CELL SUSPENSION ESTABLISHMENT AND HEAT SHOCK RESPONSE OF GOLDEN GARDENIA (*GARDENIA SOOTEPENSIS* HUTCH.)

THANAKON CHAICHANA

A Thesis Submitted to University of Phayao

in Partial Fulfillment of the Requirements

for the Bachelor of Science and Master of Science Degree in Biology

May 2025

การสร้างเซลล์แขวนลอยและการตอบสนองต่อภาวะช็อกจากความร้อนของคำมอกหลวง (*Gardenia sootepensis* Hutch.)

ธนกร ไชยชนะ

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Title

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Submitted by THANAKON CHAICHANA

Approved in partial fulfillment of the requriements for the
Bachelor of Science and Master of Science Degree in Biology
University of Phayao

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Title: CELL SUSPENSION ESTABLISHMENT AND HEAT SHOCK RESPONSE OF GOLDEN GARDENIA (*GARDENIA SOOTEPENSIS* HUTCH.)

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Keywords: Gardenia sootepensis Hutch., Plant Tissue Culture, Cell Suspension Culture, Heat Stress Response, Abiotic Stress Tolerance

ABSTRACT

Gardenia sootepensis Hutch., commonly known as golden gardenia, is a medicinal evergreen tree native to Southeast Asia, valued for its antimicrobial, anti-inflammatory, cytotoxic, and antioxidant properties. Despite its pharmacological potential, the cellular responses of *G. sootepensis* to abiotic stress, particularly heat stress, remain poorly understood. In this study, we successfully established protocols for callus induction and suspension culture using leaf explants. Murashige and Skoog (MS) medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D; 0.5–2.0 mg/L) in combination with low concentrations of kinetin (Kn; 0.1 or 0.2 mg/L) was the most effective treatment, consistently achieving a 100% callus induction rate. At 0.5–1.0 mg/L 2,4-D, the resulting callus was uniformly friable and olive-green, whereas higher concentrations (2.0 mg/L) produced brown callus. Suspension culture optimization further revealed that 2,4-D concentrations within the 0.5–1.0 mg/L range, in conjunction with 0.1 mg/L Kn, significantly enhanced cell proliferation and biomass accumulation. Heat shock experiments demonstrated that *G. sootepensis* suspension cells could withstand heat shock at temperatures up to 55°C (corresponding to an extracellular medium temperature of 46.7 ± 0.10°C) for 5 minute without exhibiting overt structural degradation. While promising advances have been made in developing *G. sootepensis* suspension cultures and identifying optimal growth conditions, further refinement of experimental protocols and extended analyses are essential to ensure long-term culture stability, assess genetic fidelity, and improve stress response metrics. These efforts will enhance the utility of suspension cultures as a platform for conservation, propagation, and future physiological and biochemical studies of this valuable medicinal species.

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Thanakon Chaichana

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CHAPTER 1

INTRODUCTION

Background

Golden gardenia (*Gardenia sootepensis* Hutch.) is the ever-green tree in Rubiaceae family which native to Cambodia, China South-Central, Laos, Myanmar, Thailand, and Vietnam. This species has several pharmacological uses mainly as antimicrobial, anti-inflammatory, cytotoxic, antioxidant and anti-angiogenic or the ability to hinder the growth of blood vessels in cancerous cells. The discovery of four new cycloartane triterpenes and two new 3,4-seco-cycloartane triterpenes, sootependial and sootepenoic acid, which have cytotoxic properties from the apical bud of *G. sootepensis*, is reported (Pudhom et al., 2012; Warinthip et al., 2022; Youn et al., 2016).

Heat stress is one of the severe environmental issues that affect growth, development and productivity of plants. Several impacts are provided on how climate change is reducing yields. Temperature stress in the temperate and tropical regions reduces the yield of wheat, rice and corn (Challinor et al., 2014). Hence, the knowledge of the cellular processes regulating plant responses to heat stress is crucial for enhancing plant tolerance when exposed to heat, especially in view of global warming (Li et al., 2021). Heat stress damages cell membranes, unravels proteins, and affects nucleotides and increases reactive oxygen species (ROS) that induce oxidative stress and can lead to programmed cell death in plants (Apoptosis) (Poór et al., 2021). In plant cells, heat stress increases membrane fluidity and induces an oxidative burst, leading to lipid peroxidation. This causes structural changes in the lipid membranes, resulting in membrane damage, electrolyte leakage, and ultimately, cell death (Narayanan et al., 2016; Prasertthai et al., 2022).

Suspension cultures of plant cells are exceptionally useful systems for studying plant reactions to heat stress. Investigative scientists can manipulate and regulate the external environment to a fine degree, which allows for detailed study of the specific molecular processes in heat stress tolerance (Yang et al., 2022).

Research on *Gardenia jasminoides*, a related species, has demonstrated the feasibility of establishing stable callus and suspension cultures on Murashige and Skoog (MS) medium supplemented with phytohormones such as kinetin and 1-naphthylacetic acid (Kim et al., 1991; Liu et al., 2018). This suggests that the cultivation of calluses and suspended cells of golden gardenia is similarly feasible.

Previous studies using heterotrophic and autotrophic suspension cultures of *Chenopodium rubrum* L. investigated the heat stress response in plant cells (Chaidee et al., 2008). These studies observed that increasing temperatures led to an increase in extracellular pH, an enhancement of extracellular proton flux, and changes in the formation of actin rings. Heat shock induced alkalinization of the extracellular environment, accompanied by changes in the actin cytoskeleton, including the formation of actin rings. However, when the heat-shocked cells were transferred back to the control medium and conditions, these changes returned to normal. Thus, suspension cells are valuable for observing the effects of heat stress on plant cells. In a similar study in heat stress response of *Pyrus communes* cv. Bartlett, by using three viability tests: regrowth (culture growth during 10 days after stress), triphenyl tetrazolium chloride reduction, and electrolyte leakage. The experimental results showed that the critical temperatures (50% injury) for 20 min of heat exposure were 42°C, 52°C and 56°C, respectively, electrolyte leakage had the lowest temperature coefficient (Wu & Wallner, 1983). The above data demonstrated the feasibility of studying heat stress in woody plant cells by observing extracellular pH.

While significant progress has been made in understanding heat stress responses in model plants like *C. rubrum* L., there is still much to learn about other

species, particularly woody plant. This study aims to bridge this knowledge gap by focusing on *Gardenia sootepensis* Hutch., an ever-green tree species with notable medicinal properties. This study aims to establish the callus and suspension cells derived from golden gardenia and to explore the cellular responses of these cells to heat stress. This includes examining changes in extracellular pH under heat shock, compared to previous research.

Objective

1. To cultivate callus and cell suspension of golden gardenia.
2. To explore heat stress response in golden gardenia cells.

Hypothesis

1. MS medium supplemented with kinetin 0.3 mg/L and 1-naphthylacetic acid 0.5 mg/L is the best media for callus inducing same as the previous study on *Gardenia jasminoides* L. (Liu et al., 2018).

2. The development of golden gardenia suspension cells will be feasible under optimized tissue culture conditions.

3. Heat stress will induce changes in the extracellular pH of golden gardenia suspension cells, pH value will be increased when plant cell exposed to higher temperatures (Chaidee et al., 2008).

Scope of Research

The objective of this experiment was to develop a suspension cell and study the heat shock response of golden gardenia (*Gardenia sootepensis* Hutch.). The experiment was divided into 3 main steps including callus culture, cell suspension culture, and heat stress response study. The scope of the experiment was determined as follows:

1. Callus Inducing Experiment

1.1 Explant: Aseptic golden gardenia leaf

1.2 Medium: MS medium supplemented with cytokinin hormones (including kinetin (Kn), and N6-Benzyladenine (BA)) and auxin hormones (including 1-Naphthylacetic acid (NAA), and 2,4-Dichlorophenoxyacetic acid (2,4-D)) in different concentration.

1.3 Number of Replicates: 5 replicated (5 bottles for each replicate)

1.4 Measurement: Observe callus formation and examine its structure under a compound light microscope and a stereo microscope.

1.5 Result: Callus induction rate (%), where higher rates indicate that the medium is suitable for callus induction, along with observations of callus structure and cell formation.

1.6 Statistical Analysis: Independent samples Kruskal-Wallis test

2. Cell Suspension Experiment

2.1 Explant: Golden gardenia callus obtained from callus inducing experiment

2.2 Medium: The best medium formula from callus inducing experiment

2.3 Number of Replicates: 3 replicated

2.4 Measurement: Record the packed cell volume (PCV, %), absorbance value at 480 nm wavelength (A480) from TTC assay, and observe cells under compound light microscope.

2.5 Result: PCV and A480 varied weekly. Morphology of suspended cells observed.

2.6 Statistical Analysis: Independent samples Kruskal-Wallis test

3. Heat Shock Experiment

3.1 Explant: Golden gardenia suspension cell obtained from cell suspension experiment

3.2 Medium: The best medium formula from cell suspension experiment

3.3 Method: Using a 6-week-old cell suspension, pipette 1 mL of the culture into a 1.5-mL centrifuge tube and place it in a water bath at different temperatures (35 ℃, 45 ℃, 55 ℃, 65 ℃, and 75 ℃) for 5 minutes.

3.3 Number of Replicates: 9 repetitions per temperature range

3.4 Measurement: Record the pH of the extracellular fluid, the absorbance value at 595 nm (A595) from the Bradford assay, the absorbance value at 480 nm (A480) from the TTC assay and observe cell changes under a compound light microscope before and after heat shock treatment.

3.5 Result: Change of extracellular fluid pH, A595, A480, and cell component after heat shock treatment.

3.6 Statistical Analysis: Independent samples Kruskal-Wallis test

Duration of Project

7 mouths for Laboratory work (April 2024 – October 2024). The thesis should be completed between November 2024 and April 2025.

Research Location

Section of Dr. rer. nat. Chatchawal Wongchai, Plant tissue Culture Laboratory, Demonstration School, University of Phayao

Definition of Specific Terms

1. Murashige and Skoog (MS) Medium: The nutrient solution that is used commonly in plant tissue culture containing certain mineral salt, vitamins, sucrose to help the plant cells and tissues to grow in tissue culture.

2. N6-Benzyladenine (BA): A synthetic plant hormone which increases mitotic activity and differentiates plant tissues in tissue culture; used to encourage shoot development and/or callus formation.

3. Kinetin (Kn): Another cytokinin which also controls the growth and differentiation of the plant, stimulation of cell divides and shoot formation in explained tissue culture experiments.

4. 1-Naphthylacetic Acid (NAA): A synthetic auxin, which is plant growth hormone and used in tissue culture to induce root formation from the stem or callus tissues or used jointly with cytokinin to make callus.

5. 2,4-Dichlorophenoxyacetic Acid (2,4-D): A synthetic auxin (plant growth hormone) commonly used in plant tissue culture to stimulate callus formation, cell division, and root initiation. It is widely used to promote plant regeneration and somatic embryogenesis. At higher concentrations, 2,4-D can also induce abnormalities or inhibit plant growth, which is why its concentration must be carefully optimized in tissue culture media to avoid toxicity.

6. Gellan Gum: An agent used in nutrition/culture media and gels involving plant tissue culture to get structural support and firmness in the culture media.

7. 2,3,5-Triphenyl Tetrazolium Chloride (TTC): A redox dye that is converted by live cells into a colored formazan product for the determination of cell's ability to support metabolism for tissue culture assays.

8. Extracellular pH and Proton Flux: Indices of the pH of the culture medium in which the plant cells are placed (extracellular pH) and the protons' concentration by cells membranes (proton flux), reflecting organism stress reaction and cell metabolism intensity.

9. Heat Shock: Trans-affect temperature change in plants or cells by applying heat shock within temperature optima resulting in alteration of gene and protein synthesis accompanied with other cellular events known as thermal stress.

10. Suspension Culture: A method used in plant tissue culture that involves the culture of cells or cell clusters in a liquid nutrient solution under controlled environmental conditions; enables identification of cell reactions, growth and metabolic processes without interference from a solid substrate of culture.

Expected Benefits

1. Development and optimization of callus and suspension culture protocols for golden gardenia.

2. Establishment of the suspension cells is useful in performing premeditated experiments involving the study of the cellular phenomena in the in vitro environment and aids in enhancing the evolutionary research in plant physiology and biology especially in heat stress responses.

3. Specific information on how the cells of golden gardenia plants respond to a heat shock at the cellular level.

4. Openings for growing thermally stable compounds from plants that can be useful in the pharma or the industrial business.

CHAPTER 2

LITERATURE REVIEW

Botanical Information of Golden Gardenia

Golden gardenia (*Gardenia sootepensis* Hutch.), also known as Thai gardenia or sootep gardenia, is an evergreen tree belonging to the Rubiaceae family. It is native to Southeast Asia, specifically Thailand, Laos, and Burma. Typically, this tree grows to a height of 5-10 meters and is characterized by its fragrant yellow flowers and glossy green leaves.

In the northern regions of Thailand, *G. sootepensis* can reach heights of 7-10 meters and often secretes a gelatinous substance. The tree features branches with both developed and shortened internodes, which are somewhat compressed to angled or subterete, and are densely puberulent, pilosulous, or tomentulose, becoming glabrescent over time. Its leaves are arranged oppositely with petioles measuring 0.6-1.2 cm and are either puberulent or tomentulose. The leaf blades are papery or thinly leathery, with an obovate to elliptic-oblong shape, measuring 7-29 cm in length and 3-16 cm in width. The adaxial surface of the leaves can be puberulent or pilosulous to glabrous, while the abaxial surface is densely tomentose. The base of the leaves is rounded to obtuse or cuneate, and the apex is shortly acuminate with an acute or obtuse tip. There are typically 12-20 pairs of secondary veins, with densely pilosulous domatia in the abaxial axils. The stipules are calyptrate, conical, 0.5-1 cm in length, sericeous outside, densely puberulent or tomentulose inside, with a triangular apical portion that is caducous and a basal portion that is truncate to broadly rounded and usually persists with the leaves, sometimes becoming hardened.

The flowers of *G. sootepensis* are pseudoaxillary, usually near the branch apices, and are typically solitary with puberulent peduncles measuring 1-1.5 cm. The calyx is densely puberulent to pilosulous externally, with the ovary portion being ellipsoid and smooth, measuring 5-6 mm. The limb is spathaceous, 13-15 mm long, splitting along one side for 2/3-3/4 of its length, with a sericeous inside that is often viscid or mucilaginous. The corolla is yellow or white and salver-form, with a cylindrical tube measuring 50-70 mm in length and 3-5 mm in diameter, sparsely puberulent outside and glabrous inside. The corolla has five broadly obovate lobes, each measuring 40-50 mm in length and 20-30 mm in width, which are glabrous on both surfaces and obtuse to acute.

The fruit of *G. sootepensis* is an ellipsoid or ellipsoid-oblong berry, measuring 2.5-5.5 cm in length and 1.5-3.5 cm in width, puberulent, and either smooth or with 5-6 longitudinal lines or very weak ridges, with a leathery to hard texture. The seeds are suborbicular, flattened, and 3-4 mm in diameter, with a foveolate surface. The tree flowers from April to August and bears fruit from June to April (The Botanical Garden Organization, 2013; Warinthip et al., 2022; Wu et al., 2011).

Figure 1 Tree of *G. sootepensis* Hutch.

Source: University of Phayao, n.d.

Figure 2 Flowers of *G. sootepensis* Hutch.

Source: Division of Building and Facilities, University of Phayao, 2021

Figure 3 Fruit of *G. sootepensis* Hutch.

