STUDY OF *IN VITRO* REGENERATION PROTOCOL OF GERBERA (*Gerbera jamesonii* Bolus) GROWN IN BANGLADESH

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE IN BIOTECHNOLOGY

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Dedicated to
My beloved parents and teachers

DECLARATION

I hereby declare that the research work embodying the results reported in this thesis entitled **'STUDY OF *IN VITRO* REGENERATION PROTOCOL OF GERBERA (*Gerbera jamesonii* Bolus) GROWN IN BANGLADESH'** submitted by SYED AKIB HOSSAIN, has been carried out under supervision of **Assistant Professor Jebunnesa Chowdhury, Biotechnology Program, Department of Mathematics and Natural Sciences, BRAC University, Dhaka.** It is further declared that the research work presented here is original and has not been submitted to any other institution for any degree or diploma.

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List of Abbreviations

The following abbreviations have been used throughout the text

Abbreviation	Full Form
BAP 6	Benzylaminopurine
EDTA	Ethylenediaminetetraacetic acid
HCl	Hydrochloric Acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
NAA	1-Naphthaleneacetic acid
Kn	Kinetin
MS	Murashige and Skoog (1962) medium
NaOH	Sodium Hydroxide
$HgCl_2$	Mercuric acid
mg	Milligram
g	Gram
ml	Millilitre
TC	Tissue culture
UV	Ultra violet

Abstract

Gerbera jamesonii Bolus is one of the most widespread decorative plants in the world. The gerbera market would benefit from an efficient and simple protocol for high rate regeneration for propagation and genetic engineering. Various explants namely leaf and capitulum were found to favorably responsive towards different culture media with different types and concentrations of growth regulators. During this study, different experiments were designed to analyze different factors affecting *in vitro* regeneration of gerbera. These experiments were optimization of the sterilization procedure for leaf and capitulum explants, study of the regeneration response of these explants towards different media combinations, selection of suitable media composition for shoot regeneration and selection of gerbera explants that can give rise to regeneration. During the study, washing the leaf and capitulum explants under tap water for 20 minutes followed by surface sterilization with 0.1% $HgCl_2$ for 6-7 minutes and 70% ethanol for 30-60 seconds respectively, found to be the best sterilization procedure. All types of explants were cultured on MS medium with different hormonal compositions and concentrations. Among the explants, capitulum was suitable and superior for callus induction and subsequent regeneration of *in vitro* shoots when cultured on MS media supplemented with 2 mg/l BAP and 0.5 mg/l NAA. Shoot proliferation was started within 6-7 weeks after inoculation of capitulum explants in the suitable medium (2 mg/l BAP and 0.5 mg/l NAA). However, callusing was observed in all leaf explants but they failed to differentiate into shoots in different media. The red variety showed best response in producing multiple shoots in comparison with the white variety. Attention should be paid to improve the regeneration percentage and reproducibility of the protocol in further studies.

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INTRODUCTION

Introduction

Gerbera (*Gerbera jamesonii* Bolus), a beautiful daisy, belongs to family compositae. Out of 40 known species, only *G. jamesonii* Bolus is cultivated. It is a native of South Africa and Asia, and has acquired commercial importance in recent years. This plant is named in the honor of German naturalist Traugott Gerber, and known as Transvaal or Barbeton daisy. Some of them show excellent agronomic characters such as flower color, floral diameter, vigor, and stem length. This plant is widely commercially produced by the floral industry both as cut flower and potted plant. The flowers are hardy and can withstand vigorous transportation. They have long-keeping quality and fetch high market price. Gerbera has a wide range of attractive flower colors such as red, pink, orange, peach, maroon etc., which makes it a valuable ornamental species; it stands among the top ten cut flowers of the world (Sujatha et al., 2002).

Gerberas can be propagated by both sexual and asexual methods. Most of the commercially grown cultivars are propagated through vegetative means, to maintain homogeneity and genetic pureness (Peper et al. 1971). Among the vegetative methods, multiplication through division of clumps has been used for several decades. These methods are however, too slow to be used commercially. Large-scale commercial production of gerbera requires an easier, quicker, and economically viable method of propagation. Micro-propagation method has been successful for rapid, large-scale multiplication of gerberas (Aswath et al. 2002; Zhang 2002).

Tissue culture allows the production of disease-free plants which are free from seasonal variations and can produce over one million plants per year of an elite variety. A tissue culture procedure has been proven to be commercially practical in gerbera propagation. This method enables a million fold expansions per year of a desired plant (Murashige et al. 1974; Aswath et al. 2002, 2003). Micro-propagation of gerbera is being used in many countries from a range of different explants. Direct shoot regeneration using shoot tips as initial explant is most convenient method for mass propagation of *Gerbera*; however, indirect shoot regeneration has been achieved from calli derived from different explants such as leaf, petals, and floral buds. Adventitious shoot regeneration from flower buds/ capitula is another favored method, followed by many researchers.

1.1 Scientific Classification of *Gerbera Jamesonii* Bolus

Kingdom: Plantae – Plants

Subkingdom: Tracheobionta – Vascular plants

Superdivision: Spermatophyta – Seed plants

Division: Magnoliophyta – Flowering plants

Class: Magnoliopsida – Dicotyledons

Subclass: Asteridae

Order: Asterales

Family: Asteraceae – Aster family

Figure 1: Two varieties of gerbera Flowers; a red gerbera flower (A) and a white gerbera flower (B)

1.2 Physiology of Gerbera

Flowers within the Compositate (Asteraceae) family are composed of morphologically differing types of flowers and genus gerbera (Gerbera hybrida) displays most of the options common to the family. Every genus of gerbera flower possesses three differing types of flowers; an outer ring of ray florets, a middle ring of trans florets, and an inner ring of disk florets. The ray and disc flowers are packed tightly into an inflorescence known as the capitulum. The marginal ray flowers powerfully ligulate and zygomorphic with united showy petals; the female ray flowers

have anthers that initiated however aborted later in development; and therefore the centrally-located disc flowers contain each anthers and carpels. The trans flowers in genus gerbera are feminine, just like the ray flowers, however the length of the petals vary among varieties. Genus gerbera flowers additionally possess hair-like structures, known as calyx. Proof that these structures are the changed sepals coming from experiments that show flower-organ determination could also be changed with MADS-box genes. The stamens of genus gerbera flowers aborted in marginal flowers, the petals and anthers united into hollow structures and therefore the plant possesses inferior ovaries. Early in development, the three main flower varieties (ray, trans and disk) are morphologically similar.

1.3 Suitable growing conditions

Gerbera jamesonii Bolus belongs to the Compositae family, and it grows well within the open in tropical and subtropical regions, and at altitudes extending between 1300 and 3200 meters higher than water level.

Gerberas typically would grow in cool and well airy conditions, with sensible lightweight, for growth, like within the greenhouse. The acceptable temperature for the cultivation of gerbera is 10-25°C. However, some gerberas need specific atmospheric condition, like cold (24°C), for flower production. Temperatures below 7°C hinder the traditional growth of this plant, and should cause inferior growth. In terms of soil preferences, gerberas typically grow well in organically wealthy, medium wet and well-drained soil.

However, the simplest quality gerbera flowers may be obtained below greenhouse conditions. A well-drained, rich, light, neutral or slightly basic soil is most fitted for gerbera production. Day temperature of 22-25°C and night temperature of 12-16°C are ideal for cultivation. Though gerberas may be found for the duration of the year, the expansion rate of this plant is higher in winter and also the flower production rate is higher in spring.

1.4 Historical Background

A Scottish named Henry Martyn Robert Jameson was accountable for discovering gerbera flower somewhere close to Barberton in South Africa. Though the flower was at first discovered in 1884, it absolutely was not till regarding forty years later that the bloom was first cultivated. Genus gerbera daisies area unit named after a German plant scientist, Traugott Gerber.

