Establishment of Micropropagation Protocol for Chrysanthemum (*Chrysanthemum morifolium* Ramat) Mutants following their Physiological Study

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of
Bachelor of Science in Biotechnology

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Declaration

It is hereby declared that

1. The thesis submitted was our original work while completing a degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material that has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. We have acknowledged all main sources of help.

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Approval

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Ethics Statement

We, Shawli Adhikari Rini and Amatullah Sugra hereby certify that the following criteria are fulfilled for the manuscript "Chrysanthemum (*Chrysanthemum morifolium* Ramat) Mutants following their Physiological Study".

1. This content is our own original work and has not been previously published.
2. All sources used are appropriately acknowledged through accurate citations and justified references.

Abstract

Micropropagation is an important technique for in vitro preservation of *Chrysanthemum morifolium* Ramat mutant types. This work developed a micropropagation methodology for chrysanthemum mutant types following analysis of chlorophyll content and few molecular investigations. In this experiment mainly hormone free *MS* medium was used for regeneration response study and the results of different mutants were evaluated. Shoot tips were collected from the ex-vivo grown plants and were subjected to sterilization. Among the used sterilization treatments B and G showed the best result for regeneration response (100%) through controlling the contamination rate. Treatment B was running tap water for 25 mins, detergent wash 10 mins and 0.8% NaClO for 10 mins and treatment G was running tap water for 25 mins, detergent wash 15 mins and 0.1% HgClO for 2 mins and 70% ethanol for 20 sec. The establishment of branching on the sterilized shoots on hormone free *MS* medium took 7 to 8 days after inoculation and roots were visible after 7 weeks. The fully rooted plantlets could stay healthy inside the flasks for 8 weeks. Once needed rooted plantlets were transferred to soil and acclimatized properly. Plantlets derived from this study showed 100% survival during acclimatization. For chlorophyll study, mutant 6 (M6) showed the best result (chlorophyll content was 0.0028685185) among all the mutants tested. Thus, this work increases knowledge for preservation of chrysanthemum mutant types by developing a micropropagation methodology. The evaluation of chlorophyll content will help to present deeper insights into their genetic and physiological properties.

Keywords: Micropropagation, *Chrysanthemum morifolium* Ramat, Preservation, DNA isolation, Liquid nitrogen, Sterilization, Mutants, Chlorophyll, Molecular study, Flower industry, Bangladesh.

Dedication

We dedicate our thesis to the mentors who have guided and encouraged us along our academic journey.

To our friends and family, we are grateful for your sacrifices and unwavering support. Without each of them, it would not have been possible.

We are really appreciative of our supervisor's assistance and support during this long term work. Without her valuable advice and encouragement, this thesis would never have been completed. We are grateful for her cooperation and patience over this whole process. She has given us this priceless chance for which we will always be grateful.

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Shawli Adhikari Rini, Amatullah Sugra

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List of Acronyms

MS	Murashige and Skoog (1962) medium
mg	Milligram
Ml	Millilitre
L	Litre
nm	Nanometer
cm	Centimeter

Chapter:1
Introduction

1.1: Background:

The Chrysanthemum (*Chrysanthemum morifolium* Ramat), with its multi-layered petals and vivid colors, has a millennium-long cultural history. Chrysanthemums were first grown in ancient China as early as the 15th century BCE, and they swiftly rose to become a very symbolic plant *(Long, 2015)*. Honored for its connection to longevity, rebirth, and honesty, it established itself as a mainstay in Chinese literature, art, and even imperial symbolism *(Shahrajabian et al.,2019)*. As time went on, its charm extended across East Asia, with Japan being one among the countries where it gained the distinction of becoming the national flower. The chrysanthemum, which is prized for its hardness and grace and which embodies both the profundity of cultural meaning and the beauty of nature, never fails to win hearts *(Shahrajabian et al.,2019)*.

1.2: Characteristic & use of Chrysanthemum:

Famous for their great range of hues, forms, and sizes, chrysanthemums have unique qualities that make them a popular option for gardens and flower arrangements all around the world *(Wang, 2023)*. Usually with thick clusters of petals arranged in various layers ranging from delicate and fragile to bold and strong, these hardy perennials look great *(Wijayani et al., 2017)*. Their blossoming seasons differ; some bloom in the spring or summer, while others bloom in the cool autumn days, bringing a burst of color to the changing seasons *(Eisa et al., 2022)*. After they are established, chrysanthemums need little care as they are a plant that can grow in a variety of soil types and climates *(Shintiavira et al.,2021)*. Some types are grown for their therapeutic qualities, thus their usefulness goes beyond simple aesthetics *(Miles, 2004)*. Chrysanthemum, which is high in antioxidants, vitamins, and minerals, has long been used in herbal treatments to treat a variety of ailments *(Sharma et al., 2023)*. One of its most noticeable advantages is its capacity to induce relaxation and peace, making it a common ingredient in herbal teas intended to reduce stress and anxiety *(Shahrajabian et al., 2019)*. The plant's anti-inflammatory qualities have also been investigated for their potential to relieve headaches and reduce inflammation caused by illnesses

such as arthritis (*Shahrajabian et al., 2019*). Furthermore, chrysanthemum tea is commonly drunk to promote eye health, as it is said to reduce eye strain and improve eyesight *(Sharma et al.,2023).* Chrysanthemums find numerous and significant uses in a variety of fields because of their remarkable beauty and rich cultural value *(Hadizadeh et al., 2022).* They adorn parks, private gardens, and landscapes in horticulture and gardening, bringing pops of color and texture to outdoor areas *(Haspolat, 2024).* Chrysanthemums are a versatile flower that florists and floral designers use to create beautiful arrangements for weddings, special occasions, and everyday decor. They are known to arouse emotions and draw attention *(Long, 2015).* Chrysanthemums are beautiful flowers, but they also have significant cultural meaning in many different cultures (weddings, auspicious occasions) *(Benan., 2024).* They represent ideas of rebirth, longevity, and purity *(Maghfiroh et al., 2023)*. Chrysanthemum petals enhance culinary experiences with their faint flower overtones by adding delicate tastes and visual appeal to teas, salads, and desserts *(Ahn et al.,2022).*

Scientific Classification:

- ❏ Kingdom: Plantae
- ❏ Subkingdom: Tracheobionta
- ❏ Superdivision: Spermatophyta
- ❏ Division; Magnoliophyta
- ❏ Class: Magnoliopsida
- ❏ Subclass: Asteridae
- ❏ Order: Asterales
- ❏ Family: Asteraceae
- ❏ Genus: Chrysanthemum
- ❏ Species: *Chrysanthemum morifolium Ramat*

1.3:Tissue Culture:

Tissue culture, often known as cell culture, is an important approach in contemporary biology and science. Cells taken from multicellular organisms are cultured in a controlled setting outside of their native habitat *(Sanghamitra. G, 2024).* This regulated environment usually comprises a culture

medium that contains vital nutrients, growth factors, and other supplements required for cell growth and proliferation. Tissue culture allows researchers to investigate cell behavior, metabolism, and responses under finely regulated circumstances *(Thakur et al., 2024).* It is widely used in many domains, including fundamental research, pharmaceutical development, tissue engineering, and biotechnology *(Singh, 2023).* Tissue culture allows for the large-scale generation of cells, which promotes research spanning In vitro regeneration is the act of cultivating plant tissues or cells in a controlled environment in order to promote their growth and development into entire plants *(Loyola-Vargas & Alejo, 2018).* This approach is commonly employed in plant biotechnology for a variety of goals, including disease-free plant production, genetic alteration, and the propagation of rare or endangered species.

The procedure usually begins with the selection and sterilization of plant tissue, such as leaf or stem segments, seeds, or meristematic cells *(Jain, 2024).* These tissues are then grown in a nutrient-rich media that includes plant growth regulators like auxins and cytokinins, which encourage cell division and differentiation. Under ideal conditions, cultured cells multiply and create callus, a mass of undifferentiated cells from basic cellular processes to the development of novel treatments and bioproducts *(Loyola-Vargas & Alejo, 2018).*

Plant regeneration occurs when plants repair or replace damaged structures based on the totipotency and pluripotency of their cells. Tissue culture is one of the most widely used regenerative technologies *(Long et al.,2022).*

Additionally, sterilization is a very important part of plant tissue culture. Sterilization means killing every bit of life, like bacteria, fungi, viruses, and spores, on an object or place. It's very important in plant tissue culture. This helps avoid any kind of unwanted life getting into the culture medium or the plant tissue we're dealing with. Plant parts, such as small bits of plant tissue which are known as explants are used to grow more plants, tools, and culture media, are usually sterilized by autoclaving. Autoclaving means exposing stuff to steam under high pressure, at around 121°C for 15-30 minutes. This step helps to remove any external contaminants from the plant surface *(Ahmadpoor et al.,2022).*

Furthermore, for new Chrysanthemum plants to grow, healthy explants like the tips of shoots, leaves, or stem parts are needed which is very important . Next, after washing, these cleaned explants are placed in agar medium *(Aslam et al., 2023).* This medium has plant growth regulators

like cytokinins and auxins which help the shoot or root formation. Though for this study we used hormone free media.. Then these new plants acclimated to get used to the new environment to keep growing *(Miler et al.,2023).* This whole process, inoculation to acclimatization is called tissue culture which can really help Bangladesh's Chrysanthemum industry. It lets us make lots of quality plants fast. Traditional methods are not needed to get more and better flowers. This makes Bangladesh's Chrysanthemum growers more competitive in markets at home and abroad. Tissue culture also gives disease-free plants. This is key to lower crop losses from bugs and disease. With tissue culture, farmers in Bangladesh lessen the risks from old methods. This means more steady crops. It makes the Chrysanthemum industry more stable *(Miler et al., 2018).*

1.4: Economical Background of Chrysanthemum in Bangladesh:

Floriculture is becoming increasingly popular in Bangladesh, where flowers are used for many social and ceremonial occasions. Flowers with unique colors, sizes, and shapes are in great demand on the market due to their appeal to flower enthusiasts *(Heuvel et al., 2023).* In Bangladesh, just a few chrysanthemum kinds are available, and they can only be produced during the winter months. Studies show that regenerating plants by organogenesis and somatic embryogenesis may produce a significant number of plants and create genetic diversity in chrysanthemum species *(Chowdhury et al., 2021).* Cultivating Chrysanthemums has substantial financial prospects for Bangladesh, as there is huge demand for the blooms for various uses such as ornamental displaying, floral designs, and spiritual ceremonies. By enhancing the effectiveness and quality of Chrysanthemum generation through tissue culture, Bangladesh can benefit from the developing request for blooms both locally and globally. This could result in increased pay for farmers and organizations included in the Chrysanthemum esteem chain. Tissue culturing is a procedure that can mass create quality seedlings of Chrysanthemums in a controlled nursery condition. This guarantees consistent creation of contamination free plants with wanted attributes *(Khandekar et., 2021).* It limits the requirement for traditional cultivating strategies like seed sowing and cutting based multiplication. Along these lines, ranchers can deliver more plants in a small portion of the land region throughout the entire year. Moreover, blooms created through tissue culturing will have more prominent resilience to sicknesses and climatic conditions *(Khan, 2021).* Subsequently, yields would expand decidedly. The high caliber plants can fulfill both nearby and outside requests. Bangladesh can send out premium