Source: The Botanical Garden Organization, 2013

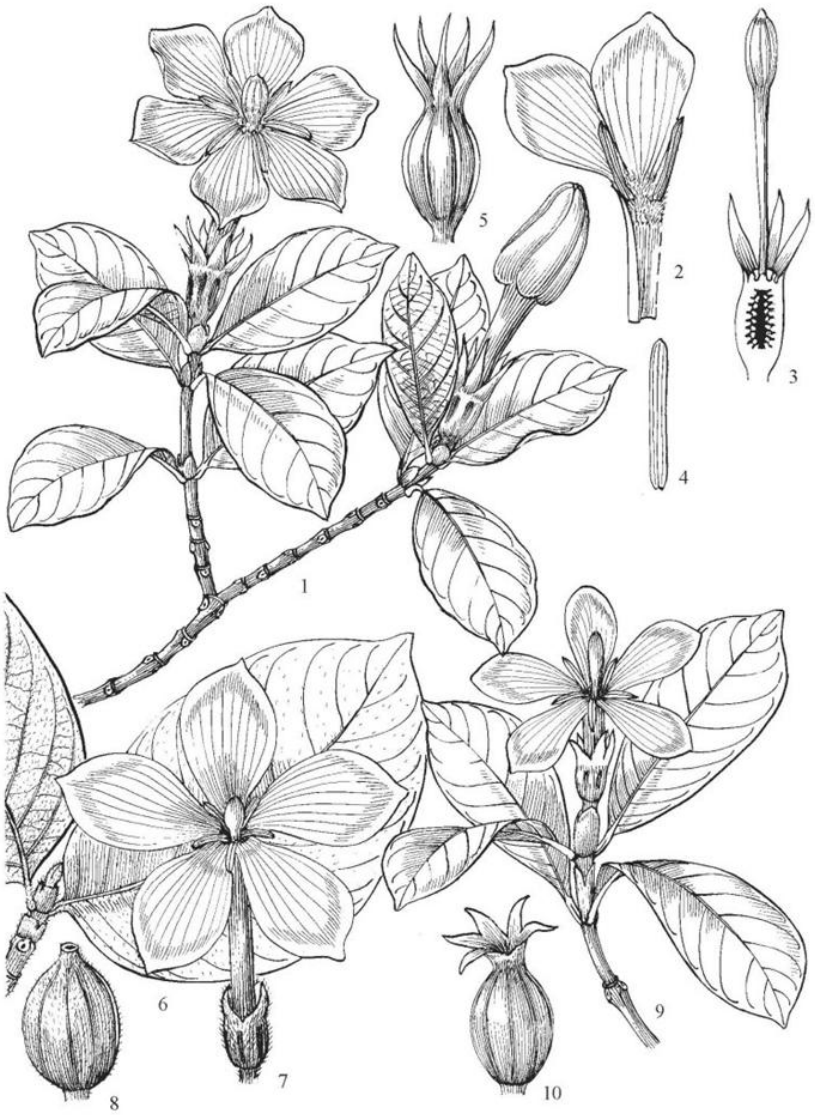

Figure 4 *Gardenia* Illustration

Note: 1-5. *Gardenia jasminoides* J.Ellis var. jasminoides, 1. flowering branch, 2. corolla spread out, 3. longitudinal section of portion of flower, 4. Anther, 5. fruit, 6-8. *Gardenia sootepensis* Hutch., 6. leafy branch, 7. flower, 8. fruit, 9-10. *Gardenia hainanensis* Merr., 9. flowering branch 10. fruit

Source: Wu et al., 2011, 141-143

Plant Heat Stress Response

Heat stress is one of the severe environmental issues that affects growth, development and productivity of plants. Some of the impacts are provided on how climate change is reducing yields: temperature stress in the temperate and tropical regions reduces the yield of wheat, rice and corn (Challinor et al., 2014). Heat stress results in the generation of ROS. While ROS is required in the body in defense mechanisms and cellular signaling, excessive levels can be destructive through oxidation. Heat stress interrupts the balance by increasing the production of ROS in plant cells above the rate at which they can be neutralized. Superoxide radicals (O^{2-}) and hydrogen peroxide (H_2O_2) can be generated when the electrons transport chain located in chloroplasts are over-reduced during photosynthesis under heat stress. Similarly, ROS can come from the mitochondrial electron transport chain; especially when high temperatures are a probable factor for the degradation of the respiratory chain. H_2O_2 can also be generated as a waste product by some organelles that are involved in other metabolic processes, the example is peroxisomes involved in photorespiration. In addition, the superoxide radicals can be formed from the NADPH oxidase of plasma membrane under the stress signals like heat shock (Poór et al., 2021).

Heat stress in plants can damage several of their cellular components due to excessive reactive oxygen species (ROS). This will increase the permeability of the outer membrane, cause ion leakages and eventually result in cell dehydration. There are also proteins which oxidative damage can affect. The ROS-induced changes in amino acids that form proteins may lead to protein denaturation, aggregation and loss of enzyme activity, all of which can disrupt cellular processes that are essential for growth. In addition, ROS can lead to strand breaks and base modifications in DNA, leading to genomic instability at transcription and replication levels. On the other

hand, carbohydrate-rich cell walls may be damaged by ROS thus affecting their structure and function (Gill & Tuteja, 2010).

Heat stress has an extremely severe effect on the cell membrane due to alterations in the membrane and intracellular or extracellular environment. This occurs because high temperatures accelerate the formation of lipid peroxides, thereby increasing the fluidity and permeability of the membranes. The consequence is that there is ion leakage, loss of cellular integrity, and eventually interference with certain cellular processes. In response to heat stress plants alter their lipid composition by lowering their membrane's fluidity. They do this specifically by increasing the ratio of saturated fatty acids (Upchurch, 2008). Heat stress can cause significant changes in pH within the intracellular and extracellular environments. Cytoplasm of cells is often acidified by heat stress because it triggers proton pumps activation and release of protons from damaged cellular compartments like vacuoles (Felle, 2005). Altering the intracellular pH can affect enzyme activities, metabolism activities, as well as general cellular homeostasis. Conversely, heat stress may mechanically alter the extracellular apoplast's pH outside of the plasma membrane depending on the type of plant and type of stress involved. In view of this, under some circumstances, for example during infection or mutualistic symbiosis with microbes that live on root surfaces or fix nitrogen, plants become more acidic while others become either more acidic or more alkaline when stressed by other means (Kiegle et al., 2000).

Previous studies using heterotrophic and autotrophic suspension cultures of *Chenopodium rubrum* L. investigated the heat stress response in plant cells. These studies observed that increasing temperatures led to an increase in extracellular pH, enhanced extracellular proton flux, and changes in the formation of actin rings. Heat shock induced alkalinization of the extracellular environment, accompanied by changes in the actin cytoskeleton, including the formation of actin rings. However,

when the heat-shocked cells were transferred back to the control medium and conditions, these changes returned to normal (Chaidee et al., 2008).

Wu and Wallner (1983) on “Heat stress responses in cultured plant cells: Development and comparison of viability tests” compared three methods for testing plant cell viability after heat stress treatment of pear (*Pyrus communes* cv. Bartlett) suspension culture: regrowth (culture growth during 10 days after stress), triphenyl tetrazolium chloride reduction, and electrolyte leakage. The experimental results showed that the critical temperatures (50% injury) for 20 min of heat exposure were 42°C, 52°C and 56°C, electrolyte leakage had the lowest temperature coefficient. Heat stress inhibition of triphenyl tetrazolium chloride reducing capacity was much greater if the viability test was conducted 3 days, rather than immediately, after the stress treatment.

When analyzing the research examples on *C. rubrum* and *P. communes* cv. Bartlett can be seen the connection between heat stress treatment makes it possible to apply them to studies with *G. sootepensis* and other woody plant species.

Callus Culture

Heat stress is one of the severe environmental issues that affect growth, development and productivity of plants. Some of the impacts are provided on how climate change is reducing yields: temperature stress in the temperate and tropical regions reduces the yield of wheat, rice and corn (Challinor et al., 2014). Callus induction refers to the process of stimulating plant cells to form a mass of undifferentiated cells, known as calluses, from plant tissues or organs under in-vitro conditions. This process involves using specific plant growth regulators (PGRs) in a culture medium to promote cell division and dedifferentiation. In general, there are 5 important processes in callus induction experiments: explant preparation, culture medium preparation, incubation, and observation

For explant preparation, healthy, young, and actively growing parts of the plant (e.g., leaves, stems, roots) were chosen to be material. The selected part was sterilization step by step with sterilizing solution (e.g., mercuric chloride, sodium hypochlorite (NaOCl), and chlorine (Cl)) and surfactants in appropriate conditions. The selection of suitable substances and methods depends on the type of tissue to be cleaned, as the dirtiness and fragility of plant tissue from different parts is a point of caution. For sterilizing *Petiveria alliacea* L. branch segments, a 1% NaOCl solution with 0.05% Tween 80 was used. The segments were disinfected under agitation for 15 minutes. Afterward, nodal explants were excised and rinsed three times with autoclaved distilled water. They were then immersed in a 1% solution of the fungicide benomyl and rinsed again three times (Castellar et al., 2011). Surface disinfected of *Swertia lawii* Burkill seeds, an aqueous solution of 0.1% HgCl2 was used (Kshirsagar et al., 2015).

The type of culture medium used in the experiment depends on the type of plant. In general, the main ingredients necessary in every plant tissue culture medium recipe can be categorized into macronutrients (nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), and sulfur (S)), micronutrients (iron (Fe), manganese (Mn), zinc (Zn), copper (Cu), boron (B), molybdenum (Mo), and cobalt (Co)), vitamins (thiamine (Vitamin B1), pyridoxine (Vitamin B6), nicotinic acid, (Vitamin B3), and myo-inositol), amino acids, and carbon source (sucrose, glucose, or fructose). Mostly used media are Murashige–Skoog (MS), Gamborg (B5), White (WH), Nitsch and Nitsch (NN), Linsmaier and Skoog (LS), Schenk and Hildebrandt (SH), McCown and Lloyd (woody plant medium, WPM). However, the proportion of macro and micronutrients is a factor that causes different effects on the same type of experimental plant (Sathyanarayana & Varghese, 2007). In this study, we use MS medium (Murashige & Skoog, 1962; Pirlak & Almokar, 2018) that widely used for a

variety of plant species, suitable for callus formation, organogenesis, and micropropagation.

To induce plant tissue to form shoots, roots, or callus, it is necessary to supplement culture media with appropriate plant growth regulators or phytohormones. Plant growth regulators known as phytohormones have significant roles in plant tissue culture as they regulate several physiological and developmental processes in plants. Generally, there are two main types of hormones used in plant tissue culture: auxins and cytokinins. Auxins primarily stimulate cell division, root formation and development, induce callus formation from explants, and influence phototropism in plants (Paciorek & Friml, 2006). Types of auxins that are used in tissue culture include: indole-3-acetic acid (IAA), indole-3-butyric Acid (IBA), naphthalene acetic acid (NAA), and 2,4-dichlorophenoxyacetic acid (2,4-D). With reference to cytokinins, their main roles include the stimulation of cell division and cytokinesis, promotion of shoot formation and growth, and inhibition of aging and senescence of tissues in culture (Bhattacharya, 2019; Howell et al., 2003). Types of cytokinins that are used in tissue culture include: benzyl aminopurine (BAP), kinetin (Kn), thidiazuron (TDZ), and zeatin. Thus, cytokinin-type hormones must be used if the goal of the developmental process is to produce shoots, whereas auxin-type hormones are used to induce root formation. To develop callus from explants, 2,4-dichlorophenoxyacetic acid or a combination of auxins and cytokinins can be used.

To induce callus, hormones such as auxin are often used or used combination with cytokinins, especially 2,4-dichlorophenoxyacetic acid that is widely used for callus induction. In *Moringa Oleifera* Lam. callus culture, 2,4-D was superior to NAA in the percentage of callus induction after three weeks of cultivation with 100% induction occurred via supplemented 0.1 mg/L 2,4-D (Al-Hamidi et al., 2023). Anther cultures of spring wheat in comparing the performance of 2,4-D and IAA for

calli induction, plant regeneration, and green plant production found that the addition of 2,4-D to the induction medium resulted in significantly higher means for all anther culture components compared to IAA. While the addition of 2,4-D significantly reduced plant regeneration, it substantially increased the percentage of green plants at a concentration of 0.3 mg/L IAA (Ball et al., 1993). Although 2,4-dichlorophenoxyacetic acid (2,4-D) performs better compared to other auxin hormones, it is generally not suitable for subsequent plant regeneration from callus. This is because 2,4-D, a synthetic auxin, tends to maintain the callus in an undifferentiated state and can inhibit the regeneration of shoots and roots (George et al., 2008). By History of Plant Tissue Culture (Thorpe, 2007) discusses the use of 2,4-D in tissue culture and highlights its effectiveness in inducing callus formation while noting its inhibitory effects on shoot and root regeneration from callus. The toxicity effects of 2,4-D have been observed in tissue-cultured *Allium* roots at a concentration of 4.02 mg/L after 48 hours of treatment. The roots tended to become toxic with prolonged exposure to 2,4-D (Özkul et al., 2016). Callus of gardenia (*Gardenia jasminoides* Ellis) can induce via combination of thidiazuron (TDZ) and indoleacetic acid (IAA) (Al-Juboory et al., 1998) or by combination of auxins (IAA, NAA, and 2,4-D) and cytokinin (BA and Kn) (Kim et al., 1991; Liu et al., 2018). Therefore, the supplementation of cytokinin hormones (including kinetin (Kn), and N6-Benzyladenine (BA)) and auxin hormones (including 1-naphthylacetic acid (NAA), and 2,4-dichlorophenoxyacetic acid (2,4-D)) into the culture media was applied and adapted from previous studies.

In the step of incubation, callus cultivation should be provided in an environment with low contamination and suitable conditions for plants. However, the controlled environment depends on the type of plant and the purpose of the experiment. In this research, the chamber temperature and lighting settings were

adapted from previous research (Liu et al., 2018), a photo period of 12/12 hours (light/dark) at 23 ± 1 °C.

There are several ways to observe callus growth, such as: weighing fresh and dry weight of callus, callus distribution area, and percentage of induction. Method to observed induction efficiency without loss of cultured callus is measuring by callus formation rate (%) and callus efficiency (g/callus) was calculated (Hagaggi et al., 2024).

Plant Suspension Culture Establishment

Plant cell suspension culture is a technique used to grow plant cells in a liquid medium under sterile and appropriate conditions, promoting rapid cell division and growth. Plant suspension cultures offer several advantages over traditional plant tissue cultures grown on solid media. One of the primary benefits is the ability to scale up cultures for large-scale production of valuable compounds, such as pharmaceuticals, flavors, fragrances, and pigments (Rao & Ravishankar, 2002). Additionally, suspension cultures provide a uniform and controlled environment for studying plant cell physiology, biochemistry, and genetic responses to various stimuli that can be used as fundamental tool in studies of plant cytology, plant physiology, pharmacology, plant genetics, and plant biotechnology (Moscatiello et al., 2012).

The first step in preparing an explant for suspension culture involves using either plant parts or callus tissue. If plant parts are used, they must be properly sterilized. Once sterilized, the plant tissue should be ground to release individual cells suitable for culture. This can be done by using a sterile medicine grinder or Potter-Elvehjem tissue grinder to grind the plant parts with liquid medium and then transfer the mixer to prepared liquid medium to suspension at appropriate conditions. In another way, callus can be directly transferred from traditional culture to suspension culture by using sterile forceps or spoon. However, the characteristics

of the callus used for culture will affect the characteristics of the cell suspension obtained. In this study, based on the study methods of previous research (Liu et al., 2018), friable callus was selected for fine cell suspension culture and incubated in incubated shaker with 120 rpm at 23 ± 1 °C under continuous light.

