Establishment of an *In Vitro* Regeneration Protocol of Gerbera (*Gerbera Jamesonii)* from Leaf, Flower bud and Flower Stalk Explants of Two Gerbera Varieties

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

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DECLARATION

This is to certify that the research work embodying the results reported in this thesis titled "**Establishment of an *In Vitro* Regeneration Protocol of Gerbera (*Gerbera Jamesonii)* from Leaf, Flower Bud and Flower Stalk Explants of Two Gerbera Varieties**", submitted by Promi Tahsin, has been carried out under the supervision of Ms. Jebunnessa Chowdhury, Assistant Professor, 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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Abstract

Gerbera Jamesonii is a flowering plant that has gained popularity and increasing commercial significance in many countries of the world, and is in great demand in the floral industry as a cut flower and a potted plant due to its beauty, color, and long vase life. In this experiment, two varieties of gerbera, containing red and white petals, were exploited for callus induction and *in-vitro* regeneration of plantlets. The aim of this experiment was to establish a reliable protocol for *in-vitro* regeneration of gerbera, including the selection of an effective sterilization procedure, the selection of suitable explants for *in-vitro* regeneration and the selection of optimal media compositions for *in-vitro* regeneration. From these varieties, three different types of explants were extracted, such as the leaf, the flower bud and the flower stalk. Among the explants used, the flower bud was superior for callus induction and subsequent regeneration of shoots when cultured on MS supplemented with 5.0 mg/l BAP and 1.0 mg/l NAA, and multiple shoot proliferation took 6 to 7 weeks after initial inoculation. Leaf explants produced a friable and non chlorophyllous callus with high growth rate at the cut surfaces of the explant, and a compact chlorophyllous callus with a low growth rate directly from whole explants. However, these calluses failed to differentiate into shoots. The highest callus induction in leaf and flower bud explants was observed with MS supplemented with 2 mg/l BAP and 0.5 mg/l NAA, and took about an average of 3 to 4 weeks to form. Further *in-vitro* regeneration studies of gerbera should be carried out to establish a procedure for large scale propagation of gerbera in Bangladesh.

Abbreviations

The following abbreviations have been used throughout the text:

- **BAP:** 6-Benzylaminopurine
- **NAA**: 1-Napthaleneacetic Acid
- **IAA:** Indole-3-Acetic Acid
- **IBA:** Indole-3-butyric Acid
- **Kn:** 6-Furfurylaminopurine
- **MS:** Murashige and Skoog (1962) Medium
- **Fe-EDTA:** Ferric ethylenediaminetetraacetic acid
- **NaOH:** Sodium Hydroxide
- **HCl:** Hydrochloric acid
- **$HgCl_2$:** Mercury Chloride
- **pH:** Power of Hydrogen
- **cm:** Centimeter
- **g:** Gram
- **mg:** Miligram
- **L:** Liter
- **ml:** Milliliter
- **Fig:** Figure
- **psi:** Pounds per square inch
- **rpm:** Rotations per minute

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Chapter 1: Introduction

Introduction

Gerbera (*Gerbera jamesonii*), an exquisite flower, belongs to the family Compositae (*Asteraceae*). It is a native of South Africa and Asia with colossal industrial importance in recent years, which is why only *G. jamesonii,* out of 40 other gerbera species, is cultivated. Originating in South Eastern Africa, this flower is commonly known as the 'Barberton Daisy,' as named by Robert Jameson in 1889. This plant is widely created for commercial use as both cut flowers and potted plants. In terms of economic value, gerbera ranks 4th in global cut flower market, after rose, chrysanthemum and tulip (Teeri et al, 2006). Some of them show wonderful agronomical characters like flower color, floral diameter, vigor, and stem length. The flowers are hardy and can withstand vigorous transportation, and because of their longevity, they fetch high market value.

Gerbera is one of the most popular ornamental flowers in the world with more than 200 varieties. (Mohamed et al, 2014). The flowers include a big selection of engaging flower colors like red, pink, orange, peach, maroon, and other vibrant colors that make it a valuable decorative species. Few renowned cultivars of gerberas currently used for commercial flower production are Cream Clementine, Maron Clementine, Delphi, Vesta, Uranus, Terraqueen, Dusty, Valentine, Diablo, Mariso and Pascal (Loeser, 1986).

Gerberas can be propagated by both sexual and nonsexual strategy. Among the vegetative strategies, multiplication through division of clumps has been used for many decades, but is too slow to be used commercially. Large-scale industrial production of gerbera requires a neater, quicker, and economically viable methodology of propagation.

Currently, micropropagation of gerbera is being employed in several countries from a variety of explants, and has been successful for speedy, large-scale multiplication of gerbera (Kumar and Kanwar, 2008). Direct shoot regeneration from shoot tips as initial explants is most convenient method for mass propagation of gerbera; however, indirect shoot regeneration has been achieved from calli derived from different explants, like leaf, petals, and floral buds (Murashige, 1977). Tissue culture permits the assembly of disease-free plants that are free of seasonal differences and might rapidly produce over a million plants (Aswath et al. 2002, 2003).

1.1 Scientific Classification of *Gerbera Jamesonii*

Kingdom: Plantae - Plants
Division: Magnoliophyte - Flowering plants
Class: Magnoliopsida - Dicotyledons
Order: Asterales
Family: Asteraceae/Compositae - Aster family
Genus: Gerbera JF Gmel - Transvaal Daisy
Species: Gerbera *Jamesonii* Bolus ex Hook f. - Baberton Daisy

1.2 Morphology of *Gerbera Jamesonii*

The morphological descriptions of *Gerbera Jamesonii* varieties (Akter et al. 2012), are described below:

- **Habit and habitat:** Herbaceous and perennial herb
- **Stem**: Cylindrical, smooth with full green pigmentation, 43-71 cm and unbranched
- **Flowers:** Plants come in varying floral colors covered with silky hairs arising from a crown. The flower size varies from 6 cm to 9.5 cm in diameter
- **Leaves**: Leaves occur in basal rosettes, petioled, oblong-spatulate and deeply lobed by half as wide; color ranges from deep green to light green in color.
- **Flowering Habits**: Flowers in between 90-150 days depending on the date of sowing and soil conditions.

1.3 Species

The genus gerbera consists of 40 species, which are of Asiatic and African origin. Some other important species are *G. aspleifolia*, *G. aurantiaca*, *G. kunzeana*, *G. viridifolia*, etc.

1.4 Suitable growing conditions

Gerbera jamesonii grows well in the open in tropical and subtropical regions, and at altitudes ranging between 1300 and 3200 meters above sea level. They usually need cool and

well ventilated conditions, with good light, for growth. The suitable temperature for the cultivation of gerbera is 10-25°C. However, some gerberas require specific climatic conditions for flower production and do not produce quality flowers when these requirements are not fulfilled. In terms of soil preferences, gerberas usually grow well in organically rich, medium moisture and well-drained soil that is neutral or slightly alkaline. However, the best quality gerbera flowers can be obtained under greenhouse conditions. Though gerberas can be found all through the year, their growth rate is higher in winter, and their flower production rate is higher in spring.

1.5 History

Robert Jameson first discovered gerbera daisies in the Transvaal area of South Africa in 1880. However, the first official description of "*Gerbera jamesonii*" was made by J. D. Hooker in 1889 in the Curtis Botanical Magazine. (Codd, 1979). The development of G. jamesonii as a floricultural crop is traced from its cultivation as a novelty in South Africa to its establishment as a commercial crop in the 1930s. Commercial breeding started in the 19th century when the *Gerbera jamesonii* species from Natal in South Africa was crossed with *Gerbera viridifolia* from the Cape Province. It was cultivated first in Europe and later planted in the Himalayan foothills in Kashmir at heights between 1,300 and 3,200 meters. (Hind, 1992).