Chrysanthemums and produce outside trade. Tissue culture techniques provide an effective means to develop novel Chrysanthemum varieties with advantageous qualities. Some desirable traits include enhanced resistance against diseases, more vibrant flower colors, and extended periods of blooming. The cultivation of a wide variety of premium Chrysanthemum cultivars through biotechnological methods can inspire novel ideas and diversification across the floral sector *(Hung et al., 2021).* This offers new avenues in the marketplace and promotes additional financial expansion throughout Bangladesh *(Khan et al., 2020).* The introduction of new cultivars with traits like disease resistance, vibrant colors and prolonged bloom times allows plant tissue culture to foster innovation within the floral industry. A diverse selection of high quality Chrysanthemum varieties stimulates creative solutions and different specializations, opening potential markets. Further, this drives continued economic growth for Bangladesh. The adoption of tissue culture techniques has much promise for revolutionizing chrysanthemum cultivation in Bangladesh. By regenerating chrysanthemum plants through tissue culture, productivity could increase substantially *(Hung et al., 2018).* Farmers may harvest higher yields of quality flowers that demand better prices in the market. This plant regeneration method further enhances product quality by facilitating disease-free propagation. Such improved varieties have the ability to boost sector profits significantly. As smallholder farmers achieve more robust incomes, rural communities and the national economy are likely to benefit in turn. Larger harvests of premium flowers mean more business opportunities and greater profits for traders and exporters as well *(Khan, 2021).* The regeneration of Chrysanthemum holds great relevance due to several key reasons. Chrysanthemums are widely grown for their ornamental beauty, making them economically important in the global flower trade. Techniques like tissue culture provide an efficient way to rapidly multiply elite varieties with desired traits, helping to ensure consistent quality and supply to meet market demands *(Khan, 2021).* Chrysanthemums serve as a vital source of income for farmers and businesses involved in the floral industry, contributing to livelihoods and rural economies. Furthermore, regeneration plays a critical role in protecting and enhancing genetic diversity within Chrysanthemum species, thereby safeguarding against disease outbreaks and issues with the environment *(Chowdhury et al., 2021).* Moreover, Chrysanthemum regeneration facilitates breeding efforts aimed at developing new cultivars with improved traits such as disease resistance, prolonged blooming, and diverse flower colors, driving innovation and competitiveness in the floral market. Overall, the regeneration of Chrysanthemum is instrumental in sustaining the floral industry's

growth and preserving biodiversity, making it a key component of horticultural and agricultural development efforts worldwide *(Khan, 2021)*.

1.5: Chlorophyll Study:

An essential component of environmental science and plant physiology is the study of chlorophyll, which entails analyzing the amount of chlorophyll in plant tissues. The green pigment chlorophyll, which is involved in photosynthesis, is a major sign of a plant's health, vitality, and ability to respond to its surroundings. Researchers can measure changes in plant metabolism, quantify photosynthetic efficiency, and identify different stresses including pollution, drought, and nutrient shortages by using chlorophyll analysis *(Pavlovic et al., 2014)*. Chlorophyll levels may be accurately measured using methods like spectrophotometry and fluorometry, which provide important information on how plants react to biotic and abiotic stimuli. Moreover, studies on chlorophyll are essential to agricultural science because they help farmers optimize crop management strategies and sustainably increase yields.

Analysis of chlorophyll concentration is important in many areas, especially environmental research, agriculture, and plant physiology. For academics, farmers, and environmentalists alike, this analytical method offers priceless insights on the health, vitality, and physiological state of plants. Scientists may monitor plant stress reactions, evaluate photosynthetic efficiency, identify nutrient shortages or environmental disturbances, and more by measuring chlorophyll levels. Chlorophyll content analysis helps farmers manage their crops more effectively, maximizing yields and sustainability through the optimization of fertilizer applications, irrigation plans, and insect control techniques. In addition, it is essential for determining how pollution, climate change, and habitat loss affect plant ecosystems, which helps to guide mitigation and conservation efforts. Chlorophyll content analysis is fundamental to our knowledge of plant health, ecosystem dynamics, and agricultural production. It helps us make educated decisions and implement sustainable management methods in a world that is changing quickly *(Li et al., 2018)*.

1.6: DNA Isolation and Quality Analysis:

Plant DNA separation includes breaking down cell walls and membranes with procedures such as liquid nitrogen grinding or mechanical force (specific buffers). Protein and impurities are removed using phenol-chloroform extraction or CTAB buffers. Following alcohol precipitation and centrifugation, the DNA is resuspended in a suitable buffer for storage or further uses. Plant DNA isolation techniques are critical for genetic research, molecular breeding, and biotechnology applications. Advances in plant DNA isolation continue to uncover plants' genetic potential for agricultural and environmental applications.

Liquid nitrogen-based plant DNA isolation is chosen because it preserves DNA integrity and is suitable for sensitive applications such as PCR and sequencing. This procedure includes crushing frozen tissue into a fine powder, which disrupts cell walls and aids in DNA extraction. However, it requires specific equipment for safe handling, which may make it inaccessible in certain labs. CTAB buffer-based isolation, on the other hand, employs a detergent to break cell membranes and solubilize proteins, therefore enabling DNA release into solution. This process is more accessible and easy than liquid nitrogen-based procedures, however it may yield less DNA and is prone to polysaccharide contamination. CTAB buffer-based DNA isolation is a flexible approach that works for a wide range of plant tissues, including those that are difficult to treat with liquid nitrogen due to secondary metabolites or rigid structures. Its scalability enables the effective processing of huge sample batches, making it suited for high-throughput research and diagnostics applications.

Accurate quantitation of DNA is essential for a wide range of molecular biology applications. In the present study, we sought to determine DNA quantity using a nanodrop and compared the result of two types of DNA isolation and analyzed the quality.

For the present study, micropropagation protocol development for different mutants of chrysanthemum was the main objective. This micropropagation study is followed by an available protocol by *Chowdhury et al., 2021* but the protocol that had been mentioned in this study is effective for preservation of available mutants. Also, evaluation of chlorophyll content and DNA measurement of different mutants was the part of present study.

On the basis of this, the specific objectives of the present study were:

- Establishment of micropropagation protocol for different types (M1, M2, M5, M6) of Chrysanthemum mutants including the control plant (CW)
- Chlorophyll content analysis of mutant varieties.
- DNA isolation and DNA quality analysis

Chapter: 2

Materials and Method

2.1 Plant materials used and plant Sample collection

Four different Chrysanthemum mutants M1, M2, M5 and M6 and CW the control or mother plant (Figure 2.1) were employed in this experiment. Every chrysanthemum plant was collected from a greenhouse at Dhaka University. Ex-vivo grown plant shoots were used for this study.

Figure 2.1: Plant materials used in the study.

A: Control Plant (CW); Different mutants of Chrysanthemum B: Mutant 1 (M1); C: Mutant 2 (M2); D: Mutant 5 (M5); E: Mutant 6 (M6)

2.2 The medium that was employed in the experiment

2.2.1 Medium used for tissue culture

In the tissue culture experiment, Murashige and Skoog (*MS*) media (1962) was utilized for the various segments. We used this medium to get the shoot regeneration and expand *(Murashige and Skoog, 1962).*

2.3 *MS* medium Preparation

The Murashige and Skoog (1962) medium (*MS*) was a commonly utilized media in plant tissue culture for plant regeneration. (*Murashige & Skoog, 1962)*

- Each of the elements of this medium (listed in table 2.1) was put into a volumetric flask.The final volume (500ml) was made by adding distilled water.
- HCl or 1 N NaOH were used to bring the medium's pH to 5.8
- An ager was added to the medium to aid in its solidification.
- The mixture was heated in the microwave for five minutes.
- A permanent marker was used to mark each 100ml conical flask containing the medium after it had been sealed with aluminum foil.

Table 2.1: Different components for preparation of *MS* medium (1000ml)

Components	Amount
MS	4.45 g
sucrose	30 g
Agar	8.5 g

2.4 Medium sterilization

Conical flasks were cleaned before pouring the medium. Additionally, the flasks were sealed with aluminum foil paper after the medium was poured into a 100 ml capacity. The conical flasks were then autoclaved for 15 mins at 121°C and 15 lb/sq. inch. media with conical shape. Flasks were kept in the culture chamber at 25°C for storage. *(Item Detail - Compendium Methods Microbiological Examination Foods, 2019).*

2.5 Maintenance of aseptic solution

A laminar airflow cabinet was used throughout the whole inoculation process. In addition to cleaning the surface with 70% ethanol to prevent contamination, the UV light was on for 30 minutes before starting work in the cabinet. The incubator was used to hold pre-autoclaved instruments such as petri dishes, filter paper, forceps, scalpels, and so on. Both the flame technique and 70% ethanol cleaning were used to sanitize such tools before work. Other items, such as the biker, the transplant carrier, etc., were cleaned with 70% ethanol to preserve the aseptic state. To ensure an aseptic environment throughout work, hands were cleansed with 70% ethanol and disinfected with soap before beginning. Each time the conical flasks were opened and closed, the lids sterilized with burner flame. To keep the flasks aseptic, parafilm paper is placed over them after inoculation or subculturing. *(Sharma et al., 2015)*

2.6 Applied culture technique

The following techniques were applied in this experiment for the various goals and segments of the investigation.

