Micropropagation of Carnation, Lilium and Gladiolus on different Growth Hormone Supplemented Media

Thesis submitted in the partial fulfilment of requirements for the degree

of

MASTER OF PHILOSOPHY

In

Plant Genomics and Biotechnology

By

Ali Asrar

Department of Plant Genomics and Biotechnology PARC Institute of Advanced Studies in Agriculture. National Agriculture Research Centre, Islamabad Quaid-I-Azam University

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CERTIFICATE

The thesis submitted by **Ali Asrar** to Plant Genomics and Biotechnology (PGB), PARC Institute of Advanced Studies in Agriculture (PIASA), National Agriculture Research Centre (NARC), Quaid-I-Azam University, Islamabad, Pakistan, is accepted in its current form. This thesis fulfills all the requirements for facilitating him with Degree of Master of Philosophy in **Plant Genomics and Biotechnology.**

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I would like to divulge that the data presented in this thesis "**Micropropagation of Carnation, Lilium and Gladiolus on different Growth Hormone Supplemented Media**" is generated by myself from original research work under the supervision of **Dr. Armghan Shahzad**at Department of Plant Genomics and Biotechnology (PGB), PARC Institute of Advanced Studies in Agriculture (PIASA), National Agriculture Research Centre (NARC), Quaid-I-Azam University Islamabad, Pakistan.

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Ali Asrar

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DEDICATION

I would like to dedicate this to my grandparents, parents, siblings and to my wife. Thank you so much to my friends as well who stuck with me throughout my life. I love and cherish each and every one of you.

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
MS Media	Murashige and Skoog media
GDP	Gross Domestic Product
mg/L	Milligram per Liter
g/L	Gram per Liter
L.A. Hybrid	Los Angeles Hybrid
PSI	Pressure per Square Inch
pH	Potential of Hydrogen
C Media	Carnation Media
LM Media	Lilium Multiplication Media
GM Media	Gladiolus Multiplication Media
2,4-D	2,4-Dichlorophenoxyacetic Acid
IBA	Indol-3-butyric Acid
NAA	1-Naphthaleneacetic Acid
IAA	Indole-3-Acetic Acid
TDZ	Thidiazuron
BAP and/or BA	6-Benzylaminopurine
GA_3	Gibberellic Acid
$D2H_2O$	Double Distilled Water
HCl	Hydrochloric Acid
g	Gram
NaOH	Sodium Hydroxide
lx	Illuminance
cm	Centimeter
mL	Milliliter
LSD	Least Significant Difference

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ABSTRACT

The cut flower industry is an untapped market in Pakistan that relies heavily on importing flowers. Using tissue culture technology, it is possible to produce different types of flowers in large quantities that can be sold to local nurseries. Multiple varieties of carnations, lilium and gladiolus were used in the experiment to study the effects of different plant growth regulators such as auxins, cytokinin and gibberellins. Different concentrations of these hormones were added in MS media to determine their effects on the phenotypic traits of cut flowers. Using optimized sterilization techniques, it was observed that maximum growth for carnation was on C-1 medium (0.5 mg/L Kinetin + MS Medium) in terms of plant height and shoot induction. While C-3 medium (0.5 mg/L 1-Napthalene Acetic Acid + MS Medium) produced the highest formation of calluses. For lilium, LM-4 medium (4 mg/L Benzylaminopurine + 1 mg/L Indole-3-Acetic Acid + MS Medium) produced the best cultivars that had the highest number of multiple shoot inductions, the most plant height and the greatest number of leaves. In case of gladiolus, contamination was present in the intercellular spaces of tissues in all types of varieties, so it was not proceeded further.

INTRODUCTION

1.1 Cut Flowers

Cut flowers refer to flowers or flower buds that have been cut from the plant bearing it. These often include the stems and leaves of the plant. Domestication of these plants has been in practice for hundreds of years resulting in a wide variety of ornamental flowers used mainly as decorations or for ceremonial events. In the modern age, the advancements in biotechnology, cultivation and trade of these plants are relatively easier and cheaper which has made floriculture an emerging field. Examples of cut flowers include Lilium, Carnations, Gladiolus, Iris, Daisies, Aster and Chrysanthemums. Flower bulbs such as Tulips, Daffodils and Freesia are also included in the cut flower taxonomy (Toumi*et al.,* 2016).

1.2 Cut Flower Industry of Pakistan

At the moment, most flowers and bulbs in Pakistan are imported from countrieslike Colombia and the Netherlands. The local flower industry, apart from roses, dandelions, marigold and daffodils, has a very limited variety of flowers. By using tissue culture technology, it is possible to grow many of the imported products domestically, free of any pathogens that can be bought and sold at affordable rates.

Statistical data has revealed that Pakistan produces8 to 12 thousand tons of flowers that grow on an estimated area of 7000 hectares. This would suggest that only 0.5% of the total agricultural area is being used by the flower industry. It is an emerging industry of about 2,500 growers and 1,000 wholesalers (Ahmad *et al*., 2018).

In Pakistan, the agricultural sector generates around 19.2% of the total GDP. 60% of the countries‟ export is also crop based. This sector provides employment to 40% of the population. Most of the regionsof Punjab and KPK is suitable for cut flower growth.Horticulture occupies 6%, while floriculture occupies only 0.5% of the land. If given more importance to the floricultural industry, it could positively impact that GDP, economy and the employment rate of the country. In larger cities, the consumption and trade of flowers is higher and different varieties

of flowers are sold. The main hub of flower cultivars in Pakistan is Pattoki where millions of flowers, mainly roses, are either locally distributed to different cities or sent for international exportation. Currently, the standards of flower cultivation in Pakistan are not very high. This is due to the lack of facilities, knowledge, absence of skilled labor and little cooperation between the public and private sectors, resulting in poor cultivation and post-harvest treatment. All of these internal and external issues has resulted in the stagnation and inactivity of the flower industry of Pakistan (Market Insider, 2015).

The production methods and practices of growing these plants are old fashioned and inefficient resulting in poor quality flowers that are not suitable for the international market. Most local growers lack an education and are hesitant of change to more modernized methods (Ahmad *et al.*, 2010).

1.3 Economic Importance of Cut Flowers

The rate at which international trade in cut flowers is growing is exponential. With an estimated worth of $11 billion USD, the cut flower industry is growing by 25% per year. Cut flowers make up to 60% of the world trade in floriculture. From 1995 to 2005, total increase in cut flower demand increased from $0.5 billion USD to $5.1 billion USD and is projected to double again by the end of 2025. In the European Union, the Netherlands is the largest producer of cut flowers, accounting for 68% of all cut flower production in Europe and exports 60 to 70% of its total production to the rest of the world (Usman*et al.,* 2015).