Different names related to this flower embrace the Barberton daisy, and also the African daisy. The name African flower is straightforward to know likewise because the name Barberton daisy. The name African daisy conjointly refers to the placement of its discovery. Transvaal means that across the Vaal (a watercourse in South Africa). This watercourse runs across the country and, to the north to the country's capital, Pretoria. The realm to the north of the watercourse was given the name Transvaal and, since Barberton is found during this space, it led to the name African daisy.

The first person to spot the flower on a scientific level was J.D. Hooker back in 1889. He stated the flower as *Gerbera jamesonii*. Different species of this flower are found in tropical Asia and South America that clearly indicates the plant's affection for warm weather. Cultivation of this flower in European country began within the 1890s. The trend followed in North America a full thirty years later.

1.5 Usage of Gerbera

Gerbera jamesonii Bolus is one of the most popular ornamental flowers in the world, both as a cut flower and as a pot plant. In gardens the *Gerbera jamesonii* Bolus is used in beds, borders or edging. As a cut flower, *Gerbera jamesonii* Bolus have a vase life of approximately 14 days. As a pot plant, *Gerbera jamesonii* Bolus is considered one of the most useful plants for improving indoor air quality to living and working spaces. Dwarf varieties of *Gerbera jamesonii* Bolus looks great on table tops in well-lit areas.

- **Medicinal uses:**

The smoke of *G. viridifolia* is inhaled by the Southern Sotho to treat head colds. In South Africa, pounded leaf infusions of *G. ambigua* are used in Zulu traditional medicine for tapeworm and stomachache and root infusions are taken orally for coughs. In Zimbabwe, root infusions are used for heart pain and for abdominal pain in babies.

- **Ornamental uses:**

Cut flowers can be used in bouquets. The plants are sold as pot plants or for special occasions/traditional events. They are also sold as dried flowers and used in artistic combinations.

1.6 Constraints of Gerbera Production

Gerbera plants are prone to attack by various insects and lack of nutrients that cause physiological disorders of the plants and also are affected by various diseases which hamper the lucrative flower production. These are discussed below:

1.6.1 Physiological Disorders

- **Flower bent:** Loss of cell turgidity and under nutrition (lack of Calcium)
- **Pre-harvest stem break:** High root pressure and high humidity in the air.
- **Premature wilting of Gerbera flower:** Cloudy weather followed by bright sun or carbohydrate depletion.
- **Double-faced Gerbera flower:** A physiological disorder caused by imbalance of nutrients. Too much growth too little flower buds.
- **Non-uniform flower blooming:** Physical injury to flower stem / pest damage / phytotoxicity.
- **Short stem length:** High salinity level, moisture stress, low soil temp.

1.6.2 Diseases of Gerbera daisies:

- **Alternaria Leaf Spot:** Brown specks form on florets and the leaves. Centers become white on the leaf spots.
- **Bacterial Leaf Spot:** Small to large spots are circular at first, then become irregular and dark brown to black. May have a concentric ring pattern.
- **Botrytis Blight:** Petioles have long brown spots. Leaves yellow and die. Petals have tan spots. Stems at soil level are killed. Infected tissues become covered with gray fungal growth.
- **Fusarium Stem Rot:** Petiole of leaves blacken at the base as the plant collapses.
- **Phytophthora Crown Rot:** Plants wilt suddenly. Leaves brown. Roots are rotted and a crown rot develops.
- **Powdery Mildew:** White fungal growth develops on the surface of leaves.
- **Pythium Root Rot:** Plants wilt and die as roots rot.
- **Rhizoctonia Crown Rot:** Stems at the soil level have a brown lesion. Plants wilt and die.
- **Thielaviopsis Root Rot:** Plants yellow, wilt, and die. Roots are dark brown to black.

1.7 Biotechnological Approach:

Conventional breeding and propagation of gerbera is not compatible with large scale commercial production and so it needs the introduction of modern methods and application of plant biotechnology. Gerbera can also be propagated asexually but the rate of multiplication is too low. Among the various methods of propagation, division of clumps is mostly used. However, it is not feasible for large number of commercial gerbera plant production. In contrast, multiplication through micro-propagation can produce millions of plants throughout the year and is also very effective and feasible (Murashige et al., 1974; Aswath et al., 2002, 2003). In tissue culture study so far, plant regeneration was uniformly achieved from different explants as the source material (Murashige, 1977). *In vitro* regeneration of Gerbera has been successful through different

explants including stem tips, floral buds, leaf, capitulum etc. Some of the examples are, Plants produce from explants of capitulum (Pierik et al., 1973, 1975), leaves (Hedtrich, 1979; Barbosa et al., 1994), floral buds (Posada et al., 1999), floral bracts (Maia et al., 1983), torus (Zhang, 2002) and inflorescence (Schum and Busold, 1985).

The nutrient medium is very important for successful regeneration and information is still scanty on the various medium used for regeneration of gerbera. 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). (Parthasarathy et al., 1996) and (Verma and Anand, 2006) used N6 (Chu, 1978) and B5 (Gamborg et al., 1968) medium for tissue culture establishment of gerbera. (Mandal and Datta, 2002) used LS (Linsmaier and Skoog, 1965) medium for establishing organogenic callus cultures from immature flower buds.

1.7 Activities regarding gerbera in Bangladesh

In Bangladesh, Gerbera is presented recently becoming more popular day by day. Due to the increasing demands of flower in local and international market; floriculture became very popular in recent years especially in the urban areas. Gerbera is a foreign flower and it has a huge demand in Bangladesh. To meet up the local demand, many nurseries started to cultivate this plant from cutting imported from India (Rahman et al., 2014). Along with nursery cultivation, biotechnological approach might be a prospective alternative for mass propagation of gerbera. As the economy of Bangladesh is largely dependent on agriculture, this flowering plant can have a great contribution to the economic growth as well as for opening a new dimension in the field of agriculture in Bangladesh. Recently cultivation of gerbera has started considerably in different districts of Bangladesh. A very few research works related to development and production of gerbera has been carried out in our country. Micro-propagation, is one propagation technique which has been done in different universities of Bangladesh and, using different explants such as leaf tip, leaf with mid-rib, leaf blade segments as well as flower buds and flower stalks. A research study from University of Dhaka concluded with flower bud and flower stalks found to

be suitable and superior for callus induction as well as *in vitro* shoots induction when cultured on MS media supplemented with specific growth hormones (Akter N., Hoque M.I. and Sarker R.H, 2012). Another experiment was done at Advanced Seed Research and Biotech Center, ACI Dhaka, Bangladesh by a student of Sher-e-Bangla Agricultural University to find out suitable explant for culture establishment to develop a standard protocol. The research mentioned that among five explants (Flower bud; Capitulum; Leaves; Peduncle, and Petiole) the flower bud was the best explant, due to higher survival rate (75.6%), early for culture establishment (16.4 days) and lowest contamination rate (7.1%) (Parvin S., 2015). But these protocols are not reliable and reproducible. So gerbera regeneration study is very necessary to develop efficient and feasible regeneration protocol.

1.8 Objective of this paper

Since the past few decades researchers have been trying to obtain an efficient and feasible regeneration protocol for gerbera. Although few protocols have been developed in foreign countries, no complete protocol has been established yet in Bangladesh for the available gerbera varieties. Hence, in this study, two varieties of *Gerbera jamesonii* Bolus were used to study the reproducible tissue culture mediated plant regeneration protocol. Leaf and capitulum were used as explants from both of the varieties for the study.