- Axenic culture
- Explant culture
- Subculture of the explants

2.6.1 Axenic culture

A major obstacle to tissue culture is fungal infection. The rainy season has an impact on the rate of fungal contamination. As a result, the explants' sterilizing procedure was unique. Compared to previous seasons, extra caution was required during the rainy period. *(Yesmin et al., 2014)*

2.6.1.1 Axenic culture (Rainy season)

The explants were collected and placed in a jar. For 30 minutes, the explants' surface was cleaned with flowing tap water. Then those explants were dipped in jet detergent for 15 minutes and then washed with distilled water. After that, the explants were submerged for five minutes in 0.2% HgCl2. Following that, those were 4 to 5 times cleaned in the laminar flow cabinet using autoclave distilled water. After that, those were cleaned for 20 seconds using 70% ethanol and then 6 to 7 times using autoclaved distilled water. *(Yesmin et al., 2014)*

2.6.1.2 Axenic culture (Other seasons)

The explants that were gathered from the garden were given a fifteen-minute shower under the running tap water. Then the explants were dipped in jet detergent for 15 minutes and washed with distilled water. The explants were submerged in 0.8% NaClO for seven minutes in the laminar flow cabinet, and then they were repeatedly rinsed with autoclaved distilled water. Lastly, autoclaved distilled water was used to wash the explants once again. *(Yesmin et al., 2014).*

2.6.2 Explant culture

Shoot tips were used as an explant (Figure 3.1 A). These tips are inoculated on nutrient-rich *MS* medium after being cleansed to eliminate surface impurities and chosen for their profusion of meristematic cells. For this study, hormone-free media has been used and the size of the explant was 2 to 2.5 cm long.

2.6.3 Subculture

Chrysanthemum tissue culture requires subculture due to the abundance of secondary metabolites in all explants. After 15 days, the direct shoot regeneration explants were moved to a *MS* medium. Explants had their death tissues removed during culturing. All of the subcultured explants were routinely observed. To optimize multiple shoot regeneration, explants were implanted in independent flasks. For the best height of the shoots, those individual explants were divided into two to three pieces following the regeneration of numerous shoots.

For this experiment, and shoot explant were used for subculture. (Figure 2.2).

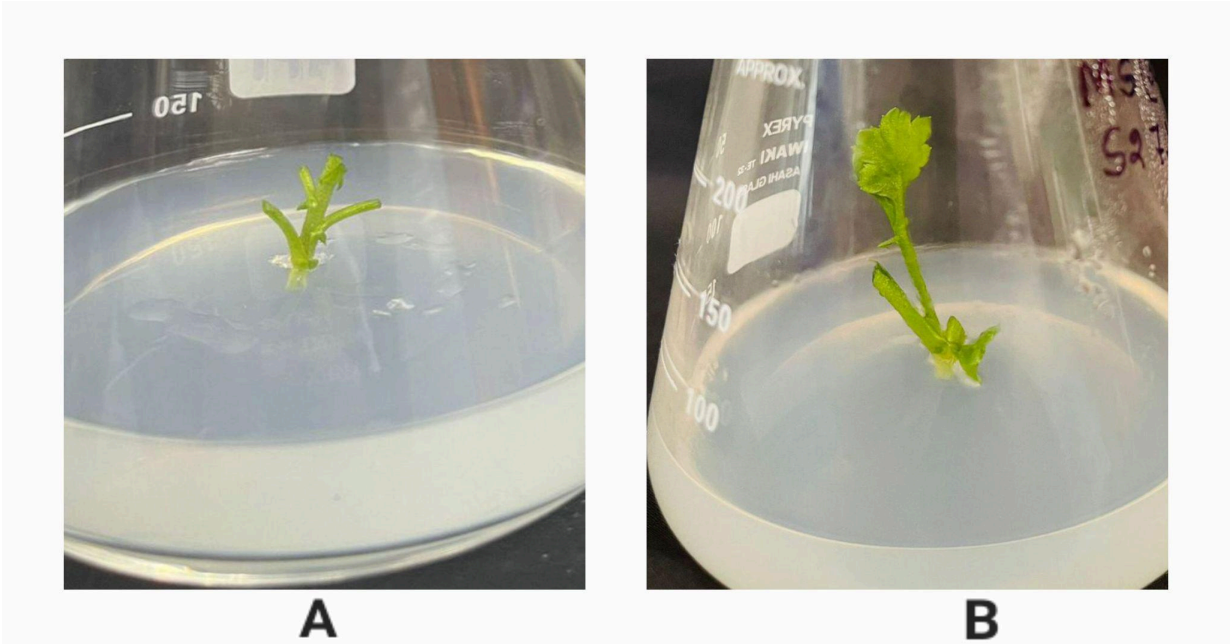


Figure 2.2: Inoculation and Subculture of shoot explant in different stages.

A) Sterilized Explant (Subcultured); B) Subcultured explant after a few days.

2.6.4 Incubation condition

The inoculated flasks were incubated in the culture room under fluorescent light of 20,000 lux intensity. The temperature of the culture room was 25 degree celsius. The culture room had a photoperiod of 16-8 (day-night) hour time period.

2.7 Measurement of chlorophyll content

When analyzing plant photosynthetic efficiency under various stress conditions, chlorophyll concentration is a crucial indication. After the leaves were cut into pieces, 50 mg were placed in a container with 12.5 ml of 80% acetone and left in the dark room for 48 hours.
Using a spectrophotometer, the absorbance of the leaf tissue extract was measured after 48 hours at wavelengths of 645, 652, and 663 nm. Initially, absorbance zero for the blank was established using 80% acetone. According to a study, the extracts of chlorophyll were put into the cuvette in order to measure absorbance at three distinct wavelengths *(Yasmin et al., 2015)*.

Chlorophyll Content Measurement Formula *(Yasmin et al., 2015)*:
$A = ECd$

Here,
A = Absorbance
C = Chlorophyll Content
E = Extinction Coefficient = 36 mL/mg.cm
d = Distance of the light path (1 cm)

2.8 DNA isolation without liquid nitrogen:

The CTAB (cetyltrimethylammonium bromide) buffer method is a popular approach for extracting DNA from plants. Collected leaves were crushed manually without liquid nitrogen. The protocol used here includes combining finely powdered plant tissue with a CTAB extraction buffer including CTAB, EDTA, NaCl, and Tris-HCl. CTAB functions as a detergent by lysing cell membranes and liberating cellular components such as DNA. EDTA chelates divalent cations, whereas NaCl precipitates proteins and polysaccharides. The solution is then extracted with chloroform/isoamyl alcohol, precipitated with isopropanol or ethanol, and washed with ethanol to remove salts and impurities. This produces pure genomic DNA that may be studied using molecular biology procedures such as PCR, restriction enzyme digestion, and sequencing.

2.9 DNA isolation using liquid nitrogen:

The liquid nitrogen approach is a way to isolate plant DNA. It entails freezing new plant tissue in liquid nitrogen to maintain its integrity. The tissue is crushed to a fine powder, combined with a DNA extraction buffer, and incubated at the appropriate temperature. The DNA is isolated from proteins and other biological components, and the aqueous phase is extracted. The DNA is precipitated with isopropanol, washed with ethanol, and then resuspended in TE buffer or water for storage. This approach produces high-quality DNA appropriate for molecular biology applications such as PCR and sequencing, while also delivering vital genetic information from plant materials.

Chapter: 3

Result

This study employed 4 types of Chrysanthemum mutant and 1 mother plant (*Chrysanthemum morifolium* Ramat) with white and purple flowers. It utilized one type of explant (leaf with midrib) to explore in vitro regeneration of Chrysanthemum and optimize various regeneration techniques.

3.1 Optimization of sterilization method for explants

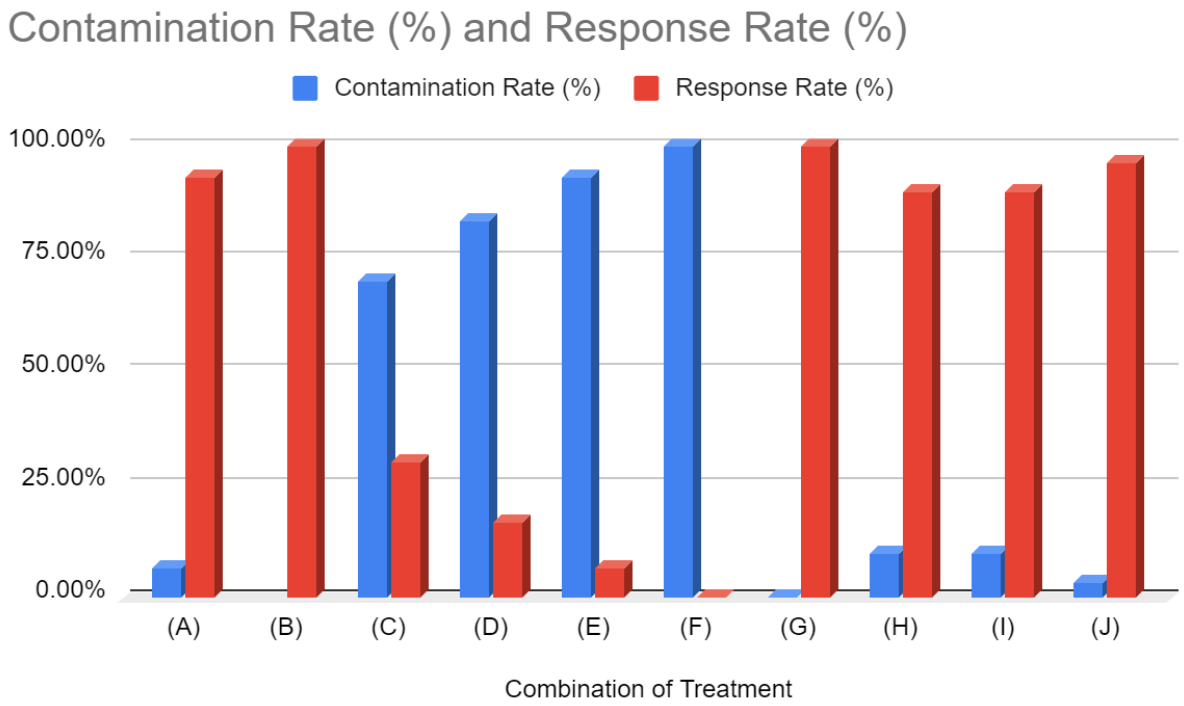
During this stage of the experiment, the explants underwent multiple rinses with flowing tap water. They were subsequently subjected to treatment with fungicide (applied exclusively during the rainy season), HgCl2, and 70% ethanol. Following this, they were washed repeatedly with autoclaved distilled water.

To prevent contamination, the explants underwent treatment with 0.8% of NaClO. The duration of washing and the timing of NaClO, and ethanol treatment were found to impact contamination rates. Optimal results were achieved with 0.2% HgCl2 for 2-3 minutes and 70% ethanol for 20 seconds (Table 3.1), resulting in minimal contamination, during the rainy season reduced contamination rates further. Prolonged ethanol exposure (less than 30 seconds) reduced explant responsiveness, and omitting 70% ethanol during sterilization increased contamination *(Stutte & Eraso, 2006)*. Higher concentrations of HgCl2 also damaged explants. The duration of tap water washing affected contamination rates; 30 minutes of tap water washing resulted in fewer contaminations but reduced responsiveness. Washing for 15 minutes with detergent and autoclaved distilled water yielded optimal results.