In Pakistan, it was estimated that the average price of flowers for bouquets for randomly selected 100 consumers was around Rs.10,000 PKR per month. In case of special events such as weddings, religious holidays and other ceremonial events, the average prices of flowers increase up to Rs. 100,000 PKR. Depending on the socioeconomic class, the average prices of flower purchases for certain customers would increase and decrease as well (Usman*et al.,* 2015).

Pakistan is a very favorable market place for the flower business since most people are keen on buying them. The flower business in Pakistan is running very successfully. By using modern methods and techniques in the floricultural business, it is possible to produce cut flowers of international standards. Over the last 20 years, some efforts are being made for the export of

floriculture products in Pakistan. Due to the lack of proper guidelines and backing from the public and private sectors, there is an unavailability of infrastructure resulting in little to no export. In 2017, the total amount of flower export was 434 tons which was worth $1.06 million USD. This ranked Pakistan at the 62nd position among the flower exporting counties in the world. Most flowers were exported to Germany and the United Arab Emirates (Farooq *et al*., 2020).

Roses, tuberose, marigold are the most common flowers being used but now people have started taking interest in Lilium and other imported flowers for decoration purposes. In Pakistan, more than 90% of lilies and other flower bulbs are imported from Holland and are grown domestically. The national flower industry of Pakistan is saturated with imported products, causing the prices of flowers to sky rocket. But by using tissue culture technology, it is possible to produce domestic lilies that can be bought and sold at much reasonable rates.

1.4 Carnation

Carnations are a species of Dianthus. Botanically known as *Dianthus caryophyllus,* they are native to the Mediterranean region and have been in cultivation over the past 2000 years. Carnations are also called clove pink. These are perennial plants that have a height of 70 to 80 cm. Their leaves are grayish green and slender in shape and can grow up to 15 cm. They can produce up to 5 flowers which are bright, pink-purple and are often fragrant. Cultivars have been produced that can blossom different colors such as red, blue, green, yellow, white or mixed (Blamey *et al*., 1989).

1.5 Lilium

Lilium is a genus of flowering plants that are herbaceous and grown from bulbs. They have large, bright and very prominent flowers. Members of the lilium genus are all known as true lilies as not all flowers with the name lily in them are true lilies. These flowers are indigenous to the northern subtropics and have a height of 2 to 6 feet. They also form bulbs underground which develop into rhizomes through which multiple small bulbs can form. Most species form these

bulbs deep underground while some form them on the surface, at the base of the plant. Some of the species form stem-roots by which bulbs can grow naturally and after every season, a new stem forms adventitious roots above the soil. (Pelkonen*et al.,* 2012).

Some of the lilium species become dormant around winter, while others that are native to hotter areas with milder winters lose their leaves, entering a short dormant stage in summer.

Lilium is one of the most sought-after ornamental flowers. It is characterized as a high value flower with a very long lifespan and has the affinity for rehydration after transportation over long distances (Zaprianova*et al.,* 2016). Lilies come in all shapes, sizes and colors. Different types of lilies are widely grown in moderate and tropical climates. The flowers are planted as bulbs during the dormant season. In the northern hemisphere, it is best to plant them facing south on a slight slope in a slightly shaded area at a depth of 2 ½ times the height of the bulb. Some species of lilium bloom in July or in August. Flowering periods range from anywhere between late spring, late summer or early autumn. The soil must be porous and have good drainage.The roots of these flowers are contractile that can pull plants to the correct depth. The stems are usually strong but in case of species with heavy flowers, the stem may need stalking, i.e., external support (Resetnik*et al.,* 2007).

1.6 Gladiolus

Gladiolus is a genus belonging to the*Iridaceae gladiolus*family. They are native to parts of Asia, Europe and tropical areas of Africa.

These plants have been in cultivation for the past 2000 years and have been extensively hybridized to point where there is a wide variety of ornamental flowers. This was achieved by crossing over 4 to 5 different species (Cantor*et al.,* 2011).

Usually, these bulbs are placed in the summer time, in the dormant season. Unlike most cut flowers, gladiolus are very hardy plants that can survive temperatures as low as 5°C to 35°C. Bulbs start to form roots and stems during winter, around November, if planted in late August. They also require very little water and partial sunlight. Their roots are thick and can spread very quickly.These flowers also grow from bulbs which have to be placed in well aerated and well-drained soil. The bulb must be planted about 1 ½ times deeper in the soil than the height of the

bulb. The plant can grow up to 30 - 40 cm in height and produce 1 to 9 leaves. Leaves themselves are narrow and grooved longitudinally, inside a sheath. Flowers from these plants are one-sided and can vary small to large which have large spikes around them. Petals and sepals are almost identical to each other due to extensive hybridization (Manning et al., 2008).

1.7 Plant Tissue Culture Technology

The aseptic culturing of cells, tissues and organs using defined physical and chemical conditions *in vitro* is known as plant tissue culture. This technology is important an important tool for both commercial and non-commercial purposes. The origin of plant tissue culture is owed to the German scientist, Haberlandt, who began to explore the fundamental applications of this technology in the late 20th century (Sathyanarayana*et al.,* 2007).

The early fruits of this study resulted in the production of the first root, embryo and callus cultures. From 1940 to 1960, these two decades were noticeable for the innovation of new techniques as well as improvement of old ones already in use (Shukla*et al.,* 2012). These techniques opened a new horizon for research in different fields of plant sciences such as cytology, pathology, plant breeding and the development of new varieties.

In the 1990s, expansion of *in vitro* technologies and their applications resulted in an increase in the number of plant species. In the fields of biochemistry and plant biology, cell cultures have become an important tool for basic plant knowledge and since then have become fundamentally important in molecular and agricultural biotechnology in the 21st century (Thrope*et al.,* 2012).

This technology involves the excision of plant tissue (explant) and growing it on a nutrient medium. Due to the totipotency of plants, the explant has the capacity to regenerate into a complete individual entity. In terms of capability, plant tissue culture can be used in a number of ways such as meristem culturing of virus/disease free plants for propagation, cell suspension, protoplast culture, tissue or organ culture, etc. This technique focuses on the inoculation of explants on growth mediums. Basic laboratories that perform biochemical experiments generally meet most requirements for tissue culturing. This technology is invaluable as it can help advance our knowledge in morphogenesis, cell signaling, physiology and molecular biology (Kumar*et al.*, 2012).

It is used in the micropropagation or plant cloning. The following are advantages of utilizing these techniques:

- Production of disease-free plants
- It helps to produce transgenic plants resulting in the development of new varieties in shorter time.
- Faster plant maturation.
- Production of plants without the need for seeds or pollinators.
- Ability to produce seeds of plants that have otherwise low chances of germination. Such as Orchids.