In this study, the specific objectives were:

- Optimization of the sterilization procedure for the gerbera explants
- Study of the regeneration responses of gerbera leaf and capitulum explants towards different media combinations
- Selection of suitable media composition for gerbera shoot regeneration
- Selection of gerbera explants that can give rise to regeneration

~~~~~ Chapter 2 ~~~~~

MATERIALS & METHODS

Chapter 2

Materials and methods

2.1 Materials

In the present study following plant materials and Medias were used.

2.1.1 Plant Materials

In this current experiment, two different types of gerbera (*Gerbera jamesonii* Bolus) plants were used. The two flower color plants were: (a) Red & (b) White. Leaf and capitulum explants were used for the in-vitro regeneration of gerbera plants (figure 2).

Figure 2: Different views of young leaf and capitulumn explants**;** A young and immature leaf of white variety(A), A young leaf explant of red variety (B), side view of 1 cm long capitulumn of white variety (C) 1 cm diameter based capitulumn explant of red variety (D).

2.1.2 Sources

The explants were collected from field grown plants from BRAC Nursery, Dhaka. During the research the plants were maintained at the balcony of our house.

2.1.3 Media used during the experiments

2.1.3.1 Tissue culture media:

In the present study, for tissue culture experiment Murashige and Skoog (MS) medium (1962) along with various concentration of different growth hormones were used for different types of experiments.

2.1.3.2 Callus induction media:

For the callus induction from different explants MS medium containing different concentrations of 6-Benzylaminopurine BAP (1, 1.5, 2, 3, 4, 5 mg/L^{-1}) hormone with different concentrations of Napthalene Acetic Acid NAA (0.5, 1 mg/L^{-1}) were used.

2.1.3.3 Shoot regeneration media:

For the initiation and development of the shoot direct from the explant, MS medium with high amount of different combination of hormones were used. On the other side MS medium with different hormones and hormonal combinations were used for the shoot development from the callus of different explants.

2.2 Methods

2.2.1 Media preparation

In the present study, Murashige and Skoog (MS) medium (1962) in full strength were used for *in-vitro* regenerations. MS medium were supplemented with different hormones to produce regeneration medium.

2.2.2 Stock solution preparation:

For all the stock solutions, the components were added in distilled water according to their concentrations and dissolved properly one after another. In case of Fe-EDTA the stock was kept in amber bottle or foil paper wrapped reagent bottle. All the stocks were kept at 4ºC for preservation.

2.2.2.1 Macro-nutrients (Stock solution-I) stock preparation for MS media

All the components of the macro nutrients (mentioned in the table 2.1) were added one after another dissolving the previous one in half of the total volume of the distilled water in a volumetric flask. The stock solution of macro-nutrient was made in such a way that its strength was 10 times (10X) more than the full strength of the medium. For this purpose, the weight of different salts required for 1 liter of stock were weighed accurately and were sequentially dissolved one after another in a beaker. The final volume of the solution was made up to 1000 ml by further addition of distilled water into the 1000ml measuring cylinder. The solution was finally poured into a Scott bottle (clear glass container). After proper labeling, the solution was stored in a refrigerator at 4°C temperature for several weeks.

Table 2.1: The components and amount of each component needed to make 1 liter (10X) of Stock solution-I:

Macro-nutrients components	Amount (mg/l)	Amount for 10X stock solution (mg/l)
KNO_3	1900	19
NH_4NO_3	1650	16.5
$MgSO_4.2H_2O$	370	3.70
$CaCl_2.2H_2O$	440	4.40
KH_2PO_4	170	1.70

2.2.2.2 Micro-nutrients (Stock solution-II) stock preparation for MS media

The components of micro nutrients (mentioned in the table 2.2) were added serially in the half of the total volume of the solution. This part of the stock solution was made with all the micronutrients. This was made 100 times (100X) the full strength of components and volume up to 1000 ml of distilled water. This solution was autoclaved and stored at 4°C in refrigerator.

Table 2.2: The components and amount of each component needed to make 1 liter of Stock solution-II:

Micro-nutrients components	Amount (mg/l)	Amount for 100X stock solution (mg/l)
KI	0.83	0.0083
H_3BO_3	6.20	0.0620
$MnSO_4.4H_2O$	22.30	0.2230
$ZnSO_4.7H_2O$	8.60	0.0860
$Na_2MoO_4.2H_2O$	0.25	0.0025
$CuSO_4.5H_2O$	0.025	0.00025
$CoCl_2.6H_2O$	0.025	0.00025

2.2.2.3 Iron-EDTA (Stock solution-III) stock preparation for MS media

Solution III was made 100 times (100X) the final strength of Iron chelate ($FeSO_4.7H_2O$) and $Na_2EDTA.2H_2O$ and volume up to 1000 ml of distilled water as described for the stock solution III. Heat was applied to dissolve the salts. The solution was autoclaved and stored at 4°C for several weeks in an amber bottle to prevent penetration of sunlight into the solution as the solution is sunlight sensitive.

Table 2.3: The components and amount of each component needed to make 1 liter (100X) of Stock solution-III:

Iron-EDTA solution components	Amount (mg/l)	Amount for 100X stock solution (mg/l)
$FeSO_4.7H_2O$	27.8	0.278
$Na_2EDTA.2H_2O$	37.3	0.373

2.2.2.4 Organic stock solution preparation:

All the components (mentioned in the table 2.4) were added to the distilled water one after dissolving another. The stock solution was made 100 times of their full strength. Components were added one by one and stirred some more time before adding next. Then it was stored at 4ºC.

Table 2.4: The components and amount of each component needed to make 1 liter of Stock solution-IV:

Organic solution components	Amount (mg/l)	Amount for 100X stock solution (mg/l)
Nicotinic acid	0.5	0.005
Pyridoxin HCl	0.5	0.005
Thiamin HCl	0.1	0.001
Glycine	2.0	0.02

2.2.2.5 Stocks solutions (Different hormone) preparation

In this experiment different types of Auxin and Cytokinin hormones were used. For the regeneration and development of plant tissues these hormones are essential. These hormones have different molecular weight. All the hormones are dissolved in 1N NaOH.

- **BAP (6-Benzylaminopurine):** 10 mg of the BAP (Sigma) was dissolved in 1-2 ml of 1 N NaOH. Distilled water was added to make the final volume (100 ml). The strength of the stock solution was 1mg/10ml. The solution was then filtered and stored in a clean flask at 4°C in the refrigerator.
- **NAA (Napthalene Acetic Acid):** 10 mg of the NAA (Sigma) was dissolved in 1-2 ml of 1 N NaOH. Distilled water was added to make the final volume (100 ml). The strength of the stock solution was 1mg/10ml. The solution was then filtered and stored in a clean flask at 4°C in the refrigerator.

2.3. Preparation of 1 liter of MS medium

MS medium was prepared from these stock solutions. The components and amount of each component are mentioned below in the following table:

Table 2.5: Components and their amount in making of 1 liter MS medium

Components	Amount (for 1000ml)
Macro-nutrients (10X)	100 ml
Micro-nutrients (100X)	10 ml
Organic (100X)	10 ml
Iron - EDTA (100X)	10 ml
Sucrose	30gm
Myo-inositol	0.10gm

The following procedure shows the general steps that should be maintained to produce MS media supplemented with the desired hormones in order to produce the regeneration medium:

1) For the preparation of MS medium, 250 ml distilled water was taken in a 1 liter volumetric flask,
2) 100 ml of Stock solution I (Macro-nutrients), 10 ml of each Stock solution II (Micro-nutrients), Stock solution III (iron-chelate) and Stock solution IV was added sequentially and mixed thoroughly,
3) 100 mg of Myo-inositol was added to this solution and was dissolved completely.
4) Then 30 gm. of sucrose was dissolved as carbon source,
5) To obtain different required concentrations of various hormone(s) the stock solutions were added individually or in case of multiple supplementations added sequentially to this solution and were mixed thoroughly,
6) The whole mixture was then made up to 1 liter with further addition of distilled water,
7) The pH of the medium was adjusted to 5.8 using a digital pH meter with the help of 1N NaOH solution or 1N HCl solution, whichever was required.

2.4 Medium sterilization

All the media were prepared and poured into conical flasks and then autoclaved for 15 minutes at 120 psi for sterilization.

2.5 Maintenance of Aseptic condition

To maintain aseptic condition, all inoculation was carried out under the laminar air flow hood (SCV, Singapore) UV light of laminar hood was on for 15 minutes to one hour. Then the laminar hood was cleaned with 70% ethanol spray and hands were washed with antimicrobial hand wash (Hexisol®, ACI Ltd.). The instruments (forcep, scalpel, Petri-dish etc.) were sterilized by using a Bunsen burner to prevent air borne bacteria and immersed into absolute alcohol during the experiment taking place. The flask and Petri-dish cover were flamed twice, once after opening and again before closing them. All contaminants and old bacterial culture were discarded after autoclaving to maintain biosafety procedure.

2.6 Applied culture technique

In this experiment depending on the purpose and experiment segments these following techniques were used:

- Axenic culture
- Explant culture
- Subculture of the explants
- Culture incubation

2.6.1 Axenic culture

Fungal contamination is one of the main barriers for tissue culture. The rate of fungal contamination varies within the seasons. And so the sterilization procedure of the different Gerbera explants were different depending on the culture type and regeneration responses. However, during rainy season the sterilization procedure needs to be more cautious than other seasons.

2.6.1 Axenic culture

The explants were excised from the mother plant at the garden and collected in a jar. Then the explants were surface sterilized and washed with the running tap water for 20 minutes. And then the explants were washed with few drops of Trepol detergent followed by wash with tap water for 5 times. After this the explants were dipped under 20% Savlon water for 2 minutes and washed by tap water for 5 times. Then the explants were washed with distilled water for three times. In the laminar flow cabinet, the explants were deepened in the $HgCl_2$ for 6 minutes and washed with the autoclaved distilled water for 5 times. After that the explants were deepened in the 70% of the ethanol for 30 seconds. And finally the explants were again washed with the autoclaved distilled water for 5 times.

2.6.2 Explant culture

Leaf segments with the midrib, leaf blades and capitulums were used as explants. The young leaf from the top of the plants was selected always for the leaf explants. The capitulums at the age of

6-7 days were collected as explants. The capitulum was separated from the stalk after sterilization process and then dissected 8-10 pieces for using as explants. The cutting size of the leaf segments with mid rib were around 1-2 cm. After sterilization and cutting those explants, they were placed on a filter paper to absorb the extra water of the surface. The leaf segments were placed on the medium in both sides. And some of the capitulums were placed on the medium with dissection into several pieces.

2.6.3 Subculture

All of the explants contain a large number of secondary metabolites so subculture is very essential part for Gerbera tissue culture. For the callus formation and also for the direct shoot regeneration explants were transferred into a fresh medium after 2-3 weeks interval depending on the progress of the experiment. During subculture, the death tissues of the explants were excised and separated from the explant. All the sub-cultured explants were kept under routine observation. During the time of multiple shoot regeneration all the explants were placed into the individual flask for better regeneration. After the regeneration of multiple shoot those individual explants were dissected into 2 to 3 pieces for the optimum height of the shoots.

2.6.4 Incubation condition

The inoculated flasks were incubated in the culture room under fluorescent light of 20,000 lux intensity. The temperature of the culture room was 25±1 ℃. The culture room had photoperiod of 16-8 (day-night) hour cycle.