To monitor, check the cultures weekly for signs of increasing the number of cells, contamination, and overall health. In general, to keep cells alive and multiply efficiently, sub-culture should be done every 7-14 days. Observing cell proliferation can be done in several ways, such as fresh cell weight, settled cell volume, %PCV (packed cell volume percentage), protein content of cells, amount of DNA and RNA, and cells viability (Mamdouh & Smetanska, 2022; Ryu et al., 1990). in this study, suspension cells growth measurement was observed by %PCV and cells viability.

Diversity of Callus and Suspension Cell Shapes

Callus refers to an unorganized, proliferative mass of parenchymatous cells, typically induced by exposing explants to plant growth regulators (PGRs), particularly auxins and cytokinins. The morphology of callus can vary significantly depending on the plant species, type of explant, and the hormonal composition of the culture medium. For example, calli may appear friable, compact, nodular, mucilaginous, or even translucent (Ikeuchi et al., 2013). In previous studies, callus cells have exhibited various shapes. In *Coffea arabica*, callus cells were arranged in clusters and included isodiametric and elongated cells, as shown in **Figure 5** (Pádua et al., 2014). Similarly, in *Camellia sinensis*, callus cells were predominantly spherical or isodiametric in shape, as shown in **Figure 6** (Esteban-Campos et al., 2024).

Suspension cultures derived from callus tissues exhibit considerable diversity in both cell shape and aggregation patterns. Cell shapes observed in suspension cultures include spherical, elongated, irregular, and star-like forms, with cells occurring as single units, small clusters, or large aggregates. The morphology of

these cells is influenced by factors such as the cytoskeleton, cell wall properties, the age of the culture, and the composition of the growth medium (George et al., 2008). *In* previous studies, various cell shapes have been reported in different species. In *Coffea arabica*, suspension culture cells included elongated and rounded cells, as well as embryogenic cells characterized by transverse and unequal division patterns, and embryogenic cellular aggregates, as shown in **Figure 7** (Silva et al., 2005). In *Sutherlandia frutescens*, suspension cells were found to be round, oval, straight and narrow, as well as curved or zigzag-shaped, as shown in **Figure 8** (Nosov et al., 2023).

Overall, the diversity of shapes observed in plant callus and suspension cells is influenced by multiple interacting factors, including the plant genotype, the type of explant used, the age of the culture, the composition of the culture medium, and the concentration and ratio of plant growth regulators (PGRs) such as auxins and cytokinins (George et al., 2008; Ikeuchi et al., 2013).

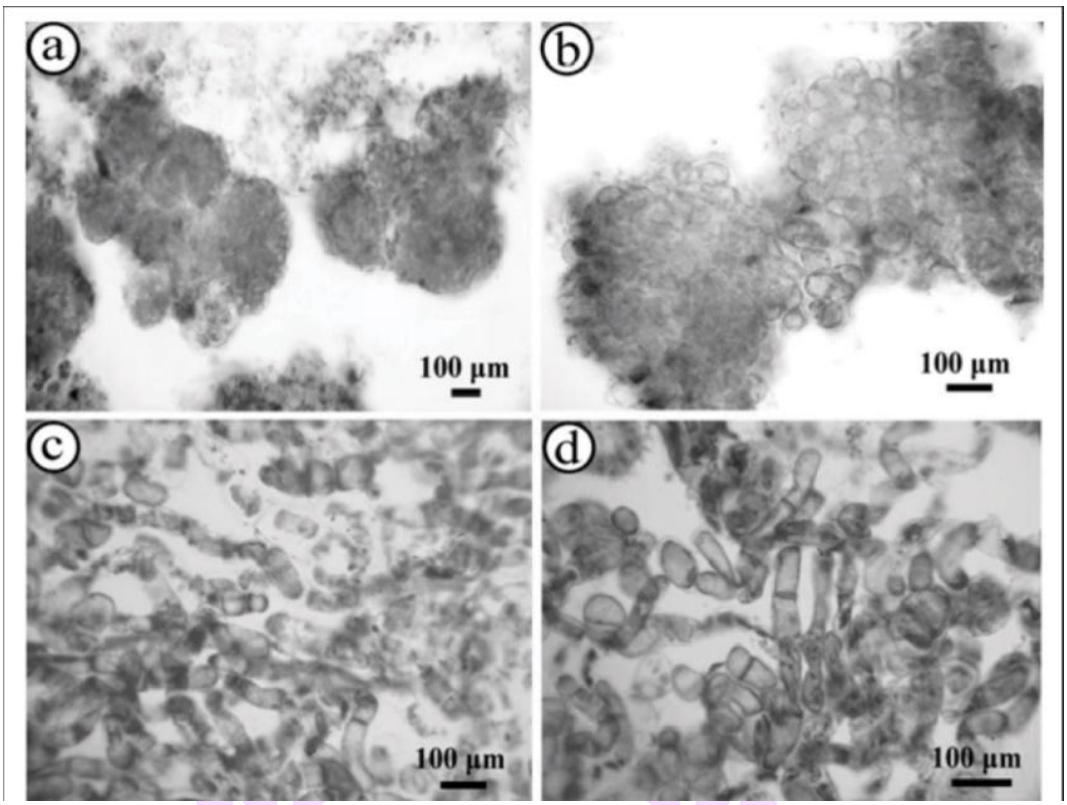

Figure 5 Diversity of *Coffea arabica* Callus Cell Shapes

Note: Double staining with Acetocarmine and Evans Blue. (a) and (b) Cell mass of yellow friable callus. (a) Deep red stained cells; (b) Red-stained isodiametric cells; (c) and (d) Cell mass of transparent watery callus: blue stained elongated cells.

Source: Pádua et al., 2014, 660-665

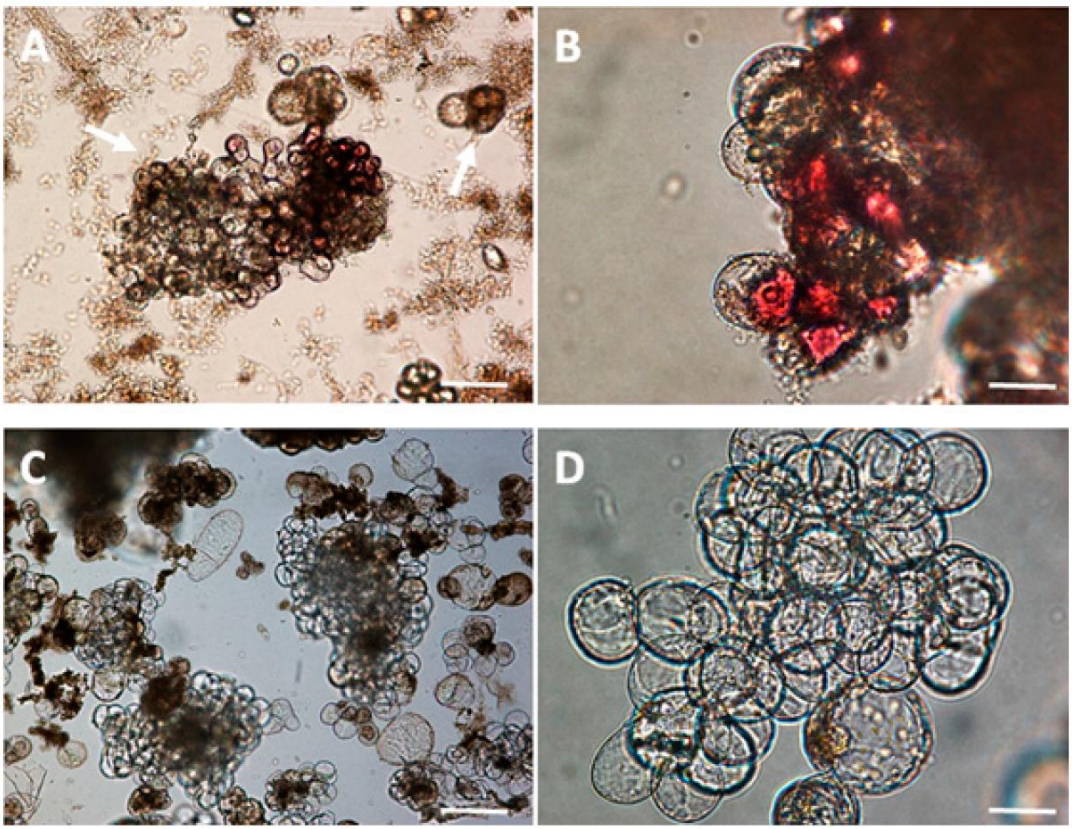

Figure 6 Diversity of *Camellia sinensis* Callus Cell Shapes

Note: Cell morphology of LSC-5R CSC callus (scale bar at 1 mm, (A), scale bar at 50 µm, (B)). Cell morphology of LSC-5Y CSC (scale bar at 1 mm, (C), scale bar at 50 µm, (D). The arrows point to necrotic cells.

Source: Esteban-Campos et al., 2024, 2461

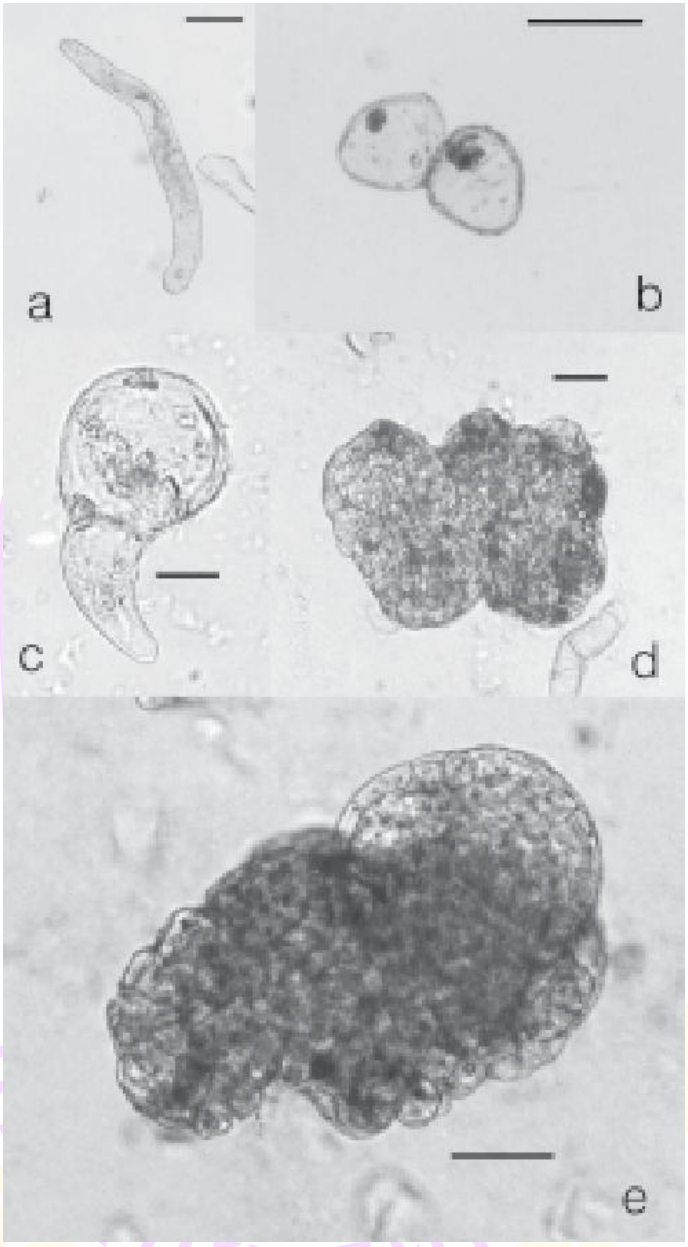

Figure 7 Diversity of *Coffea arabica* Suspension Cell Shapes

Note: Cell types in suspension cultures. a: elongated cell, b: rounded cells, c: embryogenic cell with a transverse and unequal division pattern, d: embryogenic cellular aggregate, e: compact cell aggregate with a globular embryo. Calibration bars: 100μm in a and b, 50μm in c, d and e.

Source: Silva et al., 2005, 694-698

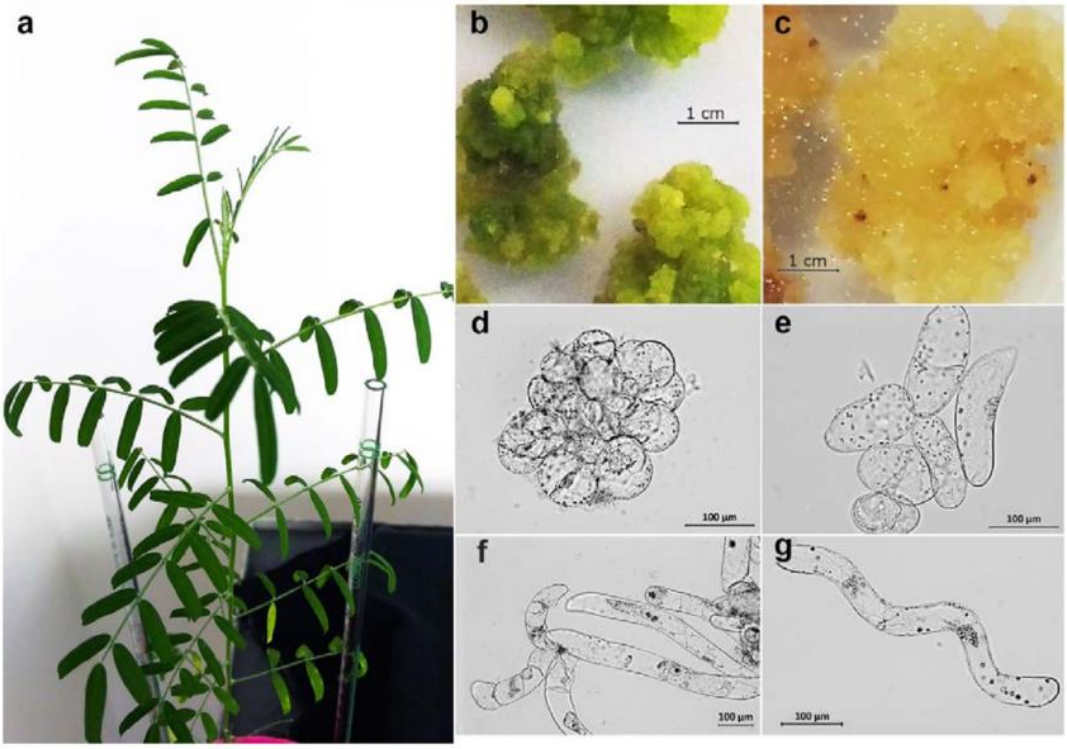

Figure 8 Diversity of *Sutherlandia frutescens* Suspension Cell Shapes

Note: Plant and cell cultures of *Sutherlandia frutescens*: (a) 9-month-old greenhouse plant raised from a sterile seedling; b callus culture originated from cotyledon explant (SfCC), sub-cultured under light; c callus culture originated from hypocotyl explant (SfHC), sub-cultured in the dark; d cell morphology of the suspension cell culture developed from SfCC callus; (e-g) cell morphology of the suspension cell culture developed from SfHC callus

Source: Nosov et al., 2023

Viability Test

Cell viability test is a general laboratory technique which offers information about the health of cells or the percentage of dead cells in the sample. This test is very important in many forms, like plant and animal tissue culture, pharmacology,

toxicological testing, biomedical research and many others. It merely assesses the ratio of healthy viable cells within a population against nonviable cells, usually defined after subjecting the cells to various treatments, including experimental drugs, chemicals, environmental stress, or genetic manipulation. The common method for cell viability testing includes colorimetric assays (triphenyl tetrazolium chloride assay, trypan blue exclusion, etc.), fluorescent assays (fluorescein diacetate (FAD) staining, propidium Iodide (PI) staining, etc.), luminescent assays (Adenosine triphosphate (ATP) assay), metabolic activity assays (resazurin assay (alamar blue), and membrane integrity assays (lactate dehydrogenase (LDH) release assay) (Kamiloglu et al., 2020; Riss et al., 2013; Strober, 2015). In this experiment, we selected colorimetric assays by using 2,3,5-triphenyl tetrazolium chloride (TTC) for testing.