Table 3.1 Optimization of sterilization process with the relation of the rate of responsive Explants (15 days)

Treatments	No of Explants	No of Contaminated Explant	Contamination Rate (%)	No of Responsive Explants	Response Rate (%)
(A)	30	2	6.67%	28	93.33%
(B)	**30**	**0**	**0 %**	**30**	**100%**
(C)	30	21	70%	9	30%
(D)	30	25	83.33%	5	16.67%
(E)	30	28	93.33%	2	6.67%
(F)	30	30	100%	0	0%
(G)	**30**	**0**	**0%**	**30**	**100%**
(H)	30	3	10%	27	90%
(I)	30	3	10%	27	90%
(J)	**30**	**1**	**3.33%**	**29**	**96.67%**

Note: Treatment B, G and J showed best results with 0%, 0% and 3.33% contamination rate respectively.



Graph 3.1: Result of sterilization optimization.

Note:

(A) = Tap water for 30 mins. + Detergent wash 15 mins. + 0.8% NaClO 10 mins.

(B) =Tap water for 25 mins. + Detergent wash 10 mins + 0.8% NaClO 10 mins.

(C) =Tap water for 25 mins. + Detergent wash 10 mins + 0.7% NaClO 12 mins.

(D) =Tap water for 25 mins. + Detergent wash 10 mins + 0.5% NaClO 15 mins.

(E) =Tap water for 25 mins. + Detergent wash 10 mins + 0.8% NaClO 5 mins.

(F) =Tap water for 30 mins. + Detergent wash 20 mins + 0.8% NaClO 10 mins. + Tween 20

(G) =Tap water for 25 mins. + Detergent wash 15 mins + 0.1% HgClO 2 mins. + 70% ethanol for 20 sec.

(H) =Tap water for 30 mins. + Detergent wash 20 mins + 0.8% NaClO 10 mins. + 70% ethanol for 20 sec.

(I) = Tap water for 20 mins + Detergent wash 10 mins + 0.8% NaClO 10 mins + 70% ethanol for 20 sec.

(J) =Tap water for 20 mins. + Detergent wash 10 mins + 0.8% NaClO 5 mins. + 70% ethanol for 15 sec.

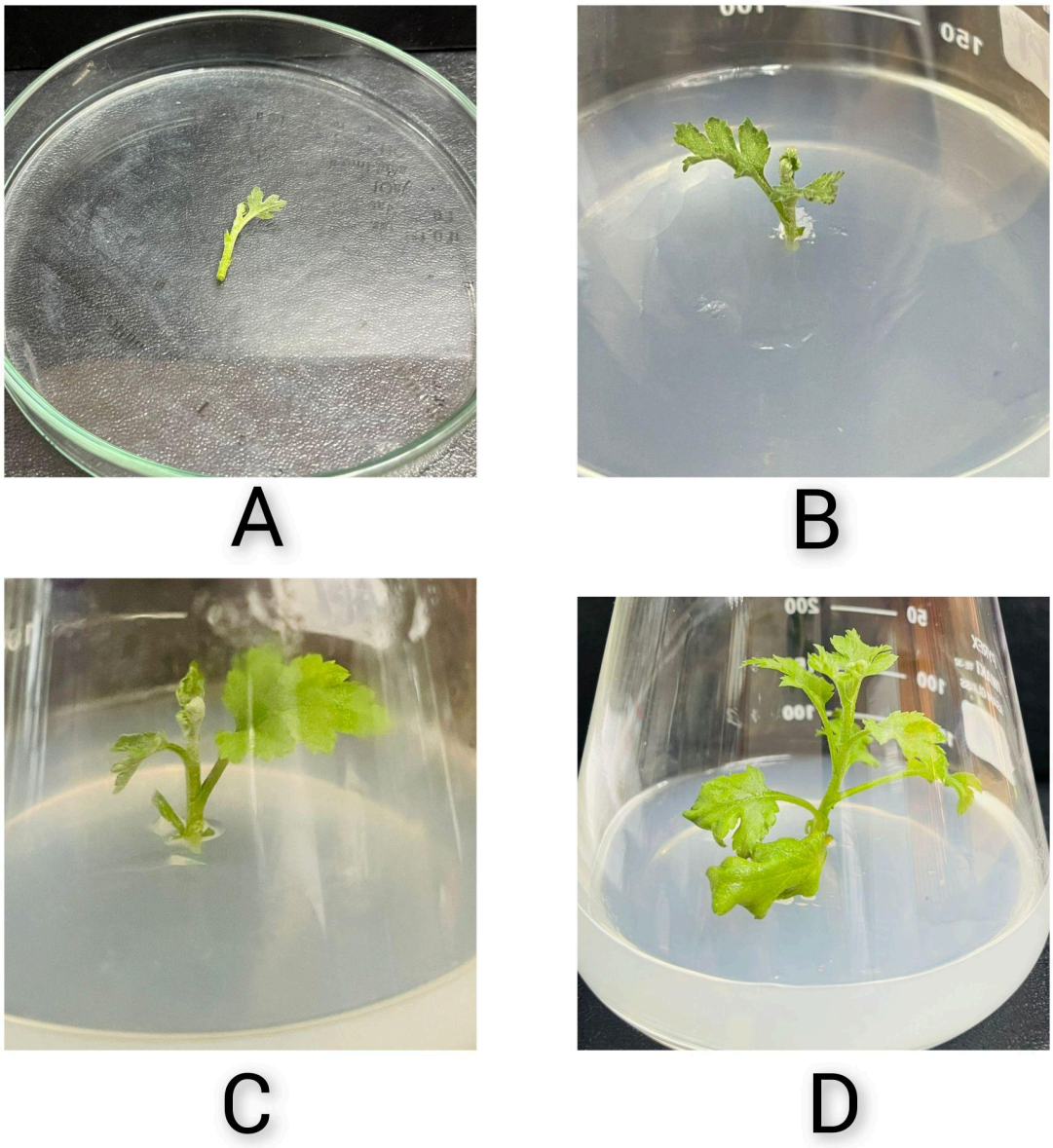


Figure 3.1: Establishment of the explant on *MS* medium showing different stages of growth. A) Sterilized explant (Shoot tip); B) Explant on hormone free *MS* medium after 7 to 8 days of inoculation; C) Explant on medium after 15 days of inoculation; D) Explant after 25 days of inoculation

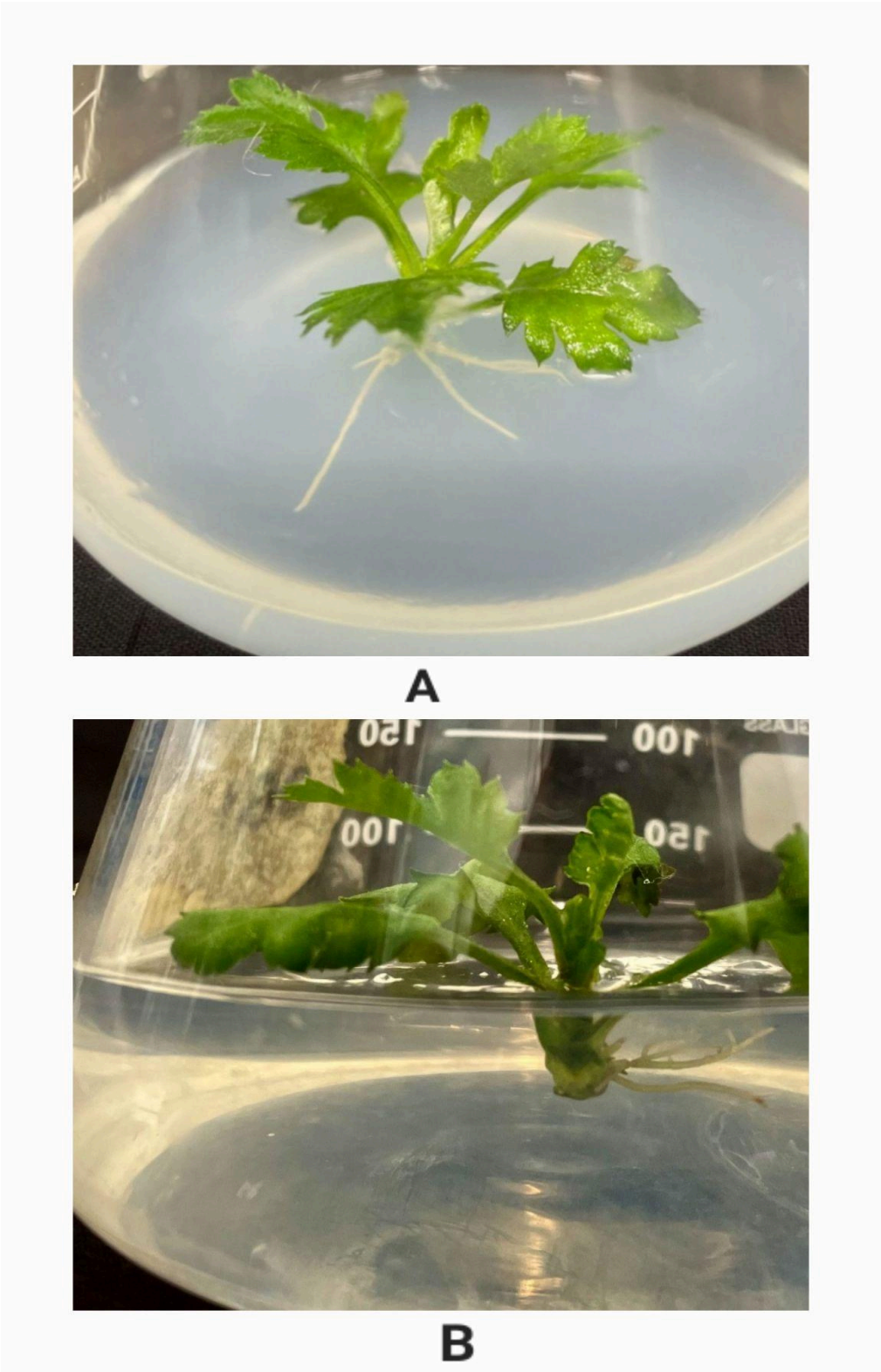


Figure 3.2: Root growth of Chrysanthemum explant on hormone free *MS* media after 7 weeks. A) Root on agar surface; B) Root on gelrite surface.