~~~~~ Chapter 3 ~~~~~

RESULTS

Results

In this present study, two varieties of the cultivar *Gerbera jamesonii* Bolus having red and white flower petals were used with different explants such as leaf tip, leaf side, and leaf with midrib and capitulum. The purpose of the study was to establish an efficient protocol for *in vitro* regeneration of *Gerbera jamesonii* Bolus and studying different parameters that affects the overall growth of the *in vitro* plantlets. In this experiment two types of explants; leaf and capitulum were used for both of the varieties. The result demonstrates the efficient treatment method for the explants to show best response rates of 85%, selection of the best responsive explants and eight different media compositions with comparisons for callus growth and shooting of responsive calluses.

3.1 Selection of the best treatment method for regeneration

In this part of the experiment, two types (e.g. leaf and capitulum) of explants were treated with different treatment methods for the selection of the most efficient method of sterilization of the explants. The explants were washed under running tap water for different periods of time, treated with 0.1% $HgCl_2$ and 70% ethanol for different time intervals with using autoclaved distilled water during surface sterilization. Then the leaf and capitulum explants were cut down to specific measurements and placed into the culture media for response observation.

During the experiment explants were treated with mercuric chloride $HgCl_2$ and 70% ethanol varying the time duration of the treatments to avoid contamination of the explants in culture media. The time duration for washing and time duration for treating with $HgCl_2$ and 70% of ethanol had effect on the contamination rate as well as response rate of the explants. It can be seen from the table 3.1 and graph 3.1, that all of our sterilization treatments have brought about responsive explants; however, the efficiencies of the sterilization methods vary widely. From all of our treatment methods, Treatment 3 (explants were washed under running tap water for 20 minutes, washed with 0.1% $HgCl_2$ for 6 minutes & washed with 70% ethanol for 30 seconds), is the most effective for leaf explants, with the greatest number of responsive explants, and the lowest rates of contamination, with 85% and 15% respectively for leaf explants (Table 3.1 and Graph 3.1). In comparison, Treatment 2 (explants were washed under running tap water for 25

minutes, washed with 0.1% $HgCl_2$ for 7 minutes & washed with 70% ethanol for 40 seconds), is the most effective for capitulum explants, with the greatest number of responsive explants, and the lowest rates of contamination, with 86% and 18% respectively (Table 3.1 and Graph 3.1). However, all other treatments have very low percentages of responsive explants and higher rates of contamination. Conversely, Treatment 5 (explants were washed under running tap water for 10 minutes, washed with 0.1% $HgCl_2$ for 5 minutes & washed with 70% ethanol for 10 seconds) had proven to be the most ineffective procedure for sterilization with both the lowest percentages of responsive explants and the highest rate of contamination, with 10% and 75% respectively for leaf explants and 15% and 70% respectively for capitulum explants. Although treatment 1 (explants were washed under running tap water for 30 minutes, washed with 0.1% $HgCl_2$ for 7 minutes & washed with 70% ethanol for 60 seconds) shows that it has quite a low percentage of contamination with 25% but it has also lower rate of response with 30% for leaf explants and 20% and 25% respectively for capitulum explants. It was found that the uses of the ethanol for longer time more than 30 seconds reduce the rate of responsive explants. The duration of washing under the running tap water also affected the contamination rate and response rate. In conclusion explants washed under running tap water for 20 minutes with additional 4 to 5 times wash with autoclaved distilled water in the shaking condition showed a great rate of responsive explants with low number of contamination. Therefore, due to its effectiveness, Treatment 3 had been chosen as the optimum sterilization method for leaf explants and treatment 2 had been chosen as the optimum sterilization method for capitulum explants in all further experiments.