Plant tissue culture relies on the ability of a plant cell to regenerate into an entire whole plant (totipotency). Explants used in these techniques range from single cells, protoplast, pieces of leaves, stems or roots. New plants can be grown on media using appropriate hormones and growth regulators (Georgiev *et al.,* 2011).

Further applications of plant tissue culture include:

- Commercial cultivation of plants that are used in landscaping, ornamentals and use different cultures to produce a large number of identical plants such as shoot culture and meristem culture (Bhatia *et al.,* 2012).
- Conservation of rare plants (Szczecińska*et al.,* 2016).
- Screening of cells for desirable traits such as tolerances/resistances (Beckles *et al.,* 2012).
- Production of plant originated metabolites and proteins used in pharmaceuticals (Yao *et al.,* 2015).
- Regeneration of novel strains and crossing of species by protoplast fusion (Wang *et al.,* 2012).
- Study of physiological, reproductive and biochemical mechanisms in plants on a molecular basis such as stress tolerance (Hasanuzzaman*et al.,* 2013).
- Tissues for transformation, followed by either short-term testing of genetic constructs or regeneration of transgenic plants. (Low *et al.,* 2018).
- Clean plant material can be produced using meristematic tissue cultures.
- Identical hybrid production through stomatic embryogenesis.

1.8 Totipotency

Plant cells have the capacity to adapt in accordance with any environmental changes. A single cell can regenerate into a whole plant via somatic embryogenesis without any fertilization. This ability of plants is known as Totipotency. Plant tissue culture technology takes advantage of this by using meristematic tissues from plants, known as explants, and grows them into entire plants *in vitro*. During this process, callus can form as well (Mitalipov *et al.,* 2009).

1.9 Importance of Tissue Culture Techniques in Cut Flower Industry

Tissue culture technology has made it possible to cultivate flowers using artificial growth mediums. Thousands of flowers can be grown inside glass apparatus" in a small growth room where temperature and light can be controlled. By using a single flower or flower bulb, it is possible to multiply and propagate it into ample amounts of plants.

The source of the explant can be initially screened for viruses/diseases which would result in creating future cultivars that are free of any pathogens.Strict sterilization guidelines ensure no contamination in future inoculations. By taking advantage of the totipotency of plants, it is possible to artificially grow flowers that are out of season and acclimatize them inside green houses. Many cut flowers can be made available all year-round using tissue culture technology. The export of tissue culture grown plants is relatively simpler as it complies with the strict quarantine rules, thus making transportation across borders hassle-free.

1.10 Potential of Tissue Culture Technology

The areas of plant tissue culture represent a promising outlook on the future at present time. These include the fields of micropropagation of ornamental plants, forest trees, production of pharmaceutical compounds, plant breeding for better nutritional value of food staple crop and the cryopreservation of rare and valuable germplasm.

Uniform, disease free and better-quality production of plants is only possible through micropropagation. This technology would generate new job opportunities for not only laboratory personnel but for farmers, producers and nursery owners as plant production would be carried

out throughout the year. High quality fruits and vegetables, ornamental flowers and other foliage would be produced at all times irrespective of the weather and season. Quality control is also very important in this field. Selection of explant sources, disease free material and the detection and elimination of soma-clonal variants are important parameters for ensuring plant quality. Consistent and well managed production would garner confidence and repute with consumers.

Invitro culture has a role in sustainable agriculture and has been successfully used in plant breeding. Since the last 20 years, considerable efforts have been made in bioproduction, biotransformation and bioconversion using this technology. Commercial production of medicinal compounds can be obtained on a large scale.

Plant tissue culture has made many advancements. One of the most significant roles is the association of transgenic plants in the future. The ability to accelerate conventional multiplication rates can benefit many countries that are facing problems such as disease or unfavorable climatic disasters which can wipe out crops. Loss of genetic resources is very common. Cryopreservation has been proposed as a solution. But by using tissue culture, a duplication collection can be created in order to preserve genetic information. To reactivate these germplasms and rapidly increase their multiplication rate, tissue culture can be used. Thus, overcoming the limitations of conventional breeding and increasing more complex genetic transformation work for future generations as technology inevitably advances (Altaf *et al.,* 2011).

1.11 Aims and Objectives

The aim of this study was to standardize a method of cut flower production through tissue culture.

- To optimize sterilization techniques for Carnation, Lilium and Gladiolus.
- To optimize growth media for tissue culturing of Carnation, Lilium and Gladiolus.
- To determine which explant would prove most successful for tissue culturing.

MATERIAL AND METHODS

2.1 SAMPLE COLLECTION

Plant material used for the experiment were seeds and flower bulbs. These were bought from various nurseries in Lahore, Pakistan. All the bulbs and seeds were grown *in vitro* conditions inside test tubes on solid Murashige-Skoog medium. (Appendix I).

The different variety of seeds and bulbs include:

2.1.1 Seed Varieties:

Carnations (*Dianthus caryophyllus)*:

- Seeds of multi-colored flowers

2.1.2 Bulb Varieties:

Lilium (*Liliaceaecandidum)*:

- Casa Blanca (white)
- L.A. Hybrid (white)

Gladiolus (*Iridaceae gladiolus*):

- Nymph (Pink)
- Robinetta (Red)
- Windsong (Yellow)
- White Prosperity (White)
- Black Beauty (Maroon)

2.2 STERLIZATION

2.2.1 Sterilization Process of Safety Cabinet and Equipment

The biosafety cabinet (ESCO, E-Series) was sterilized with a 70% ethanol solution. The work area, walls, protective shield and roof were completely cleaned. A spirit lamp was placed inside in order to heat up the air and create a sterilized zone. Scalpel set is used in plant tissue culturing

which consists of a large and small forceps and a scalpel. All equipment along with petri dishes and glass jars were autoclaved at 121°C and 15 PSI (Cassells*et al.,* 2012). After autoclave, the equipment was opened inside the biosafety cabinet.

All sterilization was done using 70% ethanol solution. Inside the biosafety cabinet, all apparatus were constantly sterilized using flame sterilization/dry heat treatment.

2.2.2 Sterilization of Flower Seeds

Carnation seeds were washed using 40% bleach solution (Appendix II) for 15 minutes and washed twice with distilled H_2O for 20 minutes. The seeds were placed out on sterilized glass petri dishes inside the biosafety cabinet for drying before inoculation.

2.2.3 Sterilization of Flower Bulbs

Lilium and Gladiolus bulbs were sterilized using the following method:

1. Bulbs were surface sterilized using commercial washing detergent (VIM) followed by multiple washings using sterilized water. The bulbs were dried at room temperature.