1.6 Uses

1.6.1: Economic Importance: Gerbera is one of the leading cut flowers, and ranks among the top ten cut-flowers of the world (Parthasarathy and Nagaraju, 1999), which is why they are a farmer's top crop choice for planting. Because of its significant economic importance, the gerbera cut flower trade is increasing exponentially across all the continents and the availability of micropropagated, clonal gerbera plants in sufficient numbers has helped commercial growers to cultivate many varieties of gerbera for the production of cut flowers (Kadu, 2013). Gerbera's rise in popularity as a cut flower is depicted by the high demand in countries like Holland, Germany and USA. The production of gerbera was approximately worth US$ 220 million in 2001, representing 70 million stems sold in US alone (Broeket et al., 2004),

and horticultural and floriculture greenhouse production of gerbera has increased 92.4% during the last 10 years (Tzouramani et al., 1995).

1.6.2: Ornamental Plant: Gerbera's are in great demand for commercial production as well as for domestic gardens and landscaping. Initially, gerbera flowers had gained popularity because of their vibrant colors, ranging from reds, yellows, whites, and purples, to many that are a mixture of two colors. Because of this attractive trait, it is widely used as a decorative garden plant, ideal for beds, borders, pots and rock gardens, and can be stored for long periods, as its' vase life is longer than other flowers, lasting from 5 to 7 days (Ranwala, 2010). Nowadays, gerberas are also indispensable items for floral arrangements and decorations for birthdays, marriages and other ceremonial functions (Shylaja et al, 2014). The flowers, of various colors, suit very well in different floral arrangements, which are highly valued by consumers as individual vase decorations and bouquet compositions (Mata *et al*., 2009). To meet the high standards of a decorative plant, *Gerbera jamesonii* is being hybridized with other gerbera species to produce great color variation in the flower, as well as the ability for a cut flower to last longer. Hundreds of new varieties are regularly tested in greenhouses each year to discover new shapes and color combinations that stay fresh for longer periods. ("The Secret of Long-Lasting Gerbera Daisies" 2008).

1.6.3: Phytoremediation Properties: As a potted plant, gerbera is considered one of the most useful plants for improving indoor air quality to living and working spaces. NASA had stated that gerberas are fantastic at removing benzene, a known cancer causing chemical (Wolverton *et al*., 1989).

1.6.4 Medicinal Properties: *Gerbera jamesonii* is also used in the preparation of traditional Chinese medicine "tu-er-feng", for curing cold and also for treating rheumatism (Ye et al., 1990). Coumarin, extracted from gerbera, was found to have broad spectrum anti-tumor and anti-bacterial activity. It is considered to be a new potential cancer chemoprevention agent (He et al., 2014).

1.7 Constraints in *Gerbera Jamesonii* production

1.7.1 Physiological Disorders: Losses from improper plant growth can have a significant economic impact, causing a reduction in income for crop producers and farmers. Some physiological disorders in gerbera include:

- Flowers becoming bent from a loss of cell turgidity
- Under-nutrition, such as from lack of calcium
- Stems of the plants breaking under high root pressure and high humidity in the air
- Premature wilting of the flower from the **c**loudy weather followed by bright sun or carbohydrate depletion
- Double-faced flowers caused by imbalance of nutrients, causing growth of too little flower buds
- Non-uniform flower blooming caused by physical injury to flower stem or by pest damage or phytotoxicity
- Short stem length resulting from a high salinity level, moisture, stress, and low soil temperature.

1.7.2 Pests: Gerbera can be afflicted by various insects and disease that can cause physical damage of the plants, and can therefore reduce flower production. Some notable pests are Whitefly *(Trialeurodes vaporariorum),* Leaf Miner fly (Liriomyzatrifolii), Mites (*Hemitarsonemus latus* and *Steneotarsonemus pallidus*), Aphids, and Nematodes (*Meloidogyne* spp.).

1.7.3: Bacteria and viruses: Infection by both bacteria and viruses cause a wide range of plant disease. In fact, the rates of viral infection in gerbera plants are much higher than any other plant. Some notable diseases in gerbera are listed here.

Root rot is a soil-borne disease caused by *Pythium irregularae* and *Rhizoctonia solani.* The infection results in stunted growth, wilting, and ultimate death of the plants. Sclerotium rot, caused by *Sclerotium rolfsii* is a fungal disease affecting the entire above-ground parts of the plants. Fusarium Stem Rot, caused by *Fusarium solani*, is symptomized by the petioles of

Gerbera leaves blackening at the base, and the plant collapsing. Phytophthora Crown Rot caused by Phytophthoracryptogea, occurs when the plants suddenly wilt, the leaves become brown and the roots begin to rot. Similarly, Rhizoctonia Crown Rot occurs when plants are infected with Rhizoctonia. Thielaviopsis, where stems at the soil level have brown lesions and plants eventually wilt and die.

Botrytis Blight**,** also known as gray mould, is caused by *Botrytis cinerea* which kills young growing tissues. Deep planting, bad drainage and poor ventilation predispose plants to infection. When a gerbera plant is infected, the petioles have long brown spots, the petals have tan spots and the leaves become yellow, and then die. The stems at soil level are killed and the infected tissues become covered with gray fungal growth. Alternaria Leaf Spot**,** caused by the fungi *Alternaria,* is characterized by brown, small scattered spots forming on the florets and the leaves that gradually become round or irregular. Eventually, the centers of the leaf spots become white and the spots coalesce to affect large areas of leaves and cause defoliation. Affected plants showed lower vitality, suppressed development and fewer, smaller, distorted in shape flowers. Bacterial Leaf Spot occurs when a gerbera plant is infected by *Pseudomonas cichorii*, and circular spots, both large and small, form in the leaves and then become irregular. The leaves turn from dark brown to black in color.

Powdery Mildew is a fungal infection caused by *Golovinomyces cichoracearum*, where a white fungal growth develops on the surface of leaves. Tobacco rattle, Gerbera mosaic, impatiens necrotic spot, and cucumber mosaic viruses can also infect Gerbera plants, but the rate of infection is low, and they can infect the plant only at a large concentration of pathogen. Once infected, these diseases show symptoms of discoloration of the flower, line or ring spots to the leaves and sometime even form mottled leaves.

1.7 Biotechnological approaches

Gerbera plants can be propagated both sexually and asexually. Sexual propagation involves the germination of seeds to grow new plants. However, one of the main problems of seed propagation is heterozygosity in the plants produced, the lack of uniformity within them (Murashige et al., 1974), and the long amount of time it takes to produce flowers (Nhut et al, 2007). Also, the improved semi-double and double cultivars do not set seeds. Vegetative propagation overcomes this problem and produce plants of better performance (Topoonyanont

and Debergh, 2001). Gerbera is generally propagated by division of clumps or rhizomes, or stem cutting, which produce true to type plants. However, vegetative propagation is too slow to be commercially utilized (Murashige et al.1974). Micropropagation is a viable alternative for large-scale multiplication of gerbera (Bhatia et al., 2008), because of its reliability, cost, and labor effectiveness. The demand for tissue cultured plants is increasing more as compared to conventional production of plants. Nowadays, *in vitro* propagation is the preferred method (Aswath and Choudhary, 2002; Kumar et al., 2004; Prasanth and Sekar, 2004; Reynoird et al., 1993; Xi and Shi, 2003).Over the years, gerbera's have been propagated by direct or indirect organogenesis using various explants, including stem tips, floral buds, leaf, capitulum etc. (Kanwar and Kumar, 2008).

1.9 Activities regarding *Gerbera Jamesonii* in Bangladesh

In the perspective of Bangladesh, *in vitro* regeneration of gerbera is important to our country because of the high demand that is created in both local and international markets. The commodity wise export shipment for cut flowers in July-April 2017-2018 was $0.07 million (collected from https://www.bb.org.bd/econdata/bop/exp_rcpt_merchandise.php). The public attraction towards gerbera is gradually increasing, particularly in urban areas, as ornamental and home decorative plants because of its attractive colors and size. Due to the increasing requirements of gerbera flowers, both as an ornamental plant and as cut flowers, floriculture has become very popular to meet the local demand, and many nurseries have started to cultivate this plant from cuttings imported from India. (Rahman et al., 2014). The reason for this rapid floriculture development in Bangladesh is the country's varied climatic conditions, where moderate climatic control at relatively cheaper cost can deliver quality products at internationally competitive prices. Along with nursery cultivation, biotechnological approach might be a prospective alternative for mass propagation of gerbera. Using methods of micropropagation, the farmers can rapidly introduce selected superior clones of gerbera in sufficient quantities, which would have a direct impact on the market potential. As the economy of Bangladesh is largely driven by agriculture, this flowering plant can have a great contribution to the economic growth as a major source of export, as well as open a new dimension in the field of agriculture in Bangladesh.