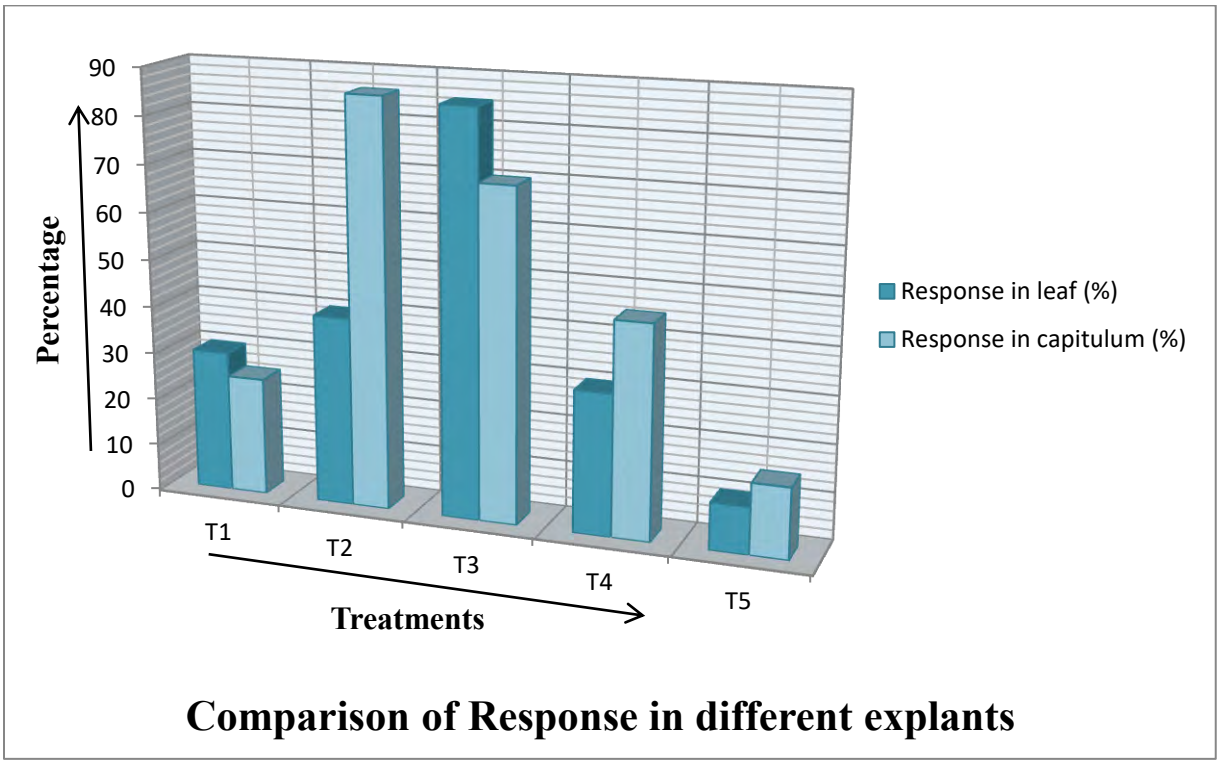
Table 3.1: Effectiveness of different treatments regarding two explants of gerbera

Treatment	No. of explants	Type of explant	No. of contaminated explants	Rate of contamination (%)	No. of responsive explants	Responsive explants (%)
T_1	100	L	25	25	30	30
	100	C	20	20	25	25
T_2	100	L	30	30	40	40
	100	C	18	18	86	**86**
T_3	100	L	15	15	85	**85**
	100	C	25	25	70	70
T_4	100	L	60	60	30	30
	100	C	66	66	45	45
T_5	100	L	75	75	10	10
	100	C	70	70	15	15

Note: Leaf Explant = L, Capitulum Explant = C

Note	Tap Water	0.1% $HgCl_2$	70% ethanol
T_1	30 minutes	7 minutes	60 seconds
T_2	25 minutes	7 minutes	40 seconds
T_3	20 minutes	6 minutes	30 seconds
T_4	15 minutes	6 minutes	20 seconds
T_5	10 minutes	5 minutes	10 seconds

Graph 3.1 Optimization of sterilization process with relation of the rate of responsive explants



3.2 Selection of suitable explants for regeneration using different media:

In this study, direct regeneration was observed from both leaf and capitulum explants and regeneration of shoots from the callus was observed only in capitulum explants. Different types of explants, namely leaf tip, leaf side, leaf with midrib, capitulum were used in this experiment. All the explants were placed on the MS medium with different hormonal supplements in different concentrations to find the highest regeneration percentage. The earliest and highest percentage of callus formation was observed on MS medium supplemented with 2 mg/l BAP and 0.5 mg/l NAA in both leaf and capitulum explants with callus induction of 95% and 90% respectively (Table 3.3).

Table 3.2: Effects of different hormonal combination for regeneration from leaf explants:

Media combination	No. of explants	Days required for callus induction	No. of responsive explants	Responsive explants (%)	Mean no. Of responsive explants
RM 1	20	15-20	16	80	0.80
RM 2	20	20-25	15	75	0.75
RM 3	20	14-18	19	**95**	**0.95**
RM 4	20	16-20	17	85	0.85
RM 5	20	14-18	16	80	0.80
RM 6	20	20-25	15	75	0.75
RM 7	20	15-20	16	80	0.80

** RM= Regeneration Media

Note: Different combinations of regeneration media

Regeneration Media	Hormonal Combination
RM 1	MS + 1 mg/l BAP+ 0.5 mg/l NAA
RM 2	MS + 1.5 mg/l BAP+ 0.5 mg/l NAA
RM 3	MS + 2 mg/l BAP+ 0.5 mg/l NAA
RM 4	MS +2 mg/l BAP+ 1 mg/l NAA
RM 5	MS + 3 mg/l BAP+ 0.5 mg/l NAA
RM 6	MS +3 mg/l BAP+ 1 mg/l NAA
RM 7	MS +5 mg/l BAP+ 1 mg/l NAA

Table 3.3: Effects of different hormonal combination for regeneration from capitulum explants:

Media combination	No. of explants	Days required for callus induction	No. of responsive explants	Responsive explants (%)	Mean no. Of responsive explants
RM 1	20	35	12	60	0.6
RM 2	20	25-30	14	70	0.7
RM 3	20	20-25	18	**90**	**0.9**
RM 4	20	25-30	16	80	0.8
RM 5	20	30-35	14	70	0.7
RM 6	20	30-35	13	65	0.65
RM 7	20	30-35	17	85	0.85

** RM= Regeneration Media

Note: Different combinations of regeneration media

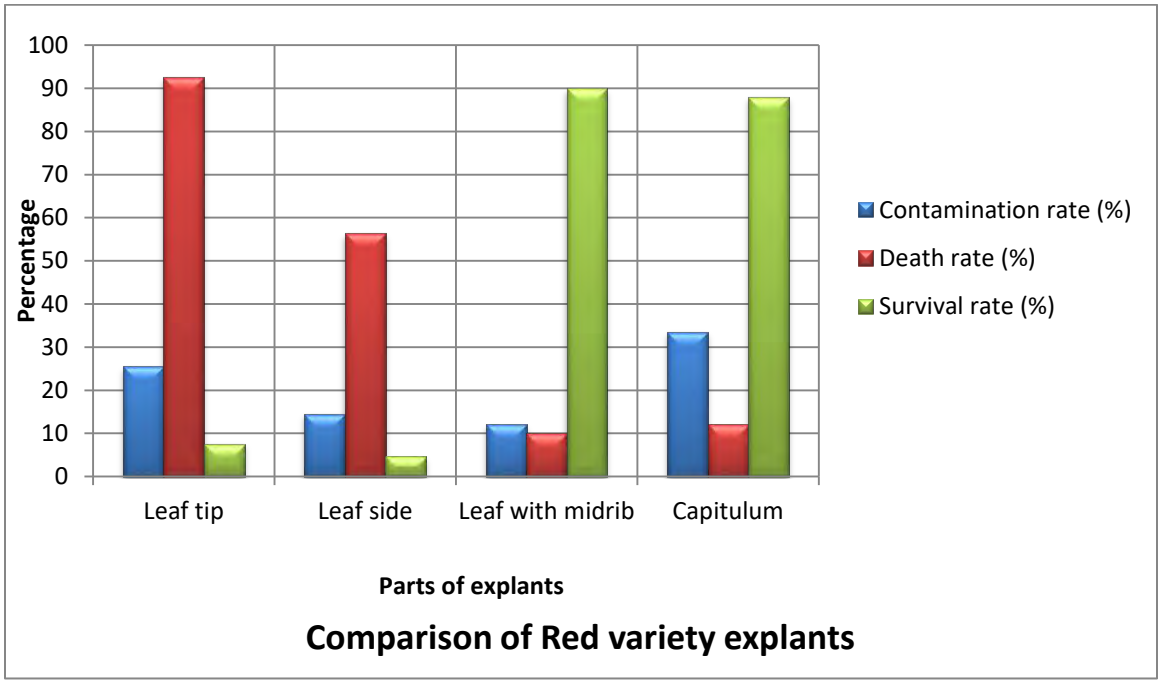
Regeneration Media	Hormonal Combination
RM 1	MS + 1 mg/l BAP+ 0.5 mg/l NAA
RM 2	MS + 1.5 mg/l BAP+ 0.5 mg/l NAA
RM 3	MS + 2 mg/l BAP+ 0.5 mg/l NAA
RM 4	MS +2 mg/l BAP+ 1 mg/l NAA
RM 5	MS + 3 mg/l BAP+ 0.5 mg/l NAA
RM 6	MS +3 mg/l BAP+ 1 mg/l NAA
RM 7	MS +5 mg/l BAP+ 1 mg/l NAA