2. Once the samples were dried, they were taken into the biosafety cabinet where the outer surface was sterilized again using 70% ethanol solution.

3. The scales and inner meristematic tissue known as the “dome” of the bulbs were separated and cut into small pieces (explants) using a sterilized scalpel set in petri dishes.

4. Explants were washed with 50% bleach solution (Appendix II) with ddH_2O and Tween-80 for 20 minutes under UV light for the first 10 minutes.

5. In the last 20 minutes the samples were shaken vigorously by hand with two-minute intervals.

6. Bleach solution from the jars was removed and explants were washed twice with ddH_2O for 15 minutes each, while constantly shaking.

7. After second washing, the water was removed and the explants were dipped in ddH_2O containing a drop of 50% bleach solution.

2.3 PREPARATION OF STANDARD MURASHIGE-SKOOG MEDIA

Murashige-Skoog media (El-Naggar *et al.,* 2016) was used for the growth of explants in all experiments. All ingredients were weighed carefully using a scale and dissolved in ddH_2O.

Recipe for Murashige-Skoog Media per liter:
Murashige-SkoogMedia (Phytotech): 4.43 g/L
Sugar (Commercial): 30 g/L
Gellan Gum (Phytotech): 2.2 g/L

Final pH: 5.80

The pH was adjusted using 0.1N HCl or 0.1N NaOH, as 5.80 to 5.85 is the essential pH range to activate gellan gum. The media was heated in microwave oven for 20 to 30 minutes until it started boiling.

The media was removed from the oven and allowed to cool down followed by gentle shaking for complete mixing and equal distribution of gellan gum throughout the solution.

The prepared media, while still hot, was poured into glass apparatus as follows:

Large test tubes (15 mL)
Flasks (60 mL)
Jars (30 mL)

After pouring, the media was autoclaved at 121°C for 20 minutes.

2.4 MULTIPLICATION AND MICROPROPAGATION OF CARNATION

2.4.1 Preparation of Media for Carnation

The following is a recipe for media used for the initial growth and germination of carnation seeds in large test tubes.

Carnation Growth Media (C Media)

The recipe of media stated below was used in the initial growth and sprouting of seeds of carnation explants in large test tubes (Thu *et al.,* 2020).

Murashige-Skoog: 4.43 g/L
Sugar: 20 g/L
Gellan Gum: 2.2 g/L

Final pH: 5.80

The following are the amounts of growth hormones added to C media for direct multiplication of carnation explants (Table 2.1).

Table 2.1 Carnation Multiplication Media (C Media)

	Kinetin (mg/L)	**BAP (6-Benzylaminopurine) (mg/L)**	**NAA (1-Naphthalene Acetic acid) (mg/L)**	**TDZ (Thidiazuron) (mg/L)**
C-1	0.5	0	0	0
C-2	0	0.5	0	0
C-3	0	0	0.5	0
C-4	0	0	0	0.5

2.4.2 Inoculation of Carnation Seeds:

Large test tubes containing media were sprayed with 70% ethanol on the surface and placed inside the safety cabinet. Sterilized seeds were inoculated by picking up each seed using forceps and dropped inside the test tube. Each test tube contained 2 seeds.

Carnation seeds were sterilized as described in 2.2.2. The test tubes were placed at 26°C in the growth room with a photoperiod of 16 hours light and 8 hours dark with a light intensity of 7000 to 9000 lx for 21 days. The test tubes were observed daily for germination.

2.4.3 Initial Growth of Carnation

In the biosafety cabinet, a total, 36 test tubes were prepared with no formation of contamination throughout the initial sprouting/growth phase.

Seeds were grown *in vitro* and in C Media for initial growth. Test tubes were placed in growth

Figure 2.1.1 (Sterilized Seeds) Figure 2.1.2 (Seed Inoculation)

2.4.4 Multiplication

Within the biosafety cabinet, the plants were taken out from test tubes, and using an autoclaved scalpel set and petri dish, the plants were cut from their nodes. Each explant was inoculated in jars containing C Media, resulting in a total of 60 jars, 15 of each type of C Media.

C1

Figure 2.1.3 (Initial
M)

Figure 2.1.4 (Initial
N ı)

Figure 2.1.5 (Initial
Multiplication of Carnation)

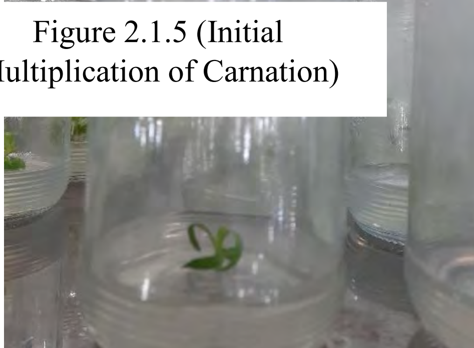


Figure 2.1.6(Initial
Multiplication of Carnation)

2.5 MULTIPLICATION AND MICROPROPAGATION OF LILIUM

2.5.1 Preparation of Media for Lilium

The recipe of medium stated below was used in the initial growth of lilium explants excised from bulbs (Mir *et al.,* 2012).

Lilium Growth Media (LM Media)
Murashige-Skoog: 4.43 g/L
Sugar: 30 g/L
Gellan Gum: 2.2 g/L

Final pH: 5.80

The following are the amount of growth hormones added to prepare LM media for direct multiplication in lilium explant (Table 2.2).

Table 2.2 Lilium Multiplication Media (LM Media)

	BAP (6-Benzylaminopurine) (mg/L)	**IAA** (Indole-3-Acetic Acid) (mg/L)
LM-1	1	1
LM-2	2	1
LM-3	3	1
LM-4	4	1

2.5.2 Inoculation of Lilium Bulbs

Plants were treated with GA_3 hormone 24 hours prior to excision in order to promote growth. Meristematic tissue of scales and domes from bulbs were inoculated and growth rate was observed in 21-day intervals.

Glass jars containing solidified media were placed inside the biosafety cabinet after spraying each of them with 70% ethanol solution.

The sterilized explants were inoculated by picking them up with the autoclaved forceps and placed inside the glass apparatus containing the media. Scales were cut width-wise and the domes were cut into halves and quarters. Stems from the bulbs were cut into 2 to 5 sections depending on the number of nodes present.

They were placed such that, the exposed explant meristematic tissue was slightly inside the growth medium. Each jar contained 1 to 3 samples while test tubes and flasks contained 1 explant sample.

