medium supplemented with 0–1 mg/dm3 IAA and 0–12 mg/dm^3 kinetin.

Hedtrich (1979) observed the regeneration of adventitious shoots from leaf blades during in it propropagation of Vulkun on modified MS medium supplemented with 1 mg/dm^3 BA and 0.1 mg/dm^3 GA^3.

Pawlowska (1977) studied the capacity for shoot formation from inflorescence buds. Explants were excised when the peduncle reached 12–20 cm in length and the ligulate flowers were yet visible or when the young buds reached 1.0–1.5 cm in length.

Pierik's medium enriched with IAA and kinetin or BA was used for the induction of shoots when the young buds reached 1.0–1.5 cm in length.

2.1.2 *In vitro* rooting of shoot

To induce rooting, individual shoots from a multiple shoot complex originated from the callus were separated after 8 weeks of shoot initiation and transferred to MS medium free of hormones or containing only NAA and IAA. In both IAA and NAA the frequency of rooting was 100%. In all media, the first roots appeared after 1–2 weeks of culture and after 4–5 weeks, the root system was well developed. On medium without NAA and IAA, the initiation of single root with secondary root formation was observed, whereas in media with NAA and IAA the formation of multiple adventitious roots without secondary roots was observed. IAA was slightly better than NAA in inducing roots in both the cultivars (Aswath and Choudhary, 2002).

Rahman *et al.*, (2014) regenerated shoots were rooted in MS medium with indole-3-acetic acid (IAA) or indole-3-buteric acid (IBA) and maximum frequency (81%) of rooting with highest number (4) of roots per shoot was achieved in MS medium fortified with 0.3 mg/l IBA

According to Akter *et al.*, (2012) for root induction at the base of the *in vitro* regenerated shoots was tried using full and half the strengths of MS containing different concentrations of IBA. Best root induction was observed on half the strength of MS supplemented with 0.2 mg/l IBA.

KAA Shabanpour *et al.*, (2011) said that the best rate of shoot rooting in orange cultivar (4.6 roots/explants with 4.8 cm length) and pink cultivar (5.2 roots/explants with 6.2 cm length) was obtained by using 3 mg/l IAA and 30 mg/l sucrose.

Among both auxins, NAA resulted in less rooting percentage as compared to IAA which is in line with the findings of Ali *et al.*, (2009) who proposed that NAA is less effective in inducing rooting.

The shoots obtained from leaf explants of gerbera were transferred to the rooting medium containing 40µg/l NAA and incubated for 3 - 4 weeks. All of the transferred shoots produced roots during the 3 - 4 weeks (Hussein *et al.*, 2008).

Salisbury and Ross (2005) observed that higher concentrations of IAA and NAA reduce root growth due to increased level of endogenous ethylene biosynthesis

Rooting of in vitro shoots derived from capitulum explants was achieved on MS medium with 0.5 mg/l IAA (Tyagi and Kothari, 2004).

According to the study of Metaxas *et al.*, (2004) metabolic changes in the rooting zones of cuttings due to auxins are capable of promoting adventitious root formation which indicates that auxin is an essential growth regulator required for the process of root formation of gerbera.

Pagnussat *et al.*, (2004) reported that IAA increases the number of roots through the development of meristematic tissues and regulation of cell differentiation. They also reported that once root primordial have been developed in the cuttings, then IAA causes a considerable metabolic activity within the cells which results in the formation of new root tissues that later grow into a root.

In the absence of NAA, the initiation of a single root with secondary root formation was observed in *in vitro* shoot tip culture derived gerbera plantlets. NAA in the media induced the formation of multiple adventitious roots without secondary roots (Aswath *et al.*, 2003).

Husen and Pal (2001) reported that IAA application strongly enhances root induction through the endogenous IAA acropetal movement from the shoot towards the central cylinder (phloem or precursor procambial cells).

Aswath and Chaudhary (2001) reported maximum root induction and average number of roots per shoot when cultured on MS medium containing 1.75 mg/l IBA.

Studies carried out by Palai *et al.*, (1998) also indicated that IBA is the best auxin for rooting in gerbera. Among the three auxins tried, IBA was found better when compared with IAA and NAA for inducing good quality roots.

Celenza *et al.*, (1995) found that IAA and NAA promoted root initiation and growth by inducing the cells to the pericycle and parenchyma to dedifferentiate and start initial cell division process.

Laskowski *et al.*, (1995) also mentioned that auxin accumulation within the root tissues may cause an increase in the number of adventitious root formation.

Barbosa *et al.*, (1992) also found a better root system in gerbera sprouts at all levels of IAA (0.5, 1.0, 2.0 and 4.0 mg L–1).

Ruffoni and Massabo (1991) indicated that *in vitro* plant, leaf petiole and shoot apices were able to regenerate some buds, whereas leaf laminas were only able to regenerate callus and roots.

The best root growth (15.2 cm) was obtained in the presence of 10mM IBA with an 85% survival rate (Meyer and van Staden, 1987).

The root formation is markedly influenced by the presence of auxins and sugars, whereas presence and absence of macro-elements did not influence rooting (Pierik *et al.*, 1975).

2.2 Mutation *in vitro*

In vitro culture methods have facilitated the use of mutation technique for improvement of both seeded and vegetatively propagated crops (Jain and Maluszynski, 2004). Mutation induction with cell and tissue culture techniques have increased progressively since a large population of haploid and diploid cells can be handled in a small space, developing new individuals in a short period of time. *In vitro* treatments with chemical mutagenic agents occur more uniformly than *in vivo* treatments in which a controlled environment and culture medium are used (Constantin, 1984).

Mutation breeding has great potential for vegetatively propagated plants such as ornamentals. The main advantage is the possibility of improving one or a few important characters of an otherwise outstanding cultivar, without basically altering the remaining genotype. Thus outstanding cultivars, often being the result of a time consuming and painstaking cross breeding programme, can be further perfected with in a relatively short period of time (Ibrahim and Debergh, 1998).

2.2.1 Chemical mutagens

Chemical mutagens are not widely used principally due to their low penetration into vegetative tissue such as buds, shoot and stem which results in a low efficiency (Van Harten, 1998). However, *in vitro* cultures have opened new avenue to use chemical mutagens on vegetative tissues. There are a number of chemical mutagens. However for practical purpose of induction of mutation, so far only a few are really useful, most of them being alkylating agents such as Ethyl methane sulphonate (EMS), Diethyl sulphate (dES), Ethyl amine (EI) etc.

EMS is a chemical mutagen of the alkylating group and has been widely used in plant because it causes high frequency of gene mutation and low frequency of chromosome aberration (Van Harten, 1998). EMS induces chemical modification of nucleotides, which results in mispairing and base changes by strong biased alkylation of guanine (G) residues. Through subsequent DNA repair, the original G/C pair can then be replaced with A/T as reported earlier (Greene *et al.*, 2003). EMS can be efficiently used to induce in vitro mutation of chrysanthemum (Latado *et al.*, 2004)

2.2.2 Plant parts used for mutagenesis

Seeds of *Solanum lycopersicum* cv. Micro-Tom were subjected to mutagenesis treatment using EMS (Watanabe *et al.*, 2007). Senapati and Rout (2008) used both apical and axillary meristems for mutation experiment in rose. In Petunia x hybrida, seeds were used for used for EMS treatment for mutagenesis treatment (Berenschot *et al.*, 2008).

2.2.3 Method of treatment

Both *in vitro* derived apical and axillary meristems of rose were treated with different concentrations of EMS along with MS liquid medium and continuous shaking on a shaker at 60 rpm for 2, 4, 6 and 12 hours at 25 ± 2°C. Following incubation, explants were rinsed three times with the sterile liquid culture medium. (Senapati and Rout, 2008).

In Petunia x hybrida, for EMS treatment, seeds were pre-imbibed in 500 μL of sterile de-ionized water for at least 14 h in the dark with mild shaking (45 rpm) at room temperature. An adequate volume of a freshly-prepared EMS stock solution at 0.5%

(v/v) was added to the pre-imbibed seeds to achieve the final concentration. The seeds were further incubated in the dark at room temperature for 24 h with mild shaking (45 rpm). After EMS treatment, the seeds were thoroughly rinsed (10 times with 1000 μL) with sterile deionized water (Berenschot *et al.*, 2008).

In tomato, the seeds were incubated in 100 ml of a solution containing EMS at 0.3, 0.5, or 1% (w/v), with gentle shaking for either 24or 48 h (Watanabe *et al.*, 2007).

Immature pedicels of chrysanthemum cv. Ingrid (dark pink colour) were treated with 0.77% (0.075 M) EMS solution for 1 h and 45 min, which was followed by rinsing in water for 15 min and surface disinfection (Latado *et al.*, 2004).

2.2.4 Application of *in vitro* mutation

The visual survey of treated populations M_1 and the bulk M_2 derived from EMS treatment resulted in isolation of a previously unreported morphological mutation in Petunia x hybrida MD that was genetically transmissible to the next generation. The mutants have increased density of trichomes on the aerial parts and abnormal regulation of branching, reduced apical dominance and disturbed patterns of determinacy. The mutant leaf blade is narrower, slightly hyponastic, exhibits undefined petioles and a more complex venation pattern in comparison to the cross-venulated pattern of the wild type. Interestingly, the stem presents leaf-like tissue-growth extensions bilaterally along its length and delayed flowering time (Berenschot, *et al.*, 2008).

Forty-eight mutants (5.2%) were obtained, deviating in petal colour (pink-salmon, light-pink, bronze, white, yellow and salmon colour) in chrysanthemum treated with EMS. Most of them (89.6% of the total) were phenotypically uniform (Latado *et al.*, 2004).

Nonomura *et al.*, (2001) obtained twenty regenerantes from petal derived embryogenic calli and almost all of them produced varied flowers similar to the shape, size, colour and number of the parental derived petals originally used for tissue culture, although only a regenerant produced more malformed flower.

2.3 Somaclonal variation studies

Tissue culture can induce so-called somaclonal variation which can be considered in the context of biological mutagens. This method might be useful, when a high enough mutation frequency and preferably, a different spectrum of mutants can be obtained. Although there is no doubt that genuine mutations occur after tissue culture, their frequency is much lower than with classical mutagens.

Shaijee *et al.*, (2006) conducted an experiment to reveal the genetic potential of African violet (*Saintpaulia ionantha*) to create and improve new traits by somaclonal variation technique. They concluded different frequencies of variation were obtained from different treatments but variegated variants were observed among plantlets obtained from leaf derived calluses of all sub culture periods. The time of callus phase influenced the rates of mutation frequency in two applied genotypes.

Bairu *et al.*, (2006) studied the effect of the type and concentration of plant growth regulators and sub culturing on somaclonal variation in Cavendish banana Cv. Zelig obtained from African Biotechnologies Ltd, South Africa. Results indicated that treatments with higher multiplication rates produced more variants, sometimes as high as 72 %. Dwarf off types accounted for 88 % of the variation.

Depending on the plant type, the number of subcultures is another important aspect that can lead to more variation. Tissue culture system itself acts as a mutagenic system because cells experience traumatic experiences from isolation, and may reprogramme during plant regeneration which are different than under natural conditions. Reprogramming or restructuring of events can create a wide range of epigenetic variation in newly regenerated plants (Jain, 2000).

Patnaik *et al.*, (1999) reported a wide variation in quantitative traits such as plant height, plant yield, tiller number, oil content, and qualitative changes in essential oil constituents of plants regenerated from cell suspension cultures of palmarosa grass, *Cymbopogon martinii* (Roxb).

The occurrence of somaclonal variation is associated with point mutations and chromosomal rearrangements and recombination, DNA methylation, altered sequence copy number, transposable elements, and seems to be influenced by the

genotype, explant type, culture medium, age of the donor plants (Veilleux & Johnson, 1998; Jain *et al.,*, 1998).

Usually gene mutations occur frequently in tissue culture-derived plants, and therefore, tissue culture system can be regarded similar to mutation process. Since somaclonal variation can broaden the genetic variation in crop plants, many plant characters can be altered, including plant height, yield, number of flowers per plant, early flowering, grain quality, resistance to diseases, insect and pests, cold and drought, and salt (Jain *et al.*, 1998).

2.4 Characteristics of *In vitro* derived plantlets

The micropropagated plants have been continuously exposed to a unique micro environment that had been selected to provide minimal stress and nearly optimal conditions for plant multiplication. Plantlets grow heterotrophic, and develop within culture vessels under conditions which are characterized by a saturated atmosphere, relative low light intensity (photosynthetic photon flux) averaging 12-70m mol m-2s-1; relatively high and constant temperature (20-28°C), low rates of gas exchange between the containers and high concentrations of carbohydrate and exogenous growth regulators in the medium. These factors often induce physiological, anatomical and morphological abnormalities which interfere with the acclimatization subsequent to transplanting resulting in low survival rates *ex vitro* (Ziv, 1991; Puthur *et al.*, 1998).

In vitro plants principally require sugar as a carbon source (Conner and Thomas, 1982), and the CO_2 uptake capability is low (Donelly *et al.*, 1985). Consequently when transplanted out of the culture vessel, plantlets suffer from severe environmental stresses and substantial losses may occur (Van Huylenbroeck and Debergh, 1996).

The relatively low light levels and a saturated internal atmosphere promote leaves in vitro that anatomically resemble shade leaves (Brainerd and Fuchigami, 1981; Lee *et al.*, 1988; Marin and Gella, 1987) and hydrophytic plant leaves (Grout and Ashton, 1977). They often have reduced or no epicuticular or cuticular wax, which lack the characteristic crystalline structure or differ in chemical composition from the control plants (Grout, 1975; Grout and Ashton, 1977; Sutter, 1984, 1985;

Short *et al.*, 1987). *In vitro* stomata have slow response times and due to an impaired function they do not close in response to stimuli such as darkness, abscisic acid application or when exposed to high levels of carbon dioxide (Brainerd and Fuchigami, 1981; Wardle *et al.*,1983; Ziv *et al.*, 1987).

In vitro plantlets are invariably diminutive and much smaller than greenhouse grown plants. The percentage of water content is increased and the dry matter accumulation per unit area is reduced compared to greenhouse grown plants (Brainerd and Fuchigami, 1981; Donelly *et al.*, 1985). This is reflected in fragile organs with reduced mechanical support tissue and thin cell walls.

2.5 Acclimatization of micropropagated plantlets

The term acclimatization is defined as the climatic adaptation of an organism, especially a plant, which has been moved to a new environment (Conouer and Poole, 1984).