2,3,5-triphenyl tetrazolium chloride or TTC is a redox indicator commonly used in viability tests with a structure in the aromatic heterocyclic compound form containing three phenyl groups attached to a tetrazolium ring. The presence of these aromatic groups makes the compound stable, yet reactive under certain conditions. TTC is a redox-sensitive compound. In its oxidized form, TTC is colorless or pale yellow. When it is reduced by cellular enzymes, particularly dehydrogenases, it is converted into triphenyl formazan, an insoluble, red-colored compound (**Figure 9**). This redox property is the basis of its use in viability assays, as only metabolically active (viable) cells have the enzymatic activity necessary to reduce TTC to formazan (França-Neto & Krzyzanowski, 2019). TTC is specifically reduced by dehydrogenase enzymes that are active in viable cells. These enzymes are involved in the electron transport chain and other metabolic processes that occur in living cells, making TTC reduction a reliable indicator of cell viability. Non-viable cells, which lack active metabolism, do not reduce TTC, and thus no red formazan is formed. The amount of formazan produced is directly proportional to the level of metabolic activity in the cells. This property makes TTC a sensitive indicator of cell viability, as cells with

higher metabolic rates will produce more formazan, resulting in a more intense red coloration.

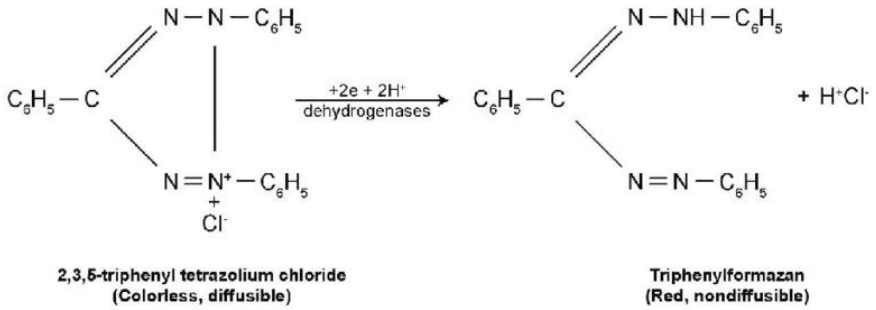

Figure 9 Reduction Reaction of 2,3,5-Triphenyl Tetrazolium Chloride with Dehydrogenases

Source: França-Neto and Krzyzanowski, 2019, 359-366

Plant Cell Heat Shock Experiment

The plant cell heat shock experiment is designed to mimic the natural heat stress conditions that plants experience in the environment. By exposing plant cells to elevated temperatures, researchers can study the cellular mechanisms that underlie the heat shock response. These experiments typically involve treating plant cell cultures or tissues with high temperatures for a defined period, followed by a recovery phase where the cells return to normal growth conditions. The effects of heat shock are then assessed through various methods, including cell viability assays, gene expression analysis, and protein profiling.

In general, when plant cells are subjected to heat stress, they undergo oxidative damage. Oxidative damage in plants can affect the pH of extracellular fluids primarily through the generation of reactive oxygen species (ROS), such as superoxide radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\bullet}OH$). When ROS

levels increase, they can cause cell damage by reacting with various cellular components, including lipids, proteins, and nucleic acids. This damage can disrupt various cellular processes, including those related to ion homeostasis and pH regulation, as shown in **Figure 10** (Hasanuzzaman et al., 2020; Zahra et al., 2023).

Oxidative stress causes damage to the cell membrane, leading to leakage of cellular proteins into the extracellular space. These leaked proteins contribute to changes in the total protein content of the extracellular fluid. In addition, cell membrane damage, including changes in the proton pump process and ion flow across the plasma membrane, also affects extracellular pH levels (Chaidee et al., 2008). Under stress conditions, plants activate various defense mechanisms to cope with oxidative stress, including programmed cell death to eliminate damaged cells and maintain tissue integrity (Petrov et al., 2015). Additionally, plants produce antioxidant enzymes, secondary metabolites, and secrete specific proteins synthesized in response to oxidative stress (Pandey et al., 2019). Therefore, the response to heat stress can be investigated by observing changes in pH and protein content in the extracellular fluid.

In this research, heat shock experiment protocol was received and adapted from the previous study in *Chenopodium* cells (Chaidee et al., 2008).

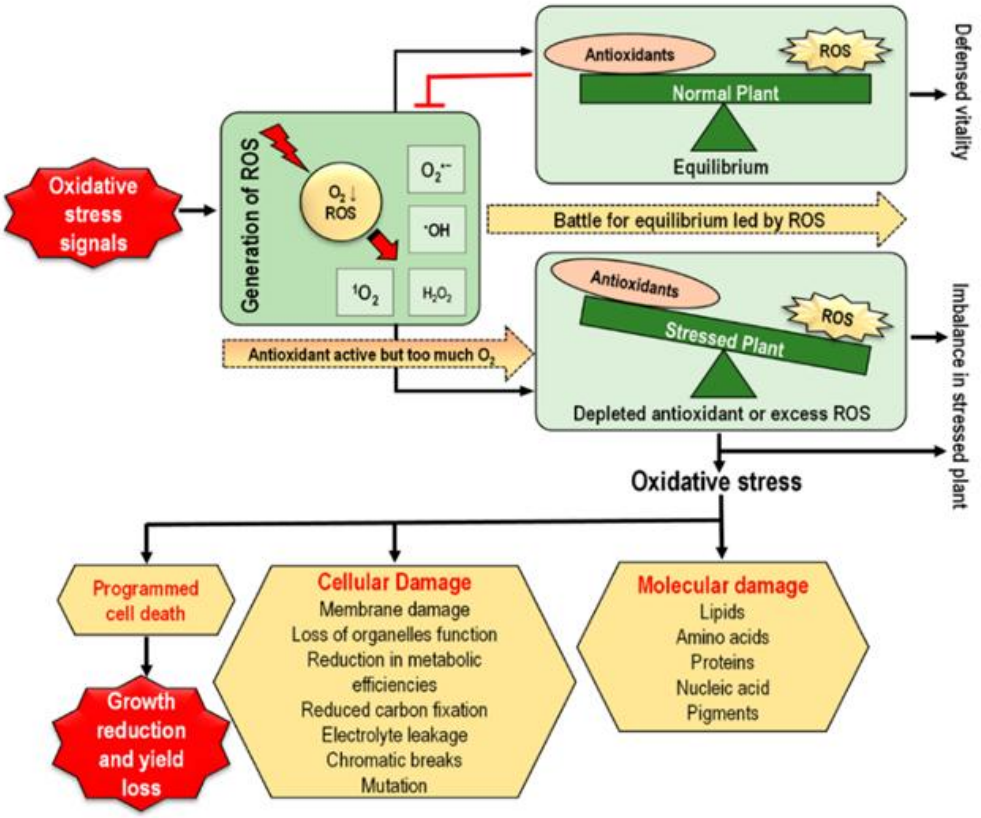

Figure 10 Oxidative Stress in Plants and Its Consequences

Source: Hasanuzzaman et al., 2020, 681

CHAPTER 3

METHODOLOGY

Materials and Equipment

1. Plant Materials

Aseptic golden gardenia plants

2. Culture Media and Reagents

Murashige and Skoog (MS) medium powder (PhytoTech)

N6-benzyladenine (SIGMA)

Kinetin (SIGMA)

1-naphthylacetic acid (SIGMA)

2,4-dichlorophenoxyacetic acid (SIGMA)

Gellan gum (KELCOGEL)

Sucrose

2,3,5-triphenyl tetrazolium chloride (TTC)

Bradford reagent (HIMEDIA)

Safranin solution (GAMMACO)

3. Laboratory Instruments

Scalpel (no.3)

Forceps

Petri dishes

Culture flasks

Pipettes (10 mL)

Micro pipettes (10-100 µL, and 100-1000 µL)

Pipette tips

Micro centrifuge tubes (1.5 mL)

Culture flasks (50, and 100 mL)

Laminar air flow hood

Autoclave (DW-FD50R, DRAWELL)

Shaker incubator (DW-SI-200B, DRAWELL)

Analytical balance (AS220.R2 PLUS, RADWAG)

Compound light microscope (BX43, OLYMPUS)

Stereo microscope (SZX10, OLYMPUS)

pH meter (STARTER3100, OHAUS)

Micro electrode (LabSen 241-3A, APERA)

Thermo-Hygrometer (with external temperature sensor) (DeltaTRAK)

Water bath (with temperature control) (LWD-106D, LabTech)

Vortex mixers (VORTEX GENIE 2, Scientific Industries)

Microcentrifuge (CF8, LABTron)

Spectrophotometer (Alpha, UV-Vis Spectrophotometer, KLAB)

Explant Preparation

The aseptic golden gardenia plants from seed culture were cultured in the section of Dr. rer. nat. Chatchawal Wongchai, tissue culture laboratory, Demonstration School, University of Phayao. The culture condition is Murashige and Skoog (MS) solid medium supplemented with 3 mg/L N6-Benzyladenine (BA) 0.2% gellan gum, and 30 g/L sucrose under a photoperiod of 12/12 hours (light/dark) at 23 ± 1 °C.

Golden Gardenia Callus Induction

Young golden gardenia leaves were obtained from the golden gardenia plants, the four edges of the plant's leaves were trimmed, and inoculated on MS solid medium supplemented with cytokinin hormones (including kinetin (Kn), and N6-benzyladenine (BA)) and auxin hormones (including 1-naphthylacetic acid (NAA), and

2,4-dichlorophenoxyacetic acid (2,4-D)) at different concentration, 0.2% gellan gum, and 30 g/L sucrose under a photoperiod of 12/12 hours (light/dark) at 23 ± 1 °C. The proportions of naphthalene acetic acid were different based on previous studies (Liu et al., 2018). Therefore, in this experiment there were a total of 14 sets of experiments (**Table 1**) including, control (only MS medium), MS medium + 0.3 mg/L Kn, MS medium + 0.3 mg/L Kn + 0.1 mg/L NAA, MS medium + 0.3 mg/L Kn + 0.3 mg/L NAA, MS medium + 0.3 mg/L Kn + 0.5 mg/L NAA, MS medium + 0.3 mg/L Kn + 1.0 mg/L NAA, MS medium + 0.5 mg/L NAA, MS medium + 0.5 mg/L Kn + 0.5 mg/L NAA, MS medium + 0.1 mg/L Kn + 0.5 mg/L NAA, MS medium + 0.1 mg/L BA + 0.5 mg/L NAA, MS medium + 0.1 mg/L Kn + 0.5 mg/L 2,4-D, MS medium + 0.1 mg/L Kn + 1.0 mg/L 2,4-D, MS medium + 0.1 mg/L Kn + 2.0 mg/L 2,4-D, and MS medium + 0.2 mg/L Kn + 1.0 mg/L 2,4-D, each set of experiments was repeated 5 replicated (5 bottles for each replicate). Observe callus formation every week. After 6 weeks observing callus structure under a compound light and a stereo microscope, callus formation rate (%) was calculated according to **equation 1**.

To observe callus cells morphology, a nail-sized piece of callus was transferred to a 1.5 mL microcentrifuge tube, followed by the addition of 1 mL of distilled water. The tube was shaken vigorously to disperse the cells, and the resulting mixture was then observed under a compound light microscope.

Table 1 Medium Formula for Callus Culture

Treatment	Hormone Concentration	
	Cytokinin (mg/L)	Auxin (mg/L)
Control	0	0
1	0.3 Kn	0 NAA
2	0.3 Kn	0.1 NAA
3	0.3 Kn	0.3 NAA
4	0.3 Kn	0.5 NAA
5	0.3 Kn	1.0 NAA
6	0	0.5 NAA
7	0.5 Kn	0.5 NAA
8	0.1 Kn	0.5 NAA
9	0.1 BA	0.5 NAA
10	0.1 Kn	0.5 2,4-D
11	0.1 Kn	1.0 2,4-D
12	0.1 Kn	2.0 2,4-D
13	0.2 Kn	1.0 2,4-D

Equation 1 Callus Inducing Rate Formula

$$Callus\ inducing\ rate\ (\%) = \frac{Number\ of\ formed\ callus}{Total\ number\ of\ culture\ plant}\ x\ 100$$

Golden Gardenia Cell Suspension Culture

Cell suspension cultures were established according to modified methods from previously reports (Kim et al., 1991; Liu et al., 2018). Friable calluses 1-2 g were selected and spread with a scalpel for inoculation into 50 mL culture flasks

containing 25 mL liquid MS medium supplemented with hormones and 30 g/L sucrose. In this study, the five formulations demonstrating the highest callus-inducing efficacy were selected (**Table 2**). Suspension cells were cultivated in incubated shaker with 120 rpm at 23 ± 1 °C under continuous light, and sub-cultured by transferring 5 mL of cell suspension into fresh media every 14-21 days. Packed cell volume (PCV, %) and viability based on absorbance value at 480 nm (A480) from TTC assay were determined every week. Morphology of suspended cells was observed.

Table 2 Medium Formula for Suspension Culture

Treatment	Hormone Concentration	
	Cytokinin (mg/L)	Auxin (mg/L)
F0	0.3 Kn	0.5 NAA
F1	0.1 Kn	0.5 2,4-D
F2	0.1 Kn	1.0 2,4-D
F3	0.1 Kn	2.0 2,4-D
F4	0.2 Kn	1.0 2,4-D

Heat Shock Experiment

Suspension culture of golden gardenia in MS medium supplemented with 0.1 mg/L Kn + 0.5 mg/L 2,4-D was pipetted (1 mL) into a 1.5 mL microcentrifuge tube. The packed cell volume (PCV, %), pH, and temperature of the extracellular medium were measured and recorded prior to the experiment. The centrifuge tubes were then floated in a water bath set to various temperatures including: 35 °C, 45 °C, 55 °C, 65 °C, and 75 °C, for 5 minutes. Following heat treatment, the pH and absorbance at 595 nm (A595) of the extracellular fluid were measured using a pH meter (Starter3100, OHAUS) equipped with a microelectrode (Labsen 241-3A) and the

Bradford assay, respectively, to assess heat stress in golden gardenia cells. In addition, the temperature of cell suspension after heat shock was immediately measured using a thermosensor (Thermo-Hygrometer – Model 13307, Deltatrak). Each temperature condition was treated as one experimental set, with nine replicates per set. Changes in pH, A595, and A480 after heat treatment were recorded to evaluate the cellular response to elevated temperature. In addition, changes in cellular components after heat shock were observed under a compound light microscope, using safranin solution and coomassie blue solution as stains to enhance visualization of structural alterations.

Determination of Packed Cell Volume

The packed cell volume (PCV, %) was determined by centrifuging 1 mL of culture broth in a 1.5 mL microcentrifuge tube at 2000 g for 5 minutes. This method was adapted from a previous study, which originally determined PCV by centrifuging 10 mL of culture broth in a 15-mL graduated conical centrifuge tube at 2000 g for 5 minutes (Ryu et al., 1990).

2,3,5-Tryphenyl Tetrazolium Chloride Assay

2,3,5-triphenyl tetrazolium chloride (TTC) is a redox-sensitive compound. In its oxidized form, TTC is colorless or pale yellow. When reduced by cellular enzymes, particularly dehydrogenases, it is converted into triphenyl formazan, an insoluble, red-colored compound. Only metabolically active (viable) cells possess the enzymatic activity required to reduce TTC to triphenyl formazan (França-Neto & Krzyzanowski, 2019). Based on this principle, the amount of formazan produced is directly proportional to the number of viable cells, making it a useful indicator of the growth and viability of suspension-cultured cells.