After inoculation of each explant, forceps were rinsed in 70% ethanol and flame sterilized. Jars and flasks were removed from the safety cabinet after inoculation, labelled and transferred into the growth room where the temperature was 25°C with a photoperiod of 16 hours light, 8 hours dark and light intensity of 7000-9000 lx.

After the first interval, whole, half and quarter domes had shown significantly better initiation compared to the scales and stems. The amount of contamination was also considerably less than other explant of the same samples.

Previous samples of Lilium of different varieties were used earlier in the experiment that had given similar results but due to contamination, these samples were discarded.

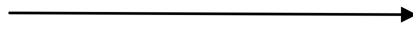
2.5.3 Initial Growth of Lilium

Lilium bulbs were sterilized and cut using the sterilization protocols mentioned above, in a Biosafety Cabinet. A new scalpel blade for each bulb was used. The shoots and domes were separated in different jars during the sterilization process.

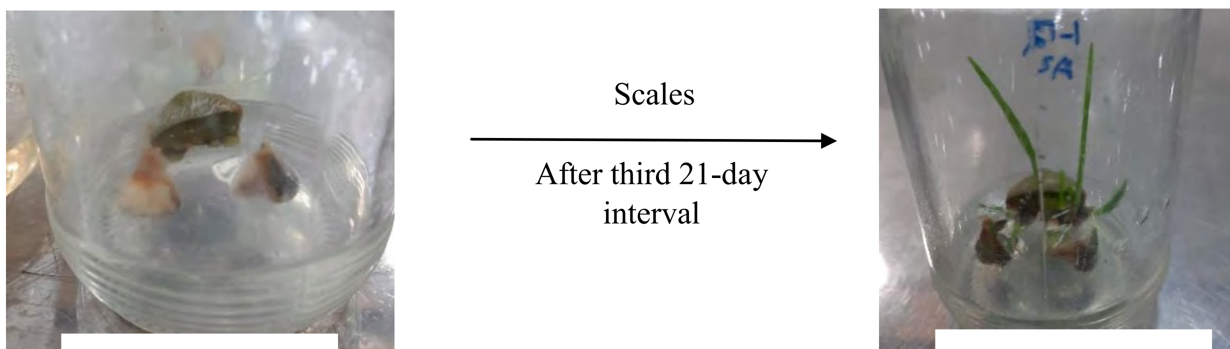
Whole dome

After first 21-day interval

Stem



After second 21-day interval



Each explant was inoculated into LM Media for general growth inside of flasks as risk of contamination in these them was less.

In the case of Lilium, a total of 5 bulbs had been used for initiation. These bulbs were of the L.A. variety. Each bulb was sterilized using the above-mentioned protocols which yielded a total of 63 usable inoculations in flasks containing LM Media. Out of the 63 samples, only 40 were left uncontaminated after 7 days in the growth room.

During each interval, samples were observed for detection of contamination and availability of light which being monitored using a lux meter and possible formation of callus was also being observed daily.

2.5.4 Multiplication

After 21 days, explants had shown growth in LM media. The inoculations were taken out of the growth room and the jars were sprayed with ethanol and placed inside the biosafety cabinet. Using a scalpel set, the explants were taken out of the jars and cut from their nodes, buds and other parts of their meristematic tissues.

These explants were inoculated into flasks containing LM media and placed into the growth room.

Figure 2.2.7All explants were washed and prepared for inoculation in LM media.

A total of 40 inoculations were prepared and placed in the growth room. Daily observations were made in order to detect any contamination and growth of the explants.

2.6 MULTIPLICATION AND MICROPROPAGATION OF GLADIOLUS

2.6.1 Preparation of Media for Gladiolus

The recipe of media stated below was used in the initial growth of gladiolus explants excised from bulbs. (Memon *et al*., 2013).

Gladiolus Growth Media (GM Media)

Murashige-Skoog: 4.43 g/L

Sugar: 30 g/L

Gellan Gum: 2.2 g/L

Final pH: 5.80

The following are the amounts of growth hormones added to GM media for direct multiplication of gladiolus explants (Table 2.3).

Table 2.3 Gladiolus Multiplication Media (GM Media)

	BAP (6-Benzylaminopurine) (mg/L)	**IAA** (Indole-3-Acetic Acid) (mg/L)
GM-1	1	1
GM-2	2	1
GM-3	3	1
GM-4	4	1
GM-5	5	1

Prior to excision, gladiolus blubs were treated with GA_3 and dried overnight in the dark. Different varieties of gladiolus bulbs were sterilized and cut inside a biosafety cabinet. Explants used consisted of meristematic tissue of scales and domes.

All bulbs were prewashed prior to explant excision using detergent and rinsed under tap water after which they were dried overnight.

2.6.2 Inoculation of Gladiolus Bulbs

Gladiolus samples were taken into the biosafety cabinet and excised using autoclaved equipment in an aseptic environment. Multiplication media was prepared with different levels of growth hormones present in each of the media and placed inside the growth room where the temperature was maintained at 25°C with a photoperiod of 16 hours light and 8 hours dark with the light intensity of 7000 to 9000 lx. In total 250 jars were made (50 jars containing each type of media LM-1, LM-2, LM-3, LM-4, LM-5).

2.7STATISTICAL ANALYSIS

In order to determine the least significant difference and level of significance between media treatments for Carnation and Lilium, ANOVA and LSD were used. By using these statistical methods, difference between media treatments were calculated.Alpha, the significance level, is the probability that you will make the mistake of rejecting the null hypothesis when in fact it is true. If the p-value is greater than alpha, you accept the null hypothesis. Therefore, the alpha value was set to 0.05.

RESULTS

3. EFFECTS OF GROWTH MEDIA

3.1 Effects of Different Growth Media on Carnation

In the case of carnations, Kinetin, BAP, NAA, TDZ (C-1, C-2, C-3, C-4 respectively) were used in order to optimize the best growth medium. After initial growth on C media, the plants were sub-cultured twice onto 4 media variants i.e., C-1, C-2, C-3, C4.

3.1.1 First sub-culture of Carnation

In a total of 100 jars of ea [illegible] 2, C-3 and C-4), inoculated samples were sub-culturec [illegible] s was observed for growth. No contamination was found during this time.

3.1.2 Second sub-culture of Carnation

[illegible] of media C-1, C-2, C-3 and [illegible] lant [illegible] ce again and were given 21 days [illegible] was found after the given time.

A total of 10 samples were selected at random and measured.

Using LSD, a grading system was used to determine the best treatment of each medium for the growth of Carnation plants with the highest-grade being A and the lowest grade being C.