To promote *ex -vitro* survival and physiological competence, especially to protect them against water stress and encourage autotrophy, a transitional environment is usually supplied for the acclimatization interval, ranging from one to several weeks (Brainerd and Fuchigami, 1981; Grout and Millam, 1985; Fabbri *et al.*, 1986). In this transitional environment the relative humidity is kept in the range of 70-100% and the light level is regulated by shading against direct sunlight. Gradually, as the plantlets acclimatize the relative humidity can be decreased and the light levels can be increased towards ambient conditions. The stage of acclimatization is the beginning of the autotrophic existence of the plant, including the initiation of the physiological processes necessary for survival.

Ray *et al.*, (2005) reported that the plantlets micropropagated in garden soil were uniform and identical to the mother plant with respect to growth characteristics and morphology.

Kumar *et al.*, (2004) reported 60–70% success in pots containing a mixture of sand : farmyard manure mixed in the ratio 1:1

The rooted plantlets exhibited 100% survival in plastic pots filled with coco peat, red soil and sand at a ratio 3:1:1 in *G. jamesonii* cv. AV 101 (Aswath, Choudhary 2002).

Olivera *et al.*, (2000) studied the effect of acclimatization on growth and plant development of gerbera under greenhouse conditions with 82.4% survival of plantlets

Kaur *et al.*, (1999) obtained 100% survival rate of *in vitro* shoots when transferred to pots filled with a mixture of soil : sand : compost in 1:1:1 ratio.

Posada *et al.*, (1999) observed 50% survival rate on the media with buried rice hull and coke scoria under high humidity.

Parthasarathy and Nagaraju (1999) achieved a 90–100% success in polythene bags containing equal amount of soil : sand : farmyard manure.

The special conditions during *in vitro* culture result in the formation of plantlets of abnormal morphology, anatomy and physiology. 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 (Pospilova *et al.*, 1999).

Difficulties in transplanting tissue cultured plantlets to soil are well documented (Puthur *et al.*, 1998; Subhan *et al.*, 1998). They appear to be as a direct result of the culture induced phenotype which reflects adaptation to *in vitro* conditions but have a harmful effect when plantlets are transferred to the greenhouse or field where the relative humidity tends to be less than 100%, the ambient light levels are much higher than in culture, the temperatures are fluctuating and the substrate has a much higher water potential.

Reynoird *et al.*,(1993) rooted *in vitro* shoots with half-strength MS containing 0.25µM NAA and acclimatized the regenerated plants in greenhouse under a plastic tunnel in trays containing peat : perlite (1:1) medium and achieved 100% success.

Laliberte *et al.*, (1985) transferred rooted plantlets to Jiffy-7 peat pellets, in glass covered acclimatization module and later to a mixture of 1 perlite: 1 sphagnum moss in greenhouse with 95% success.

Regenerants never displayed phenotypic variations during subsequent vegetative and floral development. Petru and Matouš (1984) successfully transferred the plantlets into sterilized peat: perlite (1:1) substrate and then into a standard horticultural substrate.

Chapter III
Materials and Methods

CHAPTER III

MATERIALS AND METHODS

The present investigations were carried out during 2014-2015 in the Department of Genetics and Plant Breeding, Sher-e-Bangla Agricultural University. The studies comprised of three separate experiments on *in vitro* regeneration of gerbera plant, inducing variability *in vitro* using chemical mutagen and hardening of *in vitro* gerbera plant. The materials used and the methods adopted to record the observations during the course of this study are presented in this chapter.

3.1 Experimental Material

The experimental materials were the capitulum of the following varieties of gerbera

1. Red variety
2. Yellow variety

3.1.1 Sources of plant material

All of these material were collected from my supervisors own stock. 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 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 (Murashige and Skoog, 1962) was used with different vitamins and hormonal supplementation. The composition of the MS medium has been presented in appendix 1. Hormones were added independently to different media according to the requirements. For the preparation of the media, stock solutions were prepared at the beginning and stored at $4\pm1^{\circ}$ c temperature. The relevant media were prepared from the stock solution.

Red Variety

Yellow variety

3.1.2.1 Preparation of the stock solutions

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

3.1.2.2 Stock solution of macro- nutrients (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°c for ready use.

3.1.2.3 Stock solution of micro-nutrients (Soln. B)

Stock solution of micronutrients was made up to 100 folds (100x) the final strength of the medium in 1000ml DW as described earlier for stock solution A. The stock solution was filtered, labeled and stored in a refrigerator at 4± 1°c for later use.

3.1.2.3 Stock solution of iron (Soln. C)

It was made up to 100 folds (Soln. C) the final strength of the medium in 1000ml of DW. Here two constituents, $FeSo_4$ and Na-EDTA were dissolved in 750ml of DW in a beaker by heating on a hitter cum magnetic stirrer. Then the volume was made up to 1000ml by further addition of DW. Finally the stock solution was filtered and stored by wrapping with aluminum foils in a refrigerator at 4±1°c for later use.

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

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

(a) Pyridoxine HCL (Vitamins B_6)

(b) Thiamine HCL (Vitamins B_1)

(c) Myoinositol (Inositol)

(d) Glysine

(e) Nicotinic acid (Vitmins 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 400ml of DW. Then the final volume was made up to 1000 ml by further addition of distilled water. Finally the stock solution was filtered and stored in a refrigerator at $4\pm1^{\circ}c$ 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 $4 \pm 1^{\circ}c$.

3.1.2.5 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)	70% Ethanol
Cytokinin	
Benzylaminopurine (BAP)	1N NaOH

To prepare a stock solution of growth regulator, 10 mg of the growth regulator was taken on a clean watch glass then dissolved in 1litre 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 $4\pm1^{\circ}c$ and used for a maximum period of two months.

3.1.3 Preparation of other stock solutions

Preparation of 1N NaOH

1. 1.40 g NaOH pellets was weighed
2. Then those pellets were put in dry 1 L 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.

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. Store the solution in a sterilized glass bottle
4. This solution was made fresh each time before use.

3.1.4 MS Media preparation

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

1. 500 ml double distilled water was taken into 1litre beaker
2. 100 ml of stock solution of macro-nutrients, 10 ml of stock solution of micro-nutrient, 10 ml of stock solution of Na-EDTA and 10 ml of stock solution of vitamins and growth regulators were added in this 500 ml double distilled water
3. 30 g 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. Since each hormonal stock solution contained in 100 ml of solution , to make one litre of medium, addition of 1 ml/L, 1.5 ml/L and 2.00ml/L BAP singly was added to prepare 1 litre of medium.
6. Later different combinations of these two hormones NAA and BAP respectively were used viz. (1.00+1.00), (1.00+2.00), (1.00+4.00), (1.5+1.00), (1.5+1.5), (2.00+2.00) mg/L.

7. The whole mixture was then made up to 1litre with further addition of double distilled water.

3.1.5 pH of the medium

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

3.1.6 Agar

The media WAS gelled with 8 g/L agar and the whole mixture was gently heated on microwave oven at 250°c temperature 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°c for 20 minutes. The medium was then transfer into the culture room and cooled at 24°c temperature before used. Marking is also necessary.

3.2.2 Sterilization of glassware and instruments

Glassware, culture vessels, beakers, petridishes, pipettes, slides, plastic caps, other instruments such as forceps, needles, scissor, spatula, surgical blades, brush, cotton, instrument stand and aluminum foil were sterilized in an autoclave at a temperature of 121°c for 20 minutes at 15 psi pressure. Before this, all types of glassware instrument was washed properly by liquid detergent, cleaned with running tap water and finally washed with distilled water.

3.2.3 Sterilization of culture room and transfer area

In the beginning, the culture room was spray 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% ethanol. This process of sterilization of culture room was repeated at regular intervals.

The transfer area was also cleaned with detergent and also sterilized twice in a month by 70% ethanol. Laminar air flow cabinet were 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 in empty 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 manipulations were carried out under laminar airflow cabinet. The cabinet was usually switched on with ultra violet light half an hour before use and wiped with 70% ethanol to reduce the chances of contamination. The instruments like scalpels, forceps, needles, surgical blades, scissors, pipettes, slides, plastic caps, spatula, brush, cotton etc. were presterilized by autoclaving 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, 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 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 flower buds (capitulum) of gerbera 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 and BAP to promote callus, shoot and root induction.

3.3.4 Culture conditions

The inoculated cultures were incubated in the culture room at a temperature of 25° ± 2° C 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.

39294
12.10.15

3.4 Subculture

3.4.1 Maintenance 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° c. The vials were checked daily to note the response and the development of contamination.

3.4.2 Maintenance 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.

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 per cent sucrose, 0.8 per cent agar and supplemented with NAA and BAP. The cultures were incubated for a period of 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. Then these 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 experiment consisted of two factors

1. Variety
2. Different concentration of NAA and BAP

3.7.1 Factor A

Experimental materials are two popular variety of gerbera

1. Red variety
2. Yellow variety

3.7.2 Factor B

Different concentrations of NAA and BAP

- T1=Normal MS
- T2=1.00 BAP
- T3=1.5 BAP
- T4=2 BAP
- T5=1NAA+1BAP
- T6=1NAA+2BAP
- T7=1NAA+4BAP
- T8=1.5NAA+1BAP
- T9=1.5NAA+1.5BAP
- T10=2NAA+2BAP

3.8 Experimental details

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

3.8.1.1 Experimental material

Healthy and uniform tissue cultured gerbera plantlets of cultivars, red and yellow flower buds or capitulum were used as experimental material.

3.8.1.2 Treatments

The treatments comprised of the combinations of two cultivars of gerbera with different concentration BAP and NAA on shoot multiplication and subsequent rooting of the multiplied shoots. There were 8 treatment combinations, the details of which are given below:

Treatment combinations:

- T1=Normal MS
- T2=1.00 BAP
- T3=1.5 BAP
- T4=2 BAP
- T5=1NAA+1BAP
- T6=1NAA+2BAP
- T7=1NAA+4BAP
- T8=1.5NAA+1BAP
- T9=1.5NAA+1.5BAP
- T10=2NAA+2BAP

3.8.1.3 Recording of experimental data

3.8.1.3.1 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.

3.8.1.3.2 *In-vitro* callus induction

1. **Days to callus induction**: Generally callus induction started after few days of explant incubation. Days to callus induction were recorded until callus was not induced from the explant. The mean value of the data provided the days to callus induction.

2. **Percentage of callus induction**: Percentage of callus induction were noted after 7-10 days of inoculation by using the following formula:

$$\text{Percentage of callus induction} = \frac{Number\ \ of\ explants\ \ induced\ \ calli}{Number\ \ of\ induced\ \ calli} \times 100$$

3. **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 breadth was measured vertically. The formula (Thadavong *et. al.*, 2002) used for estimating of size of callus is given below:

$$\text{Size of callus} = \frac{Breadt\ h{+}Lengt\ h}{2}$$

4. **Fresh weight of callus (gm)**: Callus weights were 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* plant regeneration

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

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.
2. **Number of shoots/plantlet**: The number of shoot proliferated was recorded at 14, 28 and 42 days after inoculation(DAI) and the number shoots/ explant was

counted and mean was recorded. The mean value was calculated using the following formula:

$$\overline{X} = \sum Xi/n$$

Where, $\bar{X}$ = Mean of shoots/plant

$\sum$ = Summation

X_i = Number of shoots/plant

N = Number of observations/replication

3. **Length 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 shoot length.
4. **Number of leaves per plantlet:** The number of fully opened leaves in each plantlet were counted and recorded by visual observation at an interval of 14, 28 and 42 days after inoculation (DAI). The mean value of the data provided the number of leaves per plantlet.
5. **Fresh weight of shoot**: The aerial part of the plant including stem and leaves were cut in to pieces avoiding senescent or necrotic tissue. It was then weighed and the weight was expressed in grams.
6. **Dry weight of shoot**: The sample observed for fresh weight was dehydrated in a hot air oven at 65°C until a constant weight was attained. It was weighed and dry weight was expressed in milligrams.
7. **Days 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.
8. **Number of roots per plantlet**: The number of roots peer plantlet was recorded at an interval 14, 28 and 42 days after inoculation of explant inoculation and it was recorded and mean was calculated.
9. **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 recorded at an interval of 14, 28 and 42 days after inoculation (DAI) of explants inoculation and it was recorded and mean was calculated.

10. **Fresh weight of the roots:** All the roots from the plant were separated, weighed and weight was expressed in grams.
11. **Dry weight of roots**: The roots observed for fresh weight were oven dried at 65° C until a constant weight was attained. The dry weight was recorded in milligrams.
12. **Subculture of *in vitro* plantlet**:

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

3.9 Experiment IV: To study the mutagen sensitivity of gerbera under *in vitro* condition.

3.9.1 Experimental material

Shoot tips from already cultured gerbera variety Red and Yellow were used as the explant for mutagen treatment.

3.9.2 Mutagen

Ethyl methane sulphonate (EMS) procured from was used for the study. It was freshly prepared by dissolving in 3 per cent dimethyl sulphoxide as suggested by IAEA (1977).

3.9.3 Treatments

Seven different concentrations of EMS were imposed on shoot tips derived from Red and Yellow variety with an untreated control, the details of which are given below:

- T_1 = Control
- T_2 =25 P M EMS
- T_3 = 50 PM EMS
- T_4 = 100 PM EMS
- T_5 = 150 P M EMS
- T_6 = 250 PM EMS

- T_7 = 375 PM EMS
- T_8 = 500 PM EMS

3.9.4 Treatment Method

The shoot tips were treated with mutagen under sterile conditions of laminar air flow chamber and kept for 6 hours as such under low temperature conditions in a refrigerator. After the treatment, shoot tips were washed four times with sterile distilled water and inoculated on MS medium with different hormone concentrations.

3.9.5 Multiplication of further generation

The surviving shoots were multiplied for two multiplication cycles.

3.9.6 Recording of experimental data

(a) Survival of shoot tips

Number of surviving shoot tips after 15 days of inoculation were counted and expressed as percent of survival of shoot tips inoculated.

(b) Number of shoots per explant

The number of shoots were counted from five explants and expressed as shoots per explant.

(c) Morphological abnormalities

The shoots were observed for different types of abnormalities such as observed percentage of mortality and percentage of abnormal leaves. During mortality observation, it was considered dead shoot when the color of shoot tip became green to brown or black. Abnormal leaves means wrinkled, distorted leaves and appearances of disfigured features of leaves.

3.10 Experiment V: To study acclimatization of *in vitro* plantlets.

3.10.1 Experimental material

Healthy and uniform tissue cultured gerbera plantlets of red and yellow variety were used as experimental material.