3.2.1 Observation of the best survival rate of red variety explants of gerbera:

The survival rates of the red variety of the different gerbera explants used in the experiment serves that the leaf with midrib has the highest survival rate of 90%, lowest death rate of 10% and the lowest contamination rate of 12% (Graph 3.3). Thus, it can be considered the explant that best survives the sterilization process.

Conversely, the leaf tip showed the worst response among all the explants used because it had a 92.5% death rate. Similarly, while the leaf sides showed a lower contamination rate of 14.5%, they had the lowest rate of survival, at 4.7% (Graph 3.3). However, the best explants used for the experiment were that from the capitulum. The capitulum had high rates of survival, lower contamination and death rate and was quick to display morphological changes.

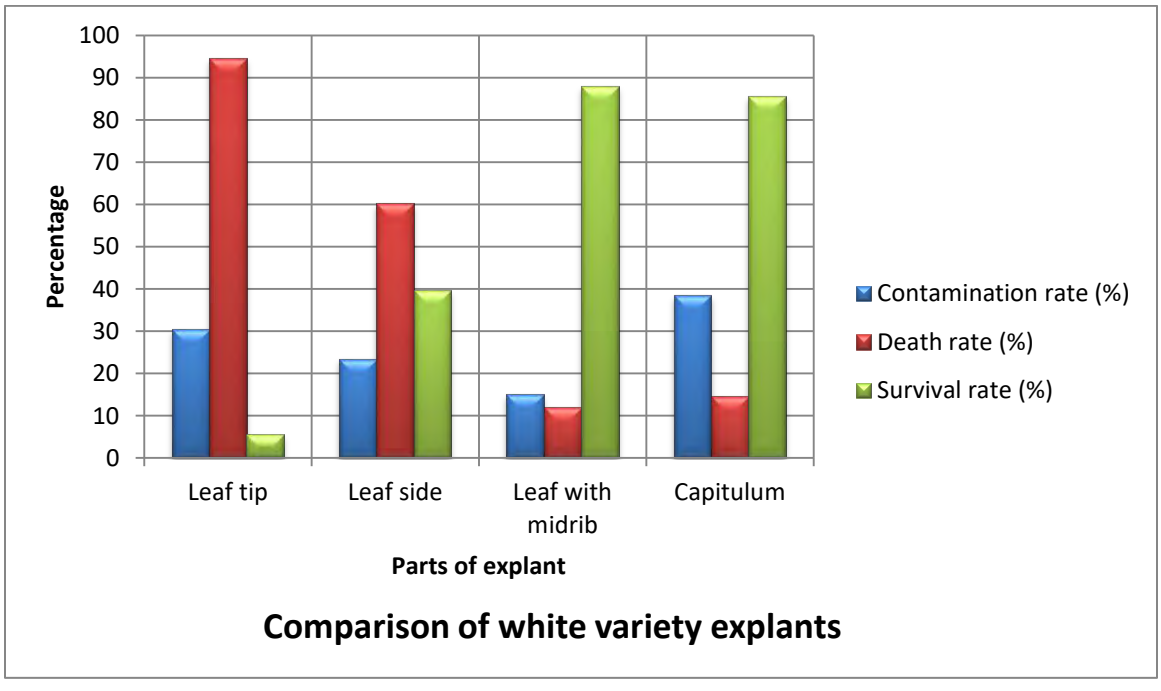
Graph 3.3: Comparison of survival rates of different parts of the red variety explants



3.2.3 Observation of the best survival rate of white variety explants of gerbera:

The survival rates of the white variety of the different gerbera explants used in the experiment serves results with that similar to the red variety, the leaf with midrib of the white variety has the highest rate of survival, lowest death rate and the lowest rate of contamination. Thus, it can be considered the explant that best survives the sterilization process. Conversely, the leaf tip showed the worst response among all the explants used because it had a 94.5% death rate and the lowest survival rate of 5.5% (Graph 3.4). However, the best explants used for the experiment were that from the capitulum. The capitulums had high rates of survival, lower death rates and were quick to display morphological changes. However, unlike the red variety, the capitulum explants in the white variety were prone to higher rates of contamination due to different factors affecting the sterilization effectiveness.

Graph 3.4: Comparison of survival rates of different parts of the white variety explants



3.3 Suitable hormonal medium for explant regeneration

In this experiment, MS medium with the different combination of growth hormones were used to observe the efficiency of the medium for successful regeneration. Firstly, only MS media without any growth regulator hormones were used as the control and study the response. All other media contained different concentration of BAP and NAA along with MS media. In this study different types of explants (leaf with mid rib, leaf sides and capitulum) were used. Hence, all those explants were cultured in different mediums to select the most efficient media for callus growth.

3.3.1 Effects of different hormonal combination for callus growth from leaf explants

The callus formation was observed in leaf and capitulum explants when MS medium was supplemented with auxin (NAA) and cytokinin (BAP) with specific concentrations. MS media containing different concentrations of BAP (1 to 5 mg/l) combined with different concentrations of NAA (0.5 to 1 mg/l) were used to study the effect of different hormonal supplements on callus growth. Those medium contained MS with combinations of 1 mg/l BAP + 0.5 mg/l NAA, 1.5 mg/l BAP + 0.5 mg/l NAA, 2 mg/l BAP + 0.5 mg/l NAA, 2 mg/l BAP + 1 mg/l NAA, 3 mg/l BAP + 0.5 mg/l NAA, 3 mg/l BAP + 1 mg/l NAA, 5 mg/l BAP + 1 mg/l NAA. The earliest and highest percentage of callus was observed on MS medium supplemented with 2 mg/l BAP and 0.5 mg/l NAA in leaf explants with callus induction of 70% (Table 3.5 and figure 3), serving it as the most suitable medium for callus induction. Callus grown in this medium showed mostly friable and compact for leaf explants. BAP less than 2 mg/l with other hormonal combination was responsible for low percentage of callus induction. All the calluses and explants were sub-cultured in the same hormonal combination for shoot induction.

3.3.2 Effects of different hormonal combination for callus growth from capitulum explants

Capitulum explants were also responsive towards *in vitro* regeneration of gerbera. The best callus growth with friable and compact callus was observed in MS media supplemented with 2 mg/l BAP and 0.5 mg/l NAA (Table 3.5). In this experiment it was found that the size of 1-2 cm diameter of the capitulum showed the best result. All the explants were inoculated in the MS medium containing BAP (1 to 5 mg/l) along with NAA (0.5 to 1 mg/l) to get the regeneration. The earliest and highest percentage of callus was observed on MS medium supplemented with 2 mg/l BAP and 0.5 mg/l NAA in capitulum explants with callus induction of 85% (Table 3.5 and Figure 4). The second highest response rate was 75% which was found with MS medium supplemented with 2 mg/l BAP and 1 mg/l NAA. The lowest response rate was 40% which was founded for the hormonal combination of MS medium supplemented with 1 mg/l BAP and 0.5 mg/l NAA (Table 3.6). The mean number of the multiple shoot regeneration was 7.38 for the hormonal combination of 2 mg/l BAP and 0.5 mg/l NAA along with MS media (Table 3.7 and figure 3).