Table 3.1 Phenotypic data of Carnation on C-1 medium

	Height of Plant (cm)	Number of Shoots	Number of Leaves
1	3.1	6	13
2	9.6	4	4
3	7	8	13
4	8	8	15
5	8.5	2	5
6	3.2	2	6

7	11.1	1	5
8	12.6	4	8
9	10	1	2
10	3.2	5	9
Mean Value	**6.30**	**4.10**	**8.00**

Table 3.2Phenotypic data of Carnation on C-2 medium

	Height of Plant (cm)	**Number of Shoots**	**Number of Leaves**
1	4.1	5	7
2	4.8	5	8
3	5.2	5	10
4	5	5	10
5	6	4	7
6	6.3	4	7
7	9.3	4	7
8	2.6	7	12
9	3	6	8
10	1.7	5	8
Mean Value	**4.80**	**5.00**	**8.40**

Table 3.3Phenotypic data of Carnation on C-3 medium

	Height of Plant (cm)	**Number of Shoots**	**Number of Leaves**
1	5.9	3	6
2	6.5	2	7
3	6.8	3	9

4	7.5	5	8
5	4.9	2	6
6	4.6	5	8
7	5.8	3	7
8	5.6	2	6
9	4.7	2	4
10	4.6	3	7
Mean Value	**5.69**	**3.00**	**6.80**

Table 3.4Phenotypic data of Carnation on C-4 medium

	Height of Plant (cm)	**Number of Shoots**	**Number of Leaves**
1	4.2	5	12
2	4.4	6	14
3	5	1	5
4	1.1	3	6
5	1.3	3	8
6	1.5	1	8
7	5.6	4	10
8	8.9	3	9
9	1.2	2	10
10	2	2	11
Mean Value	**3.52**	**3.00**	**9.30**

Height of Plant

Standard Error of a Mean: 0.7783
Standard Error (Difference of 2 Means): 1.1007

Number of Leaves

Standard Error of a Mean: 0.8884
Standard Error (Difference of 2 Means): 1.2563

Number of Shoots

Standard Error of a Mean: 0.5449
Standard Error (Difference of 2 Means): 0.7706

Table 3.5 Mean Values of Plant Height for Carnation (Mean of 10)

Variant	Mean Plant Height (cm)
C-1	7.63
C-2	4.80
C-3	5.69
C-4	3.52
LSD Value	**2.2324**

Table 3.6 Mean Values of Number of Shoots for Carnation (Mean of 10)

Variant	Mean Number of Shoots (cm)
C-1	4.10
C-2	5.00
C-3	3.00
C-4	3.00
LSD Value	**1.5629**

Table 3.7 Mean Values of Number of Leaves for Carnation (Mean of 10)

Variant	Mean Number of Leaves (cm)
C-1	8.00

C-2	8.40
C-3	6.80
C-4	9.30
LSD Value	**2.5479**

The mean values of each parameter determined that the only significant difference developed in the plants inoculated in the 4 different media was the plant height. C-1 medium had promoted the most height in the plant while C-4 medium had the highest number of leaves. All types of media generated the same rooting type.

In C-3 multiple callus" was also detected.

3.2 Effects of Different Growth Media on Lilium

The following figures distinguish the various phenotypic characteristics developed by Lilium explants in the experiment where they were inoculated in the different variants of MS media.

From each of the 4 different media, 10 sub-cultures were taken at random and measured their phenotypic data.An illuminance of 8000 lx was also optimized as the plants within that range of light intensity or higher had shown better growth.

Lilium Inoculations on LM Medium after twenty-one days: -

3.2.1First sub-culture of Lilium

All d for sub-culturing. To initiate the first sub-c ations of LM Medium were made and labeled as LM-1, LM-2, LM-3 and LM-4.

A total of 30 jars for each medium were made and the same sterilization protocol was followed as the prior inoculation. Each sample was placed in the growth room and observed for a period of 7 days to observe for contamination.

After one week in the growth room, out of the total 120 jars prepared only 63 samples were left.

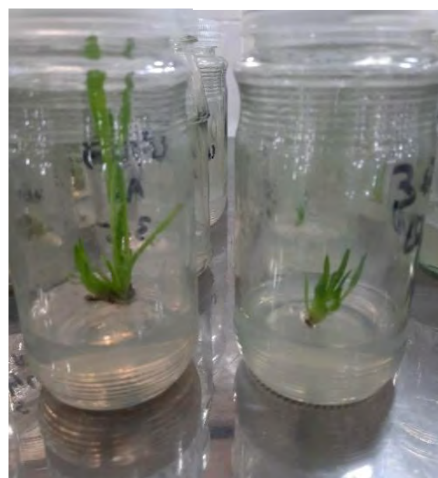
LM-1: 7

LM-2: 5

LM-3: 20

LM-4: 31

These 63 jars were kept in the growth room for an interval of twenty-one days after which they were sub-cultured for a second time.



3 um

For the second sub-culture, a total of 160 jars (40 jars of each media) were prepared. Sterilization was done for sub-culturing. After another twenty-one-day interval, a total of 111 samples were free of contamination.

LM-1: 17

LM-2: 19

LM-3: 35

LM-4: 40

The samples were kept in the growth room for further growth and root initiation.

Data was taken from 10 randomly selected plants and the same grading system that was used for carnations was implemented for Lilium media, with A being the highest grade and C being the lowest grade.

Table 3.6Phenotypic data of Lilium onLM-1 medium

	Height of Plant (cm)	Number of Shoots	Number of Leaves

1	6.2	9	9
2	6	10	14
3	7.2	4	13
4	7.8	3	11
5	6.5	4	11
6	7.7	2	10
7	6.4	2	11
8	6	10	10
9	5.6	6	12
10	6.7	5	9
Mean Value	**6.61**	**5.50**	**11.00**

Table 3.7Phenotypic data of Lilium onLM-2 medium

	Height of Plant (cm)	**Number of Shoots**	**Number of Leaves**
1	5.6	2	3
2	6.4	4	8
3	7.8	4	7
4	7.8	3	8
5	8.1	2	8
6	9.6	4	8
7	6.4	6	9
8	3.9	7	8
9	6.5	4	3
10	6.8	4	1
Mean Value	**6.89**	**4.00**	**6.30**

Table 3.8 Phenotypic data of Lilium onLM-3 medium

	Height of Plant (cm)	**Number of Shoots**	**Number of Leaves**

1	10.7	13	7
2	8.1	5	6
3	8.9	8	6
4	9.1	10	8
5	9.8	2	6
6	7.1	5	5
7	9.6	5	6
8	7.3	4	5
9	8.2	6	6
10	9.8	2	5
Mean Value	**8.86**	**6.00**	**6.00**

Table 3.9Phenotypic data of Lilium onLM-4 medium

	Height of Plant (cm)	**Number of Shoots**	**Number of Leaves**
1	12.4	16	26
2	8.7	12	18
3	12.9	15	27
4	9.2	13	16
5	9.4	14	15
6	11.5	14	21
7	10.8	13	22
8	9.7	15	19
9	10.1	14	23
10	9.2	11	20
Mean Value	**10.39**	**13.70**	**20.70**

The mean value of each of the samples was calculated and it was determined that the plants in LM-4 medium had the greatest height among the 4 types of media. This was due to the high

amount of 6-Benzylaminopurine (BAP) present, as evident by the gradual increase in height with the increase of BAP in each media.