1.9 Objective of this paper

In the present study, the experiments were designed to carry out the following objectives:

1. Optimization of the sterilization procedure for selected gerbera explants
2. Determination of the most favorable media composition for regeneration
3. Selection of suitable explants for regeneration
4. Comparison of regeneration responses between the two gerbera varieties

Chapter 2: Materials and Methods

Materials and Methods

2.1 Materials

2.1.1 Source of plant material

In the present experiment, explants were collected from two varieties of gerbera (*Gerbera Jamesonii),* the red and the white variety. The plants of gerbera were obtained from two major sources, in two distinct phases. In the initial phase, 16 gerbera plantlets were bought from local nurseries all throughout Dhaka, Bangladesh. They were then transferred into fertile soil and grown in a natural environment, with ample sunlight and water. In the second phase, all explants of gerbera were obtained from gerbera plants grown in the gardens of Dhaka University, Dhaka, Bangladesh, under constant supervision.

2.1.2 Media used

In this experiment, full strength Murashige and Skoog (MS) media (1962) was used for *in-vitro* regeneration as a source for optimal nutrition support for the growth and morphogenesis of *in-vitro* grown plant tissue. MS media, in combination with many different growth hormones, such as BAP, NAA and Kn, prepared in different concentrations, had been used as the media for both callus induction and subsequent regeneration of explants.

2.2 Methods

2.2.1 Preparation of stock solutions for media

The MS media consists of several components, including macro-nutrients, micro-nutrients, organic components and Fe-EDTA, which are required in different concentrations in the media. However, because the quantity of the reagents in a single component is often too small or difficult to weigh precisely, a stock solution for each component was used for greater convenience. Separate stock solutions for macro-nutrients, micro-nutrients, organic components and Fe-EDTA were prepared and then used to make the MS medium.

2.2.2 Macro-nutrient stock preparation for MS media

The stock solution of macro-nutrients was prepared in 10X concentration, as in the strength of the solution was 10 times more than the full strength of the medium, in 1000 ml of distilled water. The amounts of different reagents required for 1 liter of the macro-nutrient stock solution were measured accurately by a top pan balance, and sequentially added to a 1000 ml beaker, where it was dissolved in distilled water. Each reagent was fully dissolved before adding another, ensuring a clear solution. The final volume of the solution was made up to 1000 ml by adding distilled water, in a 1000 ml measuring cylinder. The solution was then poured into a large Duran bottle, after which the bottle was correctly labeled and autoclaved at 121°C for 15 minutes. It was then allowed to cool, and then stored in a refrigerator at the temperature of 4°C.

Macro-nutrient components	**Amount (mg/l)**	**Amount for 10X stock solution (mg/l)**
KNO_3	1900	19.00
NH_4NO_3	1650	16.50
$MgSO_4.7H_2O$	370	3.70
$CaCl_2.2H_20$	440	4.40
KH_2PO_4	170	1.70

Table 2.1: The components required to make 1 liter (10x) of Macronutrient Stock Solution

2.2.3 Micro-nutrient stock preparation for MS media

The stock solution of micro-nutrients was prepared in 100X concentration, as in the strength of the solution was 100 times more than the full strength of the medium, in 1000 ml of distilled water. Similar to the preparation of the macro-nutrient stock solution, the amounts of different reagents required for 1 liter of the micro-nutrient stock solution were measured accurately by a top pan balance, and sequentially added to a 1000 ml beaker, where it was dissolved in distilled water. Each reagent was fully dissolved before adding another, ensuring a clear solution. The final volume of the solution was made up to 1000 ml by adding distilled water, in a 1000 ml measuring cylinder. The solution was then poured into a large Duran bottle, after which the bottle was correctly labeled and then stored in a refrigerator at the temperature of 4°C.

Micro-nutrient 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

Table 2.2: The components required to make 1 liter (100x) of Micronutrient Stock Solution

2.2.4 Iron-EDTA stock preparation for MS media

The stock solution of Iron-EDTA was prepared in 100X concentration, as in the strength of the solution was 10 times more than the full strength of the medium, in a 1000 ml of distilled water. At first, the weight of Iron chelate ($FeSO_4.7H_2O$) required for 1 liter of the Iron-EDTA stock solution was measured accurately by a top pan balance and added to a 1000 ml beaker, where it was dissolved in distilled water by a hot plate. In succession, the correct amount of $Na_2EDTA.2H_2O$ was similarly weighed, added to the beaker, and stirred until it dissolved by the heat. The final volume of the solution was made up to 1000 ml by adding distilled water, in a 1000 ml measuring cylinder. The solution was then poured into an amber bottle, wrapped completely in aluminum foil, to prevent penetration of sunlight as it is sunlight sensitive. The bottle was then correctly labeled and then stored in a refrigerator at the temperature of 4°C.

Iron-EDTA components	Amount (mg/l)	Amount for 100X stock solution (mg/l)
$FeSO_4.7H_2O$	27.80	2.78
$Na_2EDTA.2H_2O$	37.30	3.73

Table 2.3: The components required to make 1 liter (100x) of Fe-EDTA Stock Solution

2.2.5 Organic stock preparation for MS media

The stock solution of organic components was prepared in 100X concentration, as in the strength of the solution was 100 times more than the full strength of the medium, in 1000 ml of distilled water. The amounts of different reagents required for 1 liter of the stock solution were measured accurately by a top pan balance, sequentially added to a beaker of distilled water, and dissolved. Each reagent was fully dissolved before adding another, ensuring a clear solution. The

final volume of the solution was made up to 1000 ml by adding distilled water, in a 1000 ml measuring cylinder. The solution was then poured into a large Duran bottle, after which the bottle was correctly labeled and then stored in a refrigerator at the temperature of 4°C.

Organic components	Amount (mg/l)	Amount for 100X stock solution (mg/l)
Ni acid	0.5	0.005
Pyridoxin-HCl	0.5	0.005
Thimin-HCl	0.1	0.001
Glycine	2.0	0.020

Table 2.4: The components required to make 1 liter (100x) of Organic Stock Solution

2.2.6 Preparation of stock solution for growth regulators

For the growth, differentiation, and organogenesis of tissues, growth regulators, such as the auxin NAA (α-napthalene acetic acid), and the cytokinin BAP (6-benzyl amino purine) were used in this experiment in various concentrations, by preparation of prior stock solutions which were then added to the MS medium. The preparation for the different hormone stock solutions follows the same method, and is made in the concentration of 1 mg per liter (10 mg/100 ml). At first, 10 mg of the powdered hormone (BAP/NAA) was accurately measured, and dissolved in 1-2 ml of 1N NaOH. The final volume of the dissolved solution was made up to 100 ml by adding distilled water. The solution was then poured into a clear plastic bottle, correctly labeled and then stored in a refrigerator at the temperature of 4°C.

Hormone	Molecular weight (g/mol)	Solvent
BAP	225.3	1N NaOH
NAA	186.2	1N NaOH

Table 2.5: The molecular weights and solvents of the plant growth hormones used

2.2.7 Preparation of 1 liter of MS medium

The following procedures were carried out in the making of 1 liter of MS media with a desired hormone composition:

- Initially, 500 ml of distilled water was measured and added to a 1000 ml beaker
- To it, 100 ml of the macro-nutrient stock solution, 10 ml of the micro-nutrient stock solution, 10 ml of the Fe-EDTA stock solution, and 10ml of the organic

component stock solution were accurately measured by a pipette, and then added sequentially into the solution.