Table 3.5: Gerbera explants producing calli and callus growth in response to growth regulators

Media combinations	No. of explants	Callus induction (%)		Type of Callus	
		Leaf	Capitulum	Leaf	Capitulum
RM 0	20	0	0	-	-
RM 1	20	45	40	Friable	Compact
RM 2	20	40	42	Friable	Compact
RM 3	20	**70**	**85**	Friable and Compact	Friable and Compact
RM 4	20	65	75	Friable	Compact
RM 5	20	50	70	Friable and Compact	Compact
RM 6	20	55	50	Friable and Compact	Compact
RM 7	20	60	55	Friable	Compact

** RM= Regeneration Media

Note: Different combinations of regeneration media

Regeneration Media	Hormonal Combination
RM 0	MS
RM 1	MS + 1 mg/l BAP+ 0.5 mg/l NAA
RM 2	MS + 1.5 mg/l BAP+ 0.5 mg/l NAA
RM 3	MS + 2 mg/l BAP+ 0.5 mg/l NAA
RM 4	MS +2 mg/l BAP+ 1 mg/l NAA
RM 5	MS + 3 mg/l BAP+ 0.5 mg/l NAA
RM 6	MS +3 mg/l BAP+ 1 mg/l NAA
RM 7	MS +5 mg/l BAP+ 1 mg/l NAA

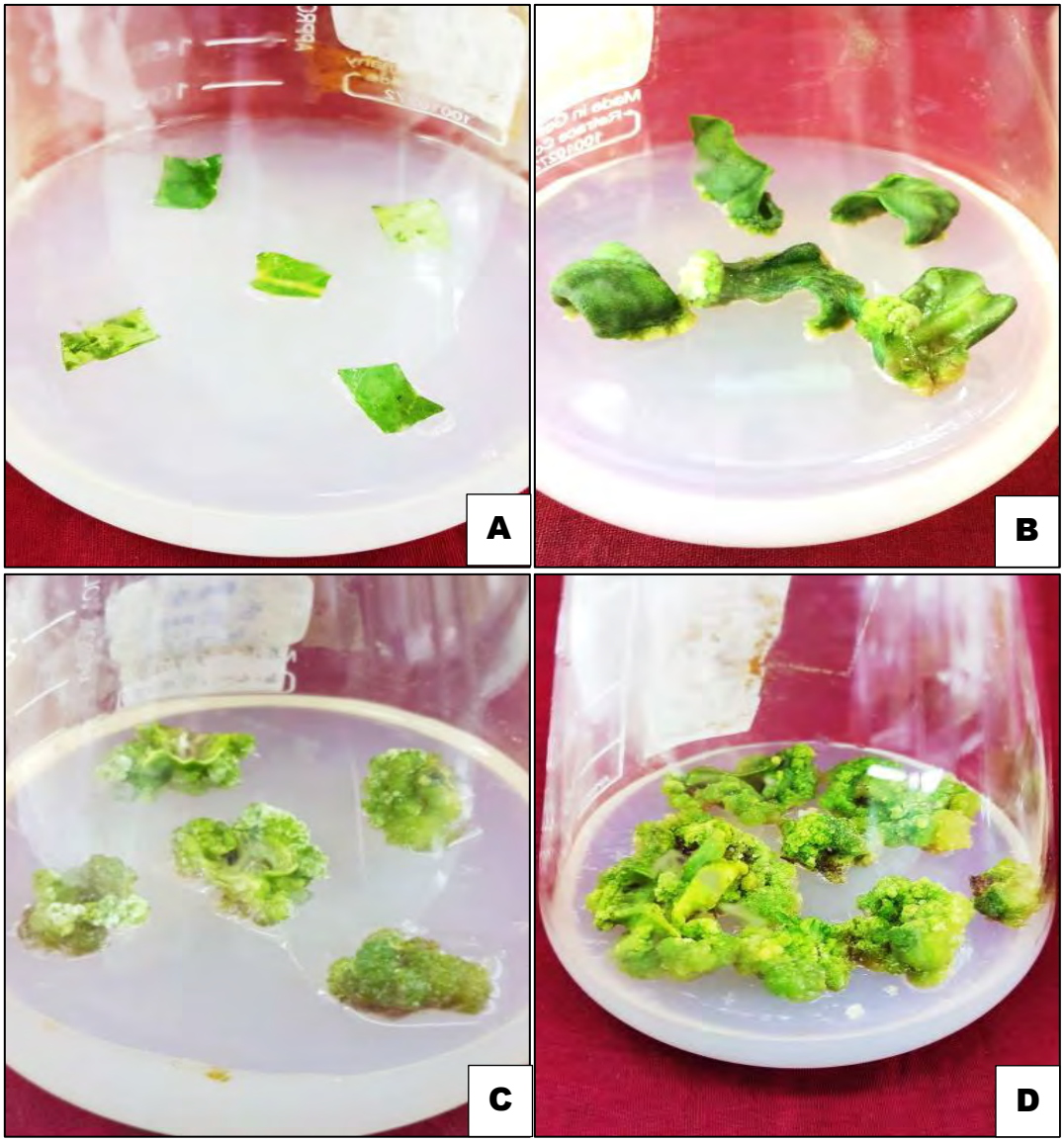


Figure 3: Different growth phases for leaf explants; Initial inoculation in MS+BAP 2 mg/L+ NAA 0.5 mg/L media (A), After two weeks of inoculation and response initiation on MS+BAP 2 mg/L+ NAA 0.5 mg/L media (B), After four weeks of inoculation and callus growth on MS+BAP 2 mg/L+ NAA 1 mg/L media (C), After six Weeks of inoculation callus turning mature phase MS+BAP 2 mg/L+ NAA 0.5 mg/L media.

Table 3.6: Effects of different hormonal combination for regeneration from capitulum explants

Media Combination	No. of explants	Days required for callus induction	Type of responsive explants	Responsive explants (%)	Days to shoot initiation	Mean no. of shoots per explant
RM 1	20	14 Days	Only callus	-	-	-
RM 2	20	20 Days	Only callus	-	-	-
RM 3	20	12 Days	Shoot	75	25-27	7.38
RM 4	20	14 Days	Only callus	-	-	-
RM 5	20	20 Days	Only callus	-	-	-
RM 6	20	14 Days	Only callus	-	-	-
RM 7	20	14 Days	Only callus	-	-	-

Note: Different combinations of regeneration media

Regeneration Media	Hormonal Combination
RM 1	MS + 1 mg/l BAP+ 0.5 mg/l NAA
RM 2	MS + 1.5 mg/l BAP+ 0.5 mg/l NAA
RM 3	MS + 2 mg/l BAP+ 0.5 mg/l NAA
RM 4	MS +2 mg/l BAP+ 1 mg/l NAA
RM 5	MS + 3 mg/l BAP+ 0.5 mg/l NAA
RM 6	MS +3 mg/l BAP+ 1 mg/l NAA
RM 7	MS +5 mg/l BAP+ 1 mg/l NAA