The amount of triphenyl formazan in solution can typically be measured photometrically using a spectrophotometer at various wavelengths, depending on excitation conditions, solvent environment, and the isomeric forms of triphenyl formazan. The steady-state UV-Vis absorption spectra of triphenyl formazan in methanol and acetonitrile solutions are nearly identical, showing strong absorption peaks around 300 nm and 480 nm (Wortmann et al., 2022). Therefore, 480 nm can be used for preliminary measurements of formazan concentration. However, to accurately quantify the amount of formazan present, a standard curve must be generated at the selected wavelength.

The TTC assay in this study was conducted by pipetting 1 mL of cell suspension into a 1.5 mL microcentrifuge tube. The cells were centrifuged at 2000 g for 5 minutes, and the supernatant was gently removed. Then, 500 µL of 1% TTC solution (prepared by dissolving 0.1 g of TTC in deionized water and adjusting the final volume to 10 mL) was added. The mixture was gently vortexed and incubated in the dark at room temperature for 6 hours. After incubation, the cells were centrifuged again, and the reaction mixture was carefully removed. Subsequently, 1 mL of absolute ethanol (99.5%) was added to the cells, and the suspension was incubated at 120 rpm at 23 °C for 16 hours. After incubation, the suspension was gently centrifuged to obtain a cell-free supernatant, and the absorbance of the supernatant was measured at 480 nm via using a UV-Vis spectrophotometer. This method was adapted from a previous study, which originally used 50 mg of vacuum-filtered cell culture (fresh weight) placed into a 5-mL Eppendorf tube. To this, 500 µL of TTC solution (1% TTC in 0.05 M Tris-HCl buffer, pH 7.5) was added. The suspension was incubated in the dark at room temperature for 6 hours, followed by gentle centrifugation to remove the reaction mixture. Then, 3 mL of ethanol was added to the cells, and the mixture was incubated overnight at room temperature. After incubation, the suspension was gently centrifuged to obtain a cell-free supernatant,

and the absorbance of the supernatant was measured at 500 nm. If the A500 is less than 0.05, the cell cultures are non-viable while they have low viability if the A500 is between 0.05 and 0.15. The cells are considered viable in case of A500 higher than 0.15 after 2 weeks of culture (Mamdouh & Smetanska, 2022).

A comparison between the TTC assay used in this study and previously established methods revealed several key differences. In this study, the amount of cells tested was determined based on the volume of the suspension culture rather than a fixed cell mass. As a result, the actual number of cells tested varied weekly, depending on the growth of the suspension culture. Consequently, the absorbance values obtained reflect relative changes in the number of viable cells over time, making this approach suitable for analyzing growth trends based on absorbance at 480 nm. However, unlike methods used in previous studies, this approach does not allow for accurate quantification of the absolute number of viable cells. Additionally, the volume of solvent used to extract formazan from the cells prior to absorbance measurement differed. While the original study used 3 mL of absolute ethanol for extraction, the current study used only 1 mL. This difference likely contributed to higher absorbance values per unit cell volume in this study compared to the referenced method.

To evaluate cell viability using the same standards as in previous studies, the absorbance values (A480) were normalized by the packed cell volume (PCV, %), as shown in **Equation 2**. After normalization, the A480 value was expressed per 1% PCV, which is approximately equivalent to 10 µL of cell volume or about 10 mg of fresh cell weight. The normalized absorbance was then further corrected based on fresh cell weight (50 mg) and the dilution factor of the solvent used in previous studies, as shown in **Equation 3**.

Equation 2 Absorbance Normalization

$$\text{Normalized absorbance} = \frac{\text{Absorbance (A480)}}{\text{Packed cell volume (PCV, \%)}}$$

Equation 3 Corrected Absorbance for Assessing Cell Viability

$$\text{Corrected absorbance} = \text{Normalized absorbance x} \left(\frac{\text{Reference cell weight (50 mg)}}{\text{Approximate cell weight (10 mg)}} \right) \text{x Dilution factor} \left(\frac{1}{3} \right)$$

Bradford Assay for Heat Shock Experiment

The Bradford assay in this study was conducted by mixing 500 µL of cell-free supernatant with 500 µL of Bradford reagent. The mixture was then incubated at room temperature for 10 minutes. Finally, the absorbance was measured at 595 nm using a spectrophotometer. The absorbance values at 595 nm obtained from the Bradford assay can be interpreted as an indication of increased total protein content in the solution. However, to accurately determine the actual protein concentration, a standard curve must be prepared and used as a reference.

Statistical Data Analysis

Data analysis was performed using IBM SPSS Statistics version 26. Nonparametric tests were used for the callus induction, cell suspension, and heat stress experiments. An independent samples Kruskal-Wallis test , followed by a stepwise step-down procedure, was employed for comparative analysis at a 95% confidence level ($P \leq 0.05$).

CHAPTER 4

RESULT AND DISCUSSION

Golden Gardenia Callus Induction

Callus induction from golden gardenia leaf explant was performed using a various combination of plant growth regulators, namely cytokinin (kinetin, Kn and N6-benzyladenine, BA) and auxin (1-naphthylacetic acid, NAA and 2,4-dichlorophenoxyacetic acid, 2,4-D). It was hypothesized that the formula supplemented with 0.3 mg/L kinetin and 0.5 mg/L 1-naphthylacetic acid was the best combination for callus induction according to the previous study on *Gardenia jasminoides* L. (Liu et al., 2018). After 3-week cultivation, callus formation was successfully induced with different callus induction rate depending on formula (**Table 3**).

The application of 2,4-D in combination with low concentrations of Kn (0.1 or 0.2 mg/L) proved most effective for callus induction. All treatments incorporating 2,4-D (treatments 10, 11, 12, and 13) achieved a 100% average induction rate. These treatments were statistically superior to all other formula. The callus produced was uniformly friable. Callus color was generally olive green (treatments 10, 11, and 13), however, the highest concentration of 2,4-D tested (2.0 mg/L, treatment 12) resulted in brown, friable callus.

In contrast to previous study (Liu et al., 2018), the formula with 0.3 mg/L kinetin and 0.5 mg/L NAA was not the most suitable for golden gardenia in this study. Treatments utilizing NAA resulted in varying degrees of callus induction, with induction rates ranging from 12 ± 5% to 60 ± 0%. The most effective groups were treatment 6 (0.5 mg/L NAA alone, 56% induction rate), treatment 8 (1.0 mg/L Kn + 0.5 mg/L NAA, 48% induction rate), and treatment 9 (0.1 mg/L BA + 0.5 mg/L NAA,

60% induction rate). Callus induced under NAA treatments was consistently friable in texture. Treatments 4, 5, 7, 8, and 9 produced light green, friable callus, while treatment 6 resulted in olive green, friable callus. In contrast, no callus was formed when using lower concentrations of NAA (0.1 or 0.3 mg/L; treatments 2 and 3). In addition, callus was not formed in the control group (lacking plant growth regulators) or in treatments containing only 0.3 mg/L Kn (treatment 1). These treatments exhibited a 0% induction rate, and as a result, callus characteristics could not be evaluated.

The successful induction of callus from *G. sootepensis* leaf explants was significantly influenced by the type and concentration of auxin and cytokinin used in the culture medium. The absence of callus formation in the control group (no plant growth regulator hormones) and in treatments containing only cytokinin highlights the essential role of auxin in initiating callus development. Specifically, the application of either NAA or 2,4-D successfully triggered callus formation, indicating that auxin is required for this process. This finding aligns with studies on *G. jasminoides*, where auxin (NAA) plays a more significant role than cytokinin (Kn) in inducing callus, with callus formation occurring at NAA concentrations of 0.25–0.50 mg/L, while higher or lower concentrations were found to decrease the induction rate (Liu et al., 2018).

However, the efficacy of the two auxins varied considerably. While NAA induced callus formation with induction rates ranging from 12% to 60%, 2,4-D consistently achieved a 100% induction rate across all tested concentrations. This suggests that *G. sootepensis* cells are more responsive to 2,4-D, which aligns with studies on *Moringa oleifera* that explicitly reported 2,4-D as superior to NAA in terms of callus induction. In those studies, 100% of explants cultured in media supplemented with 0.1 mg/L 2,4-D formed callus (Al-Hamidi et al., 2023).

Although this study could not clearly demonstrate the effects of increasing auxin and cytokinin concentrations on callus formation from *G. sootepensis* leaf explants, it was observed that excessive doses of either auxin or cytokinin led to a reduced callus induction rate and altered certain callus characteristics. This is in line with previous research on *Atropa acuminata* Royle ex Lindl., where MS medium supplemented with 1.0 mg/L NAA and 1.0 mg/L BAP was found to be most suitable for inducing callus from leaves, while higher concentrations of NAA and BA reduced the induction rate (Dar et al., 2021). Similarly, in *Curcuma longa* Linn., MS medium supplemented with 0.5 mg/L 2,4-D yielded the best results for inducing callus from the leaf sheath, whereas increasing the concentration of 2,4-D or adding cytokinin reduced the callus induction rate (Kaewthip et al., 2021). These reports emphasized the importance of hormonal balance.

Golden Gardenia Callus Cell Morphology

To observe *G. sootepensis* callus cells, a nail-sized piece of callus was mixed with distilled water (1 mL) and the tube was then shaken vigorously to disperse the cells. Various cell shapes were observed in the callus of *G. sootepensis* cultured on media supplemented with Kn and NAA (**Figure 11**), including coiled (**Figure 11A**), elongated (**Figures 11B and 11C**), curved (**Figure 11D**), kidney-shaped (**Figure 11E**), free-form (**Figure 11F**), circlet (**Figure 11G**), and stretched forms (**Figure 11H**). In addition, callus grown with Kn and 2,4-D also exhibited diverse cell morphologies (**Figure 12**), such as elongated (**Figure 12A**), curved (**Figure 12B**), oval (**Figure 12C**), kidney-shaped (**Figure 12D**), filamentous (**Figure 12E**), and stretched forms (**Figure 12F**). The diversity of cell shapes observed in these callus cultures is typical during active cell proliferation and is consistent with previous finding In *Coffea arabica* (Pádua et al., 2014) and *Camellia sinensis* (Esteban-Campos et al., 2024). The diversity of cell shapes observed in the *G. sootepensis* callus cultures reflects

dynamic cellular activity typical of dedifferentiated and actively proliferating plant cells. Morphological variations such as coiled, elongated, curved, and kidney-shaped cells suggest that the callus contains a heterogeneous population at different stages of the cell cycle or differentiation (Kruglova et al., 2023).

Table 3 *G. sootepensis* Formation from Leaf After 3 Weeks Culture

Treatment	Hormone concentration		Response			
	Cytokinin (mg/L)	Auxin (mg/L)	Callus image	Callus color	Callus texture	Callus inducing rate
Control	0	0		nd	nd	0 ± 0.00 [f]
1	0.3 Kn	0 NAA		nd	nd	0 ± 0.00 [f]
2	0.3 Kn	0.1 NAA		nd	nd	0 ± 0.00 [f]
3	0.3 Kn	0.3 NAA		nd	nd	0 ± 0.00 [f]
4 (F0)	0.3 Kn	0.5 NAA		Light green	friable	32 ± 4.99 [cd]

Table 3 (cont.)

Treatment	Hormone concentration		Response			
	Cytokinin (mg/L)	Auxin (mg/L)	Callus image	Callus color	Callus texture	Callus inducing rate
5	0.3 Kn	1.0 NAA		Light green	friable	24 ± 4.00 ^{de}
6	0	0.5 NAA		Olive green	friable	56 ± 4.00 ^{b}
7	0.5 Kn	0.5 NAA		Light green	friable	12 ± 4.99 ^{e}
8	0.1 Kn	0.5 NAA		Light green	friable	48 ± 4.99 ^{bc}

Table 3 (cont.)

Treatment	Hormone concentration		Response			
	Cytokinin (mg/L)	Auxin (mg/L)	Callus image	Callus color	Callus texture	Callus inducing rate
9	0.1 BA	0.5 NAA		Light green	friable	60 ± 0.00 [b]
10 (F1)	0.1 Kn	0.5 2,4-D		Olive green	friable	100 ± 0.00 [a]
11 (F2)	0.1 Kn	1.0 2,4-D		Olive green	friable	100 ± 0.00 [a]
12 (F3)	0.1 Kn	2.0 2,4-D		Brown	friable	100 ± 0.00 [a]
13 (F4)	0.2 Kn	1.0 2,4-D		Olive green	friable	100 ± 0.00 [a]

Note: Each treatment was performed with five replicates (N = 5 per treatment). The callus inducing rate was reported (%) via Mean ± Std. Eror. Superscript letters indicate statistically significant groupings based on the Kruskal–Wallis test for independent samples ($p < 0.05$).

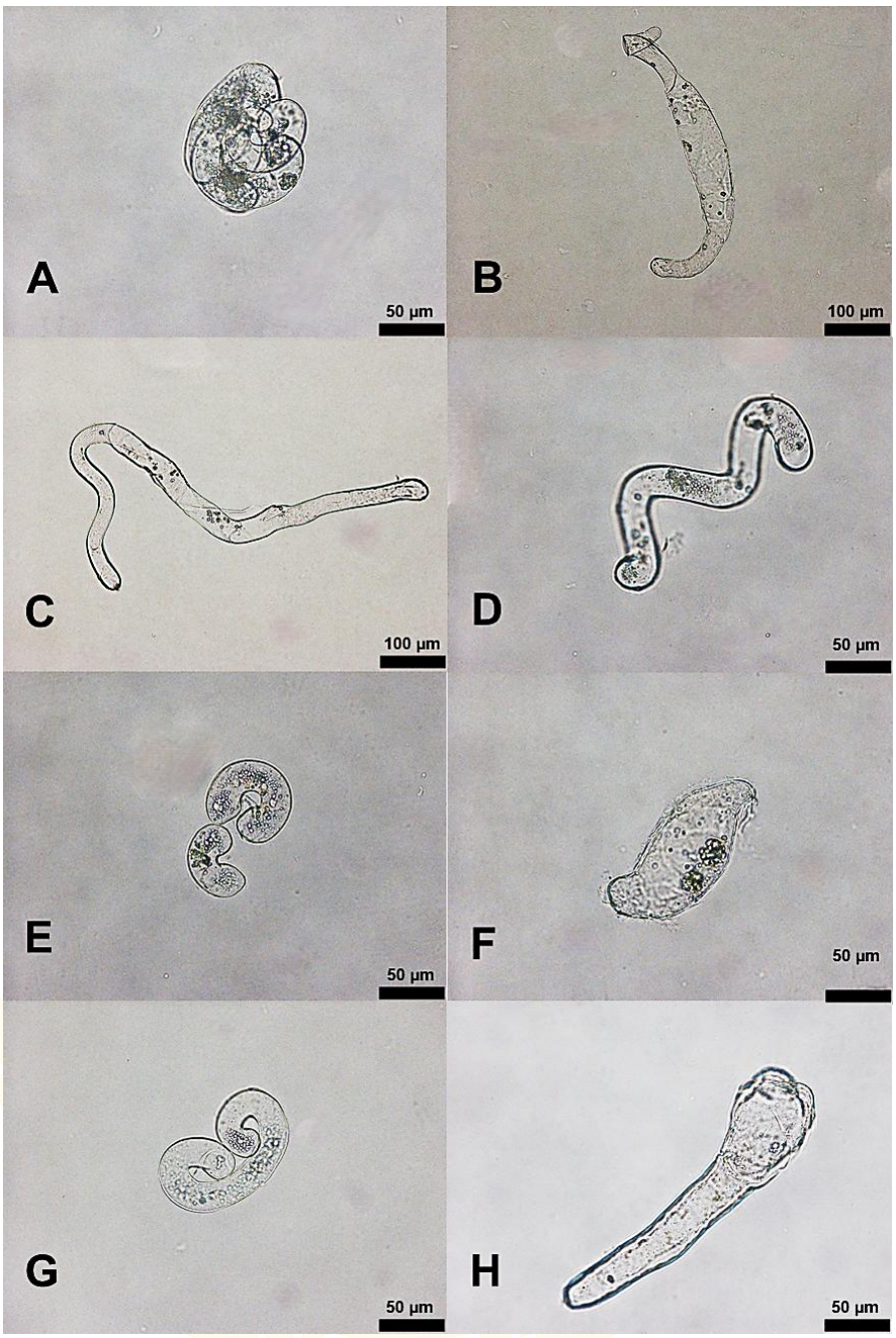

Figure 11 Various Cell Shapes Observed in The Callus of *G. sootepensis* Cultured on MS Medium Supplemented with Kn and NAA

Note: Various cell shapes, including coiled (A), elongated (B and C), curved (D), kidney-shaped (E), free-form (F), circlet (G), and stretched forms (H).