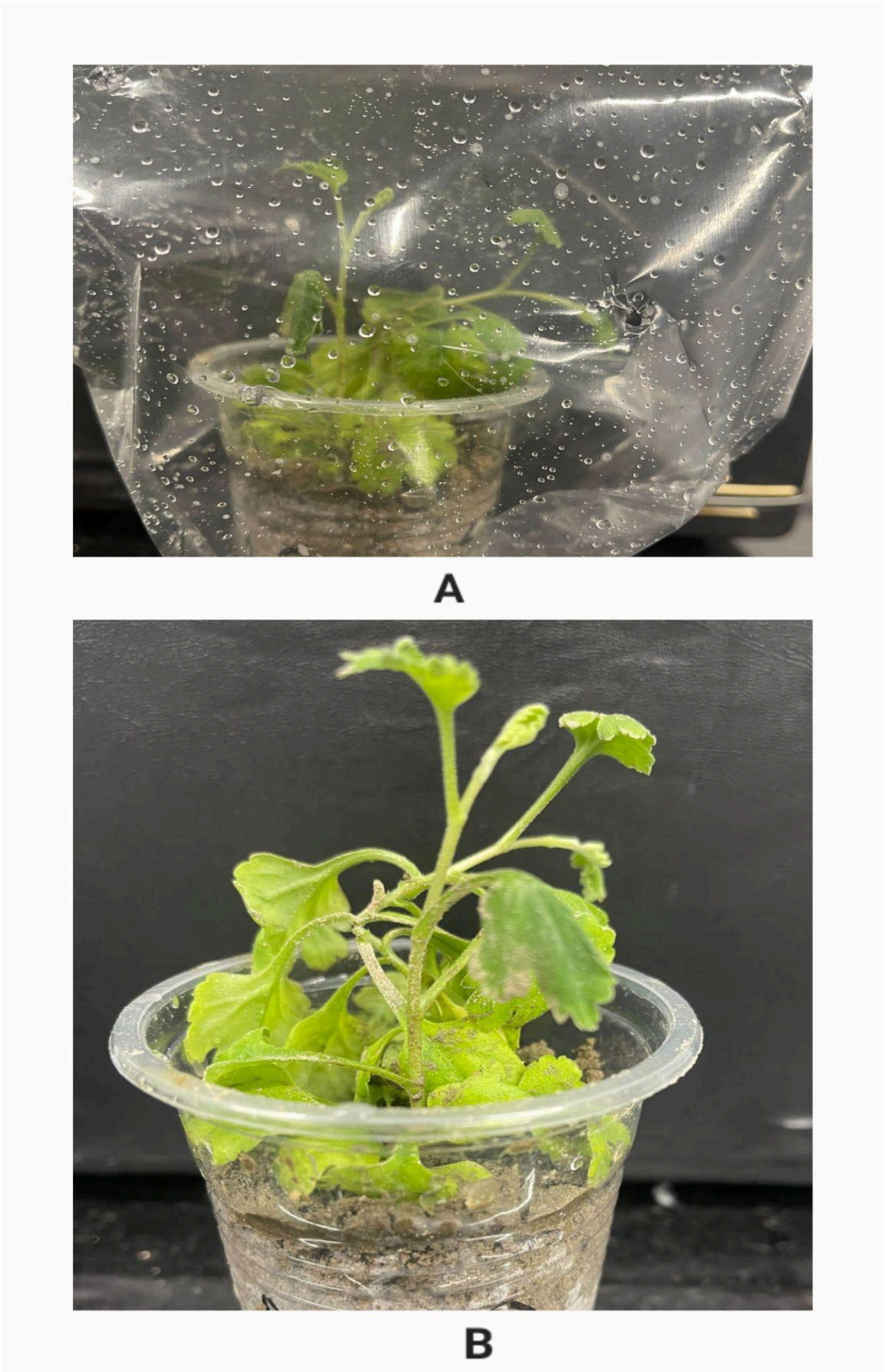


Figure 3.3: Plant transferred in soil.

A) Rooted plant transferred to soil after 8 weeks from inoculation and plant was covered with polythene for 3 to 4 days; B) Plant after removing the polythene.

3.2 Optimized Sterilization Treatments on Different Mutants

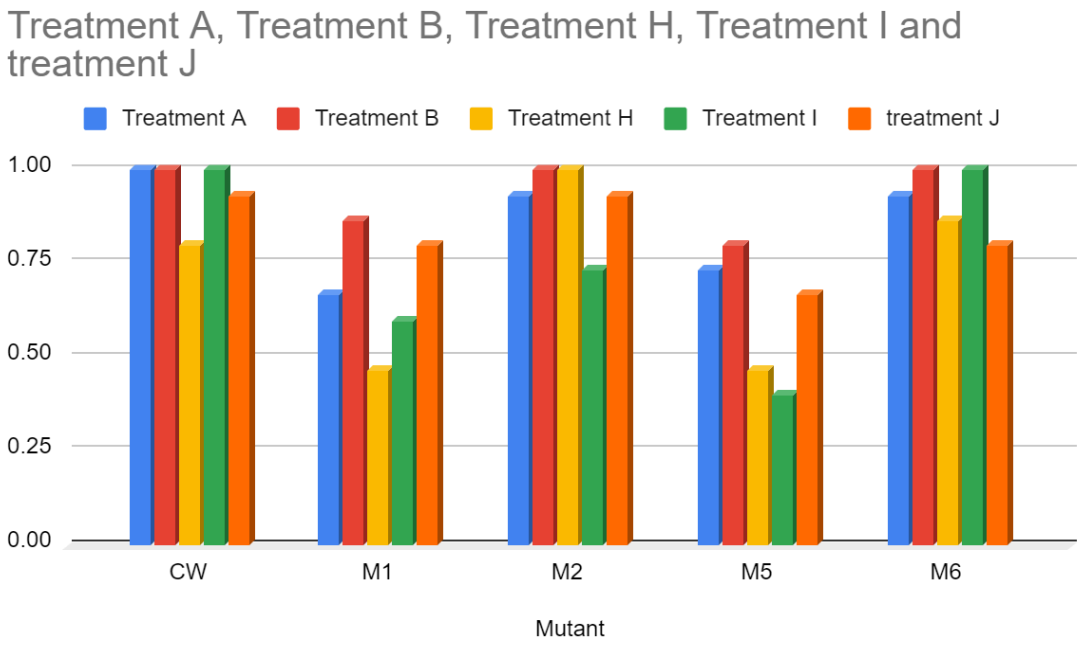
As the main purpose of this study is to preserve the available mutants, we also identified the survival rate of these treatments on different mutants. All the treatments were not tested. Mainly the high response rate treatments were applied on these specific mutants.

The study analyzed the survival rates of various treatments on CW, with treatment A and B showing 100%, treatment H, I, and J showing 80%, 100%, and 93.33% respectively. Mutant 1 (M1) showed 66.67%, 86.67%, and 46.67% survival rates, while treatment I and J showed 60% and 80% survival rates. Mutant 2 showed 100% survival rates for treatment B and H, while mutant 5 showed 73.33%, 80%, 46.67%, 40%, and 66.67% survival rates. Mutant 6 had 100% survival rates for treatment B and I.

Table 3.2 The survival rate of different lines after the application of different treatments

Mutant	Treatment A	Treatment B	Treatment H	Treatment I	treatment J
CW	**100%**	**100%**	**80%**	**100%**	**93.33%**
M1	66.67%	86.67%	46.67%	60%	80%
M2	**93.33%**	**100%**	**100%**	**73.33%**	**93.33%**
M5	73.33%	80%	46.67%	40%	66.67%
M6	**93.33%**	**100%**	**86.67%**	**100%**	**80%**

Note: CW, M2 and M6 showed 100% survival rate on treatment B.

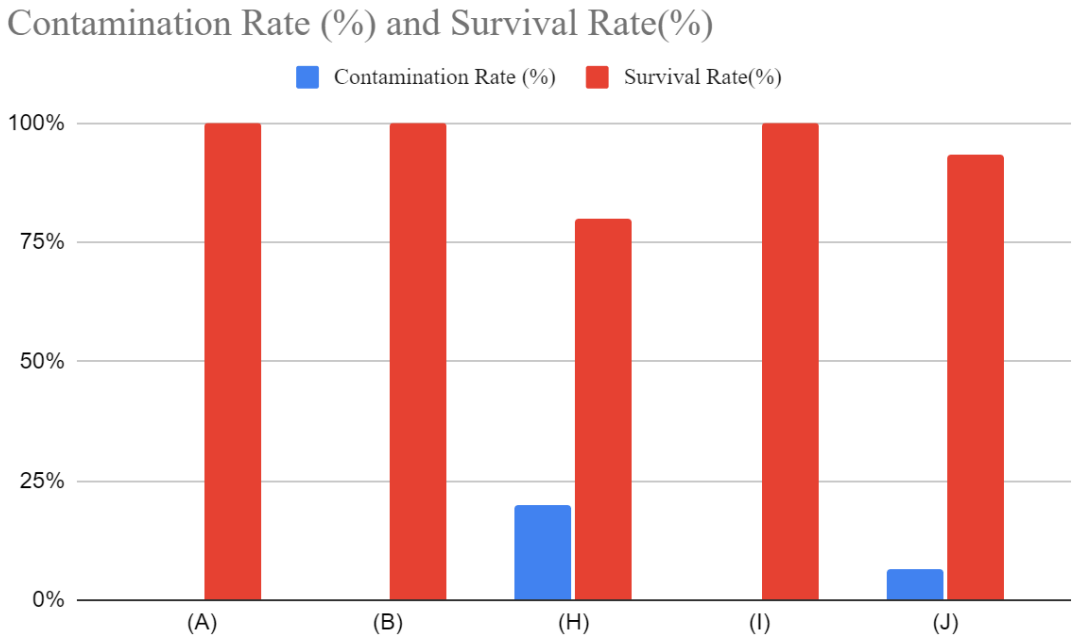


Graph 3.2: Graphical representation of survival rate of mutants towards different treatments

Table 3.2.1 Table of Survival Rate of control plant (CW) after the application of different treatments (15 Days)

Treatments	No of Explants	No of Contaminated Explant	Contamination Rate (%)	No of Survived Explants	Survival Rate (%)
(A)	**15**	**0**	**0%**	**15**	**100%**
(B)	**15**	**0**	**0 %**	**15**	**100%**
(H)	15	3	20%	12	80%
(I)	**15**	**0**	**0%**	**15**	**100%**
(J)	15	1	6.67%	14	93.33%

Note: Control plant (CW) showed 100% survival rate on treatment A, B and I.