3.10.2 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.

3.10.3 Transfer plantlet from culture vessels to soil

During *in vivo* acclimatization and the establishment of the previously regenerated and sub-cultured plantlets in to the soil, data were collected for the following parameter.

3.10.4 Survival rate of plantlets

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

$$\text{Survival rate (\%) of plant} = \frac{\text{Number of establis hed plantlets}}{\text{Total number of plantlets}} \times 100$$

3.11 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 **IBM SPSS Statistics 20** statistical package programme. The analysis of variance was performed and differences among the means were compared by the Least Significant Different test at 1% level of significance.

Chapter IV
Experimental Results and Discussion

CHAPTER IV

EXPERIMENTAL RESULTS AND DISCUSSION

The results of the investigation on the studies on *in vitro* regeneration by using hormone and inducing variability by using of mutagen and hardening of gerbera plantlets conducted during 2014 in the department of Genetics and Plant Breeding, Sher-e-Bangla Agricultural University.

4.1 Experiment 1: Responses of the age of flower bud explants towards callus formation.

4.1.1 Flower bud explants response towards callus induction

Various explants, namely flower bud, flower stalk, leaf tip, leaf with midrib, leaf blade, petiole were used for callus induction and in vitro growth of plantlets. Among the different explants, flower bud exhibited the best response towards callus formation, shoot regeneration and subsequent development of plantlets in two varieties of gerbera. The age of the flower bud was found to be an important factor in callus induction and shoot proliferation. In Red varieties 9 - 11 days old flower buds were more responsive (80 - 85%) and in Yellow varieties 7-8 days old flower buds were more responsive (65 - 75%) in callus induction and shoot development (Table 1). There are some previous reports on using of flower bud as explants for shoot regeneration in gerbera (Pierik *et al.*, 1975, Laliberte *et al.*, 1985, Aswath and Choudhary 2002, Tyagi and Kothari 2004, Ray *et al.*, 2005, Kumar and Kanwar 2006, 2007 and Nhut *et al.*, 2007).

(a) Sources of Explants

(b) Flower bud in source material (c) Flower bud before collecting as explants

Plate 1. Explants collection from source materials

(a) Inoculation

(b) Initiation

(c) Few days after initiation

(d) Initiation at lab conditions

Plate 2. Progress of micropropagation of gerbera

Table 1. Responses between the age of flower bud explants and callus formation

Variety	Age of flower bud explants (days)	No. of explants inoculated	No. of explants responded to callus induction	% of responsive explants
Red variety	7	20	8	40
	8	20	12	60
	9	20	16	80
	10	20	13	65
	11	20	17	85
Yellow variety	7	20	15	75
	8	20	13	65
	9	20	10	50
	10	20	8	40
	11	20	6	30

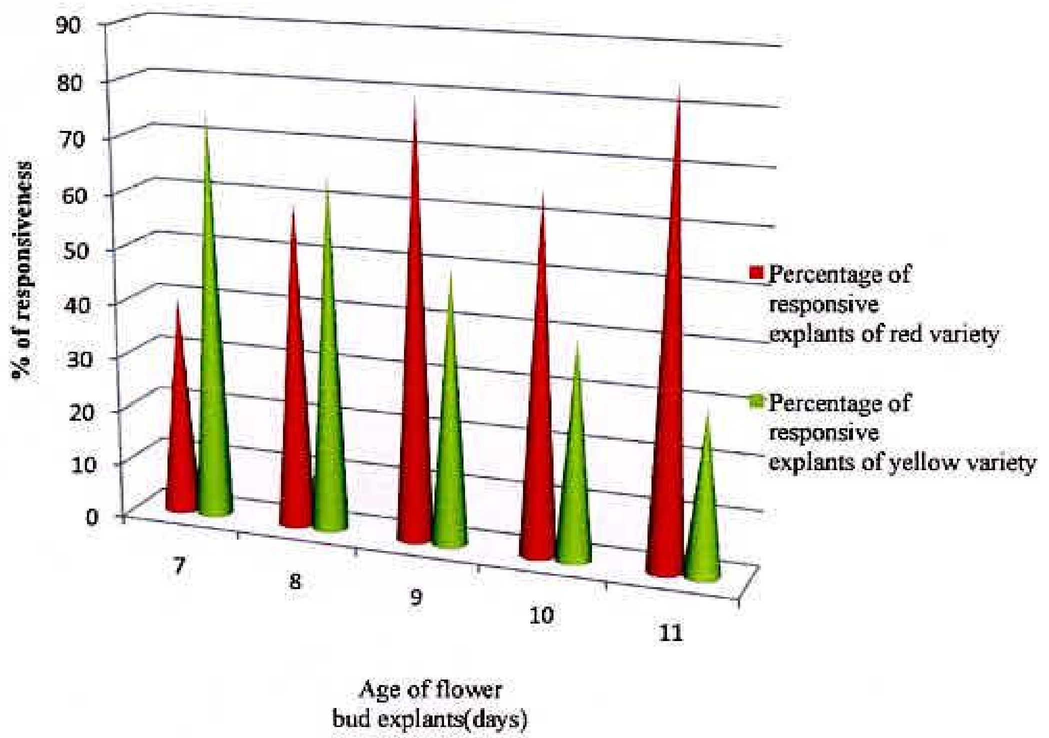


Figure 1. Percentage of responsiveness at different age of flower bud

In case of red variety, highest percentage (82%) of responsive explants were found at 11 days and in case of yellow variety, lowest percentage (26%) of responsive explants were found at 11 days (Figure1).

4.1.2 Callus induction of two gerbera verities supplemented with different concentrations of NAA and BAP

4.1.2.1 Callus color and callus texture:

Two gerbera verities red and yellow were cultured on Ms media supplemented with different concentration of NAA and BAP. Color of the calli were white from the beginning but DAI, calli were mostly change into green, light green, deep green, brown, pink and yellow. Most of the calli were non friable in texture. The effect of NAA and BAP for callus induction is presented in table 2. There was wide range of variation in days to callus induction. The result of the study have been presented and discussed under the following headings.

4.1.2.2 Days to callus induction:

The results of major effect of varieties on days to callus induction have been presented in table 2. The flower bud from the gerbera varieties were used as explants and cultured on MS medium supplemented with different concentrations of NAA and BAP. The main effect of varieties revealed that there was significant difference on days to callus induction. The maximum days to callus induction (7.5 days) and the lowest (4.00) days were note in yellow and red variety respectively.

The combined effect of different gerbera varieties and hormone showed significant variation on days to callus induction (**Appendix II**). The maximum days (7.5days) to callus induction was noticed in the treatment combination 1 mg/L NAA+2 mg/L BAP in variety of yellow. The minimum days (4.00 days) to callus induction was found in variety red with 1.5 mg/L NAA+1.5 mg/L BAP (Table 2). Auxin is an important media supplements for callus induction in culture. No callus was formed when explants of different varieties were culture in fresh MS media without hormone and BAP alone (1.00, 1.50 and 2.00 mg/L).

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

Variety	Hormone	days to callus induction	Size of callus (cm)			Fresh weight of callus (gm)		
			14 DAI	28 DAI	42 DAI	14 DAI	28 DAI	42 DAI
Red variety	T1=Normal MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	T2=1.00 BAP	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	T3=1.5 BAP	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	T4=2 BAP	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	T5=1NAA+1BAP	5.2	0.78	1.52	2.63	0.82	1.60	3.12
	T6=1NAA+2BAP	4.8	0.80	1.49	2.50	0.77	1.57	2.97
	T7=1NAA+4BAP	4.5	0.87	1.58	2.65	0.85	1.64	3.08
	T8=1.5NAA+1BAP	4.7	0.81	1.62	2.75	0.78	1.45	2.80
	T9=1.5NAA+1.5BAP	4.00	0.98	1.65	2.77	0.94	1.52	2.90
	T10=2NAA+2BAP	4.30	0.92	1.43	2.52	0.62	1.34	2.03
Yellow variety	T1=Normal MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	T2=1.00 BAP	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	T3=1.5 BAP	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	T4=2 BAP	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	T5=1NAA+1BAP	6.8	0.34	0.98	1.62	0.55	1.53	2.96
	T6=1NAA+2BAP	7.5	0.88	1.53	2.72	0.87	1.34	2.22
	T7=1NAA+4BAP	5.6	0.92	1.77	2.57	0.82	1.62	2.98
	T8=1.5NAA+1BAP	6.4	0.77	1.11	2.02	0.57	1.44	2.50
	T9=1.5NAA+1.5BAP	7.2	0.56	1.30	2.02	0.45	1.04	2.04
	T10=2NAA+2BAP	6.9	0.67	1.44	2.33	0.75	1.64	2.80
	SE±	.478	0.057	0.037	0.026	0.00	0.026	0.026
	Max	7.50	0.98	1.77	2.77	0.94	1.64	3.12
	Min	4.00	0.34	0.98	1.62	0.45	1.04	2.03
	LSD (0.01)	1.293	0.156	0.098	0.069	0.00	0.069	0.069
	Level of Significance	**	**	**	**	**	**	**

** = Significant at 1% level of probability

4.1.2.3 Relation between callus induction and days after inoculation

Callus induction varied among the varieties used in the present study. NAA and BAP, these two hormones viz. 1 mg/L NAA+1 mg/L BAP (T_5), 1 mg/L NAA+2 mg/L BAP (T_6), 1 mg/L NAA+4 mg/L BAP (T_7), 1.5 mg/L NAA+1 mg/L BAP (T_8), 1.5 mg/L NAA+1.5 mg/L BAP (T_9), 2 mg/L NAA+2 mg/L BAP (T_{10}) were also induced callus. MS media (T_1) without hormone and BAP 1.00 mg/L (T_2), 1.5 mg/L (T_3), 2 mg/L (T_4) alone did not induce callus.

In case of Red and Yellow variety no callus was formed in MS media (T_1) and BAP 1.00 mg/L (T_2), 1.5 mg/L (T_3), 2 mg/L (T_4). Maximum days (7.50) required for callus induction in treatment 1 mg/L NAA+2 mg/L BAP (T_6) for yellow variety and minimum days (4.90) required for callus induction in treatment 1.5 mg/L NAA+1.5 mg/L BAP (T_9) for Red variety (Figure 2).

4.1.2.4 Size of callus

The size of callus was recorded after 14, 28 and 42 days of cultured on MS media containing different concentrations of NAA and BAP. The results have been presented in Table 2 and plate 3.

The combined effect of varieties and different concentrations of hormone showed significant variations on size of callus at different DAI (**Appendix II**). The highest size of callus was found in the variety Red (0.98, 1.65, 2.77cm at 14, 28 and 42 DAI, respectively) was observed at 1.5 mg/L NAA+1.5 mg/L BAP. The size of the callus increased gradually with advancement of DAI in this variety. The minimum size of callus was found in the variety Yellow (0.34, 0.98 and 1.62 cm at 14, 28 and 42 DAI, respectively) at 1 mg/L NAA+1 mg/L BAP.

In figure 3, treatment 9 has given best result on the size of callus in Red variety and treatment 5 has given minimum result on the size of callus in Yellow variety at 42 days after inoculation.

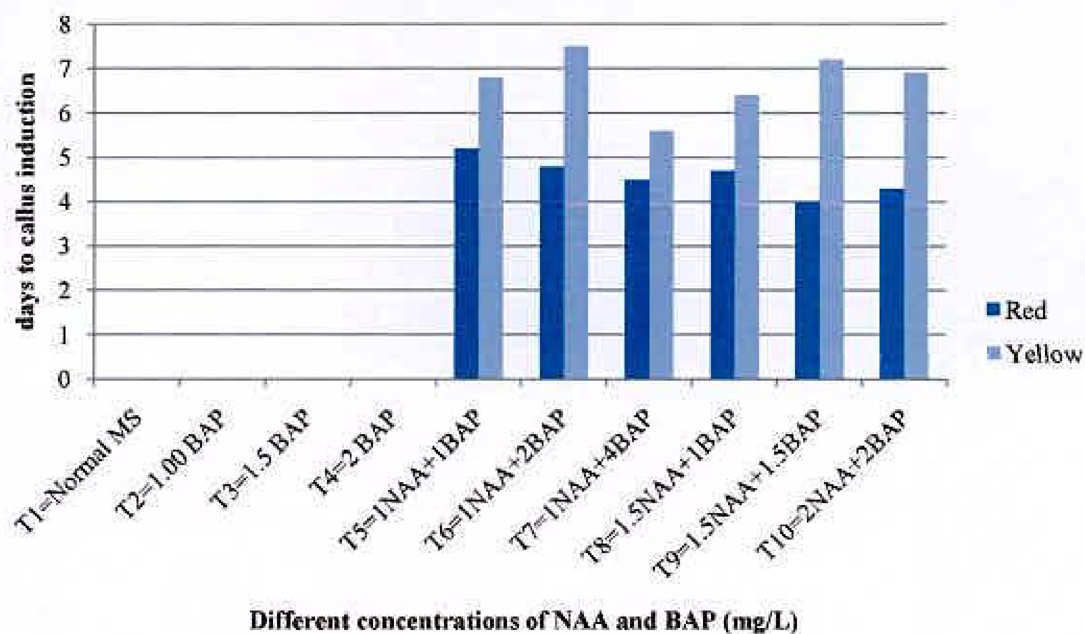


Figure 2. Combined effect of varieties and different concentrations of NAA and BAP on days to callus induction

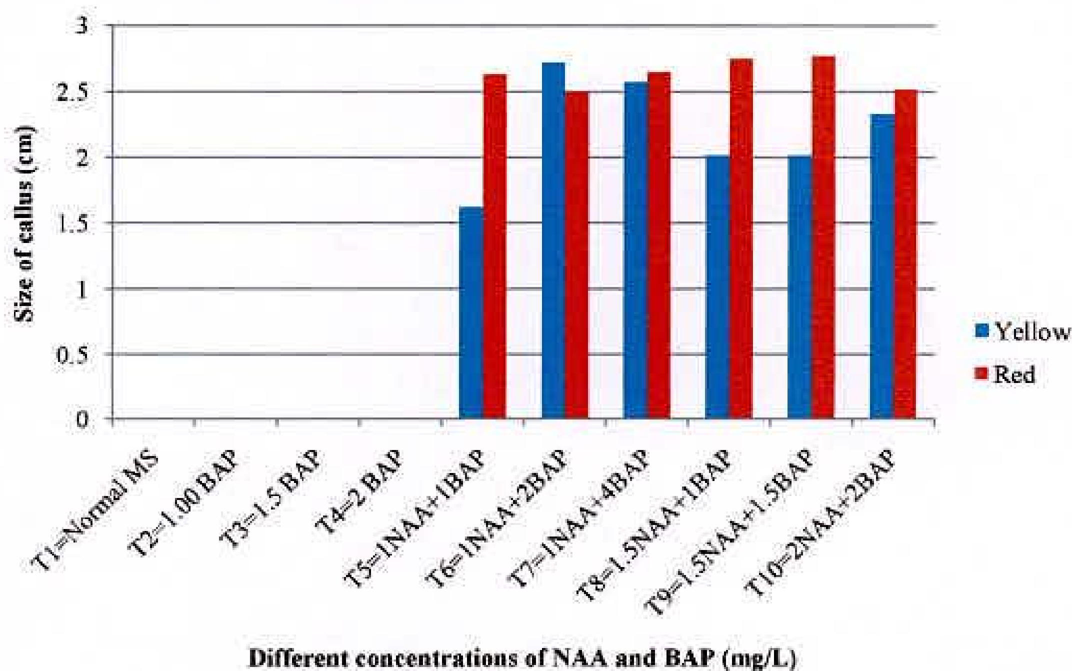


Figure 3. Combined effect of varieties and different concentrations of NAA and BAP on size of callus at 42 days after inoculation

4.2.5 Fresh weight of callus

There was significant interaction effect between the varieties and hormone concentrations on callus weight at 14, 28 and 42 DAI **(Appendix III)**. The highest weight of callus (0.94 gm at 14 DAI) was found in the variety of Red with the treatment combination 1.5 mg/L NAA+1.5 mg/L BAP and the lowest weight 0.45gm 0.04gm and 2.04 gm at 14, 28 and 42 DAI in the variety of Yellow with the treatment combination 1.5 mg/L NAA+1.5 mg/L BAP. Whereas the maximum weight (3.12 gm) was observed in the variety of Red with the treatment combination of 1mg/L NAA+1mg/L BAP (Table 2). Fomenko *et al.*, (1998) also showed that different genotypes reacted differently to the growth regulators in terms of callus induction.