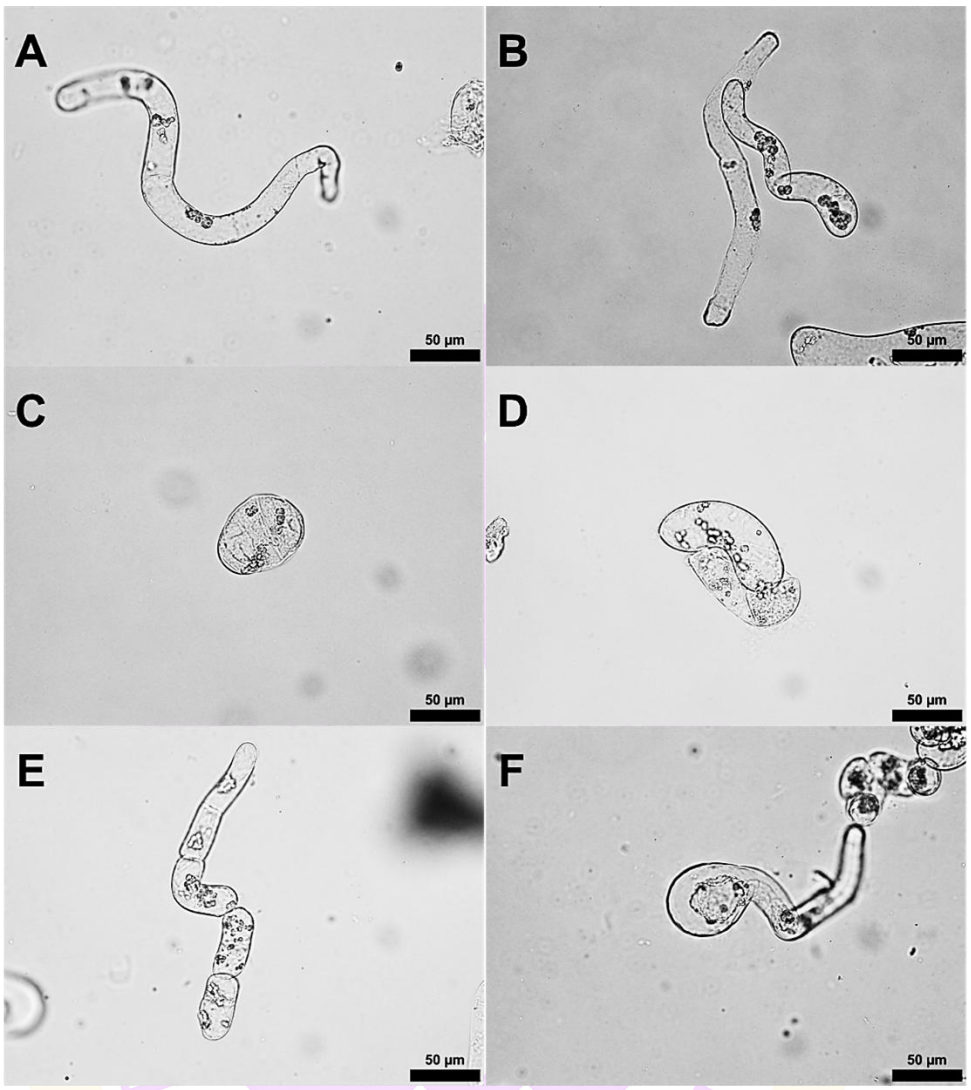

Figure 12 Various Cell Shapes Observed in The Callus of *G. sootepensis* Cultured on MS Medium Supplemented with Kn and 2,4-D

Note: Various cell shapes, including elongated (A), curved (B), oval (C), kidney-shaped (D), filamentous (E), and stretched forms (F).

Golden Gardenia Cell Suspension Culture

To initiate cell suspension, MS medium supplemented with four combinations of kinetin (Kn) and 2,4-dichlorophenoxyacetic acid (2,4-D) achieving a 100% callus induction rate were selected as the culture medium in the first subculture. Additionally, to represent the combined use of kinetin and 1-naphthylacetic acid (NAA), MS medium supplemented with 0.3 mg/L Kn and 0.5 mg/L NAA was chosen, following the protocol from the previous study by Liu et al. (2018). For the second subculture, MS medium supplemented with 0.1 mg/L Kn and 0.5 mg/L 2,4-D, as well as 0.1 mg/L Kn and 1.0 mg/L 2,4-D, were selected for further evaluation of cell growth and viability.

The first subculture of *G. sootepensis* suspension cells in MS medium supplemented with 0.1 mg/L Kn and 0.5 mg/L 2,4-D showed a significant increase in packed cell volume (PCV), rising from 1.83 ± 0.17% at the beginning (week 0) to 9.83 ± 0.17% at week 4, representing a 5.4-fold increase. Although no statistically significant differences were observed in corrected absorbance at 480 nm (corrected A480), viability assessment based on reference criteria indicated an improvement from low viability at week 0 to viable status by week 2. Similarly, cells cultured with 0.1 mg/L Kn and 1.0 mg/L 2,4-D exhibited a 5.8-fold increase in PCV, from 1.50 ± 0.00% to 8.67 ± 1.33% over four weeks. However, viability declined from viable in week 0 to low viability by week 4, despite the increase in biomass. In the treatment with 0.1 mg/L Kn and 2.0 mg/L 2,4-D, PCV increased 2.8-fold from 1.67 ± 0.17% to 4.67 ± 0.33%. In contrast, corrected A480 decreased significantly from 0.2922 ± 0.0275 to 0.0819 ± 0.0066, suggesting a steady decline in viability, shifting from viable at week 0 to low viability by week 3. For the 0.2 mg/L Kn and 1.0 mg/L 2,4-D treatment, PCV increased modestly from 5.33 ± 0.33% to 9.00 ± 0.30% (1.7 folds), while cell viability remained consistently low throughout the

culture period, with no significant changes in corrected A480. For the formula using NAA (0.1 mg/L Kn and 0.5 mg/L NAA), there were no significant changes in either PCV or corrected A480. In addition, cells remained non-viable throughout the experimental period (**Table 4-8**).

Repeat sub cultivation is needed to obtain a consistent and homogenous population of plant cell lines (Mustafa et al., 2011). A cell suspension culture with uniform fine cells is preferable to one containing a mixture of cell aggregates of different sizes (Mustafa et al., 2011; Yann et al., 2012). In this study, the second subculture experiment aimed to induce homogeneity and to monitor the capability of golden gardenia cells to actively divide when cultured with a suitable medium formula (**Table 9-10**). The results showed that using the combination of 0.1 mg/L Kn and 0.5 mg/L 2,4-D resulted in a 5.9-fold increase in PCV (2.33 ± 0.17% to 13.83 ± 0.17%) and a 1.9-fold increase in corrected A480 (0.1065 ± 0.0029 to 0.2065 ± 0.0230), with viability shifting from low at week 0 to viable by week 3. A similar response was observed in the second subculture with 0.1 mg/L Kn and 1.0 mg/L 2,4-D, where PCV increased 8.2 folds from 2.00 ± 0.00% to 16.33 ± 0.17%, corrected absorbance at 480 nm (Corrected A480) also increased 1.9-fold, from 0.0987 ± 0.0023 to 0.1840 ± 0.0023. Viability improved progressively, achieving viable status by week 3.

From the above experimental results, treatments involving 2,4-D consistently led to greater increases in %PCV and viability. This suggests a potent stimulatory effect of 2,4-D on *G. sootepensis* cell growth and proliferation, implying that 2,4-D is an effective auxin for promoting cell division and expansion in this species under these experimental conditions. While 2,4-D generally promoted growth, its concentration had a significant impact. Higher concentrations (2.0 mg/L) tended to negatively affect viability. Lower 2,4-D concentrations (0.5 mg/L and 1.0

mg/L) generally yielded better results for growth and viability, suggesting an optimal range for promoting growth without inducing stress. This highlights the importance of carefully optimizing 2,4-D concentrations to avoid potential toxicity. This finding aligns with studies on *Swertia lawii* demonstrated that a 2,4-D concentration of 3.0 mg/L resulted in the highest callus induction frequency (95%) and maximum fresh weight of callus (1.7 g). However, increasing the 2,4-D concentration to 5.0 mg/L led to a decline in both callus induction frequency and fresh weight, indicating potential toxicity at higher concentrations (Kshirsagar et al., 2015). Similarly, studies on *Datura innoxia* revealed that suspension cultures exhibited optimal growth at 2,4-D concentrations of 10^{-6} M, with doubling times of 1.1 days. Conversely, at higher concentrations of 10^{-5} M, the doubling time increased to 5.1 days, suggesting reduced proliferation rates. These findings underscore the importance of carefully optimizing 2,4-D concentrations to promote growth while minimizing potential toxicity in plant cell suspension cultures (Engvild, 1974).

2,4-D is a synthetic auxin commonly used in plant tissue culture due to its effectiveness in promoting cell division and growth. However, at elevated concentrations, 2,4-D can exhibit phytotoxic effects, primarily through the induction of oxidative stress caused by the excessive accumulation of reactive oxygen species (ROS). Previous studies have reported that high levels of 2,4-D can activate enzymes such as xanthine oxidoreductase (XOR), acyl-CoA oxidase (ACX), and lipoxygenase (LOX), which are involved in ureide metabolism, fatty acid β-oxidation, and jasmonic acid biosynthesis, respectively. The upregulation of these enzymes contributes to increased ROS production, potentially overwhelming the plant's antioxidant defense systems and resulting in oxidative damage (Pazmiño et al., 2011). In addition, 2,4-D has been shown to affect the biosynthesis of other phytohormones, particularly ethylene and abscisic acid (ABA). Excessive 2,4-D can stimulate the de novo synthesis of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, a key enzyme in ethylene

biosynthesis, leading to ethylene overproduction. Elevated ethylene levels can cause the accumulation of cyanide, which inhibits essential plant enzymes and impairs metabolic processes. Furthermore, ethylene can enhance ABA biosynthesis, leading to stomatal closure, reduced biomass production, and further ROS accumulation, compounding the stress effects on plant cells (Pazmiño et al., 2012).

The results of this study also suggest that kinetin (Kn) concentration plays a significant role in suspension culture performance. The highest Kn concentration (0.2 mg/L) with 1.0 mg/L 2,4-D resulted in the lowest PCV increase and cell viability remained consistently low throughout the culture period (**Table 5 and Table 7**), indicating a potential inhibitory effect at higher concentrations. This underscores the importance of the auxin-cytokinin balance. This finding aligns studies on *Taxus baccata* embryo cultures reporting that increasing kinetin concentrations beyond 0.1 mg/L, in combination with 2,4-D, did not enhance callus growth, suggesting a potential inhibitory effect at higher cytokinin levels (Karimian et al., 2015).

Interestingly, when comparing the first and second subcultures, it was found that the second subculture generally exhibited higher increase of PCV, and viability compared to the first subculture (**Tables 4 – 10**). This suggests a potential adaptability and enhanced responsiveness of the cells to medium phase change from the solid to liquid type, and hormone treatments over successive subcultures. Cells may become more acclimated to the media and hormone conditions, leading to improved growth and viability. This finding aligns with studies on *Artemisia annua* L., where continuous subculturing of callus and cells on callus induction medium, in both solid and liquid forms, increases callus and cell biomass (Yann et al., 2012).

When plant cells are transitioned from a complex, organized tissue environment to a relatively homogeneous suspension culture, they undergo a dramatic shift. Initially, the cells may experience stress due to altered nutrient availability, hormonal gradients, and physical conditions. However, over successive

subcultures, the cells can gradually acclimate to these conditions. This process involves the selection of cell lines that are better equipped to thrive in the specific culture environment. Cells that exhibit higher rates of division and metabolic efficiency are more likely to dominate the culture over time. This selection pressure can result in the establishment of cell lines with enhanced responsiveness to applied hormone treatments (George et al., 2008).

Further research is therefore needed to elucidate the underlying mechanisms of 2,4-D and Kn action in *G. sootepensis* cells to consider the effects of these hormones on suspension cell culture when designing experiments and developing propagation protocols, emphasizing the importance of careful adjustment of hormone concentrations.

Golden Gardenia Suspension Culture Growth Curve

The growth curve of *G. sootepensis*, constructed from PCV values, revealed distinct growth patterns under different hormonal treatments. In the first subculture, *G. sootepensis* cell suspension cultured in MS medium supplemented with 0.1 mg/L Kn and 0.5 mg/L 2,4-D exhibited a steady increase in PCV, showing a consistent upward trend over time. This indicates a strong positive response, possibly due to active cell proliferation or high metabolic activity (**Figure 13A**). In contrast, cultures supplemented with 0.1 mg/L Kn and 1.0 mg/L 2,4-D showed a gradual increase in PCV from Week 0 to Week 3, followed by a sharp rise at Week 4, suggesting a lag phase followed by rapid growth, potentially due to a delayed cellular response or adaptation to the medium (**Figure 13B**). Cultures grown in MS medium with 0.1 mg/L Kn and 2.0 mg/L 2,4-D showed a linear and consistent increase in PCV throughout the culture period, indicating uniform growth under stable conditions (**Figure 13C**). The culture supplemented with 0.2 mg/L Kn and 1.0 mg/L 2,4-D displayed a rapid

increase in PCV up to Week 2, followed by a plateau from Week 3 to Week 4. This pattern reflects an early growth phase that levels off, possibly due to inhibitory conditions or hormonal limitations (**Figure 13D**). Lastly, cultures grown in MS medium with 0.3 mg/L Kn and 0.5 mg/L NAA showed no significant change in PCV, represented by a flat growth curve, suggesting no cell proliferation, likely due to an unfavorable hormonal balance (**Figure 13E**).

The growth curves of *G. sootepensis* in the second subculture, maintained in both medium formula, MS medium supplemented with 0.1 mg/L Kn and 0.5 mg/L 2,4-D (**Figure 14A**) and with 0.1 mg/L Kn and 1.0 mg/L 2,4-D (**Figure 14B**), were assessed using PCV. The results showed a positive growth trend, with a linear increase in PCV over the four-week culture period.

Golden Gardenia Suspension Cell Morphology

Various cell shapes were observed in *G. sootepensis* cell suspension cultured on medium supplemented with Kn and NAA (**Figure 15**), including filamentous (**Figure 15A and B**), elongated (**Figure 15C**), kidney-shaped (**Figure 15D**), oval (**Figure 15E**), and curved (**Figure 15F**). In addition, suspension culture with Kn and 2,4-D also exhibited diverse cell morphologies (**Figure 16**), such as elongated (**Figure 16A**), filamentous (**Figure 16B**), oval (**Figure 16C**), coiled (**Figure 16D**), stretched forms (**Figure 16E**), and kidney-shaped (**Figure 16F**). The diversity of cell shapes observed in this suspension culture is typical during active cell proliferation and is consistent with previous findings in *Coffea arabica*, suspension (Silva et al., 2005) and *Sutherlandia frutescens* (Nosov et al., 2023). The diverse cell morphologies observed in *G. sootepensis* suspension cultures reflect active cellular proliferation and dynamic growth, suggesting a heterogeneous cell population at various physiological states or stages of the cell cycle.