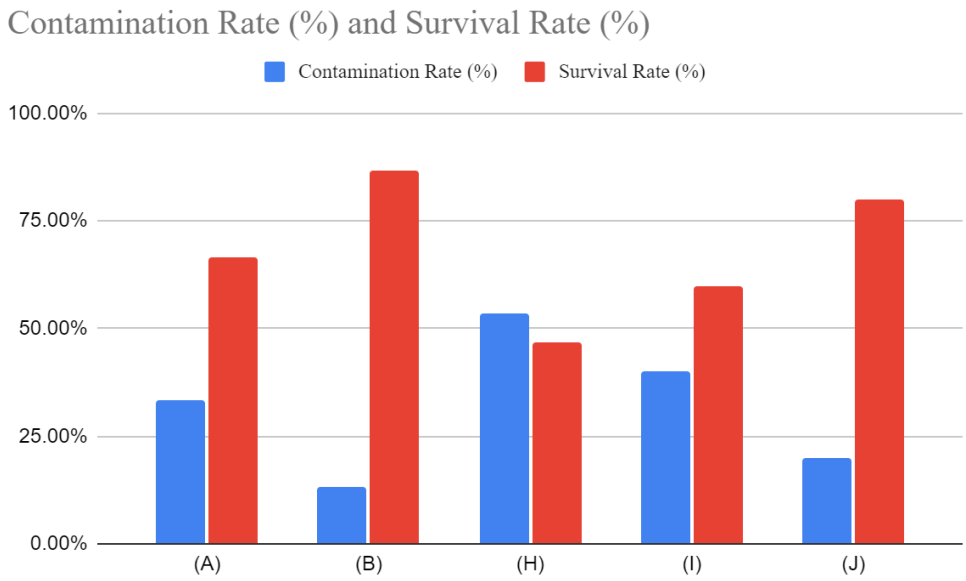


Graph 3.3: Graphical representation of survival rate of control plant towards different treatments

Table 3.2.2 Table of Survival Rate of M1 mutant plant after the application of different treatments (15 Days)

Treatments	No of Explants	No of Contaminated Explant	Contamination Rate (%)	No of Survived Explants	Survival Rate (%)
(A)	15	5	33.33%	10	66.67%
(B)	**15**	**2**	**13.33%**	**13**	**86.67%**
(H)	15	8	53.33%	7	46.67%
(I)	15	6	40%	9	60%
(J)	15	3	20%	12	80%

Note: M1 mutant showed 86.67% survival rate on treatment B.

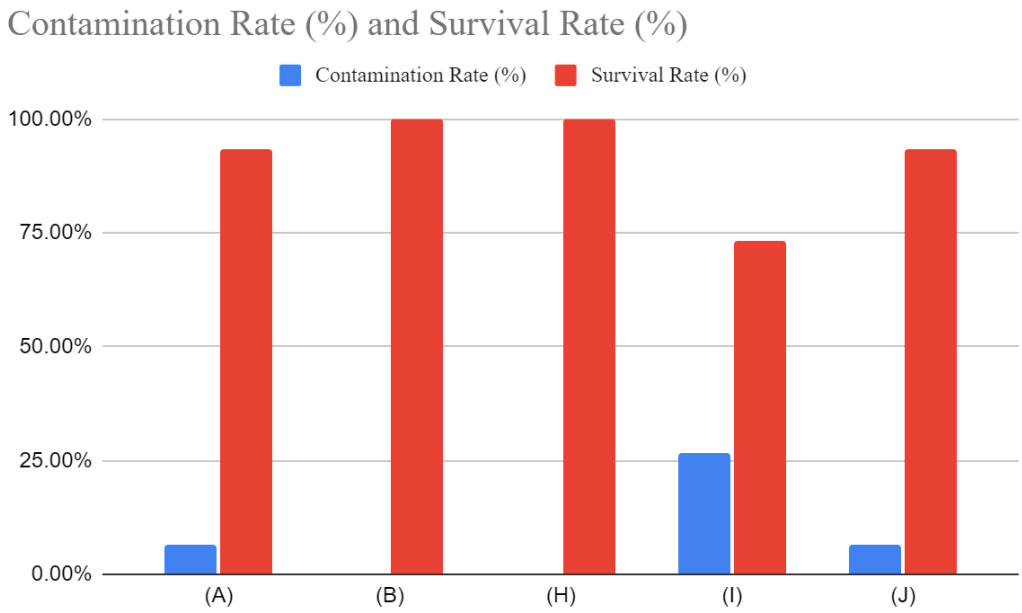


Graph 3.4: Graphical representation of survival rate of M1 mutant plant towards different treatments

Table 3.2.3 Table of Survival Rate of M2 mutant plant after the application of different treatments (15 Days)

Treatments	No of Explants	No of Contaminated Explant	Contamination Rate (%)	No of Survived Explants	Survival Rate (%)
(A)	15	1	6.67%	14	93.33%
(B)	**15**	**0**	**0 %**	**15**	**100%**
(H)	**15**	**0**	**0%**	**15**	**100%**
(I)	15	4	26.67%	11	73.33%
(J)	15	1	6.67%	14	93.33%

Note: M2 mutant showed 100% survival rate on treatment B and H.

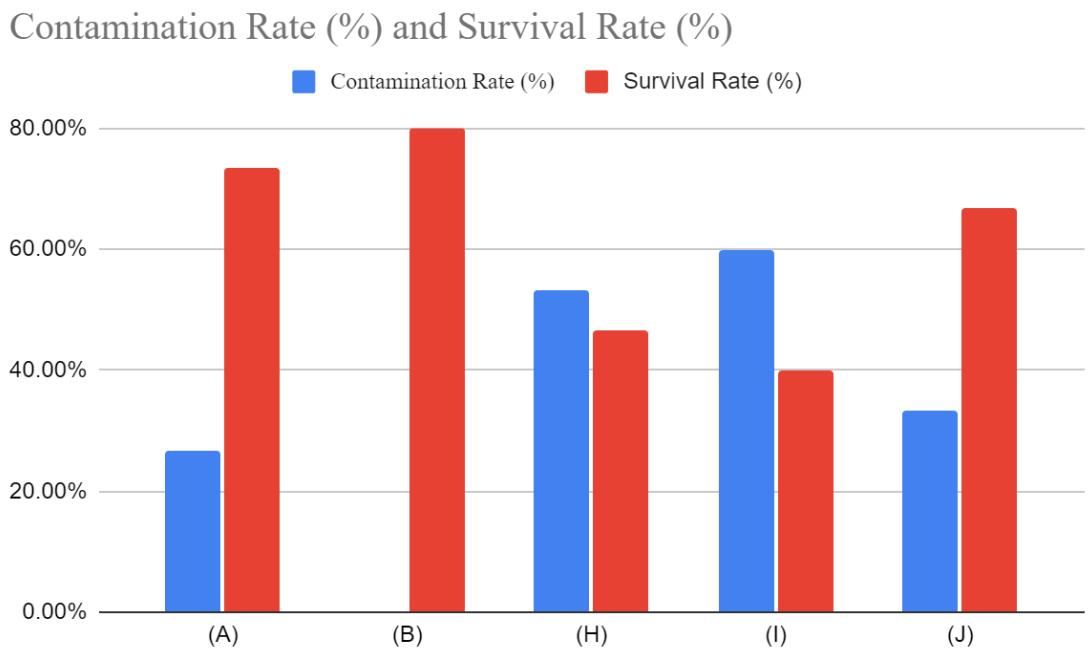


Graph 3.5: Graphical representation of survival rate of M2 mutant plant towards different treatments

Table 3.2.4 Table of Survival Rate of M5 mutant plant after the application of different treatments (15 Days)

Treatments	No of Explants	No of Contaminated Explant	Contamination Rate (%)	No of Survived Explants	Survival Rate (%)
(A)	15	4	26.67%	11	73.33%
(B)	**15**	**3**	**20 %**	**12**	**80%**
(H)	15	8	53.33%	7	46.67%
(I)	15	9	60%	6	40%
(J)	15	5	33.33%	10	66.67%

Note: M5 mutant showed 80% survival rate on treatment B.

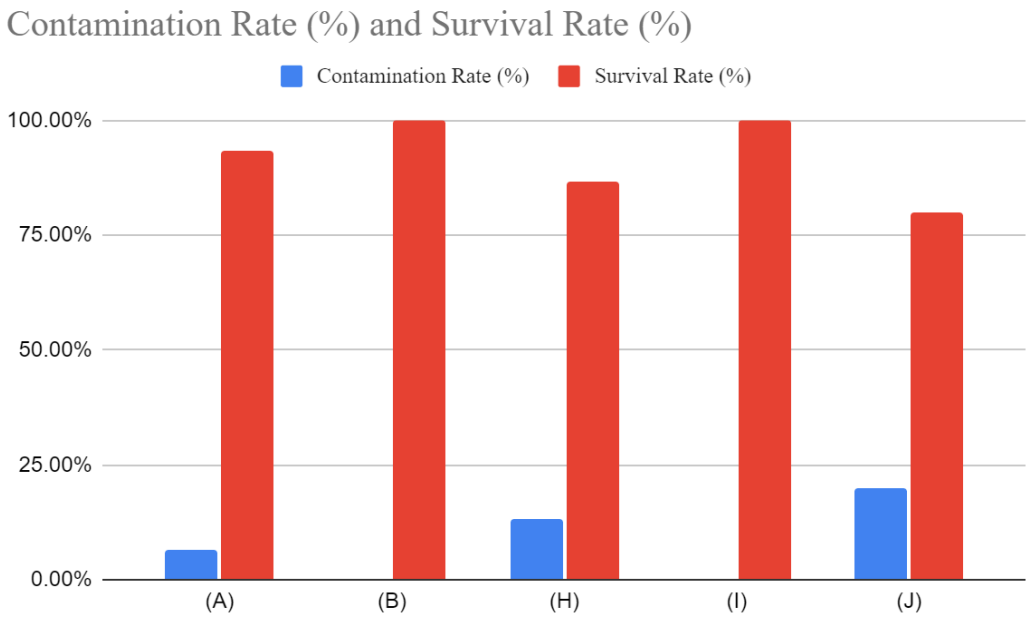


Graph 3.6: Graphical representation of survival rate of M5 mutant plant towards different treatments

Table 3.2.5 Table of Survival Rate of M6 mutant plant after the application of different treatments (15 Days)

Treatments	No of Explants	No of Contaminated Explant	Contamination Rate (%)	No of Survived Explants	Survival Rate (%)
(A)	15	1	6.67%	14	93.33%
(B)	**15**	**0**	**0%**	**15**	**100%**
(H)	15	2	13.33%	13	86.67%
(I)	**15**	**0**	**0%**	**15**	**100%**
(J)	15	3	20%	12	80%

Note: M6 mutant showed 100% survival rate on treatment B and I.