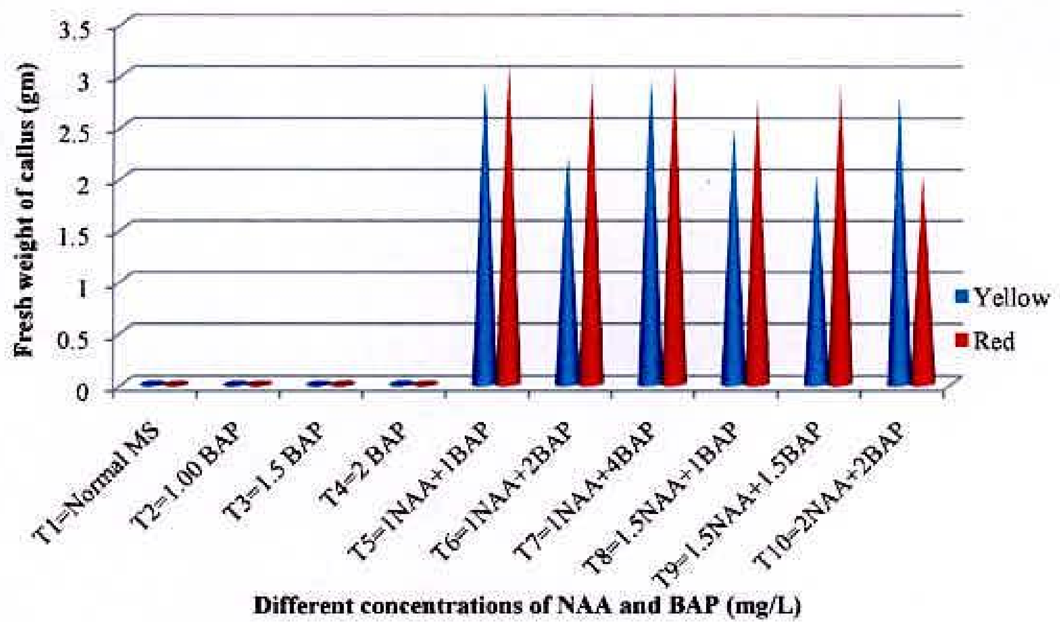
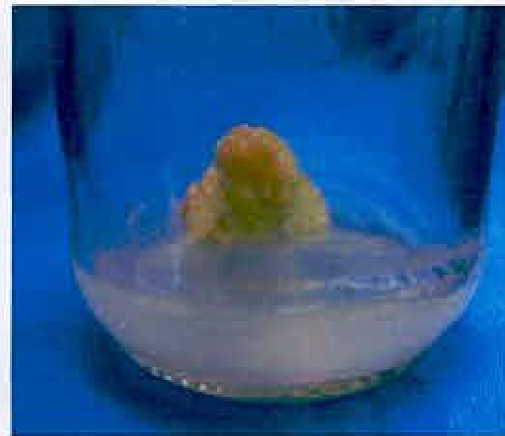


Figure 4. Combined effect of varieties and different concentrations of NAA and BAP on fresh weight of callus at 42 days after inoculation



(a) (b)

(a) Maximum size of callus at 14 DAI on MS media supplemented with1.5 mg/L NAA+1.5 mg/L BAP in Red variety

(b) Maximum size of callus at 28 DAI on MS media supplemented with1.00 mg/L NAA+4 mg/L BAP in Yellow variety

(c) (d)

(c) Maximum size of callus at 42 DAI on MS media supplemented with1.5 mg/L NAA+1.5 mg/L BAP in Red variety

(d) Maximum size of callus at 42 DAI on MS media supplemented with1.5 mg/L NAA+1.5 mg/L BAP in Red variety

Plate 3. Different sizes of callus at 14, 28 and 42 DAI on Red and Yellow variety

4.2 Experiment II: Effect of different concentration of NAA and BAP on shoot proliferation from flower bud/capitulum explants.

4.2.1 Days to shoot initiation

To achieve the goal of plant regeneration and subsequent shoot proliferation from flower bud/capitulum derived plantlets of gerbera varieties, MS medium supplemented with different concentration of NAA and BAP were used. Days to shoot initiation were recorded after inoculation in the media. The results have been presented in (Table 3). The combined effect of varieties and hormonal combination on days to shoot initiation has been presented in the **(Appendix IV)**. In Table 3 the maximum days to shoot initiation was found in variety Yellow (22.98 days) with 1.5 mg/L NAA+1.5 mg/L BAP and the minimum days to shoot initiation in variety Red (5.60 days) at 1 mg/L NAA+4 mg/L BAP. Maximum days (23.00) required for shoot initiation in treatment 1.5 mg/L NAA+1.5 mg/L BAP (T_9) for yellow variety and minimum days (6.00) required for shoot initiation in treatment 1.00 mg/L NAA+4.00 mg/L BAP (T_9) for Red variety (Figure 5).

4.2.2 Number of shoots per plantlet

Different varieties and concentrations of NAA and BAP significantly influenced the number of shoots produced per plantlet. Data were recorded at 14, 28 and 42 DAI. The response of varieties on shoot proliferation was found significant. The results showed (Table 3) and plate 4. The red variety produced 8.68 shoots per plantlet at 14 DAI whereas; in some cases no response was observed in Yellow and Red variety. Red variety has given the highest number of shoots while the lowest number of shoot was found in Yellow variety at 42 DAI. The number shoots produced per explants increased with the increase of DAI but the rate of increase was higher in Red variety than Yellow variety. These results indicated that the Red variety was superior to Yellow variety for this parameter.

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Table 3. Effect of different varieties and different hormones on shoot initiation and number of shoots/plantlet at different days after inoculation

Variety	Hormone	Days to shoot initiation	number of shoots/plantlet		
			14 DAI	28 DAI	42 DAI
Red variety	T1=Normal MS	7.00	1.30	2.22	4.77
	T2=1.00 BAP	11.50	3.32	5.80	8.36
	T3=1.5 BAP	8.90	6.68	10.52	13.65
	T4=2 BAP	12.70	4.54	8.45	12.62
	T5=1NAA+1BAP	7.80	6.72	8.24	10.94
	T6=1NAA+2BAP	7.20	4.32	6.25	9.87
	T7=1NAA+4BAP	5.60	6.87	11.22	16.80
	T8=1.5NAA+1BAP	18.20	0.00	2.45	4.62
	T9=1.5NAA+1.5BAP	12.30	1.70	4.68	6.43
	T10=2NAA+2BAP	22.65	0.00	0.78	1.90
Yellow variety	T1=Normal MS	10.80	2.76	4.00	4.59
	T2=1.00 BAP	15.73	0.00	2.34	5.96
	T3=1.5 BAP	12.43	2.89	4.10	7.20
	T4=2 BAP	20.21	0.00	7.67	9.45
	T5=1NAA+1BAP	10.67	1.57	3.50	6.43
	T6=1NAA+2BAP	8.74	3.34	7.67	8.46
	T7=1NAA+4BAP	9.56	4.86	7.87	13.29
	T8=1.5NAA+1BAP	17.50	0.00	5.32	8.50
	T9=1.5NAA+1.5BAP	22.98	0.00	2.98	4.34
	T10=2NAA+2BAP	18.58	0.00	3.42	7.52
	SE±	0.359	0.037	0.073	0.165
	Max	22.98	6.87	11.22	16.80
	Min	5.60	1.30	0.78	1.90
	LSD (0.01)	0.972	0.098	0.197	0.447
	Level of Significance	**	**	**	**

** = Significant at 1% level of probability

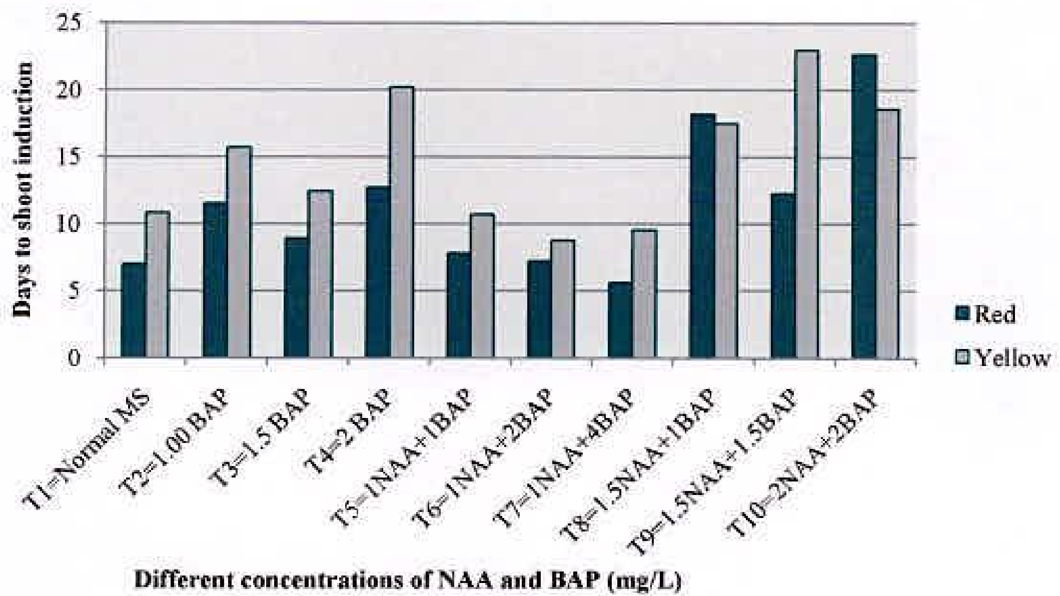

Figure 5. Combined effect of varieties and different concentrations of NAA and BAP on days to callus induction

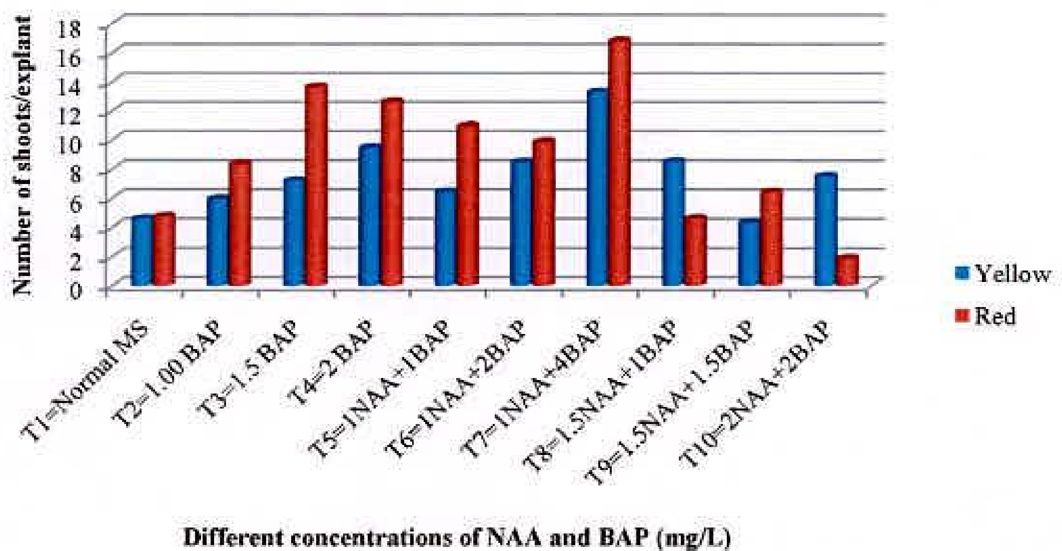

Figure 6. Combined effect of varieties and different concentrations of NAA and BAP on number of shoots/explant at 42 days after inoculation

(a)

(b)

(a) Initiated shoot at 14 DAI on MS media supplemented with 1.00 mg/L NAA+4 mg/L BAP in Red variety

(b) Initiated shoot at 14 DAI on MS media supplemented with 1.00 mg/L NAA+2.00 mg/L BAP in Yellow variety

(c)

(d)

(c) Maximum number of shoot initiated at 42 DAI on MS media supplemented with 1.00 mg/L NAA+4 mg/L BAP in Red variety

(d) Maximum number of shoot initiated at 42 DAI on MS media supplemented with 1.00 mg/L NAA+4 mg/L BAP in Yellow variety

Plate 4. Different days of shoot initiation at 14 and 42 DAI on Red and Yellow variety

Combined effect of varieties and different concentrations of hormones showed significant variation at different days on number shoots/plantlet (**Appendix IV**). The highest number of shoot/plantlet (6.87, 11.22 and 16.80 shoots/plantlet at 14, 28 and 42 DAI respectively) was recorded in variety of Red at 1 mg/L NAA+4 mg/L BAP and the lowest number of shoots/plantlet (0.00, 2.98 and 4.34) at 14, 28 and 42 DAI respectively was recorded in variety Yellow at 1.5 mg/L NAA+1.5 mg/L BAP (Table 3).