Table 4 Packed Cell Volume (PCV, %) and Viability of *G. sootepensis* Cell Suspension Culture in MS Medium Supplemented with 0.1 mg/L Kn and 0.5 mg/L 2,4-D, The First Sub-culture

Duration	Response			
	PCV (%)	A480	Corrected A480	Viability
Week 0	1.83 ± 0.167 ^{d}	0.1441 ± 0.0007 ^{e}	0.1333 ± 0.0128 ^{ns}	Low viable
Week 1	5.67 ± 0.167 ^{c}	0.4505 ± 0.0001 ^{d}	0.1327 ± 0.0038 ^{ns}	Low viable
Week 2	7.33 ± 0.667 ^{cb}	0.7345 ± 0.0014 ^{c}	0.1700 ± 0.0167 ^{ns}	Viable
Week 3	8.67 ± 0.333 ^{b}	0.9186 ± 0.0002 ^{b}	0.1772 ± 0.0071 ^{ns}	Viable
Week 4	9.83 ± 0.167 ^{a}	1.0689 ± 0.0005 ^{a}	0.1813 ± 0.0030 ^{ns}	Viable

Note: Data are presented as mean ± standard error (SE), N = 3. Superscript letters indicate significant groupings based on the Kruskal–Wallis test. "ns" indicates no significant difference ($p > 0.05$).

Table 5 Packed Cell Volume (PCV, %) and Viability of *G. sootepensis* Cell Suspension Culture in MS Medium Supplemented with 0.1 mg/L Kn and 1.0 mg/L 2,4-D, The First Sub-culture

Duration	Response			
	PCV (%)	A480	Corrected A480	Viability
Week 0	1.50 ± 0.000 ^{c}	0.3263 ± 0.0617 ^{ns}	0.3626 ± 0.0686 ^{ns}	Viable
Week 1	2.50 ± 0.000 ^{b}	0.4770 ± 0.0610 ^{ns}	0.3180 ± 0.0407 ^{ns}	Viable
Week 2	3.67 ± 0.333 ^{a}	0.5470 ± 0.0586 ^{ns}	0.2478 ± 0.0047 ^{ns}	Viable
Week 3	4.33 ± 0.667 ^{a}	0.5730 ± 0.0499 ^{ns}	0.2261 ± 0.0187 ^{ns}	Viable
Week 4	8.67 ± 1.333 ^{a}	0.6803 ± 0.0587 ^{ns}	0.1342 ± 0.0111 ^{ns}	Low viable

Note: Data are presented as mean ± standard error (SE), N = 3. Superscript letters indicate significant groupings based on the Kruskal–Wallis test. "ns" indicates no significant difference ($p > 0.05$).

Table 6 Packed Cell Volume (PCV, %) and Viability of *G. sootepensis* Cell Suspension Culture in MS Medium Supplemented with 0.1 mg/L Kn and 2.0 mg/L 2,4-D, The First Sub-culture

Duration	Response			
	PCV (%)	A480	Corrected A480	Viability
Week 0	1.67 ± 0.167 ^{d}	0.2867 ± 0.0011 ^{ab}	0.2922 ± 0.0275 ^{a}	Viable
Week 1	2.50 ± 0.000 ^{c}	0.2869 ± 0.0004 ^{ab}	0.1913 ± 0.0003 ^{b}	Viable
Week 2	3.00 ± 0.000 ^{b}	0.2971 ± 0.0004 ^{a}	0.1651 ± 0.0002 ^{c}	Viable
Week 3	4.00 ± 0.000 ^{a}	0.2193 ± 0.0002 ^{c}	0.0914 ± 0.0001 ^{c}	Low viable
Week 4	4.67 ± 0.333 ^{a}	0.2268 ± 0.0009 ^{b}	0.0819 ± 0.0066 ^{c}	Low viable

Note: Data are presented as mean ± standard error (SE), N = 3. Superscript letters indicate significant groupings based on the Kruskal–Wallis test. "ns" indicates no significant difference ($p > 0.05$).

Table 7 Packed Cell Volume (PCV, %) and Viability of *G. sootepensis* Cell Suspension Culture in MS Medium Supplemented with 0.2 mg/L Kn and 1.0 mg/L 2,4-D, The First Sub-culture

Duration	Response			
	PCV (%)	A480	Corrected A480	Viability
Week 0	5.33 ± 0.333 ^{b}	0.2648 ± 0.0218 ^{e}	0.0839 ± 0.0108 ^{ab}	Low viable
Week 1	6.83 ± 0.167 ^{a}	0.4197 ± 0.0009 ^{b}	0.1025 ± 0.0027 ^{a}	Low viable
Week 2	8.33 ± 0.667 ^{a}	0.5107 ± 0.0005 ^{a}	0.1036 ± 0.0089 ^{ab}	Low viable
Week 3	8.83 ± 0.441 ^{a}	0.3995 ± 0.0013 ^{c}	0.0758 ± 0.0040 ^{ab}	Low viable
Week 4	9.00 ± 0.298 ^{a}	0.3505 ± 0.0001 ^{d}	0.0650 ± 0.0021 ^{b}	Low viable

Note: Data are presented as mean ± standard error (SE), N = 3. Superscript letters indicate significant groupings based on the Kruskal–Wallis test. "ns" indicates no significant difference ($p > 0.05$).

Table 8 Packed Cell Volume (PCV, %) and Viability of *G. sootepensis* Cell Suspension Culture in MS Medium Supplemented with 0.3 mg/L Kn and 0.5 mg/L NAA, The First Sub-culture

Duration	Response			
	PCV (%)	A480	Corrected A480	Viability
Week 0	3.33 ± 0.167 ^{ns}	0.0352 ± 0.0005 ^{b}	0.0177 ± 0.0011 ^{ns}	Non-viable
Week 1	3.33 ± 0.167 ^{ns}	0.0355 ± 0.0010 ^{b}	0.0178 ± 0.0007 ^{ns}	Non-viable
Week 2	3.33 ± 0.167 ^{ns}	0.0402 ± 0.0006 ^{ab}	0.0202 ± 0.0008 ^{ns}	Non-viable
Week 3	3.50 ± 0.000 ^{ns}	0.0432 ± 0.0011 ^{a}	0.0206 ± 0.0005 ^{ns}	Non-viable

Note: Data are presented as mean ± standard error (SE), N = 3. Superscript letters indicate significant groupings based on the Kruskal–Wallis test. "ns" indicates no significant difference ($p > 0.05$).

Table 9 Packed Cell Volume (PCV, %) and Viability of *G. sootepensis* Cell Suspension Culture in MS Medium Supplemented with 0.1 mg/L Kn and 0.5 mg/L 2,4-D, The Second Sub-culture

Duration	Response			
	PCV (%)	A480	Corrected A480	Viability
Week 0	2.33 ± 0.167 ^{e}	0.1491 ± 0.0120 ^{e}	0.1065 ± 0.0029 ^{c}	Low viable
Week 1	5.33 ± 0.167 ^{d}	0.4070 ± 0.0142 ^{d}	0.1272 ± 0.0023 ^{bc}	Low viable
Week 2	8.83 ± 0.167 ^{c}	0.6770 ± 0.0761 ^{c}	0.1273 ± 0.0122 ^{abc}	Low viable
Week 3	10.67 ± 0.333 ^{b}	1.2047 ± 0.0814 ^{b}	0.1878 ± 0.0071 ^{ab}	Viable
Week 4	13.83 ± 0.167 ^{a}	1.7113 ± 0.1800 ^{a}	0.2065 ± 0.0230 ^{a}	Viable

Note: Data are presented as mean ± standard error (SE), N = 3. Superscript letters indicate significant groupings based on the Kruskal–Wallis test. "ns" indicates no significant difference ($p > 0.05$).

Table 10 Packed Cell Volume (PCV, %) and Viability of *G. sootepensis* Cell Suspension Culture in MS Medium Supplemented with 0.1 mg/L Kn and 1.0 mg/L 2,4-D, The Second Sub-culture

Duration	Response			
	PCV (%)	A480	Corrected A480	Viability
Week 0	2.00 ± 0.000 ^{e}	0.1184 ± 0.0023 ^{e}	0.0987 ± 0.0023 ^{b}	Low viable
Week 1	6.00 ± 0.000 ^{d}	0.3710 ± 0.0023 ^{d}	0.1031 ± 0.0023 ^{b}	Low viable
Week 2	9.67 ± 0.333 ^{c}	0.6030 ± 0.0023 ^{c}	0.1039 ± 0.0023 ^{ab}	Low viable
Week 3	13.33 ± 0.167 ^{b}	1.3560 ± 0.0023 ^{b}	0.1699 ± 0.0023 ^{ab}	Viable
Week 4	16.33 ± 0.167 ^{a}	1.8034 ± 0.0023 ^{a}	0.1840 ± 0.0023 ^{a}	Viable

Note: Data are presented as mean ± standard error (SE), N = 3. Superscript letters indicate significant groupings based on the Kruskal–Wallis test. "ns" indicates no significant difference ($p > 0.05$).

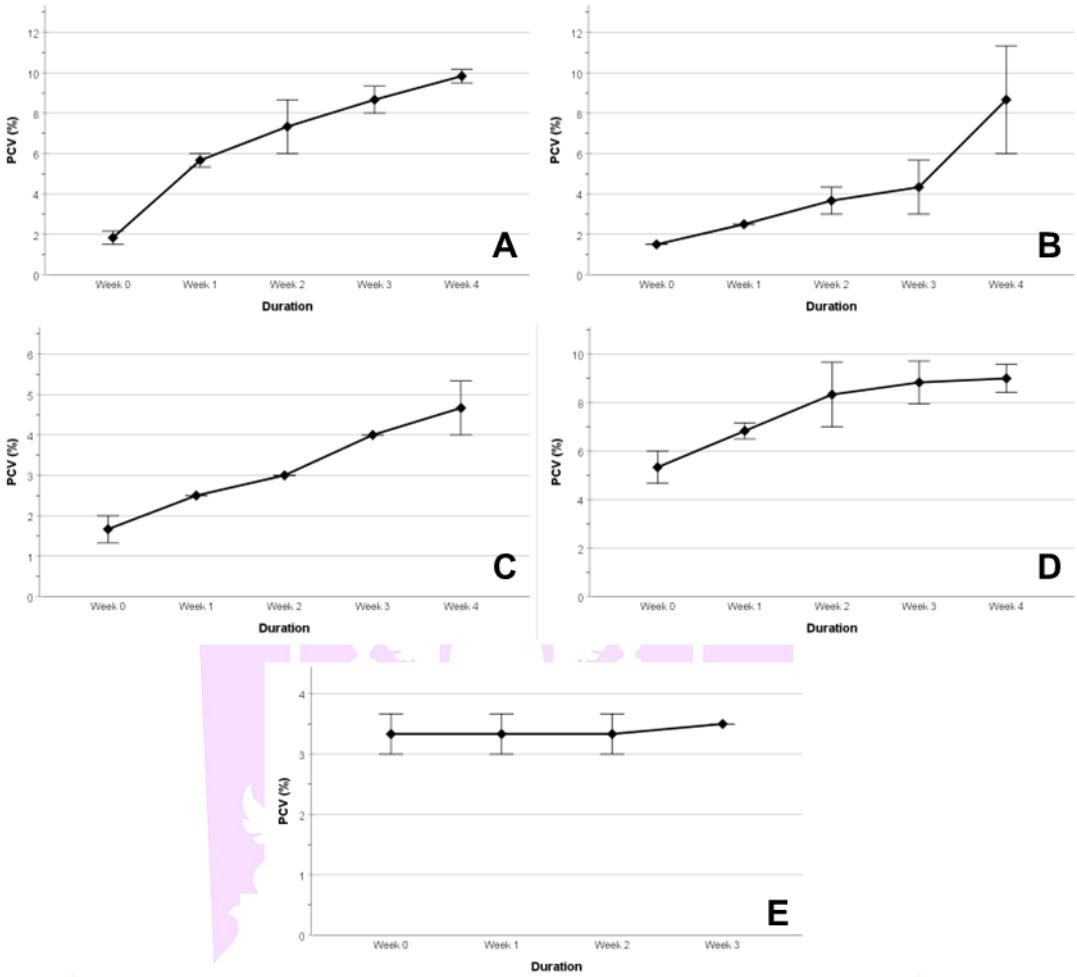

Figure 13 Growth Curve of *G. sootepensis* Cell Suspension Cultured by Packed Cell Volume (PCV, %), The First Sub-culture

Note: Culture medium formula, including MS medium supplemented with 0.1 mg/L Kn and 0.5 mg/L 2,4-D (A), MS medium supplemented with 0.1 mg/L Kn and 1.0 mg/L 2,4-D (B), MS medium supplemented with 0.1 mg/L Kn and 2.0 mg/L 2,4-D (C), MS medium supplemented with 0.2 mg/L Kn and 1.0 mg/L 2,4-D (D), MS medium supplemented with 0.3 mg/L Kn and 0.5 mg/L NAA (E).

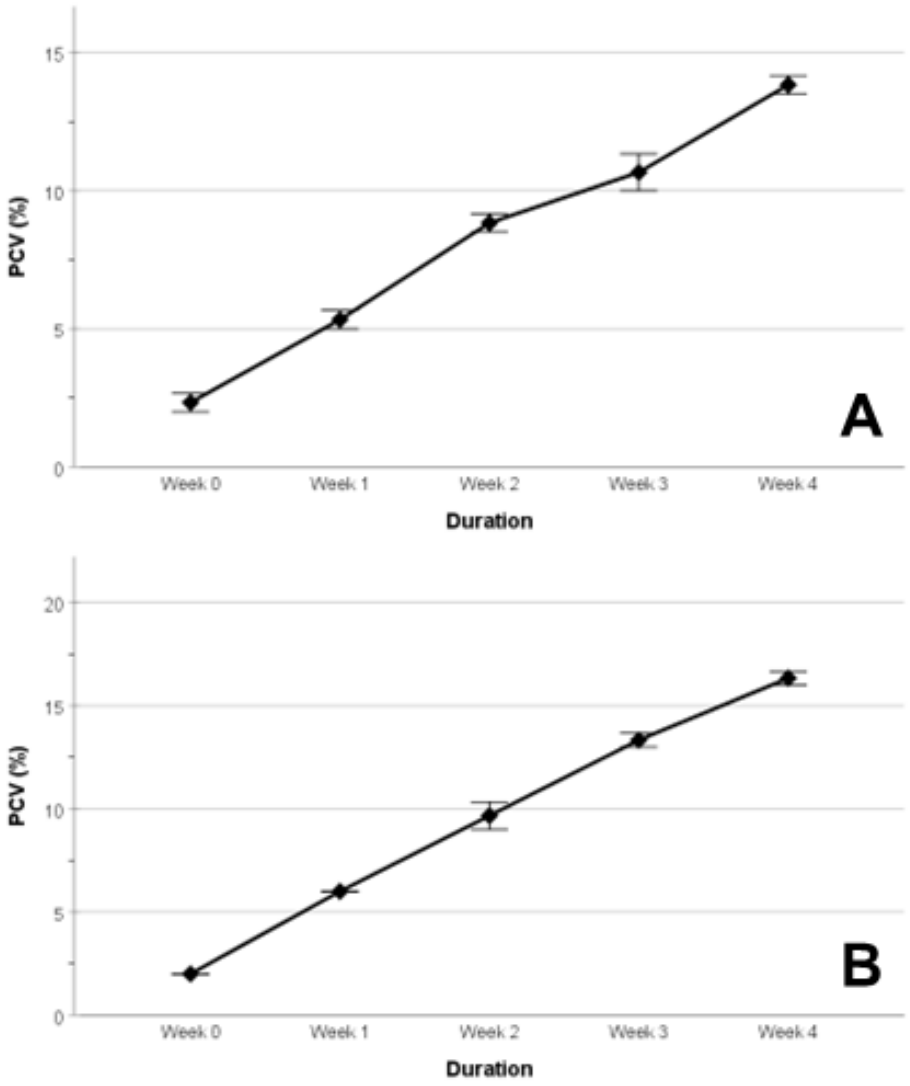

Figure 14 Growth Curve of *G. sootepensis* Cell Suspension Cultured by Packed Cell Volume (PCV, %), The Second Sub-culture

Note: Culture medium formula, including MS medium supplemented with 0.1 mg/L Kn and 0.5 mg/L 2,4-D (A) and MS medium supplemented with 0.1 mg/L Kn and 1.0 mg/L 2,4-D (B).