- A 100 mg of Myo-inositol was precisely weighed by a measuring balance, and added to the solution, which was then continuously stirred until it completely dissolved.
- Similarly, 30 g of sucrose was exactly measured and dissolved into the solution.
- The desired amount of hormone stock solution was measured and then added to obtain a certain concentration of hormone in the media. In case of multiple hormones, the varying hormone stock solutions are accurately measured, then added one after another into the media, and stirred to create a clear solution.
- The final volume of the mixture was made up to 1000 ml by adding distilled water, in a 1000 ml measuring cylinder.
- The pH of the medium was adjusted to 5.8, using a digital pH meter. To the solution, acid and alkaline solutions of 1N NaOH and/or 1N HCl were added drop-wise until the desired pH was reached.
- Subsequently, 8 g of Agar was accurately weighed by a measuring balance and added to the mixture and stirred. The media was then heated in a microwave until the agar had completely melted and the media had come to a boil.
- Finally, the molten media was poured into conical flasks, with approximately a 100 ml of media in each flask. The mouths of the flasks were sealed by wrapping with large cut pieces of aluminum foil.

Components	**Amount (for 1L)**
Macro-nutrients (10X)	100ml
Micro-nutrients (100X)	10ml
Organic (100X)	10ml
Iron-EDTA (100X)	10ml
Myo-inositol	100mg
Sucrose	30g

Table 2.6: The components required to make 1 liter of MS media

2.2.8 Media sterilization

For sterilization purposes, all conical flasks containing MS media were sterilized by sterilized by autoclaving at 15 psi pressure at 121ºC temperature for 30 minutes.

2.2.9 Precaution for aseptic culture

All inoculations and subcultures were carried out in a laminar airflow cabinet. Prior to its use, the Ultra-Violet (UV) light in cabinet was switched 'ON' for 30 minutes, and then its surfaces were wiped thrice with 70% ethanol to free it from contaminants. Instruments like scalpels, forceps, petri dishes, beakers and conical flasks, and materials like cotton wool, filter papers were autoclaved before their use, and stored in an incubator. Additionally the distilled water used for inoculations, were also autoclaved and stowed in large sealed conical flasks. All the necessary equipments were vigorously wiped with 70% ethanol before their use, and put before the flames of an ethanol burner for one to two minutes, to kill off contamination by heat. 70% ethanol and flame sterilization of equipments was also carried out in between inoculations to reduce contamination of explants. Additionally, mouths of the flask containing explants were flamed prior to covering them in aluminum foil and wrapping the mouth of the flask with Parafilm tape. Before work in the cabinet, hands were thoroughly washed with antibacterial soap, rubbed with both hexisol solution (ACI) and 70% ethanol, and then dried in the air. All measures were taken to obtain maximum contamination-free conditions during the work. Furthermore, any contaminated culture and older non-regenerative explants were autoclaved before disposal, as according to bio-safety rules.

2.2.10 Explant sterilization

2.2.10.1 Preparation of 20% Savlon solution: In a conical flask, 20ml of the antiseptic disinfectant liquid Savlon, containing chlorohexidine gluconate and cetrimide (Novartis Consumer health UK limited), measured by a measuring cylinder, was added and mixed with 80 ml of distilled water, such that the final volume of the solution was 100 ml.

2.2.10.2 Preparation of 0.1% $HgCl_2$ solution: In a beaker, 1 g of powdered Mercury Chloride was weighed accurately by a measuring balance and stirred until it completely dissolved in distilled water. The final volume was made to 1000 ml in a measuring cylinder by addition of more distilled water. The solution was then correctly labeled and stored in a large bottle wrapped in aluminum foil, to prevent sunlight from entering, as the reagent is sunlight sensitive.

2.2.10.3 Process of explants sterilization: To reduce the level of surface organisms, the collected explants (leaves, flower bud and flower stalk) were first washed in a beaker under running tap water for 30 minutes. They were washed with the dish detergent 'Trix' under running tap water until the detergent was completely washed out. The explants were subsequently immersed in 20% Savlon solution for 1 minute, after which they were rinsed five times in tap water, followed by two times with distilled water. In the laminar airflow cabinet, after transferring the explants to an autoclaved beaker, they were initially washed three times with sterilized distilled water. The explants were then surface sterilized by dipping them into 70% ethanol for 30 seconds, followed by washing 5 times with sterilized distilled water. Finally, the explants were submerged in 0.1% $HgCl_2$ solution for 6 to 7 minutes, where the beaker was continually shaken to ensure all surfaces of the explants being sterilized, and washed 7 times with sterilized distilled water.

2.2.11 Explant culture

For this experiment, tender leaf segments, such as with midrib, sides and tip, flower buds and flower stalks were used as explants. In case of leaf explants, leaves, 9-30 days old and about 4 cm to 15 cm in size, were taken from the first and second positions in the gerbera plant. Once sterilized, portions of a leaf were cut into small 1-2 cm squares, each including parts of the leaf tip, the leaf sides, the petiole and the midrib portion in the leaf. For flower bud and flower stalk explants, young flower buds, with about 1 to 2 cm diameter, along with flower stalks, of 2.5 to 6 cm, both of which were 9-11 days old, were collected. After sterilization, the flower bud and the flower stalk were separated. The flower bud was dissected down the middle, had its whorl of petals removed, and was further cut into 6 equal pieces, while the flower stalk was cut into small

1-2 cm explants segments. After the cutting of the explants, they were placed on sterile filter paper to absorb unnecessary water after surface sterilization. Finally, the explants were inoculated by vertically placing the explants onto the media such that one of its surfaces was firmly dipped into the agar to receive nutrition. During this experiment, a variable kept as a constant was that each conical flask was inoculated with five explants segments.

2.2.12 Incubation conditions

The inoculated flasks were incubated in the controlled environment of a culture room, under fluorescent lights of 20,000 lux intensity, and temperatures of 25°C±2. The light period of the room was maintained at 16/8 (dark/light) hours.

2.2.13 Subculture

After two weeks, regenerated callus were transferred to conical flasks containing fresh media of the same hormonal composition. During this time, dead tissues within the explants were cut out, and the explants are further dissected to reduce their size, for good callus and shoot growth. Inoculated cultures were sub-cultured regularly within 12-15 days for maintenance and optimal response of explants. The cultures were also routinely examined to collect data on any morphological changes observed.

Figure 2.1: The different explants of gerbera used in the experiment. (A) A young leaf explant, 9-11 days old, 4 cm in size. (B) Side view of a young flower bud with flower stalk, 9-11 days old, with bud diameter of 1 cm. (C) Mature leaf explants of gerbera, 15-21 days old to the right, followed by young leaf explants, 9-11 days old and young flower buds, also 9-11 days old, to the left.

Chapter 3: Results

Results

This study had aimed to develop an effective *in vitro* regeneration protocol for two varieties of Gerbera *Jamesonii,* the red variety and the white variety. For this purpose, three parts of the gerbera plant were used as explants from each variety; the leaf, the flower bud and the flower stalk. Multiple trials were carried out to establish the best hormonal concentrations and/or combinations for inducing the growth of calluses and shoots during the tissue culture procedure.

3.1 Selection of the most suitable explant for regeneration

In the present study, three different types of explants were used for in-vitro regeneration of Gerbera *jamesonii*. In order to select the most responsive explant from among them, explants from the two varieties were cultured on MS medium, supplemented with different concentrations of BAP and NAA, and the behavior of the explants in culture conditions were recorded.

Gerbera Variety	Type of explant	Parts of explants	Number of explants	No. of responsive explants	Responsive explant (%)
Red	Leaf	Leaf sides	20	18	90
		Leaf with midrib	20	20	100
		Leaf with petiole	20	5	25
		Leaf tip	20	3	15
	Flower	Flower bud	20	20	100
		Flower stalk	20	19	95
White	Leaf	Leaf sides	20	17	85
		Leaf with midrib	20	19	95

	Leaf with petiole	20	6	30
	Leaf tip	20	4	20
Flower	Flower bud	20	20	100
	Flower stalk	20	20	100

Table 3.1.1: A comparative analysis between the morphological responses displayed by the explants of the red and white varieties of Gerbera *jamesonii*

The results show that all explants of gerbera, for both varieties are responsive to morphological changes, some more than others. Morphological changes are attributed to the increased size of the explant, observed after several days of incubation, and any other visible changes to the explant in its shape or form. The highest rate of response (100%) had been observed in the flower buds for both the red and the white variety, thus it can be considered the most responsive explant of gerbera. A high rate of response was also observed in flower stalks, with a 100% response rate for the white variety and a 95% response rate for the red variety. From the leaf explants, the leaf with midrib had shown high rates of response, with 100% in the red variety and 95% in the white variety. On the contrary, the least responsive explant is the leaf tip, with only a 15% response rate in the red variety and a 20% response rate in the white variety. Overall, based on their large difference in rate of responses, it can be concluded that the flower bud and flower stalk explants are more responsive to morphological changes than the leaf explants in both varieties. The average time for an explant to display any morphological change was recorded to be 7 days.