In figure 6, the Red variety produced significantly higher number of shoots than yellow plant. Treatment 7 has given maximum number shoots in red variety. On the other hand treatment 10 gave minimum number shoots in red variety.

4.2.3 Length of shoots/plantlet

Shoot length also influenced by varieties and different concentrations of NAA and BAP. Data were recorded at 14, 28 and 42 DAI and results have been presented in Table 4 and plate 5. The response of varieties showed that Red variety produced longer shoot (8.60 cm) compared to Yellow (6.90 cm) at 42 DAI. Besides Red variety produced longer shoot than Yellow at 14 and 28 DAI. These result indicated that Red variety performed better than Yellow for the increment of shoot.

The combined effect of varieties different concentrations of NAA and BAP showed that the length of shoot varied due to varieties and concentrations (**Appendix V**). Among the concentrations of NAA and BAP, 2 mg/L BAP produced the longest shoot in both varieties but the varieties Red produced longer shoot compared to Yellow at 14, 28 and 42 DAI. The red verities produced shoot (2.79, 5.72 and 8.60cm) and Yellow varieties produced shoot (3.88, 4.47 and 6.90 cm) at 14, 28 and 42 DAI respectively with 2 mg/L BAP. The lowest length of shoot (0.39, 2.13 and 3.78 cm) was obtained from 1 mg/L NAA+1 mg/L BAP with the variety of Yellow.

These results indicated that 2 mg/L BAP was the best among all concentrations of NAA and BAP and Red variety showed better performance over Yellow for this parameter (Figure 7).

Table 4. Effect of different varieties and different hormones on length of shoots/plantlet and number of leaves/plantlet at different days after inoculation.

Variety	Hormone	Length of shoots/plantlet(cm)			number of eaves/plantlet		
		14 DAI	28 DAI	42 DAI	14 DAI	28 DAI	42 DAI
Red variety	T1=Normal MS	0.82	1.98	4.62	0.00	1.60	5.60
	T2=1.00 BAP	1.54	3.70	5.22	0.00	0.00	8.90
	T3=1.5 BAP	1.60	3.77	6.60	0.76	7.65	14.70
	T4=2 BAP	2.79	5.72	8.60	1.00	8.90	22.40
	T5=1NAA+1BAP	0.52	1.52	3.36	0.00	0.00	4.20
	T6=1NAA+2BAP	1.85	3.23	5.00	0.00	1.30	5.80
	T7=1NAA+4BAP	2.89	4.39	6.50	0.00	1.20	8.30
	T8=1.5NAA+1BAP	0.52	2.43	4.23	0.00	0.00	3.40
	T9=1.5NAA+1.5BAP	3.26	4.89	6.80	0.00	2.26	8.00
	T10=2NAA+2BAP	0.87	2.76	4.87	0.00	2.00	7.80
Yellow variety	T1=Normal MS	2.26	3.64	5.80	0.00	0.00	4.80
	T2=1.00 BAP	2.10	4.10	5.77	0.00	4.30	10.60
	T3=1.5 BAP	2.98	4.26	6.45	0.00	6.90	16.00
	T4=2 BAP	3.88	4.47	6.90	0.00	5.40	18.60
	T5=1NAA+1BAP	0.39	2.13	3.78	0.00	1.20	7.50
	T6=1NAA+2BAP	0.00	1.78	2.90	0.00	0.00	0.00
	T7=1NAA+4BAP	1.48	3.87	5.96	0.00	2.00	12.50
	T8=1.5NAA+1BAP	0.00	0.00	0.00	0.00	0.00	0.00
	T9=1.5NAA+1.5BAP	0.75	1.35	2.63	0.00	0.00	4.54
	T10=2NAA+2BAP	0.65	2.28	3.98	0.00	1.30	3.80
	SE±	0.037	0.045	0.045	0.026	0.103	0.225
	Max	3.88	5.72	8.60	1.00	8.90	22.40
	Min	0.39	1.35	2.63	0.76	1.20	3.40
	LSD (0.01)	0.098	0.121	0.121	0.069	0.279	0.609
	Level of Significance	**	**	**	**	**	**

** = Significant at 1% level of probability

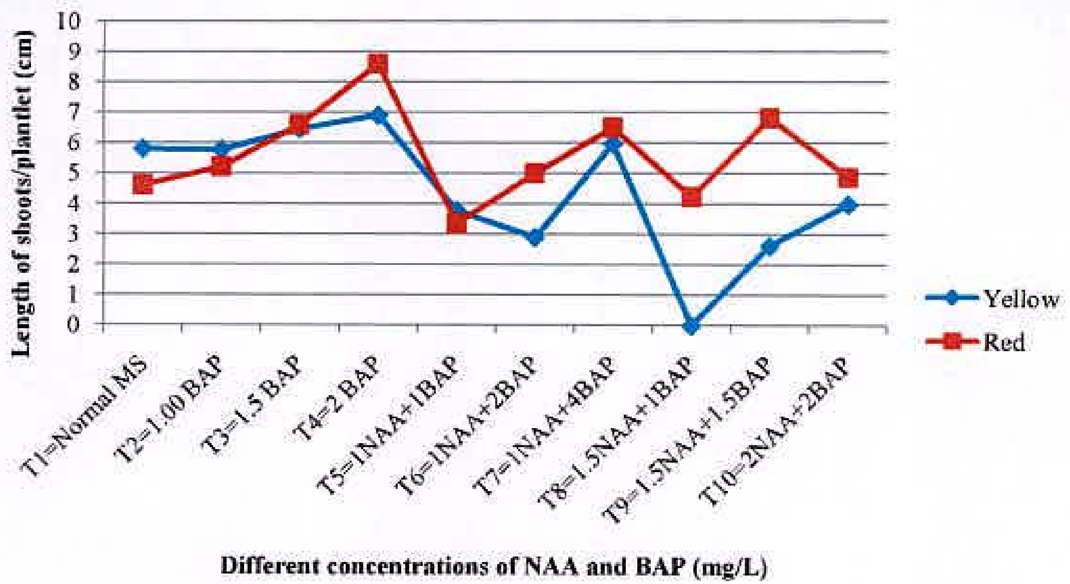

Figure 7. Combined effect of varieties and different concentrations of NAA and BAP on length of shoots/explant at 42 days after inoculation

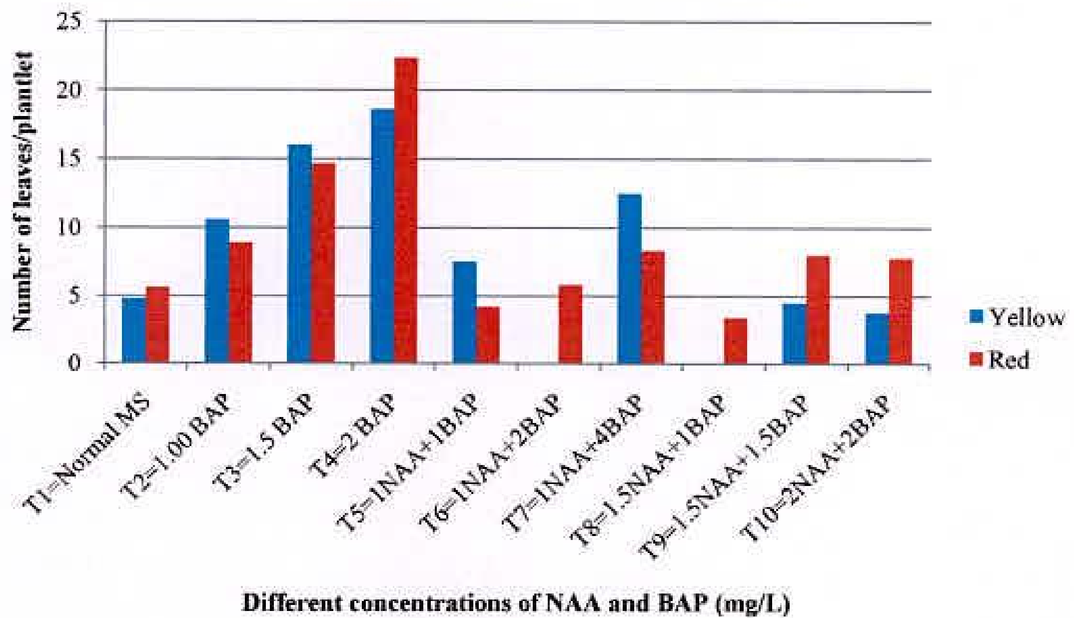

Figure 8. Combined effect of varieties and different concentrations of NAA and BAP on number of leaves/plantlet at 42 days after inoculation

(a) Maximum number of leaf initiated at 28 DAI on MS media with 2.00 mg/L BAP in Red variety

(b)Maximum length of shoot at 28 DAI on MS media with 2.00 mg/L BAP in Yellow variety

(c) Maximum length of shoot at 42 DAI on MS media with 2.00 mg/L BAP in Red variety

(d) Maximum length of shoot at 42 DAI on MS media with 2.00 mg/L BAP in Yellow variety

Plate 5. Different days to shoot length and number of leaves at 28 and 42 DAI on Red and Yellow variety

4.2.4 Number of leaves per plantlet

Number of leaves per plantlet varied significantly on MS medium supplemented with different concentrations of NAA and BAP used. The variety red produced 0.18 leaves per plantlet at 14 DAI but no response took place in case of Yellow variety. Besides Red variety responded more in the formation of leaves (8.91/plantlet) compared to Yellow (7.93/plantlet) at 42 DAI (Table 4). These result indicated that Red variety was superior to Yellow in respect to production of leaves per plantlet.

The combined effect of variety and different concentrations of BAP and NAA on leaf formation has been presented in table 4 (**Appendix VI**). Among the concentrations of BAP and NAA, 2 mg/L BAP showed the highest number of leaves in both the varieties at different DAI. The Red variety produced 22.40 leaves per plantlet at 2 mg/L BAP at 42 DAI, while the variety Yellow produced 18.60 leaves per plant at the same level of BAP and DAI (Figure 8). From the results it was found that leaf number per plantlet increased with the increase of BAP concentration up to 2 mg/L and then decreased in both the varieties.

4.2.5 Fresh weight of shoot

The combined effect of varieties and different concentrations of NAA and BAP on fresh weight of shoot was statistically significant at 14 days after inoculation Table 5 (**Appendix VII**). The plantlet of Red variety produced significantly higher fresh biomass compared to Yellow variety. Highest fresh weight has been obtained with the treatment of 2 mg/L BAP. After 14 days of inoculation red and yellow plantlet had a highest fresh weight 78.20 mg and 58.20 mg respectively.

Table 5. Effect of different verities and different hormone on fresh weight of shoot and dry weight of shoot at 14 days after inoculation

Variety	Hormone	Fresh weight of shoot (mg)	Dry weight of shoot (mg)
Red variety	T1=Normal MS	20.60	3.20
	T2=1.00 BAP	40.80	5.78
	T3=1.5 BAP	56.10	5.35
	T4=2 BAP	78.20	9.96
	T5=1NAA+1BAP	70.80	9.14
	T6=1NAA+2BAP	54.40	7.12
	T7=1NAA+4BAP	72.56	9.36
	T8=1.5NAA+1BAP	0.00	0.00
	T9=1.5NAA+1.5BAP	24.45	3.68
	T10=2NAA+2BAP	0.00	0.00
Yellow variety	T1=Normal MS	35.70	5.06
	T2=1.00 BAP	0.00	0.00
	T3=1.5 BAP	38.35	5.20
	T4=2 BAP	0.00	0.00
	T5=1NAA+1BAP	25.56	3.76
	T6=1NAA+2BAP	41.25	5.88
	T7=1NAA+4BAP	58.20	7.56
	T8=1.5NAA+1BAP	0.00	0.00
	T9=1.5NAA+1.5BAP	0.00	0.00
	T10=2NAA+2BAP	0.00	0.00
	SE±	0.242	0.126
	Max	78.20	9.96
	Min	20.60	3.20
	LSD (0.01)	0.655	0.342
	Level of Significance	**	**

** = Significant at 1% level of probability

4.2.6 Dry weight of shoot

The combined effect of varieties and different concentrations of NAA and BAP on dry weight of shoot was statistically significant at 14 days after inoculation Table 5 **(Appendix VII).** The dry matter production in the shoot was significantly higher in Red variety plantlet compared to Yellow plantlet. With the treatment of 2 mg/ L BAP, it increases both fresh shoot weight and dry shoot weight after 14 days of inoculation in Red variety plantlet. Highest dry shoot weight (9.96 mg and 7.56 mg) in Red and Yellow variety plantlet respectively are found. In some cases there was no dry shoot weight after 14 days of inoculation.