Height of Plant

Standard Error of a Mean: 0.4011
Standard Error (Difference of 2 Means): 0.5673

Number of Leaves

Standard Error of a Mean 0.8141
Standard Error (Difference of 2 Means): 1.1513

Number of Shoots

Standard Error of a Mean: 0.8236
Standard Error (Difference of 2 Means): 1.1648

Table 4.0 Mean Values of Plant Height for Lilium (Mean of 10)

Variant	Mean Plant Height (cm)
LM-1	6.61
LM-2	6.89
LM-3	8.86
LM-4	10.39
LSD Value	**1.1506**

Table 4.1 Mean Values of Leaves for Lilium (Mean of 10)

Variant	Mean Number of Leaves (cm)
LM-1	5.50
LM-2	4.00
LM-3	6.00
LM-4	13.70
LSD Value	**2.3350**

Table 4.2 Mean Values of Number of Shoots(Mean of 10)

Variant	Mean Number of Shoots (cm)
LM-1	11.00
LM-2	6.30
LM-3	6.00
LM-4	20.70
LSD Value	**2.3622**

The highest number of shoot induction was present in LM-4 medium. Each of the plants contained more than 10 shoots with the greatest number of leaves. This leads to believe that increasing the number of BAP while keeping IAA constant at 1mg/L would result in a better plant. An upper limit to the addition of BAP was not determined.

3.3 Effects of Different Growth Media on Gladiolus

After a twenty-one-day interval, all samples were contaminated by fungus. More samples had been inoculated for initiation, however after another interval, they had all developed contamination. Stronger sterilization techniques had been used but yielded the same results. Further analysis had revealed that the sterilization protocols had been sufficient but the samples had contained fungal spores in the inter-cellular spaces of the samples making it impossible to remove the possibility of preparing clean inoculations.

The experiment for gladiolus had been repeated multiple times using different sterilization strategies. Each time contamination had occurred which led to the hypothesis that the fungal and bacterial contamination was present within the intercellular spaces of the bulbs.

DISCUSSION

The optimization and multiplication of flowers has shown that it is possible to grow these imported ornamentals locally in Pakistan. Tissue culture technology is proving to be more and more essential to countries that are spending millions of dollars annually on imported goods. By implementing and encouraging the use of this technology, the flower market will be able to see a rise in locally grown ornamentals. The geological disadvantages, countries have with the growth of some plants has essentially been addressed thanks to this form of biotechnology.The abiotic limitations of water, sunlight, air, soil, climate have become non-existent. Using a small explant, it is possible to grow virus free, healthy plants inside laboratories (Sadeghia *et al.,* 2015).

Plant tissue culture technology is a form of biotechnology in which a single cell can be used to grow (regeneration) an entire plant on a nutrient rich medium. The objective of this research is the micropropagation and multiplication of cut flowers artificially using different ratios of plant growth regulators and various sterilization techniques.

The addition of BAP influenced the growth of plants in terms of height, number of leaves and flowers produced. It also influenced early flowering (Nambiar*et al.,* 2012). By adding kinetin, the length & number of shoots and roots increased as well as the number of nodes (Hesar*et al.,* 2011). GA_3 had effects that promoted cell division and cell elongation (Sharifuzzaman*et al.,* 2011). 2,4-D is an effective auxin which is used to promote the growth of broadleaf plants and induce callus formation (Özkul*et al.,* 2016). IBA is an auxin which was used for the initiation of roots, leaves and stems (Frick*et al.,* 2018). NAA was used to increase the number of roots and height of the plant (Khandaker*et al.,* 2017). IAA was added for its ability to induce root formation and its positive effects on rhizobium (Li *et al*., 2014). TDZ was used for inducing early flowering in plants as well as shoot proliferation (Dewir*et al.,* 2018).

Carnation seeds were germinated *in vitro.* Four different variants of MS media were prepared, C-1, C-2, C-3, C4, containing Kinetin, BAP, NAA and TDZ as growth regulators, respectively. Height was measured, number of leaves and shoots were counted and root formation was observed. C-1 media was found to be best for multiple shoot inductions while C-3 was effective in callus formation.

Lilium bulbs were sterilized and grown on four different variants of MS media, LM-1, LM-2, LM-3 and LM-4. These contained a fixed amount of IAA along with BAP at different concentrations ranging from 1 mg/L to 4 mg/L, respectively. LM-4 was found to be the best as plants grown in this medium had the maximum number of shoots, leaves and height.

Gladiolus bulbs were inoculated on five different variants of MS media. GM-1, GM-2, GM-3, GM-4 and GM-5. All these variants were made by adding different concentrations of BAP, ranging from 1 mg/L to 5 mg/L, respectively, while IAA was kept constant. Results were inconclusive after multiple tries and over 1000 inoculations. After constant alteration of the sterilization protocols to the point where 100% bleach solution was being used, the inoculations still developed fungal contamination. This led to the hypothesis that contaminants were present in the inter-cellular spaces of the bulbs. Therefore, this work was not pursued.

A total ~350 plants were artificially grown using 72 seeds of carnation plants and ~141 plants of lilium were cultivated using 5 bulbs. This proves that this technology has the potential to give a positive outcome to the cut flower industry.

Future Prospects

The floral industry of Pakistan is still an untapped market. The importation of different flowers from Europe has stagnated the potential growth of the local flower industry in Pakistan. As seen in the case of gladiolus in the experiment, contaminated bulbs are being transported within the country with foreign diseases and contaminations which will eventually disturb the resident biome leading to all sorts of problems within the soil and flora growing on it.

By using tissue culture technology, the production of local flowers can directly impact the economy by marginalizing the import of flowers and promoting home grown flora which can be produced cheaper, larger quantities in a smaller space and selling them to local nurseries at a cheaper rate.

Early detection and analysis of viruses and contaminations using Double Sandwich Enzyme Linked Immuno Sorbent Assay (DS-ELISA), can limit the number of possible pathogenic seed borne diseases and help in developing a “heathier” flower market.