Graph 3.7: Graphical representation of survival rate of M6 mutant plant towards different treatments

3.3: Chlorophyll content measurement

The quantity of chlorophyll per unit area indicates a plant's photosynthetic potential. The amount of chlorophyll in leaf tissue is affected by nutritional availability and environmental conditions such as drought, salt, cold, and heat *(Palta, 2009)*. Formula has been used to measure the content of chlorophyll of these mutants.

Table 3.3.1 Chlorophyll Content Measurement for CW mutant.

645 nm	652 nm	663 nm
0.016 nm	0.024 nm	0.042 nm
0.001 nm	0.008 nm	0.026 nm
0.064 nm	0.008 nm	0.137 nm
0.098 nm	0.121nm	0.177 nm
0.063 nm	0.071 nm	0.1 nm
0.027 nm	0.036 nm	0.059 nm
0.023 nm	0.03 nm	0.046 nm
0.025 nm	0.032 nm	0.05 nm
0.022 nm	0.031 nm	0.053 nm
0.025 nm	0.032 nm	0.05 nm
0.0364 nm	**0.0393 nm**	**0.074 nm**

Note (Calculation):

For CW variant

Absorbance = 0.0499

Chlorophyll Content = 0.0013861

Table 3.3.2 Chlorophyll Content Measurement for M1 mutant.

645 nm	652 nm	663 nm
0.009 nm	0.024 nm	0.042 nm
0.025 nm	0.047 nm	0.033 nm
0.101 nm	0.118 nm	0.148 nm
0.075 nm	0.089 nm	0.121 nm
0.05 nm	0.066 nm	0.095 nm
0.01 nm	0.006 nm	0.021 nm
0.003 nm	0.011 nm	0.027 nm
0.015 nm	0.025 nm	0.049 nm
0.001 nm	0.006 nm	0.019 nm
0.006 nm	0.015 nm	0.036 nm
0.0295 nm	**0.0407 nm**	**0.0591 nm**

Note (Calculation):

For M1 variant

Absorbance = 0.0431

Chlorophyll Content = 0.0011972

Table 3.3.3 Chlorophyll Content Measurement for M2 mutant.

645 nm	652 nm	663 nm
0.035 nm	0.038 nm	0.057 nm
0.021 nm	0.026 nm	0.034 nm
0.031 nm	0.047 nm	0.076 nm
0.049 nm	0.059 nm	0.081 nm
0.045 nm	0.065 nm	0.092 nm
0.042 nm	0.042 nm	0.063 nm
0.037 nm	0.049 nm	0.072 nm
0.029 nm	0.036 nm	0.052 nm
0.035 nm	0.046 nm	0.068 nm
0.033 nm	0.043 nm	0.063 nm
0.0357 nm	**0.0451 nm**	**0.0658 nm**

Note (Calculation):

For M2 variant

Absorbance = 0.04867

Chlorophyll Content = 0.001357407

Table 3.3.4 Chlorophyll Content Measurement for M5 mutant.

645 nm	652 nm	663 nm
0.006 nm	0.01 nm	0.018 nm
0.013 nm	0.028 nm	0.017 nm
0.058 nm	0.072 nm	0.091 nm
0.016 nm	0.04 nm	0.058 nm
0.043 nm	0.053 nm	0.076 nm
0.043 nm	0.049 nm	0.081 nm
0.031 nm	0.046 nm	0.072 nm
0.013 nm	0.018 nm	0.034 nm
0.013 nm	0.022 nm	0.039 nm
0.014 nm	0.021 nm	0.037 nm
0.025 nm	**0.0359 nm**	**0.0523 nm**

Note (Calculation):

For M5 variant

Absorbance = 0.0377333333

Chlorophyll Content = 0.001048148

Table 3.3.5 Chlorophyll Content Measurement for M6 mutant.

645 nm	652 nm	663 nm
0.042 nm	0.061 nm	0.098 nm
0.023 nm	0.073 nm	0.038 nm
0.1 nm	0.135 nm	0.186 nm
0.092 nm	0.119 nm	0.175 nm
0.056 nm	0.08 nm	0.13 nm
0.099 nm	0.12 nm	0.182 nm
0.08 nm	0.107 nm	0.152 nm
0.055 nm	0.071 nm	0.11 nm
0.076 nm	0.101 nm	0.153 nm
0.092 nm	0.119 nm	0.173 nm
0.0715 nm	**0.0986 nm**	**0.1397 nm**

Note (Calculation):

For M6 variant

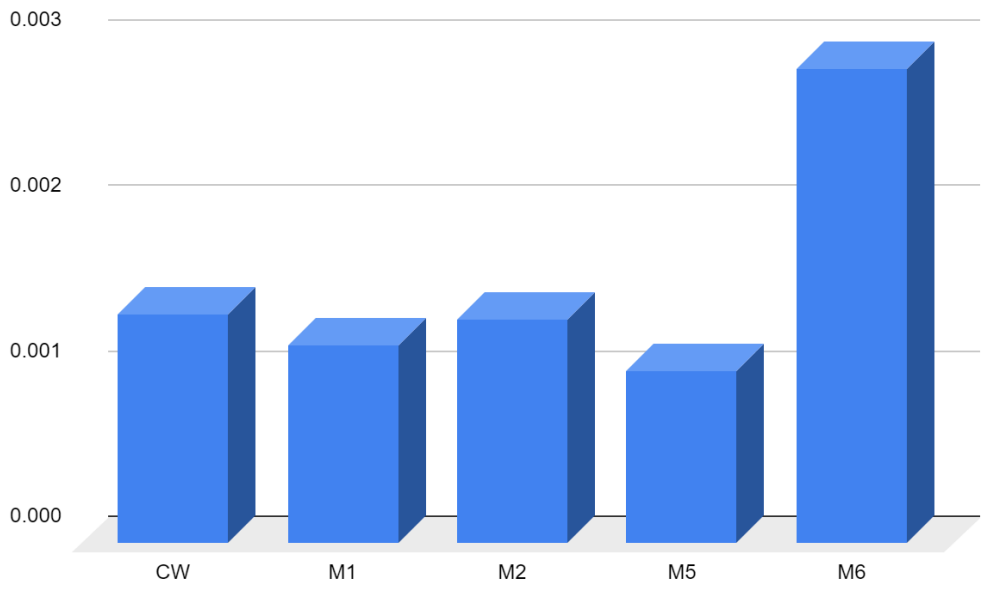
Absorbance = 0.1032666667

Chlorophyll Content = 0.0028685185

Table 3.3.6 Comparison between chlorophyll content of different mutants

CW	M1	M2	M5	M6
0.0013861	0.0011972	0.001357407	0.001048148	**0.0028685185**

Note: M6 has high chlorophyll content.



Graph 3.8 : Chlorophyll content of different mutants

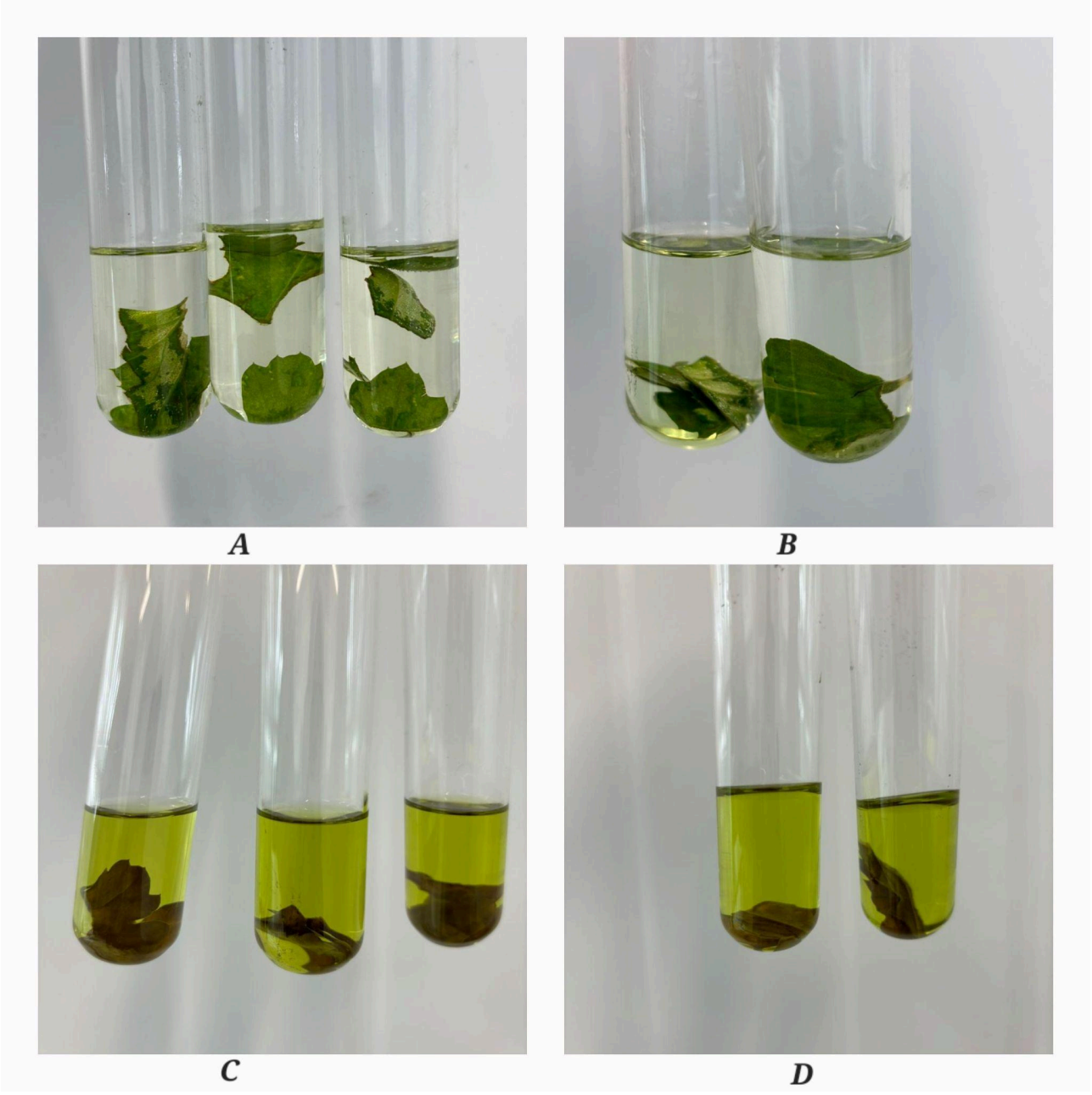


Figure 3.4: Chlorophyll Content Measurement study.