3.2 Determination of the effectiveness of the sterilization procedure used on different explants

The explants used in these experiments, the leaf, the flower bud and the flower stalk, were collected from the tender and actively growing parts of the gerbera plant. To avoid contamination of explants, 20% Savlon solution, Trix detergent, 70% alcohol and 0.1% $HgCl_2$ were used as surface sterilizing agents. For the procedure of explant sterilization, all the explants were washed under running tap water for 30 minutes, followed by washing with Trix detergent

and 20% Savlon solution, after which the explants were treated with 0.1% $HgCl_2$ for six minutes and 70% ethanol for 30 seconds, in between many autoclaved distilled water washes, for surface sterilization. The efficiency of the surface sterilization method in controlling contamination, and in producing responsive explants, was examined.

Gerbera Variety	Type of explant	Part of explant	Contamination rate (%)	Death rate (%)	Survival rate (%)	Average time until contamination (days)
Red	Leaf	Leaf sides	14.5	56.3	4.7	9
		Leaf with midrib	12	10	90	7
		Leaf with petiole	30.0	16.6	33.3	8
		Leaf tip	25.5	92.5	7.5	10
	Flower	Flower bud	33.5	12	88	7
		Flower Stalk	38.7	15.5	84.5	8
White	Leaf	Leaf sides	23.5	60.3	39.7	7
		Leaf with midrib	15	12	88	9
		Leaf with petiole	37.5	25.0	27.5	8
		Leaf tip	30.5	94.5	5.5	10
	Flower	Flower bud	38.5	14.5	85.5	7
		Flower stalk	38.7	15.5	84.5	9

Table 3.2.1: Efficiency of the sterilization procedure used

From Table 3.2.1, it can be seen that although the sterilization treatment used had brought about responsive explants, it had also left room for high rates of explant death and contamination. It can be noted that the greatest rate of total contamination occur in flower stalks of both the red and the white varieties (33.5% and 38.7% respectively). The highest death rate observed had occurred in the leaf tips, with the white leaf tip having a death rate of 94.5% and the red leaf tip with a death rate of 92.5%. Consequently, leaf tips are also the explants with the lowest survival rates, the white variety having the least survival rate of 5.5% and the red variety having the survival rate of 7.5%. The explant best suited for this sterilization method was observed to be the leaf with midrib for the red variety, having the lowest death rate (10%), the lowest contamination rate (12%) and the highest survival rate (90%). However, the flower buds, despite having the highest rate of contamination among all other explants, were considered as a very suitable explant for regeneration because of their lower death rates and high survival rates, which made them more likely to display morphological responses in culture. The overall average rate of contamination is 8.25 days, with the greatest duration for contamination to occur taking 10 days, and the least duration being 7 days.

3.3 Selection of a suitable medium for regeneration

To select the most suitable medium for successful plant regeneration, MS medium with different concentrations of hormonal supplements were used for explant inoculation. The efficiency of the medium towards callusing and in-vitro shoot regeneration was tested, and MS media containing different concentrations of BAP (1 to 10 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 an explants' morphological changes, and subsequent callus growth.

Sl No.	MS+PGRs* (mg/l)		No. of explants	Variety of Gerbera	No. of responsive explants	Responsive explants (%)	Mean no. of responsive explants
	BAP	NAA		Color			
1	1	0.5	20	Red	17	85	0.85
				White	18	90	0.90
2	1.5	0.5	20	Red	20	100	1.00
				White	19	95	0.95
3	2	0.5	20	Red	19	95	0.95
				White	20	100	1.00
4	2	1	20	Red	20	100	1.00
				White	18	90	0.90
5	3	0.5	20	Red	19	95	0.95
				White	20	100	1.00
6	3	1	20	Red	20	100	1.00
				White	15	75	0.75
7	5	1	20	Red	20	100	1
				White	16	80	0.80

***PGR = Plant Growth Regulator**

Table 3.3.1: A comparative analysis on the effects of different plant growth hormones (BAP and NAA) on leaf explants of the red and white variety of *Gerbera jamesonii*

The results show that all of the media compositions used in this experiment had brought about morphological change within the leaf explants. From Table 3.3.1, it can be seen that the rate of responses of both the red variety and the white variety are very similar in each media composition tried. The highest rate of response in the red variety (100%) had been recorded with four media compositions: MS + 1.5 mg/l BAP + 0.5 mg/l NAA, MS + 2 mg/l BAP + 1 mg/l NAA, MS + 3 mg/l BAP + 1 mg/l NAA, and MS + 5 mg/l BAP + 1 mg/l NAA. Similarly, the highest rate of response in the white variety (100%) had been recorded with two media compositions: MS + 2 mg/l BAP + 0.5 mg/l NAA and MS + 3 mg/l BAP + 0.5 mg/l NAA. On the contrary, the lowest rate of response (75%) had been observed in the white variety with the

media composition MS + 3 mg/l BAP + 1 mg/l NAA. The overall duration required for morphological changes was counted to be 7 days.

Sl No.	MS + PGRs* (mg/l)		No. of explants	Variety of Gerbera	No. of responsive explants	Responsive explants (%)	Mean no. of responsive explants
	BAP	NAA		Color			
1	2	0.5	20	Red	20	100	1.00
				White	18	80	0.80
2	2	1	20	Red	20	100	1.00
				White	19	95	0.95
3	3	0.5	20	Red	18	80	0.80
				White	20	100	1.00
4	3	1	20	Red	20	100	1.00
				White	18	80	0.80
5	4	1	20	Red	17	85	0.85
				White	19	95	0.95
6	5	1	20	Red	20	100	1.00
				White	19	95	0.95
7	6	1	20	Red	18	80	0.80
				White	15	75	0.75
8	10	1	20	Red	17	85	0.85
				White	19	95	0.95

***PGR = Plant Growth regulator**

Table 3.3.2: A comparative analysis on the effects of different plant growth hormones (BAP and NAA) on flower bud explants of the red and white variety of *Gerbera jamesonii*

Similar to the results of the leaf explants, the Table 3.3.2 shows that all of the media compositions used in this experiment had brought about morphological change within the flower bud explants. The rate of responses of both the red variety and the white variety are very similar in each media composition tried. The highest rate of response (100%) in the red variety had been

recorded with four media compositions: MS + 2 mg/l BAP + 0.5 mg/l NAA, MS + 2 mg/l BAP + 1 mg/l NAA, MS + 3 mg/l BAP + 1 mg/l NAA, and MS + 6 mg/l BAP + 1 mg/l NAA. Similarly, the highest rate of response (100%) in the white variety had been recorded with one media composition: MS + 3 mg/l BAP + 0.5 mg/l NAA. On the contrary, the lowest rate of response (75%) had been observed in the white variety with the media composition MS + 6 mg/l BAP + 1 mg/l NAA. The overall duration required for morphological changes was counted to be 7 days.