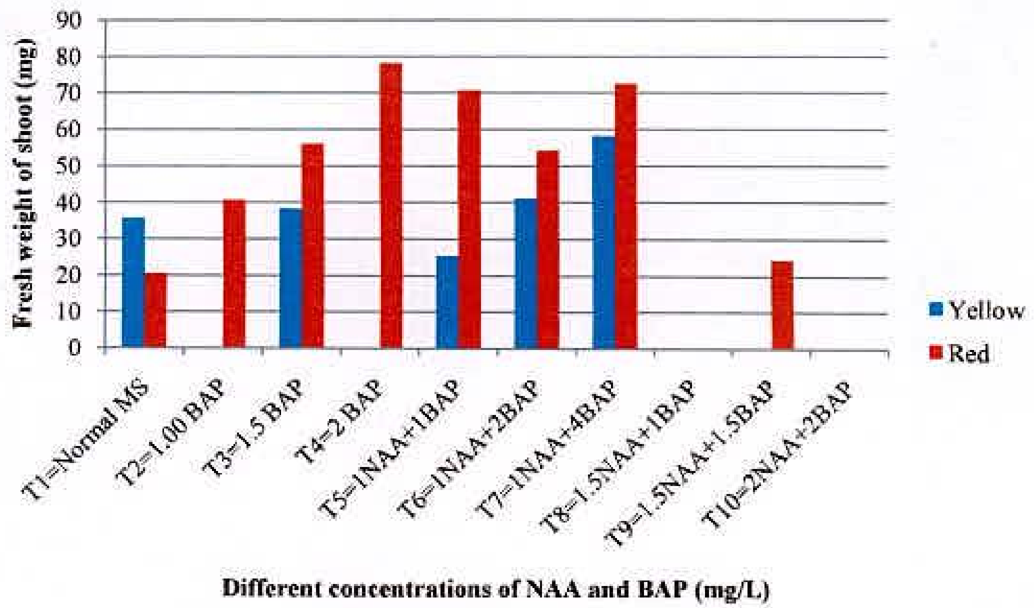

Figure 9. Effect of different verities and different hormone on fresh weight of shoot at 14 days after inoculation

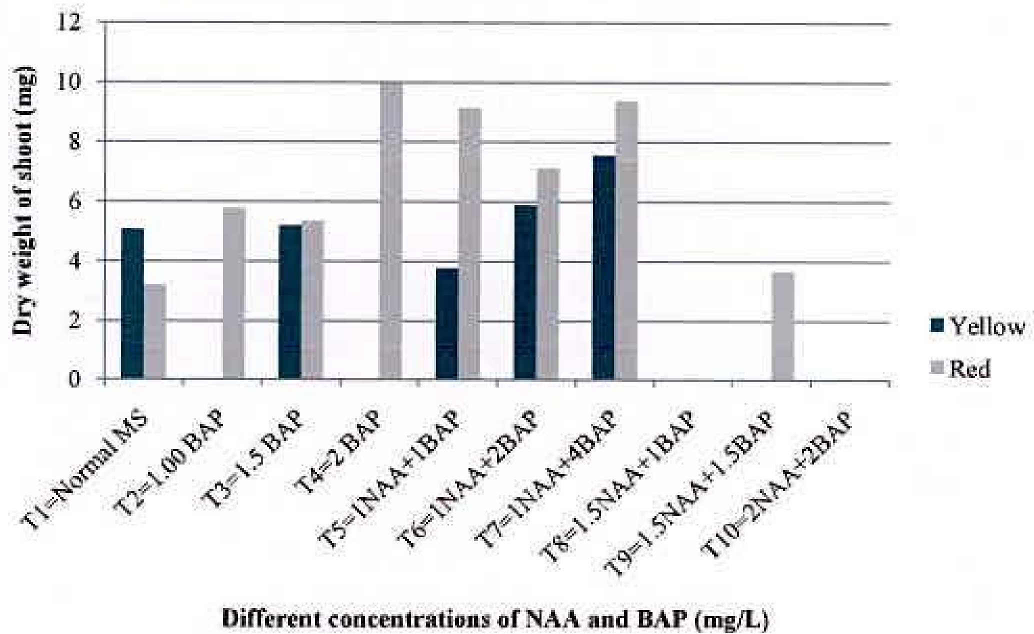

Figure 10. Effect of different verities and different hormone on dry weight of shoot at 14 days after inoculation

(a) Initial shoot initiation

(b) Shoot initiation at 14 DAI on MS media supplemented with 2.00 mg/L BAP in Red varieties

(c) Shoot initiation at 14 DAI on MS media supplemented with 2.00 mg/L BAP in Yellow varieties

Plate 6. Shoot initiation at 14 DAI on Red and Yellow variety

4.3 Experiment III: Effect of NAA and BAP on the induction of roots from multiplied shoots of gerbera varieties Red and Yellow

4.3.1 Days to root initiation

Days to root initiation was recorded after inoculation in the cultured on MS media containing different concentrations of NAA and BAP. The results have been presented in Table 6 and **AppendixVIII** .

The gerbera varieties and different levels of hormones showed significant interaction in relation to the days to root initiation. The maximum days (30.40 days) to root initiation is found on MS medium containing 1.5mg/L BAP in the variety red plantlet. The minimum days observed in the variety Yellow (9.30 days) on MS media containing 2 mg/L NAA+2 mg/L BAP (Table 6) and Figure 11.

4.3.2 Number of roots per plantlet

The number of roots produced per plantlet varied on MS medium supplemented with different concentrations of NAA and BAP were used. The response of varieties on number of roots per plantlet at different DAI was found significant. The variety Red produced more number of roots per plantlet compared to Yellow at different DAI. The Red produced 6.68 roots per plantlet at 42 DAI while the variety Yellow produced 6.57 roots per plantlet at the same DAI (Figure 12). Besides, the number of roots per plantlet increased with the increase of DAI. These result indicated that Red variety showed better performance in root formation over Yellow.

The combined effect of varieties and different concentration of NAA and BAP on root formation at different DAI were found significant (**Appendix VIII**). The results (Table 6) showed that among the concentration of NAA and BAP, 1.5 mg/L NAA+1.5 mg/L BAP gave the highest number of roots per plantlet and 1.00 mg/L BAP +2 mg/L BAP gave no response to produce roots. The Red variety produced roots (2.20, 6.90 and 14.70) per plantlet at 14, 28 and 42 days DAI.

Table 6. Effect of different varieties and different hormone on days to root initiation and number of roots/plantlet at different days after inoculation

Variety	Hormone	Days to root initiation	number of roots/plantlet		
			14 DAI	28 DAI	42 DAI
Red variety	T1=Normal MS	28.90	0.00	0.00	1.40
	T2=1.00 BAP	29.70	0.00	0.00	1.00
	T3=1.5 BAP	30.40	0.00	0.00	3.20
	T4=2 BAP	28.80	0.00	0.00	2.40
	T5=1NAA+1BAP	22.30	0.00	3.80	7.20
	T6=1NAA+2BAP	20.20	0.00	3.20	6.80
	T7=1NAA+4BAP	27.60	0.00	0.80	9.40
	T8=1.5NAA+1BAP	18.40	0.00	4.70	11.30
	T9=1.5NAA+1.5BAP	11.20	2.20	6.90	14.70
	T10=2NAA+2BAP	13.20	1.90	5.20	9.40
Yellow variety	T1=Normal MS	30.30	0.00	0.00	2.80
	T2=1.00 BAP	0.00	0.00	0.00	0.00
	T3=1.5 BAP	28.20	0.00	0.00	2.80
	T4=2 BAP	0.00	0.00	0.00	0.00
	T5=1NAA+1BAP	22.56	0.00	3.78	5.54
	T6=1NAA+2BAP	25.70	0.00	2.40	7.34
	T7=1NAA+4BAP	22.40	0.00	4.40	10.00
	T8=1.5NAA+1BAP	10.90	4.76	9.56	12.80
	T9=1.5NAA+1.5BAP	16.70	0.00	7.40	11.78
	T10=2NAA+2BAP	9.30	5.60	9.61	12.70
	SE±	0.482	0.073	0.153	0.349
	Max	30.40	5.60	9.61	14.70
	Min	9.30	1.90	0.80	1.00
	LSD (0.01)	1.304	0.197	0.413	0.944
	Level of Significance	**	**	**	**

** = Significant at 1% level of probability

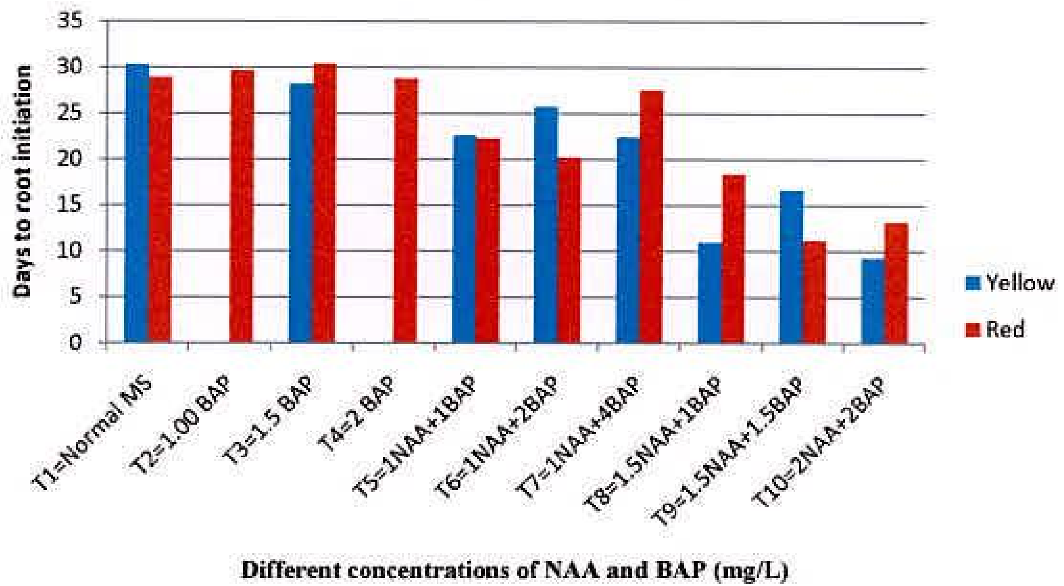

Figure 11. **Effect of different verities and different hormone on days to root initiation**

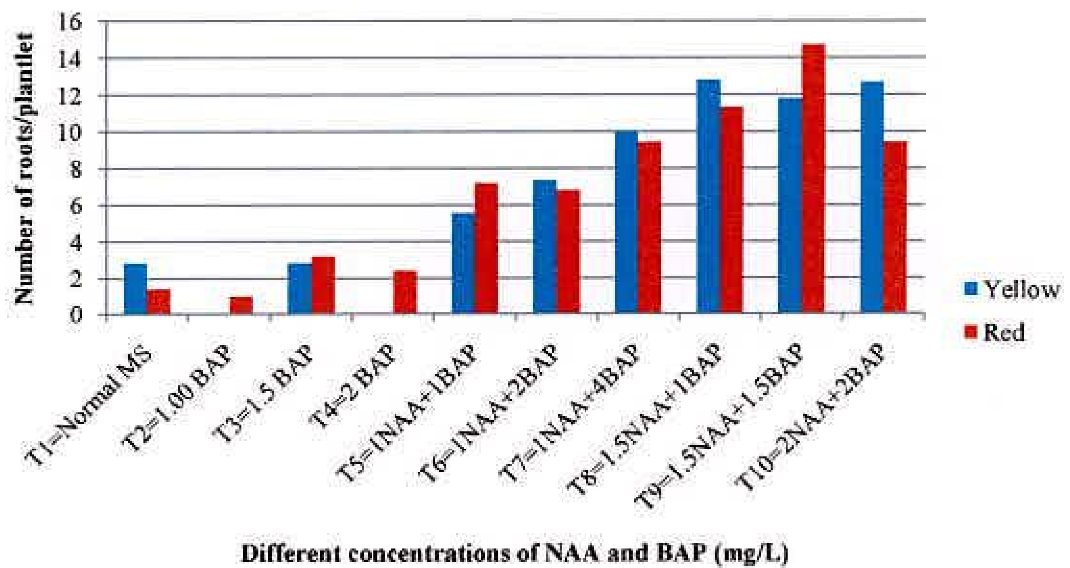

Figure 12. **Effect of different verities and different hormone on number of roots/plantlet at 42 days after inoculation**

4.3.3 Length of roots/plantlet

The response of varieties on the increase of root in length at DAI was found significant difference (**Appendix IX**). There was sharp increasing trend in root length at different DAI in both the varieties but the increment of root length of Red variety was superior to Yellow at 14, 28 and 42 DAI (Table 7). At 42 DAI, the Red variety produced roots 4.79 cm in length while the Yellow variety produced 3.73 cm roots (Figure 13). These result indicated that the Red variety was better than Yellow on the increment of root length. A significant variation was found among the concentrations of NAA and BAP used on MS medium. Among the concentrations of NAA and BAP, 1.5 mg/L NAA+1 mg/L BAP produced the longest root in Red variety. Maximum length of root (2.20, 6.90 and 14.70cm at 14, 28 and 42 DAI) was recorded in the variety of Red. There were no root formation in the concentrations 1.00 mg/L BAP and 2.00 mg/L BAP.

4.3.4 Fresh weight of root

The data on fresh root biomass production by the plantlet at 42 DAI are given in Table 8. The plantlet of Yellow variety showed a significantly higher root fresh weight at 42 DAI.

The combined effect of varieties and different concentration of NAA and BAP on fresh weight of root at different DAI were found significant **(Appendix X)**.Maximum fresh weight of yellow variety is 0.92 gm at 42 days after inoculation with the treatment of 1.5 mg/L NAA+1 mg/L BAP. Maximum fresh weight of Red variety is 0.80 gm at 42 days after inoculation with the treatment of 1.5 mg/L NAA+1.5 mg/L BAP (Figure 14). Therefore, Yellow variety has been performed better than Red variety.

4.3.5 Dry weight of root

The data regarding the dry weight of the root system are presented in Table 8. The plantlet of yellow variety showed a significantly higher dry weight of root at 42 days after inoculation compared to Red variety.

The combined effect of varieties and different concentration of NAA and BAP on dry weight of root at different DAI were found significant **(Appendix X)**. Maximum dry weight of Yellow variety is 0.16 gm at 42 days after inoculation with the treatment 1.5 mg/L NAA+ 1.00 mg/L BAP (Figure 15).