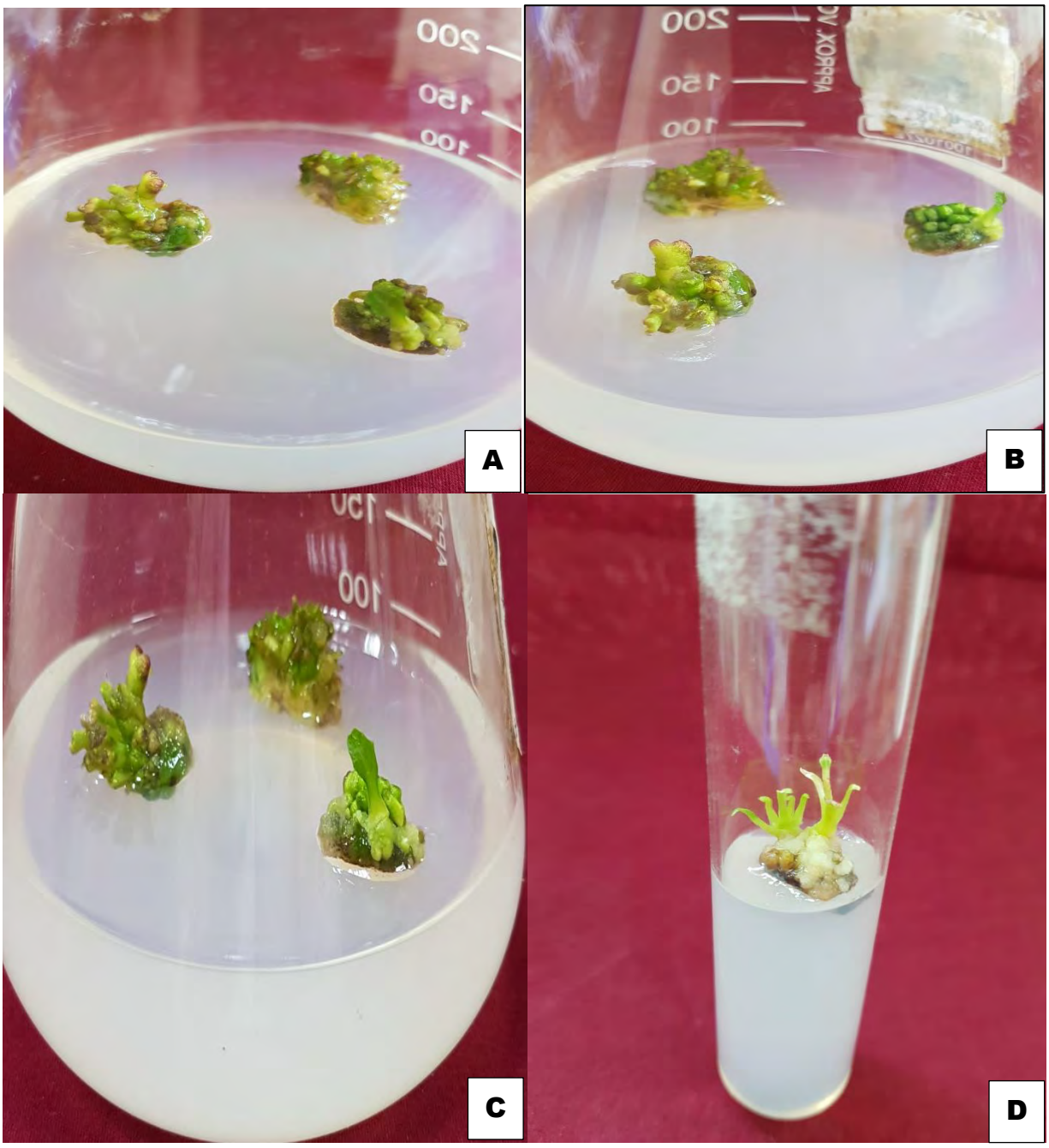


Figure 4: Different growth phases of Capitulum explants; after four weeks of inoculation in MS+BAP 2 mg/L+ NAA 0.5 mg/L media (A), And After Six weeks of inoculation in MS+BAP 2 mg/L+ NAA 0.5 mg/L media (B), Eight weeks of inoculation (C) and developed multiple shoots in MS+BAP 2 mg/L+ NAA 0.5 mg/L media (D).

~~~~~ Chapter 4 ~~~~~

DISCUSSION

Discussion

The study was conducted with different types of explants of *Gerbera jamesoni* Bolus for development of an optimized *in vitro* regeneration protocol. For regeneration the best response was obtained from capitulum explants in comparison with leaf explants. The specific aspects of the study were to optimize the sterilization treatment method, source of explants and media composition for successful regeneration. In this regard different types and parts of explants were tried for successful regeneration. Among all different explants, capitulum exhibited the best response towards callus formation, shoot regeneration and subsequent development in two varieties of gerbera. The size of the capitulum was found to be an important factor in callus induction and shoot proliferation. In case of capitulum explants, the number of shoots per flower was much smaller, but loss by infection was only 10 %. The use of capitulum explants had the advantage of leaving the mother plant intact. There are number of previous reports on using capitulum as explant source for shoot regeneration in Gerbera found similar result (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).

The selection of a safe sterling agent is of prime importance for the control of bacterial and fungal infection and establishment of explant culture. Furthermore, appropriate concentration of sterilizing agent is required for the removal of fungal and bacterial contamination (Ahmad et al., 2003). In this investigation, an effective control of contamination was developed with maximum survival percentage (75%) and minimum necrosis (24.5%) was observed from leaf explants of *G. jamesonii* Bolus by using 0.1% $HgCl_2$ solution.

The development of an efficient and reproductive *in vitro* regeneration protocol depends on several factors including culture condition and regeneration medium with proper hormonal supplements. Previous studies showed that different medium with different hormonal supplements were used for successful *in vitro* regeneration of gerbera. In this study, the earliest and highest percentage of callus was observed on MS medium supplemented with 2 mg/l BAP and 0.5 mg/l NAA in both leaf and capitulum explants with callus induction of 70% and 85% respectively, serving it as the most suitable medium for callus growth. These results were in close agreement with those of Reeta Bhatia et al., showing that induction of callus formation by cytokinin was promoted by the addition of an auxin in combination of BAP. Aswath and

Choudhary, also found combination of NAA and BAP was more effective for callus induction than either NAA or BAP alone. Modh et al. (2002) reported that BAP stimulate RNA and protein synthesis which activate enzyme activity for cell division and cell wall loosening. Previously, poor callus induction on MS medium supplemented with different concentrations of Kn from leaf explants of *G. jamesonii* Bolus was also reported by Kumar and Kanwar (2007).

In case of both varieties, all the leaf derived explants induced callus formation and subsequent callus growth in almost all the medium except the MS media without any growth hormones. The induced calli were sub-cultured initially on the same medium having the same hormonal supplements for shoot regeneration. Following such subculture the induced calli were found to increase their volume, but failed to induce shoots irrespective of variety as well as explants used. However, a few reports are available about *in vitro* shoot regeneration from different parts of leaf including petiole (Orlikowska et al. 1999). The regeneration efficiency of the calli depends significantly on the type of explant and medium components. Vardja and Vardja (2001) inferred that all cells of the plants normally carry the same genetic information, but the morphogenetic responses vary according to the spatial and temporal distribution of the cells and their physiological and developmental stages. The genetic make-up, varied endogenous concentrations of growth hormones and response of the genotype to different concentration of growth hormones play a significant role.

The variation in callus induction/differentiation in different explants might have resulted due to the maturity of leaf and capitulum explants in response to different growth regulators, since the explants (leaf and petiole) were collected just before anthesis. Bonga (1987) reported that the type of explant used for induction of callus mainly depends upon the juvenility of the explants.

In the perspective of Bangladesh *in vitro* regeneration of gerbera is important in recent era because of the demand in the local and international market. The climate and condition is not very suitable for Gerbera cultivation in Bangladesh. So, commercially reproducible *in vitro* regeneration protocol is very essential for large scale plantlet production. This research also opens the windows for further new research like; shoot induction from leaf explants, root induction of *in vitro* regenerated shoot from capitulum explants to get variation through somaclonal variation in flowers.

~~~~~ Chapter 5 ~~~~~

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