Sl no.	**MS + PGRs* (mg/l)**		**No. of explants inoculated**	**Explants inoculated**	**Callus induction (%)**
	BAP	**NAA**			
1	0	0	10	Leaf	-
				Flower bud	-
2	1	0.5	10	Leaf	45
				Flower bud	40
3	1.5	0.5	10	Leaf	40
				Flower bud	42
4	2	0.5	10	Leaf	70
				Flower bud	85
5	2	1	10	Leaf	65
				Flower bud	75
6	3	0.5	10	Leaf	50
				Flower bud	70
7	3	1	10	Leaf	55
				Flower bud	50
8	5	1	10	Leaf	60
				Flower bud	55
9	6	1	10	Flower bud	70
10	10	1	10	Flower bud	60

***PGR = Plant Growth Regulators**

Table 3.3.3: A comparative analysis on the effects of different plant growth hormones (BAP and NAA) for callus induction in explants of the red and white variety of Gerbera *jamesonii*

In terms of the most suitable media composition for callus induction in explants, the results from Table 3.3.3 show that, other than the control media without any growth regulators, all the media compositions used were successful in inducing callus in both leaf and flower bud explants. The greatest percentage of callus induction (85%) occurred in flower buds in the media composition MS + 2 mg/l BAP + 0.5 mg/l NAA. The lowest percentage of callus induction (40%) occurred in both flower buds and leaves with the media compositions MS + 1 mg/l BAP + 0.5 mg/l NAA and MS + 1.5 mg/l BAP + 0.5 mg/l NAA. The results also show that the best media composition for callusing is MS + 2 mg/l BAP + 0.5 mg/l NAA, which gave the highest percentages of callus induction for both the leaves (70%) and the flower buds (85%). Conversely, the worst media composition for callusing is MS + 1.5 mg/l BAP + 0.5 mg/l NAA, which gave the lowest percentages of callus induction for both the leaves (40%) and the flower buds (42%). It had taken an average of 2-3 weeks for callus induction in all explants, and the calluses induced were subcultured in the same media to promote further growth.

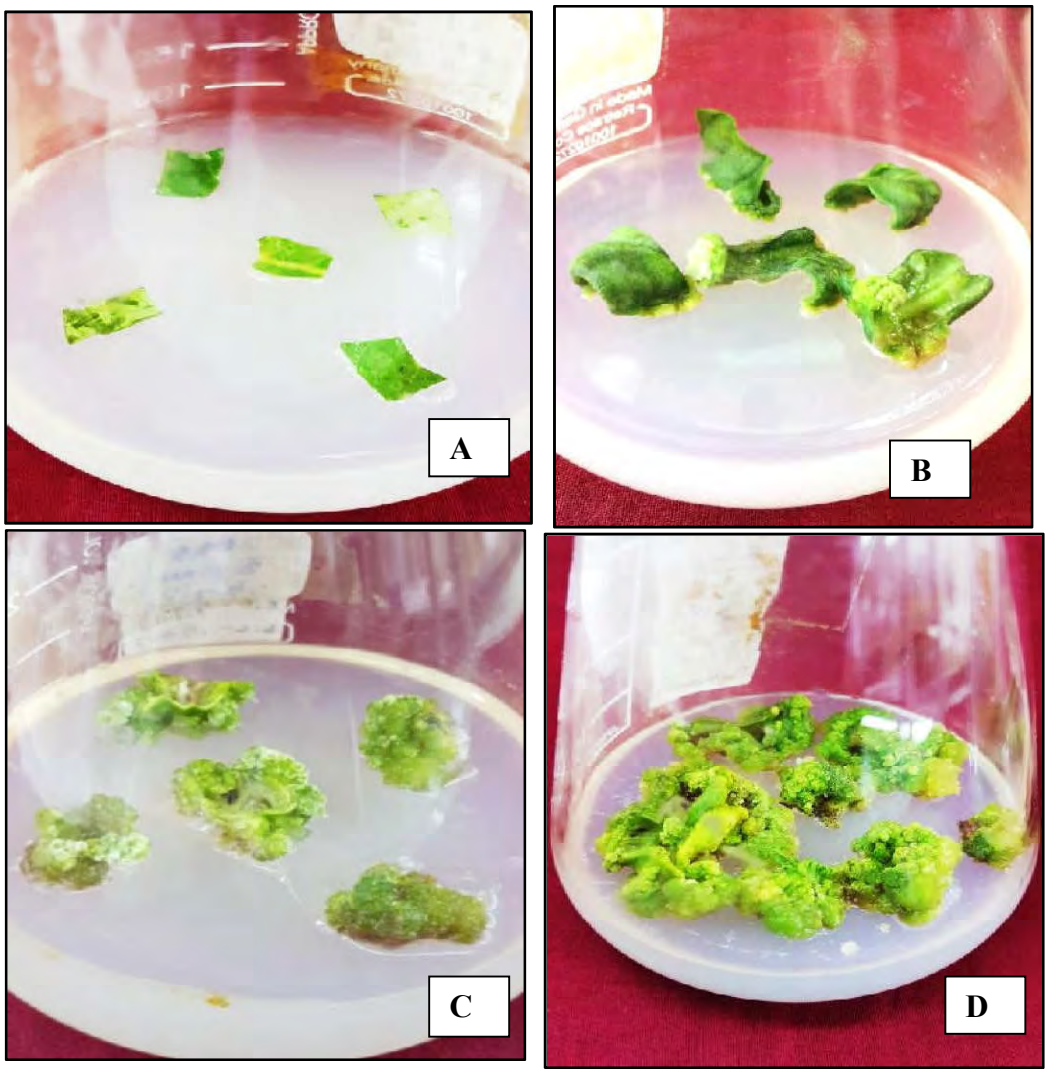

Figure 3.1: Different growth phases in varying media compositions for red leaf explants; (A) Initial inoculation (B) After three weeks of inoculation in MS + 2 mg/l BAP + 0.5 mg/l NAA, (C) After four weeks of inoculation and callus growth in MS + 2 mg/l BAP + 1 mg/l NAA, (D) After six weeks of inoculation, compact and friable callusing in MS + 3 mg/l BAP + 0.5 mg/l NAA.

3.4 Determining the effect of leaf size and flower bud diameter on the rate of callusing in explants of the red and white variety of Gerbera *jamesonii.*

In order to select a suitable size of explant for optimal callus induction, explants of differing sizes were measured prior to their subsequent sterilization and inoculation in MS media. The effect of leaf size and flower bud diameter, for both varieties, on the rate of callus formation was calculated.

Gerbera variety	Average size of leaf (cm)	Number of explants inoculated	Rate of responsive explants (%)	Rate of callus formation (%)	Average time taken for callusing to occur (days)
Red	6.4	20	94	85	18
White	5.5	20	83	75	20

Table 3.4.1: The effect of leaf sizes on the rate of callusing

Table 3.4.1 shows that the red leaf of gerbera, having a larger average size, was more responsive to morphological changes than the white leaf, and had a greater rate of callus formation; the red leaf explants also took less time for callus induction than the white leaves.

Gerbera Variety	Average diameter of bud (cm)	Number of explants inoculated	Rate of responsive explants (%)	Rate of callus formation (%)	Average time taken for callusing to occur (days)
Red	1.3	20	45	95	20
White	1.2	20	52	85	21

Table 3.4.2: The effect of flower bud size on the rate of callusing

Table 3.4.2 shows that white flower buds, being smaller in average diameter, were more responsive to morphological changes than the red flower buds. However, the red flower bud had a higher rate of callus formation, and took less time for callus induction than the white flower buds.

3.5 Determining the effect of age of explant on the rate of callusing in explants of Gerbera *jamesonii.*

During this study, in order to select a suitable age of explant for optimal callus induction, explants of differing ages were obtained for subsequent sterilization and inoculation in MS

media. Explants that had been excised at 9-11 days were considered young, at 15- 21 days, were considered old, and at 25-30 days, were considered mature. The effect of the age of explant on the rate of callusing was calculated for both varieties.

Gerbera Variety	Explant Type	Age of explant	No. of inoculated explants	No. of callus formation	Callus formation (%)
		Young	20	17	85
Red		Matured	20	9	45
	Leaf	Old	20	7	35
		Young Bud	20	19	95
	Flower	Young Stalk	20	12	60
		Young	20	15	75
White		Matured	20	8	40
	Leaf	Old	20	6	30
		Young Bud	20	17	85
	Flower	Young Stalk	20	10	50

Table 3.5.1 Effect of the age of explant on the rate of response

Table 3.5.1 shows that explants of gerbera, from both the red variety and the white variety had formed calluses, regardless of their age. However, there were many differences noted in the regeneration capacity between the younger explants and the older explants. For both the red and white variety, it can be seen that the young bud, or capitulum, had the highest percentages of callus formation among all explants, with 95% callus formation on the red variety and 85% callus formation on the white variety. Similarly, for both the varieties, it can be seen that there is a greater percentage of callus formation for younger leaves, with 85% of callus formation for the red variety and 75% of callus formation for the white variety. In contrast, older leaves and mature leaves show far less percentages of callus formation in both varieties.