Table 7. Effect of different varieties and different hormones on length of roots/plantlet at different days after inoculation

Variety	Hormone	Length of roots/plantlet(cm)		
		14 DAI	28 DAI	42 DAI
Red variety	T1=Normal MS	0.00	0.00	4.20
	T2=1.00 BAP	0.00	0.00	1.45
	T3=1.5 BAP	0.00	0.00	2.03
	T4=2 BAP	0.00	0.00	1.80
	T5=1NAA+1BAP	0.00	3.30	6.20
	T6=1NAA+2BAP	0.00	1.97	5.80
	T7=1NAA+4BAP	0.00	0.48	6.00
	T8=1.5NAA+1BAP	0.00	4.50	7.50
	T9=1.5NAA+1.5BAP	2.34	4.87	7.00
	T10=2NAA+2BAP	1.00	3.02	6.00
Yellow variety	T1=Normal MS	0.00	0.00	2.75
	T2=1.00 BAP	0.00	0.00	0.00
	T3=1.5 BAP	0.00	0.00	3.42
	T4=2 BAP	0.00	0.00	0.00
	T5=1NAA+1BAP	0.00	0.46	2.50
	T6=1NAA+2BAP	0.00	3.16	6.30
	T7=1NAA+4BAP	0.00	2.40	5.00
	T8=1.5NAA+1BAP	2.30	4.90	6.00
	T9=1.5NAA+1.5BAP	0.00	2.88	5.36
	T10=2NAA+2BAP	2.40	4.69	6.00
	SE±	0.425	0.728	0.548
	Max	2.40	4.90	7.50
	Min	1.00	0.46	1.45
	LSD (0.01)	1.149	1.969	1.483
	Level of Significance	**	**	**

** = Significant at 1% level of probability

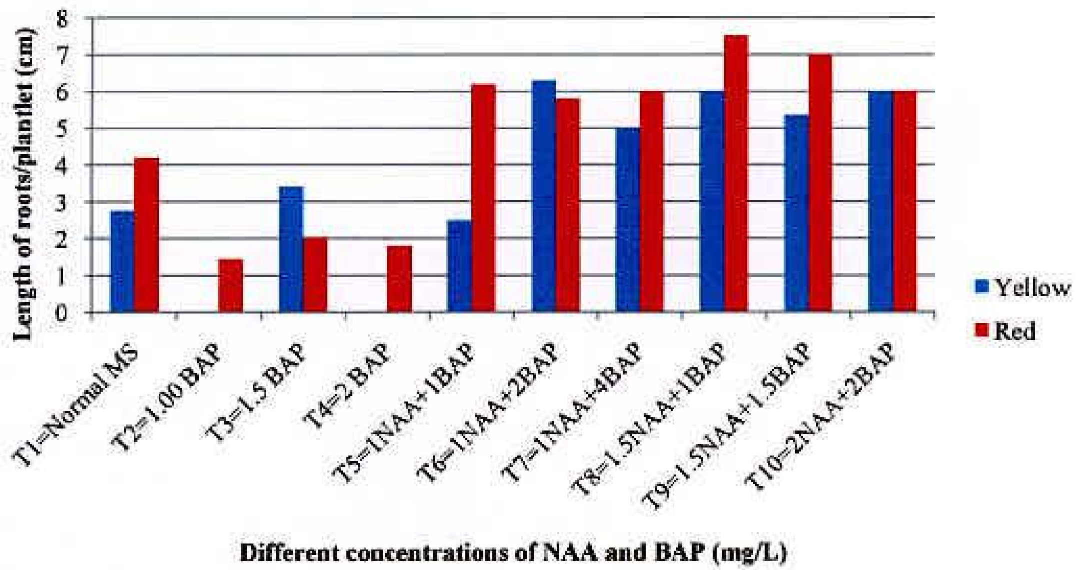

Figure 13. Effect of different verities and different hormone on length of roots/plantlet at 42 days after inoculation.

(a) Maximum length of root 28 DAI on MS media supplemented with 1.5 mg/L NAA+1.00 mg/L BAP in Yellow variety.

(b)

(c)

(b) Maximum number of roots 42 DAI on MS media supplemented with 1.5 mg/L NAA+1.5mg/L BAP in Red variety.

(c) Maximum length of roots 42 DAI on MS media supplemented with 1.5 mg/L NAA+1.00 mg/L BAP in Red variety.

Plate 7. Different length and number of roots at 28 and 42 DAI on Red and Yellow variety

Table 8. Effect of different verities and different hormone on fresh weight of root and dry weight of root at 42 days after inoculation

Variety	Hormone	Fresh weight of root (gm)	Dry weight of root (gm)
Red variety	T1=Normal MS	0.23	0.05
	T2=1.00 BAP	0.17	0.04
	T3=1.5 BAP	0.45	0.08
	T4=2 BAP	0.34	0.06
	T5=1NAA+1BAP	0.63	0.10
	T6=1NAA+2BAP	0.59	0.09
	T7=1NAA+4BAP	0.71	0.12
	T8=1.5NAA+1BAP	0.75	0.12
	T9=1.5NAA+1.5BAP	0.80	0.13
	T10=2NAA+2BAP	0.49	0.08
Yellow variety	T1=Normal MS	0.39	0.07
	T2=1.00 BAP	0.00	0.00
	T3=1.5 BAP	0.29	0.06
	T4=2 BAP	0.00	0.00
	T5=1NAA+1BAP	0.51	0.08
	T6=1NAA+2BAP	0.71	0.12
	T7=1NAA+4BAP	0.70	0.12
	T8=1.5NAA+1BAP	0.92	0.16
	T9=1.5NAA+1.5BAP	0.72	0.12
	T10=2NAA+2BAP	0.75	0.13
	SE±	0.037	0.00
	Max	0.92	0.16
	Min	0.17	0.04
	LSD (0.01)	0.098	0.00
	Level of Significance	**	**

** = Significant at 1% level of probability

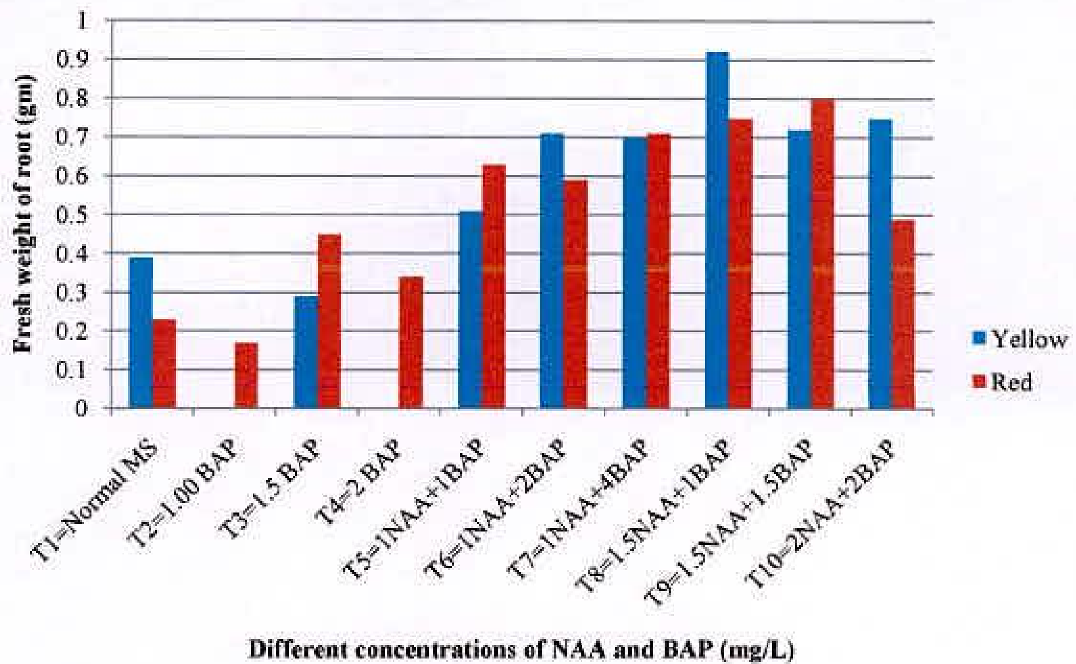

Figure 14. Effect of different varieties and different hormone on fresh weight of root at 42 days after inoculation

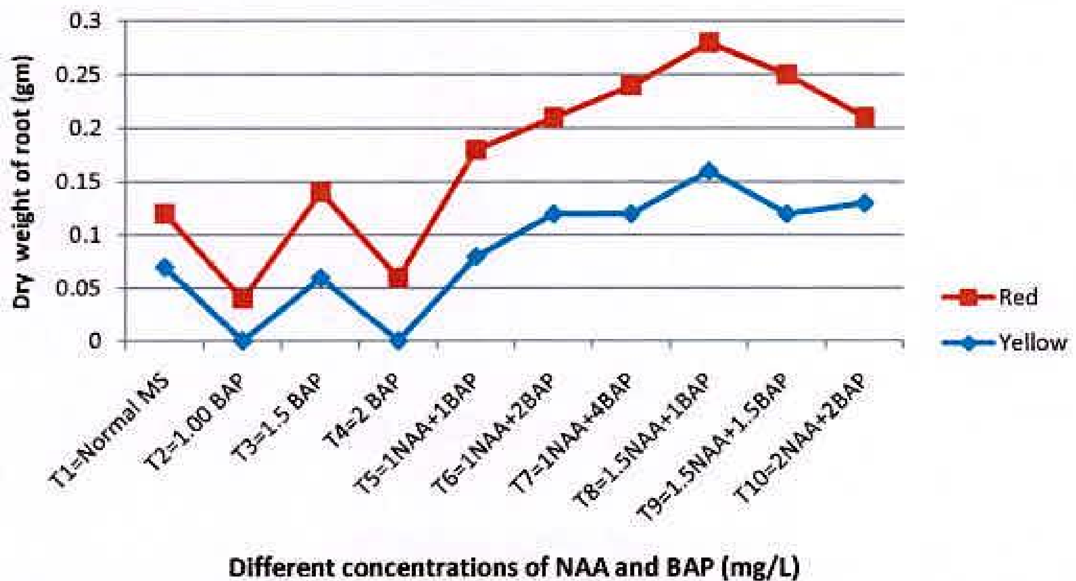

Figure 15. Effect of different verities and different hormone on dry weight of root at 42 days after inoculation

4.4 Experiment IV: Mutation *In vitro* using chemical mutagen

4.4.1 Percent survival of shoot tips

Survival of shoot tips treated with chemical mutagens decreased with increasing concentrations (Table 9). Among the EMS treatments, the highest survival rate (84.21%) was at 25 µM. At 50 µM the per cent survival was reduced to 81.74 where as 500µM it was the least with 41.60% survival in red variety. In case of Yellow variety, the highest survival rate (82.56%) was at 25 µM. The lowest survival rate (39.59%) was found at 500µM concentration (Figure 16).

4.4.2 Number of shoots per explant

The observations on the number of shoots per explant as influenced by mutagen concentrations are presented in Table 9. Untreated shoot tips gave the highest number of shoots. There were reductions in number of shoots produced with increasing concentrations. The lowest number of shoots was observed in 500 µM EMS (3.12) in red variety and (2.12) in Yellow variety (Figure 17).

4.4.3 Observation of percentage mortality of shoot tips and abnormalities in leaves

Thirty number of shoot tips were treated for each concentration EMS for two varieties. Data were recorded in Table 10 for percentage mortality and abnormalities of leaves. The combined effect of varieties and different concentration of EMS on percentage of observed mortality and percentage of abnormalities in leaves were found significant **(Appendix XII).**

In Red and Yellow variety, the highest mortality and abnormalities were found with the treatment 500 µM EMS concentration. Maximum mortality rate (60.41%) and minimum mortality (1.21%) were found in Yellow and Red variety respectively. Maximum leaf abnormalities in Red and Yellow variety were found almost same.

Table 9. Effect of different varieties and different concentration of EMS on the survival and multiplication of shoots

Variety	EMS Concentration(μM)	Percent survival of shoot tips	Number of shoots/plantlet
Red variety	0	98.79	11.54
	25	84.21	9.76
	50	81.74	8.95
	100	76.23	8.34
	150	69.56	7.05
	250	66.87	6.42
	375	61.50	4.85
	500	41.60	3.12
Yellow variety	0	98.20	10.45
	25	82.56	9.80
	50	80.07	8.79
	100	73.03	7.34
	150	64.34	6.67
	250	57.78	4.48
	375	46.56	3.38
	500	39.59	2.12
	SE±	2.746	0.563
	Max	98.79	11.54
	Min	39.59	2.12
	LSD (0.01)	7.426	1.523
	Level of Significance	**	**

** = Significant at 1% level of probability

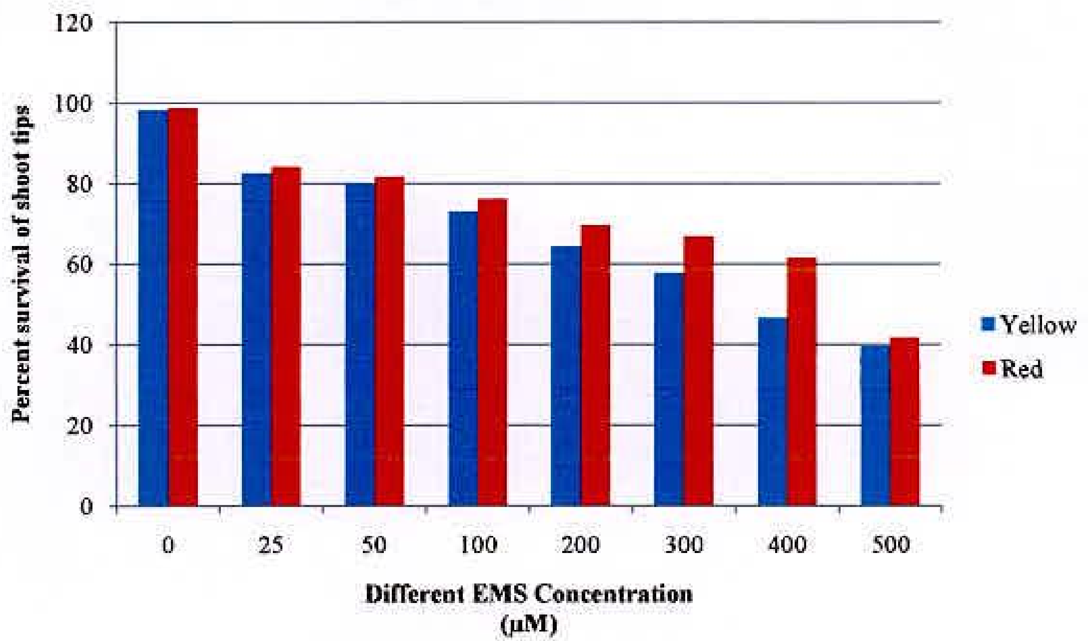

Figure 16. Effect of different varieties and different concentration of EMS on the percentage of survival of shoot tips