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APPENDICES

Appendix-I (Murashige-Skoog Medium)

Murashige-Skoog Medium

MS Medium: 4.43g/L (Phytotech Lab)

Sugar: 30g/L (Commercial)

Gellan Gum: 2.2g/L (Phytotech Lab)

Water: 1000mL

Final pH: 5.8

Plastic container placed on magnetic stirrer. One-liter ddH2O, 4.43g MS Medium and 30g sugar were added to the container and mixed. pH Meter (Fisher, USA) was used to check initial pH. Final pH was adjusted to 5.80 using 1N HCl and 1N NaOH solution. 2.2g gellan gum was dissolved afterwards. Container was placed in microwave and solution was heated till boiling. Medium was stirred and poured inside glass apparatus. All medium was then autoclaved.

Appendix-II (Bleach Solution)

40% Bleach Solution

Bleach: 400mL (Clorox, USA)

Water (Distilled): 600mL

50% Bleach Solution

Bleach: 500mL (Clorox, USA)

Water (ddH2O): 500mL

Tween-80: 1mL to 2mL (Riedel-Haen)

Appendix-III (Growth Hormones)

Growth hormone powders were weighed on microbalance and dissolved in ethanol inside falcon tubes. 25mL to 30mL distilled water (ddH2O) was added to each falcon tube. Different concentrations were prepared using various amounts of growth hormones, creating stock solutions. All growth hormones were manufactured by Phytotech Labs.

Auxins:

- 2,4-Dichlorophenoxyacetic Acid (2,4-D)

Formula: $C_8H_6Cl_2O_3$

- Indol-3-butyric Acid (IBA)

Formula: $C_{12}H_{13}NO_2$

- 1-Naphthaleneacetic Acid (NAA)

Formula: $C_{12}H_{10}O_2$

- Indole-3-Acetic Acid (IAA)

Formula: $C_{10}H_9NO_2$

- Thidiazuron (TDZ)

Formula: $C_9H_8O_4$

Cytokinin:

- Kinetin

Formula: $C_{10}H_9N_5O$

- 6-Benzylaminopurine (BAP or BA)

Formula: $C_{12}H_{11}N_5$

Gibberellin:

- Gibberellic Acid (GA_3)

Formula: $C_{19}H_{22}O_6$

Appendix-IV (ANOVA-Carnation)

Completely Randomized AOV for Height of Plant

Source	DF	SS	MS	F	P
Treatment	3	89.510	29.8367	4.93	0.0057

1. Grand Mean: 5.4100
2. CV: 45.50
3. Bartlett's Test of Equal Variances: **Chi-Sq (**10.9), **DF** (3), **P** (0.0121)
4. Cochran's Q: 0.4920
5. Largest Var / Smallest Var: 11.652
6. Component of variance for between groups: 2.37787
7. Effective cell size: 10.0
8. Alpha: 0.05
9. Standard Error for Comparison: 1.1007
10. Critical T Value: 2.028
11. Critical Value for Comparison: 2.2324
12. There are 2 groups (A and B) in which the means are not significantly different from one another.

Completely Randomized AOV for Number of Leaves

Source	DF	SS	MF	F	P
Treatment	3	32.275	10.7583	1.36	0.2695

1. Grand Mean: 8.1250
2. CV: 34.57
3. Bartlett's Test of Equal Variances: **Chi-Sq** (13.4), **DF** (3), **P** (0.0038)
4. Cochran's Q: 0.6125
5. Largest Var / Smallest Var: 9.8864
6. Component of variance for between groups: 0.28667
7. Effective cell size: 10.0
8. Alpha: 0.05
9. Standard Error for Comparison: 1.2563
10. Critical T Value: 2.028
11. Critical Value for Comparison: 2.5479
12. There are no significant pairwise differences among the means.

Completely Randomized AOV for Number of Shoots

Source	DF	SS	MF	F	P
Treatment	3	28.075	9.35833	3.15	0.0366

1. Grand Mean: 3.7750

2. CV: 45.65
3. Bartlett's Test of Equal Variances: **Chi-Sq** (10.8), **DF** (3), **P** (0.0127)
4. Cochran's Q: 0.5884
5. Largest Var / Smallest Var: 7.8625
6. Component of variance for between groups: 0.63889
7. Effective cell size: 10.0
8. Alpha: 0.05
9. Standard Error for Comparison: 0.7706
10. Critical T Value: 2.028
11. Critical Value for Comparison: 1.5629
12. There are 2 groups (A and B) in which the means are not significantly different from one another.

APPENDIX-V (ANOVA-Lilium)

Completely Randomized AOV for Height of Plant

Source	DF	SS	MF	F	P
Treatment	3	94.753	31.5842	19.6	0.0000

1. Grand Mean: 8.1875
2. CV: 15.49
3. Bartlett's Test of Equal Variances: **Chi-Sq** (4.84), **DF** (3), **P** (0.1842)
4. Cochran's Q: 0.3747
5. Largest Var / Smallest Var: 4.3865
6. Component of variance for between groups: 2.99751
7. Effective cell size: 10.0
8. Alpha: 0.05
9. Standard Error for Comparison: 0.5673
10. Critical T Value: 2.028
11. Critical Value for Comparison: 1.1506
12. There are 3 groups (A, B, etc.) in which the means are not significantly different from one another.

Completely Randomized AOV for Number of Leaves

Source	DF	SS	MF	F	P
Treatment	3	567.800	189.267	28.6	0.0000

1. Grand Mean: 7.3000
2. CV: 35.27
3. Bartlett's Test of Equal Variances: **Chi-Sq** (9.44), **DF** (3), **P** (0.0240).

4. Cochran's Q: 0.4526
5. Largest Var / Smallest Var: 5.3731
6. Component of variance for between groups: 18.2639
7. Effective cell size 10.0
8. Alpha: 0.05
9. Standard Error for Comparison: 1.1513
10. Critical T Value: 2.028
11. Critical Value for Comparison: 2.3350
12. There are 2 groups (A and B) in which the means are not significantly different from one another.

Completely Randomized AOV for Number of Shoots

Source	DF	SS	MF	F	P
Treatment	3	1411.80	470.600	69.4	0.0000

1. Grand Mean: 11.000
2. CV: 23.68
3. Bartlett's Test of Equal Variances: **Chi-Sq** (16.9), **DF** (3), **P** (0.0007).
4. Cochran's Q: 0.5737
5. Largest Var / Smallest Var: 17.513
6. Component of variance for between groups: 46.3817
7. Effective cell size: 10.0
8. Alpha: 0.05
9. Standard Error for Comparison: 1.1648
10. Critical T Value: 2.028
11. Critical Value for Comparison: 2.3622
12. There are 3 groups (A, B, etc.) in which the means are not significantly different from one another.