3.6 Selection of explants in the two varieties of Gerbera *jamesonii* on the basis of regenerative callusing

For the induction and development of callus, leaf, flower bud and flower stalk explants from both varieties were used. The surface sterilized explants were inoculated in MS medium supplemented with different concentrations of BAP (1.0 to 10.0 mg/l) and NAA (0.5 to 1 mg/l), and the rate of their callus formation was observed.

Variety	Type of Explant	Number of explants inoculated	Percentage of callus formation (%)	Type of callus	Time to callus formation (days)
Leaf	Leaf with Midrib	20	93%	Compact and Friable	18 Days
	Leaf side	20	82%	Compact and Friable	18 Days
	Leaf tip	20	15%	Compact	21 Days
Flower	Flower Bud	20	95%	Compact and Friable	20 Days
	Flower Stalk	20	75%	Compact	21 Days

Table 3.6.1: Selection of leaf and flower explants based on callusing

The results show that direct growth of two types of calluses occurred in both leaf and flower bud explants. A friable and non chlorophyllous callus with high growth rate appeared at the cut surfaces of the explant, and a compact chlorophyllous callus with a low growth rate was formed directly from whole explants. Callus formations from explants were observed 3 weeks after the initial culture initiation. Among the leaf explants, Table 3.6.1 shows that leaf with midrib showed highest percentage of callus formation (93%), whereas leaf tips showed the lowest percentage of callus formation (15%). On the other hand, flower bud showed the overall highest percentage of callus formation (95%).

3.7 Selection of explants based on formation of shoots

During this study, shoot regeneration was achieved through callus formation from flower bud explants from both the red and the white variety. Adventitious shoots from flower buds were developed from the tissues of the compact calluses when inoculated in MS media supplemented with 5 mg/l BAP and 1 mg/l NAA.

Variety	Type of explants	Number of explants inoculated	Percentage of shoot formation (%)	Type of shoot formation	Time to shoot formation (days)
Red	Leaf	20	0	-	-
	Flower bud	20	12%	Multiple	25 Days
	Flower Stalk	20	0	-	-
White	Leaf	20	0	-	-
	Flower bud	20	10%	Multiple	27 Days
	Flower Stalk	20	0	-	-

Table 3.7.1: Selection of explants of leaf and flower based on shooting

In terms of shoot regeneration, flower buds of the red variety showed a greater percentage of shoot formation (12%) when compared to the white variety (10%). Both varieties had successfully produced multiple shoots per explant; however the duration for the formation of shoots in the red variety (25 days) is faster than that of the white variety (27 days).

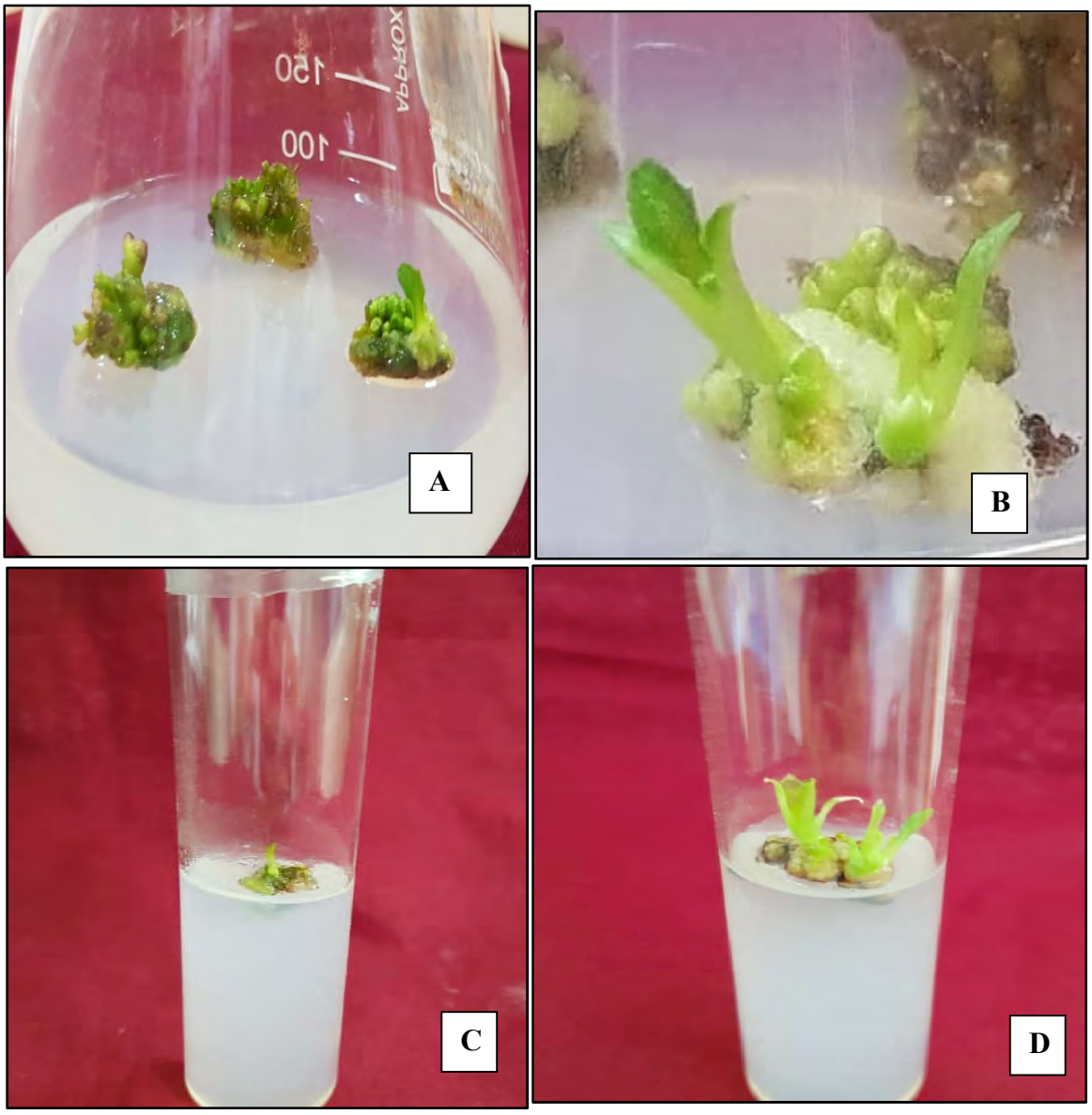

Figure 3.2: Different growth phases of red flower bud explant for shoot initiation; (A) After six weeks of inoculation in MS + 5 mg/l BAP + 1 mg/l NAA, (B) After eight weeks of inoculation in MS + 5 mg/l BAP + 1 mg/l NAA, (C) Excised callus with shoot subcultured on MS + 5 mg/l BAP + 1 mg/l NAA, (D) Multiple shoots in MS+BAP 5 mg/l + 1 mg/L NAA after 11 weeks

3.8 Comparison of morphological changes, callusing and shoot formation between the red variety and white variety of Gerbera *Jamesonii*

During the experiment, an overall comparison between the red and the white variety of Gerbera was conducted to conclude which variety was better for in-vitro regeneration protocols.

Variety	Number of explants inoculated	Percentage of responsive explants (%)	Percentage of callused explants (%)	Percentage of explants with shoot (%)
Red	20	98%	85%	18%
White	20	90%	78.5%	15%

Table 3.8.1: Comparison between the red and the white variety of *Gerbera jamesonii*

Both species of gerbera had shown *in vitro* indirect regeneration by forming calluses and shoots. However, the red variety of gerbera showed a greater response towards *in vitro* regeneration with 98% of responsive explants, 85% of callusing and 18% of shoot formation whereas white variety showed less response with 90% of responsive explants, 78.5% of callused explants and 15% of shoot formation.