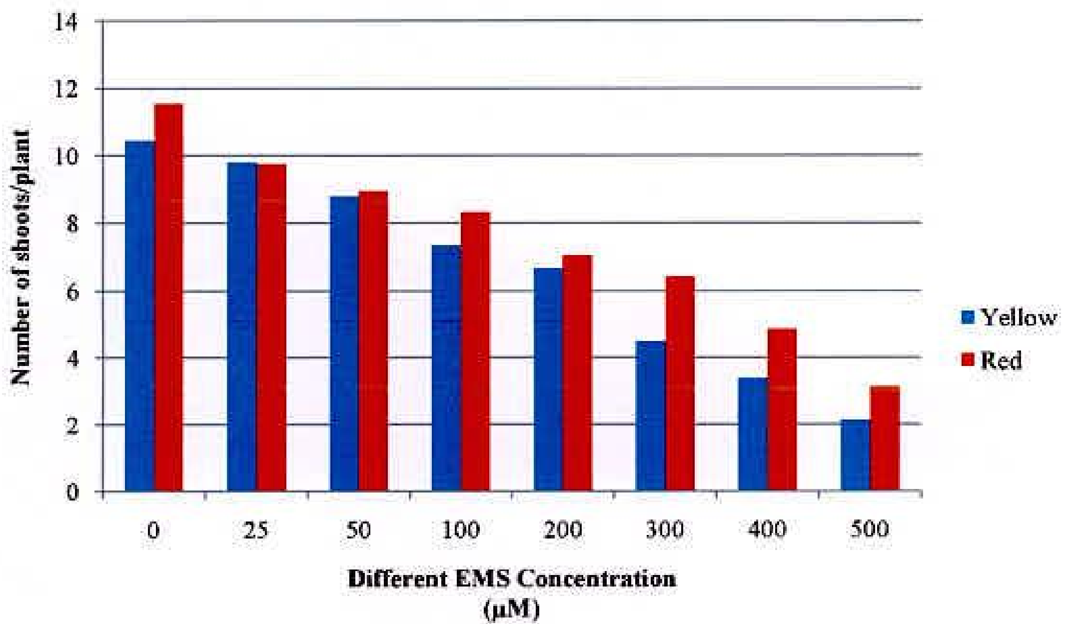

Figure 17. Effect of different varieties and different concentration of EMS on number of shoots/plant

(a) Observed normal leaves

(b) Observed abnormalities in leaves

Plate 8. Observation of abnormalities in leaves after mutagen application

Table 10. Effect of different varieties and different concentration of EMS on the mortality of shoot tips and abnormalities in leaves

Variety	EMS Concentration (μM)	Number of shoot tips treated	Observed mortality %	Abnormalities in leaves %
Red variety	0	30	1.21	2.34
	25	30	15.79	11.67
	50	30	18.26	19.23
	100	30	23.77	26.98
	150	30	30.44	35.56
	250	30	33.13	46.89
	375	30	38.50	57.67
	500	30	58.40	69.87
Yellow variety	0	30	1.80	1.96
	25	30	17.44	8.87
	50	30	19.93	17.65
	100	30	26.97	27.34
	150	30	35.66	45.64
	250	30	42.22	55.39
	375	30	53.44	61.34
	500	30	60.41	69.49
	SE±		1.649	1.285
	Max		60.41	69.87
	Min		1.21	1.96
	LSD (0.01)		4.461	3.476
	Level of Significance		**	**

** = Significant at 1% level of probability

4.5 Experiment V: Acclimatization and establishment of plantlets in soil

4.5.1 *Ex-vitro* hardening of plantlets

Hardening is a time-consuming and labour intensive process 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 desiccate 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 and Conover, 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 are susceptible to transplantation shocks leading 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 al.*, 1998). Acclimatization units have been developed with temperature, humidity, irradiance, CO_2 concentration and air flow rate controlled by computer (Hayashi *et al.*, 1988).

4.5.2 Multiplication of plantlet for acclimatization

From the overall experiment, it was revealed that three treatments were best in respect with rapid callus induction (1.5 mg/L NAA+1.5 mg/L BAP), maximum shoot/plantlet (1 mg/L NAA+4 mg/L BAP) and rapid root (2.00 mg/L NAA+2.00 mg/L BAP) initiation. Plants from these treatments were sub-cultured on normal MS media for further multiplication.

4.5.3 Transplantation

Potting mixture (sand: soil: compost, 1:1:1) was mixed properly and autoclaved one hour in 121°c for 20 minutes. After cooling, the soil mixtures were taken into 10 cm pots for growing the pots *in vivo* condition. When the plantlets become 5-8 cm in height with sufficient shoot and root system, they 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 translation 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 high humidity around the plantlets.

Table 11. Survival rate of in vitro regenerated plantlets of two gerbera varieties in earthen pot

Variety	Number of trans plated plants	Number of plants survived	Survival rate (%)
Red	30	25	83.33
Yellow	30	23	76.66

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 9. Transplantation of gerbera plantlets in earthen pot

Chapter V
Summary and Conclusion

CHAPTER V

SUMMARY AND CONCLUSION

The present experiment was conducted in the tissue culture laboratory of Genetics and Plant Breeding Department, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka, during the period of 2014-2015 from flower bud or capitulum explants of gerbera variety Red and Yellow to establish *in vitro* callus induction, plant regeneration and mutagen sensitivity using different concentration and combination of NAA and BAP. The experiment was conducted at Completely Randomized Design (CRD) with 3 replications.

Flower buds excised from source material were used as experimental materials. Explants were sterilized by 0.1% $HgCl_2$ for 10 minutes. Explant were cultured on MS medium with the concentrations of BAP 1.0 mg/L, 1.5 mg/L and 2.0mg/L and the concentration for combined NAA and BAP were1.0 mg/L NAA+1.0 mg/L BAP, 1.0mg/L NAA+2.0 mg/LBAP, 1.0 mg/L NAA+4.0 mg/L BAP, 1.5 mg/L NAA+1.0 mg/L BAP and 1.50 mg/L NAA+1.5 mg/L BAP. To investigate the effect of different treatments of this experiment, the parameters were recorded on, callus color, days to callus induction, percentage of callus induction, size of callus, fresh weight of callus, days to shoot initiation, number of shoots/plantlet, number of leaves/plantlet, length of shoots/plantlet, days to root initiation, number roots/plantlet, survival of shoot tips, number of shoots per explant, morphological abnormalities and survival rate of plantlets.

The combined effect of different gerbera varieties and hormone showed significant variation on days to callus induction, size of callus and weight of callus. The minimum days (4.00 days) to callus induction was found in variety red with the treatment 1.5 mg/L NAA+1.5 mg/L BAP . The highest size of callus was found in the variety Red (0.98, 1.65, 2.77cm at 14, 28 and 42 DAI, respectively) was observed at 1.5 mg/L NAA+1.5 mg/L BAP. The minimum size of callus was found in the variety Yellow (0.34, 0.98 and 1.62 cm at 14, 28 and 42 DAI, respectively) at 1 mg/L NAA+1 mg/L BAP. The highest weight of callus (0.94 gm at 14 DAI) was found in the variety of Red with the treatment combination 1.5 mg/L NAA+1.5 mg/L BAP

The different concentrations of hormone significantly influenced the days to shoot initiation and days to root initiation with number of shoots and roots/plantlet. The

minimum days to shoot initiation in variety Red is 5.60 days at 1 mg/L NAA+4 mg/L BAP. The highest number of shoots/plantlet (6.87, 11.22 and 16.80 shoots/plantlet at 14, 28 and 42 DAI respectively) was recorded in variety of Red at 1 mg/L NAA+4 mg/L BAP and longest shoot 8.60 cm. The minimum days observed in the variety Yellow (9.30 days) on MS media containing 2 mg/L NAA+2 mg/L BAP. Maximum length of root (2.20, 6.90 and 14.70cm at 14, 28 and 42 DAI) was recorded in the variety of Red.

The combined effect of varieties and different concentration of NAA and BAP on fresh weight dry weight of root at different DAI were found significant. Maximum fresh weight of yellow variety is 0.92 gm at 42 days after inoculation with the treatment of 1.5 mg/L NAA+1 mg/L BAP. Maximum dry weight of Yellow variety is 0.16 gm at 42 days after inoculation with the treatment 1.5 mg/L NAA+ 1.00 mg/L BAP.

The investigation on induction of mutation *in vitro* was aimed for understanding the effect of the highest survival rate (84.21%) was at 25 µM. Chemical mutagens on the survival percentage of the gerbera shoot tips at the laboratory level. The survival percentage of the shoot tips and the number of shoots produced from the treated shoot tips of gerbera decreased with different concentrations of the mutagen used. In Red and Yellow variety, the highest mortality and abnormalities were found with the treatment 500 µM EMS concentration.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 gerbera commercially. This protocol could be used for *in vitro* breeding programme.

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Appendices

APPENDICES

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

Components	**Concentrations (mg/l)**
Macronutrients	
KNO_3	1900.00
NH_4NO_3	1650.00
$MgSO_4.7H_2O$	370.00
$CaCl_2.2H_2O$	440.00
KH_2PO_4	170.00
Micronutrients	
$MnSO_4.7H_2O$	22.30
H_3BO_3	6.20
$ZnSO_4.7H_2O_4$	8.60
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
KI	0.83
Iron Sources	
$FeSO_4.7H_2O$	27.80
$Na_2EDTA.2H_2O$	37.30
Vitamin and Organic nutrients	
Nicotine Acid	0.50
Pyriodoxine HCl	0.50
Thaimine HCl	0.50
Glycine	2.00
Myo inositol	100.00
Sucrose	30000.00
Agar	7000.00
pH is adjusted to 5.8 before autoclaving	

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

Source of variance	Degrees of freedom (df)	Mean square of days to callus induction	Mean square of size of callus (cm)		
			14 DAI	28 DAI	42 DAI
Factor A (Variety)	1	16.716**	.133**	.190**	.993**
Factor B (Treatment)	9	51.133**	.938**	3.397**	9.309**
AB (Variety×Treatment)	9	1.826**	.042**	.103**	.233**
Error	40	.343	.005	.002	.001

** = Significant at 1% level of probability

* = Significant at 5% level of probability

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

Source of variance	Degrees of freedom (df)	Mean square of fresh weight of callus (gm)		
		14 DAI	28 DAI	42 DAI
Factor A (Variety)	1	.080**	.027**	.276**
Factor B (Treatment)	9	.876**	3.546**	11.942**
AB (Variety×Treatment)	9	.053**	.051*	.284**
Error	40	.000	.001	.001

** = 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/plantlet.

Source of variance	Degrees of freedom (df)	Mean square of days to shoot initiation	Mean square of number of shoots/plantlet		
			14 DAI	28 DAI	42 DAI
Factor A (Variety)	1	171.975**	59.441**	19.403**	26.653**
Factor B (Treatment)	9	132.385**	24.618**	34.732**	64.088**
AB (Variety×Treatment)	9	25.324**	6.997**	15.479**	19.656**
Error	40	.194	.002	.008	.041

** = Significant at 1% level of probability

* = Significant at 5% level of probability

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

Source of variance	Degrees of freedom (df)	Mean square of length of shoots/plantlet (cm)		
		14 DAI	28 DAI	42 DAI
Factor A (Variety)	1	.724**	6.370**	6.370**
Factor B (Treatment)	9	5.381**	8.189**	8.189**
AB (Variety×Treatment)	9	2.741**	3.618**	3.618**
Error	40	.002	.003	.003

** = Significant at 1% level of probability

* = Significant at 5% level of probability

Appendix VI. Analysis of variance of number of leaves/plantlet.

Source of variance	Degrees of freedom (df)	Mean square of number of leaves/plantlet		
		14 DAI	28 DAI	42 DAI
Factor A (Variety)	1	.504**	1.817*	19.278**
Factor B (Treatment)	9	.206**	42.466**	199.160**
AB (Variety×Treatment)	9	.206**	6.818**	18.133**
Error	40	.001	.016	.076

** = Significant at 1% level of probability

* = Significant at 5% level of probability

Appendix VII. Analysis of variance of fresh weight of shoot and dry weight of shoot at 14 days after inoculation.

Source of variance	Degrees of freedom (df)	fresh weight of shoot at 14 days after inoculation (mg)	dry weight of shoot at 14 days after inoculation (mg)
Factor A (Variety)	1	7152.382**	107.201**
Factor B (Treatment)	9	2963.400**	46.541**
AB (Variety×Treatment)	9	1091.709**	18.865**
Error	40	.088	.024

** = Significant at 1% level of probability

* = Significant at 5% level of probability

Appendix VIII. Analysis of variance of days to root initiation and number of roots/plantlet

Source of variance	Degrees of freedom (df)	Mean square of days to root initiation	Mean square of number of roots/plantlet		
			14 DAI	28 DAI	42 DAI
Factor A (Variety)	1	671.609**	5.741**	23.002**	.231^{NS}
Factor B (Treatment)	9	278.791**	10.401**	60.819**	133.165**
AB (Variety×Treatment)	9	235.780**	6.156**	6.686**	6.166*
Error	40	.349	.008	.035	.183

** = Significant at 1% level of probability

* = Significant at 5% level of probability

NS = Non-significant

Appendix IX. Analysis of variance of length of roots/plantlet

Source of variance	Degrees of freedom (df)	Mean square of length of roots/plantlet		
		14 DAI	28 DAI	42 DAI
Factor A (Variety)	1	.920^{NS}	.329^{NS}	4.521**
Factor B (Treatment)	9	6.049**	30.624**	33.111**
AB (Variety×Treatment)	9	1.369**	2.032**	2.557**
Error	40	.271	.796	.451

** = Significant at 1% level of probability

* = Significant at 5% level of probability

NS = Non-significant

Appendix X. Analysis of variance of fresh weight of root and dry weight of root at 42 days after inoculation

Source of variance	Degrees of freedom (df)	fresh weight of root (gm)	dry weight of root (gm)
Factor A (Variety)	1	$.004^{NS}$	$4.167E\text{-}005^{NS}$
Factor B (Treatment)	9	$.494^{**}$	$.015^{**}$
AB (Variety×Treatment)	9	$.053^{**}$	$.002^{**}$
Error	40	.002	.000

** = Significant at 1% level of probability

* = Significant at 5% level of probability; NS = Non-significant

Appendix XI. Analysis of variance of survival of shoot tips and number of shoots/plantlet

Source of variance	Degrees of freedom (df)	Survival of shoot tips (%)	number of shoots/plantlet
Factor A (Variety)	1	98.613^{**}	2.960^{**}
Factor B (Treatment)	7	2381.205^{**}	50.261^{**}
AB (Variety×Treatment)	7	25.561^{NS}	$.326^{NS}$
Error	32	11.313	.476

** = Significant at 1% level of probability

* = Significant at 5% level of probability

NS = Non-significant

Appendix XII. Analysis of variance of the mortality of shoot tips and abnormalities in leaves

Source of variance	Degrees of freedom (df)	Observed mortality (%)	Abnormalities in leaves (%)
Factor A (Variety)	1	352.679**	81.146**
Factor B (Treatment)	7	1897.811**	3483.284*
AB (Variety×Treatment)	7	48.594**	45.680*
Error	32	4.083	2.479

** = Significant at 1% level of probability

* = Significant at 5% level of probability

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