A), B) Leaves of different mutants on 80% acetone. C), D) Color change after 48 hours.

3.4 DNA Isolation Result: Comparison between the use of mechanical force and liquid Nitrogen:

In nanodrop results, an absorbance 260/280 ratio of 1.8 to 2.0 is generally accepted as "pure" for DNA, whereas less than or equal to 1.6 is considered as protein or phenol contamination and more than 2.0 as RNA contamination.

In the experiment, using liquid nitrogen yielded superior results overall. Four out of five samples showed good outcomes when treated with liquid nitrogen, highlighting its efficacy in DNA preservation and isolation. However, Sample 1 exhibited a poor result, suggesting a procedural error specific to that sample. In contrast, when liquid nitrogen was not used, only two samples demonstrated good results, while the remaining three were found to be highly contaminated. This underscores the importance of liquid nitrogen in minimizing contamination and ensuring successful DNA isolation. Moving forward, to address the presence of secondary metabolites in the isolated DNA, additional treatments will be necessary. These treatments aim to enhance the purity of the DNA and may involve methods such as phenol-chloroform extraction or commercial purification kits tailored for removing secondary metabolites. Implementing these steps will be crucial for obtaining reliable and uncontaminated DNA samples suitable for molecular applications.

Table 3.4.1 Quality Check of Isolated DNA (Nanodrop Result)

Sample	With Liquid Nitrogen (A 260/280)	Without Liquid Nitrogen (A 260/280)
Sample 1 (CW)	1.1	2.16
Sample 2 (M1)	1.9	2.07
Sample 3 (M2)	1.82	1.86
Sample 4 (M5)	1.89	1.51
Sample 5 (M6)	1.98	1.92

Table 3.4.2 Quality Comparison between the Isolated DNA

Sample	With Liquid Nitrogen	Without Liquid Nitrogen
Sample 1	Protein/Phenol Contamination	RNA Contamination
Sample 2	Pure	RNA Contamination
Sample 3	Pure	Pure
Sample 4	Pure	Protein/Phenol Contamination
Sample 5	Pure	Pure

Note: Liquid Nitrogen based method showed better results.

Chapter: 4

Discussion

4.1 Discussion:

This study's primary goal was to develop a micropropagation sterilizing procedure for the preservation of Chrysanthemum mutants. Four distinct mutant kinds (M1, M2, M5, M6) and one control variety (CW) were used in this investigation. There were two main components to this regeneration attempt. First, all varieties were subjected to 10 distinct sterilization procedures. Second, several types were subjected to the five most successful treatments. The findings on different combinations of treatments for explants show considerable differences in their efficacy. For example, for Chrysanthemum micropropagation winter is the best season as we have seen the most successful optimizing result of that season. Whereas, in the rainy season we needed to change the sterilization procedure. The best options for achieving the best outcomes are combinations A, B, H, and I since they are the least contaminated and fully responsive. Hormone-free *MS* medium was used to test shoot elongation, proliferation, and multiplication according to *Chowdhury et al., 2021*. Treatment A consisted of a 30-minute tap water wash, 15-minute detergent wash, and a 10-minute 0.8% NaClO wash *(Yesmin et al., 2014)*. Furthermore, the M1 survival rate for this treatment was 66.67%, while the CW survival rate was 100%. M5 survival rate was 73.33%, whereas M2 and M6 survival rates were 93.33% and 93.33%, respectively.

Treatment B consisted of a 25-minute tap water wash, a 10-minute detergent wash, and a 10-minute 0.8% NaClO wash. Although the survival rates for the M1 and M5 variants were 86.67% and 80%, respectively, the overall survival rate was 100%. On the other hand, 100% survived to the CW, M2, and M6 variations.

Treatments C, D, and E involved a 10-minute detergent wash and a 25-minute tap water wash. However, the NaClO treatment was 0.7% and 0.5% in treatments C and D, and 0.8% once more in treatment E. Treatment C had the highest survival rate at 30%, followed by treatment D at 16.67% and treatment E at 6.67%, which had the lowest reaction rate. Furthermore, treatment F had a 0% survival rate, indicating that it was another unsuccessful optimal sterilization treatment.

Additionally, treatment G had a 100% survival rate, however the development of the roots was sluggish. Treatment G consisted of a 25-minute tap water wash, a 15-minute detergent wash, 0.1%

HgClO, and 70% ethanol for 20 seconds *(Akter et al.,2012)*. During the rainy season, when microbiological contamination rates are higher, surface sterilization is required as regular treatment was not effective in this season. Due to its strong antibacterial properties, mercuric chloride is frequently employed as a surface sterilizer *(Gu et al., 2022).* As a result, this form of treatment is mostly employed during the rainy season. Mercury, one of the poisonous elements discharged into the environment, is thought to be extremely harmful to plant development. According to a study, the effects of varying mercury concentrations on shoot germination and growth performance in various plants (Mung bean) *(Iqbql et al., 2015).*

Treatment I and J were 20-minute tap water washes. Treatment I and J required only 10 minutes. For treatments I, 0.8% NaClO was used for 10 minutes and 70% ethanol for 20 seconds and for treatment J, 0.8% NaClO was used for 5 minutes and 70% ethanol for 15 seconds. The survival rates for therapy I, and J were 90%, and 96.67%, respectively. The CW treatment I, and J response rates were 100%, and 93.33%, respectively. For M2 variant therapy H, I, and J, the survival rates were 100%, 73.33%, and 93.33%, respectively. Additionally, for M6 variant therapy H, I, and J, the survival rates were 86.67%, 100%, and 80%, respectively.

For this study, hormone-free *MS* medium was utilized for regeneration. The commercial usage of plant hormones has certain potential downsides, including the risk of damage to the ecosystem and nontarget species. Overuse of plant hormones can also cause resistance in plants, reducing their efficacy over time. Additionally, hormone-free media is cost effective and time consuming. *(Bucker-Neto et al., 2007).*

In the current study, in hormone free media branching was visible after 1 week (7 to 8 days). Roots were visible after 5 to 6 weeks (35 to 42 days). In hormone free media Chrysanthemum takes the same duration for rooting and shoot multiplication *(Chowdhury et al., 2021).* According to *Chowdhury at., al 2021*, roots were visible after 7 to 8 weeks in hormone free media and in hormone induced media the rooting of regeneration shoot was fast also, hormone free media is cost effective and environmentally friendly.

Moreover, measuring the amount of chlorophyll can provide valuable information about the presence of biotic stress factors as well as abiotic problems like light, drought, and pigment that prevents herbicide damage (all of which have a significant impact on leaf chlorophyll content) *(Kalaji et al., 2017)*. Chlorophyll content is vital to measure since it is strongly tied to the plant's nutritional state. Furthermore, chlorophyll content can be utilized as an indirect measure of nitrogen levels in fertilizer to avoid overfertilization. For environmental stress study purposes these chlorophyll data measurement was necessary *(Kalaji et al., 2017)*.

From data, M6 has the highest chlorophyll content and CW and M2 contain the same amount of chlorophyll. Whereas, M5 contains the lowest amount of chlorophyll and M1 chlorophyll content is near CW and M2 content.

DNA extraction methods are critical in genetic studies, and two approaches work well for isolating DNA from Chrysanthemum mutants. The mechanical force approach, which includes grinding or shearing plant tissue, produces somewhat more protein contamination than the liquid nitrogen-based method. This is due to physical disturbance of cells and organelles, which might result in higher protein or RNA contamination. In contrast, the liquid nitrogen approach includes flash-freezing plant tissue to maintain cellular integrity and avoid nucleic acid damage. The greater protein contamination of this approach underscores the need of selecting a method based on unique research needs and desired DNA purity *(Lucina-Aguilar et al., 2016)*.

The trial examined DNA extraction methods with and without liquid nitrogen among five Chrysanthemum mutant samples. Liquid nitrogen consistently produced superior results, with four of five samples yielding positive findings, demonstrating its efficiency in DNA preservation and isolation. In contrast, samples not treated with liquid nitrogen yielded only two positive findings, while the rest were heavily contaminated, emphasizing the method's usefulness in reducing contamination. Moving further, to assure purity for molecular applications, additional procedures such as phenol-chloroform extraction or commercial purification kits designed to remove secondary metabolites are advised. Overall, DNA isolation using liquid nitrogen is extremely advantageous for future molecular reasons since it produces purer DNA samples that can be used reliably in

experiments.

In comparison between mechanical force and liquid Nitrogen based isolation methods, liquid nitrogen showed better results. Whereas in mechanical force RNA and Protein or Phenol contamination was more. For future molecular use, isolated DNA using liquid nitrogen will be more beneficial because of purity quality.

The initial step in doing many genetic investigations is to extract DNA from a plant. For example, we may determine whether the plant has any intriguing (helpful or detrimental) mutations in any of its genes, which may make it more adapted to flourish under a particular environment. When determining how closely related two plant species are, variations in their DNA might provide useful information *(Eswaran et al., 2004)*.

In other circumstances, we may wish to generate a clone of an essential gene in one plant (such as one that confers resistance to specific insects) and insert it into the genetic sequence of another plant, providing the same resistance *(Eswaran et al., 2004)*.

Quality checking DNA is crucial for various operations such as restriction digests, PCR, and RAPDs (Chapman & Hall, 1998), where knowing the quantity and integrity of DNA is essential. These quality assessments are invaluable for ensuring reliable results in subsequent studies and experiments.

Chapter: 5

Conclusion

5.1 Conclusion:

The study of Chrysanthemum micropropagation, Chrysanthemum mutant preservation and chlorophyll content is critical for improving agricultural methods. It allows farmers to mass-produce high-quality plants in regulated conditions, improving production while keeping desired features. The study also underlines the relevance of sterilizing processes and growth media selection in increasing regeneration efficiency and reducing contamination hazards. Chlorophyll content analysis assesses plant metabolism, photosynthetic efficiency, and reactions to environmental stresses, assuring long-term agricultural productivity. Overall, the outcomes of this study help to improve Chrysanthemum cultivation practices, genetic conservation, and breeding initiatives. By combining micropropagation, preservation, molecular analysis, and chlorophyll investigations, the study gives a comprehensive understanding of chrysanthemum biology and practical solutions for improving crop yield, resilience, and sustainability in agricultural systems.

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