Chapter 4: Discussion

Discussion

The main objective of the current study was to establish a reliable protocol for the in-vitro regeneration of Gerbera *jamesonii.* Three different types of explants, the leaf, the flower bud and the flower stalk, were collected from two varieties of gerbera containing red and white petals. The experiments conducted were carried out to monitor the response of the selected gerbera varieties *in-vitro* callus induction and shoot regeneration. Additionally, the study aimed to select an optimal sterilization method for the gerbera explants, the best explant for plant regeneration, and the most suitable media composition for *in-vitro* plant regeneration.

For this experiment, *ex-vitro* derived plantlets were used as the source of explants for *in-vitro* plant regeneration. Because field grown plant materials are normally infested with different microbes, it is necessary to create contamination free explants prior to tissue culture. Thus, during the sterilization process, the various surface contaminants of the explants were removed with the sterilants, Mercury Chloride ($HgCl_2$) and 70% ethanol. However, despite decreasing the rate of external bacterial and fungal contamination, the sterilants greatly hampered the regeneration ability of the explants upon longer exposure. Altaf et al. (2009) had stated that explants from field grown plants, such as shoot tips, leaf sections and capitulum, were prone to slow growth due to their treatment with harsh sterilizing chemicals that damage their growing regions, therefore limiting their rate of regeneration; they were also afflicted with severe contamination problems, leading to high bacterial and fungal growth in cultures.

The selection of an effective sterilizing agent is a key factor to reduce contamination and enable responsive explants. Therefore, in this experiment, long exposure of the explants to the harmful chemicals like Mercury Chloride ($HgCl_2$) and 70% ethanol was avoided. For optimizing the surface sterilization process, 20% Savlon solution and Trix detergent acted as pre-treating agents that helped reduced the contamination rate and also maintained the regeneration ability. Many sterilization treatments, with varying times of the explant exposure to the sterilizing agents, were conducted and monitored for the lowest rates of contamination. The best sterilization treatment found from this study was to wash the explant in running tap water for 30 minutes, followed by washing with Trix detergent and 20% Savlon solution, after which the explants were submerged in 70% ethanol for 30 seconds and 0.1% $HgCl_2$ solution for 5 minutes

in between many autoclaved distilled water washes. Using this treatment, the regeneration ability of the explants was also not hampered after the surface sterilization process.

Culture establishment is the most critical factor for *in vitro* morphogenesis and the success depends largely upon several factors like correct choice of explants, physiological state of the explants, growth, and biochemical composition, coupled with the presence of phytohormones, their ratio and level (Nugent et al. 1991).

In this experiment, three different types of explants, the leaf, the flower bud and the flower stalk were used for *in-vitro* regeneration of plantlets. Kanwar and Kumar (2008) have reported that gerberas are highly amenable to *in-vitro* studies and favourably respond to tissue culture media. Therefore, there have been a lot of documented reports involving in-vitro regeneration of gerbera. Some of them have used different parts of the leaf as a source of explant, such as shoot tip and petiole (Huang and Chu 1985 and Orlikowska et al. 1999). Similarly, many previous reports have also used flower bud as explant source for shoot regeneration (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, Nhut et al. 2007 and Akter et al. 2012).

Among all the explants, the flower bud, for both varieties of gerbera, showed the greatest response towards callus formation and further shoot regeneration. The surface sterilized flower buds were dissected into 1-2 cm explants and cultured on MS medium supplemented with various combinations of BAP (2.0 to 6.0 mg/l) and NAA (0.5 to 1 mg/l). Among all other explants, flower buds showed the overall highest percentage of callus formation (95%). Both of the gerbera varieties had also formed multiple adventitious shoots when inoculated in MS supplemented with 5 mg/l BAP and 1 mg/l NAA within an average of 26 days. The formation of shoots might be due to the creation of meristematic tissues in segment of immature flower heads (Mandal et al. 2002).

The age and size of the leaf and flower bud explants were important factors in callus induction and shoot proliferation. Bonga (1987) reported that the type of explant used for induction of callus mainly depends upon the juvenility of the explants. For flower buds, in both the varieties tested, 9-11 days old flower buds, with diameters of 1.0 to 1.3 cm, were more responsive in terms of shoot regeneration (Akter et al, 2012). Similarly, 9-11 days old leaves with sizes of 6 cm to 7 cm were more responsive to growing green chlorophyllous compact

calluses, and at a quicker pace, than older and mature leaves. This shows that the variation in callus differentiation in different explants might have resulted due to the maturity of leaf and flower bud explants in response to different innate growth regulators, since the explants were collected just before anthesis.

For leaf explants, the surface sterilized leaves were cut into five different segments, the leaf tip, the leaf with midrib, the leaf blade and the leaf with petiole, and were used as explants for callus induction and shoot regeneration. About 1-2 cm long segments of leaf explants were cultured on MS medium supplemented with various combinations of BAP (1.0 to 5 mg/l) and NAA (0.5 to 1.0 mg/l) for *in vitro* shoot regeneration via callus formation. Apart from the control media with no plant growth regulators, all the explants had initiated calluses within 21 to 28 days of inoculation.

Corresponding to the findings of Aswath and Choudhary (2002), there were two types of calluses formed from the explants. A friable and non chlorophyllous callus with high growth rate appeared at the cut surfaces of the explant, and a compact chlorophyllous callus with a low growth rate was formed directly from whole explants. However, only the second type of callus contained shoot primordia. There was a greater amount of friable callusing than compact callusing in the inoculated explants, but no shoots had developed from friable calluses. The induced calluses from the leaf explants of both varieties of gerbera were subcultured on the same medium composition for shoot regeneration, and while the calluses increased in their volume and thickness, there was no sign of shoot initiation from these calluses irrespective of the variety as well as the explants used, and cultures showing initial stages of differentiation didn't develop further on the same medium. This finding agreed with the inference of Vardja and Vardja (2001), who stated that all plant cells 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 in the production of adventitious shoots.

Flower stalks were also quite responsive to callusing (55%), and had formed both compact and friable calluses within 3 weeks of incubation in cultures, but the calluses also failed to regenerate into shoots. The flower stalks that were collected were of the same age as the flower buds.

The different concentrations of cytokinins and auxins in the media had improved the initiation of callus growth and shoot proliferation, as the induction media had significant effect on initial culture establishment from capitulum explants (Bhargava et al. 2013). In fact, the stimulation of callus formation by cytokinin such as BAP was promoted by the addition of a strong auxin such as NAA, and the combination of BAP and NAA was more effective for callus and shoot induction than NAA or BAP alone (Pierek and Segers 1973, Bhatia et al. 2008, Hasbullah et al. 2008, and Aswath and Choudhary, 2002). In our experiment, and in accordance to the results of Akter et al. (2008), MS supplemented with 5 mg/l BAP and 1 mg/l NAA, was selected as the best medium for shoot induction in flower buds of both varieties. Additionally, MS supplemented with 2 mg/l BAP and 0.5 mg/l NAA was selected as the best media for callus induction, as it induced great percentages of regenerative compact calluses in both leaf and flower bud explants.

However, when the regenerated shoots were cultured for a longer duration on MS media supplemented with 5.0 mg/l BAP and 1.0 mg/l NAA for shoot multiplication, the shoots became stunted in growth, lanky, showed signs of vitrification, and did not multiply any further. Li et al (2003) anticipated that an excess in cytokinins along with the high water potential of the medium were the major reasons for vitrification of shoots. Although BAP is the best cytokinin for *in vitro* propagation of gerbera, its positive effect on shoot multiplication is related to the detrimental effect on their growth. High concentration of BAP coupled with high humidity often result in vitrification of *in vitro* raised shoots (Kataeva et al. 1991, Jerzy and Lumbosky 1991).

With the results of this current study, it can eventually be possible to develop an efficient and reproducible *in vitro* mass propagation system from different explants of gerbera, which could be profitably used for commercial utilization. In order to meet the export demands of the local and global markets, *Gerbera Jamesonii*, a highly sought out ornamental plant, could be a major source of foreign exchange as a non-traditional export item, opening a new dimension to our economy. This research also opens the windows for further new research to create an efficient and reliable large-scale plant regeneration system that can be exploited for improvement in quality and productivity through genetic transformation and other genetic techniques.

Chapter 5: References

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