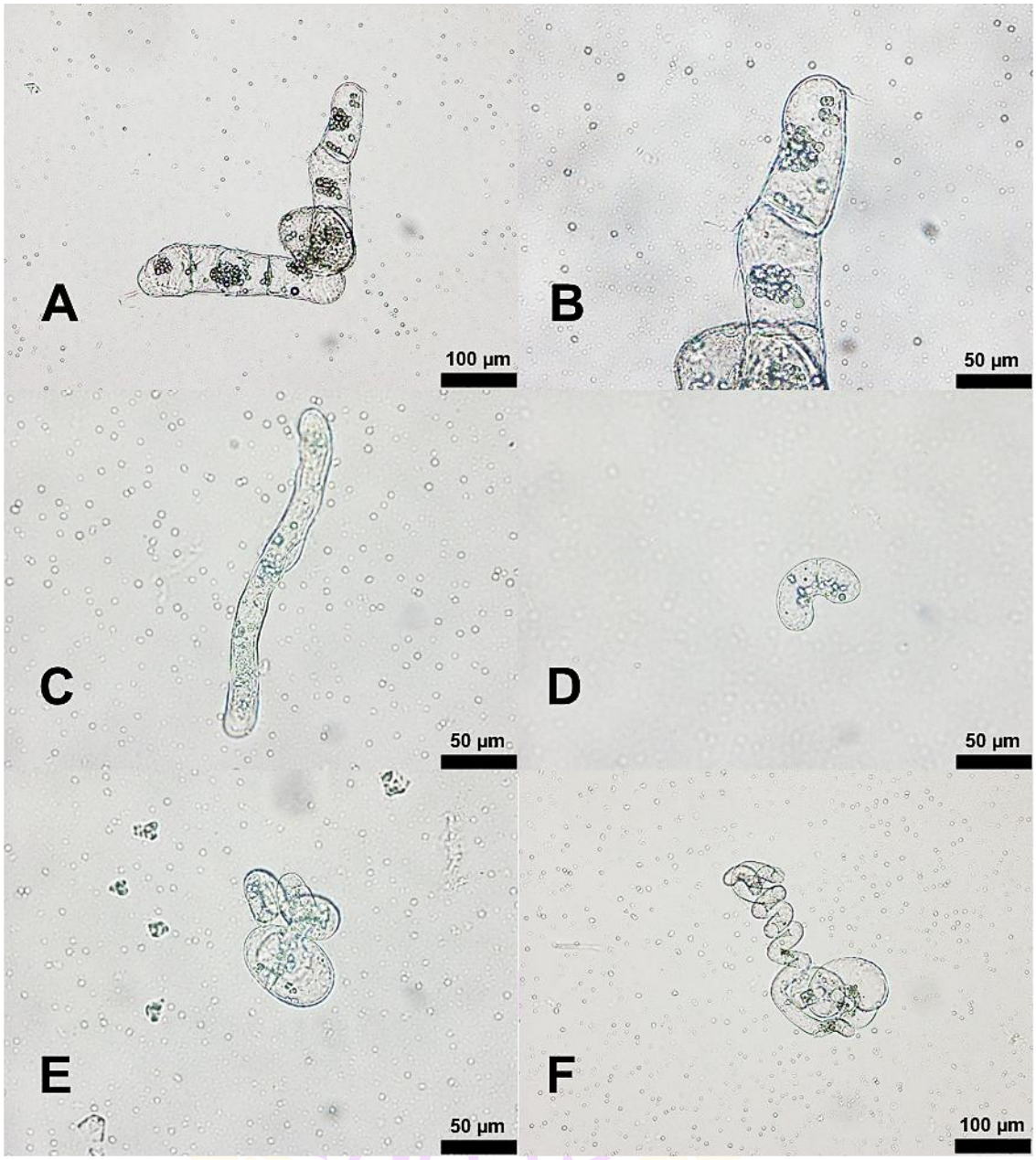

Figure 15 Various Cell Shapes were Observed in *G. sootepensis* Cell Suspension Cultured on MS Medium Supplemented with Kn and NAA

Note: Various cell shapes, including filamentous (A and B), elongated (C), kidney-shaped (D), oval (E), and curved (F).

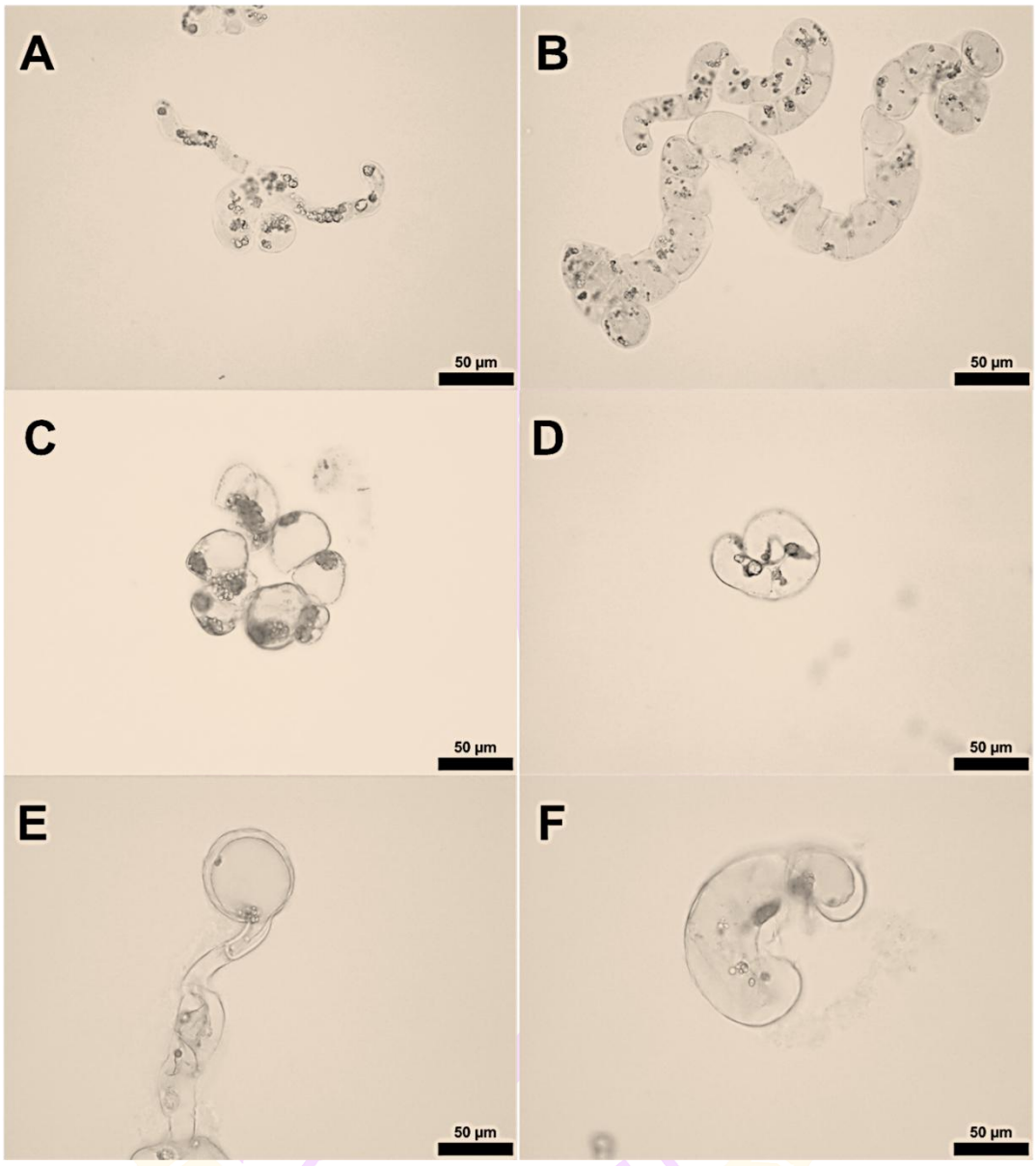

Figure 16 Various Cell Shapes were Observed in *G. sootepensis* Cell Suspension Cultured on MS Medium Supplemented with Kn and 2,4-D

Note: Various cell shapes, including elongated (A), filamentous (B), oval (C), coiled (D), stretched forms (E), and kidney-shaped (F).

Golden Gardenia Heat Shock Response

The heat stress experiment investigated the effects of elevated temperatures on sudden changes in extracellular pH, absorbance value at 595 nm (A595) from the Bradford assay, and cell viability via TTC assay in *G. sootepensis* suspension cultures. The primary objective was to assess how increasing temperatures from 35 °C to specified temperatures in 5 min influenced the plant cells. Extracellular temperatures, representing the actual temperatures experienced by the cells, were also recorded for comparison **(Table 11)**. The packed cell volume percentage (PCV, %) was measured to ensure consistent cell density across treatments, thereby minimizing cell number as a confounding factor.

The experimental results showed that the A595 value increased with temperature, reaching a 1.23-fold increase from 0.378 ± 0.0049 at 35 °C (34.1 ± 0.11 °C extracellular temperatures) to 0.466 ± 0.0072 at 65 °C (53.2 ± 0.17 °C extracellular temperatures). However, at 75 °C (59.5 ± 0.10 °C extracellular temperatures), the A595 value was not significantly different from that at 35 °C (**Table 12**). Conversely, no significant differences in extracellular pH were observed before or after heat shock across all temperature treatments (**Table 13**). Interestingly, higher temperatures were found to have a significant effect on cell viability. The corrected A480 value decreased sharply from 0.134 ± 0.0498 at 55 °C (46.7 ± 0.10 °C extracellular temperatures), identified as the critical point, to 0.002 ± 0.0111 at 65 °C (53.2 ± 0.17 °C extracellular temperatures). According to reference criteria (Mamdouh & Smetanska, 2022), this indicates a shift from low viability to non-viable status (**Table 14**).

Golden Gardenia Suspension Cell Structure after Heat Shock

Microscopic examination of the structure of *G. sootepensis* suspension cells after heat shock experiment revealed some changes when compared to the cells in normal condition. At room temperature (**Figure 17**), cells contain a number of chloroplasts distributed throughout the cytoplasm, mostly surrounding or near the nucleus. In addition, the cell wall and membrane appeared to be structurally intact. These cell characteristics were similarly observed at 35 °C to 55 °C, indicating that the temperature range of the water bath, 35 °C to 55 °C, did not affect the structures that could be observed under the compound light microscope.

However, when cells were exposed to 65 °C, the green coloration of chloroplasts within the cells decreased significantly or nearly disappeared, leaving behind only transparent, round structures, presumed to be remnants of chloroplasts damaged by heat. This suggests that at temperatures exceeding the tolerance threshold of plant cells, essential pigments are degraded. Additionally, the cell membrane appears to be severely damaged by heat, as evidenced by the detachment from the cell wall (**Figure 18**). These observations align with studies indicating that chloroplasts are highly sensitive to heat stress, which affects photosynthetic processes and can lead to chlorophyll degradation and structural disorganization. High temperatures can cause alterations in chloroplast morphology, including reduced size and disrupted thylakoid membranes, leading to decreased photosynthetic efficiency and pigment loss (Hu et al., 2020). Additionally, heat stress can compromise cell wall polymers and anatomy, resulting in structural damage (Lima et al., 2013). The observed changes in cell structure are consistent with results obtained from heat stress experiments in *G. sootepensis* cell suspension and indicate that the critical temperature that this plant can tolerate is 55 °C in a water bath, or approximately 46.7 ± 0.10 °C the actual temperature felt by the plant.

Table 11 Extracellular Temperature Changes of *G. sootepensis* Suspension Culture after Heat Shock Treatment at Different Temperatures for 5 min

Water bath Temp. (°C)	Extracellular Temp. (°C)
35	34.1 ± 0.11 ^{e}
45	39.9 ± 0.11 ^{d}
55	46.7 ± 0.10 ^{c}
65	53.2 ± 0.17 ^{b}
75	59.5 ± 0.10 ^{a}

Note: Data are presented as mean ± standard error (SE), N = 9. Superscript letters indicate significant groupings based on the Kruskal–Wallis test ($p > 0.05$).

Table 12 Absorbance Value at 595 nm (A595) of Culture Medium Collected after Treating *G. sootepensis* Cell Suspension in 5-min Heat Shock at Different Temperatures

Water bath Temp. (°C)	%PCV (%)	A595
35	22 ± 0.8 ^{ns}	0.378 ± 0.0049 ^{b}
45	22 ± 0.8 ^{ns}	0.398 ± 0.0060 ^{ab}
55	22 ± 0.8 ^{ns}	0.449 ± 0.0101 ^{ab}
65	22 ± 0.8 ^{ns}	0.466 ± 0.0072 ^{a}
75	22 ± 0.8 ^{ns}	0.425 ± 0.0037 ^{ab}

Note: Data are presented as mean ± standard error (SE), N = 9. Superscript letters indicate significant groupings based on the Kruskal–Wallis test ($p > 0.05$).

Table 13 Extracellular pH Changes of *G. sootepensis* Suspension Culture after 5-min Heat Shock at Different Temperatures

Water bath Temp. (°C)	PCV (%)	Start pH	Final pH
35	22 ± 0.8 ^{ns}	4.71 ± 0.069 ^{ns}	4.72 ± 0.072 ^{ns}
45	22 ± 0.8 ^{ns}	4.72 ± 0.068 ^{ns}	4.69 ± 0.072 ^{ns}
55	22 ± 0.8 ^{ns}	4.72 ± 0.068 ^{ns}	4.69 ± 0.078 ^{ns}
65	22 ± 0.8 ^{ns}	4.72 ± 0.068 ^{ns}	4.74 ± 0.075 ^{ns}
75	22 ± 0.8 ^{ns}	4.73 ± 0.069 ^{ns}	4.70 ± 0.077 ^{ns}

Note: Data are presented as mean ± standard error (SE), N = 9. Superscript letters indicate significant groupings based on the Kruskal–Wallis test ($p > 0.05$).

Table 14 Viability Changes of *G. sootepensis* Cell Suspension after 5-min Heat Shock at Different Temperatures

Water bath Temp. (°C)	PCV (%)	A480	Corrected A481	Viability
35	22 ± 0.8 ^{ns}	0.793 ± 0.0498 ^{a}	0.064 ± 0.0048 ^{a}	Low viable
45	22 ± 0.8 ^{ns}	0.798 ± 0.0416 ^{a}	0.065 ± 0.0042 ^{a}	Low viable
55	22 ± 0.8 ^{ns}	1.651 ± 0.1173 ^{a}	0.134 ± 0.0498 ^{a}	Low viable
65	22 ± 0.8 ^{ns}	0.020 ± 0.0015 ^{b}	0.002 ± 0.0111 ^{b}	Non-viable
75	22 ± 0.8 ^{ns}	0.017 ± 0.0008 ^{b}	0.001 ± 0.0001 ^{b}	Non-viable

Note: Data are presented as mean ± standard error (SE), N = 9. Superscript letters indicate significant groupings based on the Kruskal–Wallis test ($p > 0.05$).

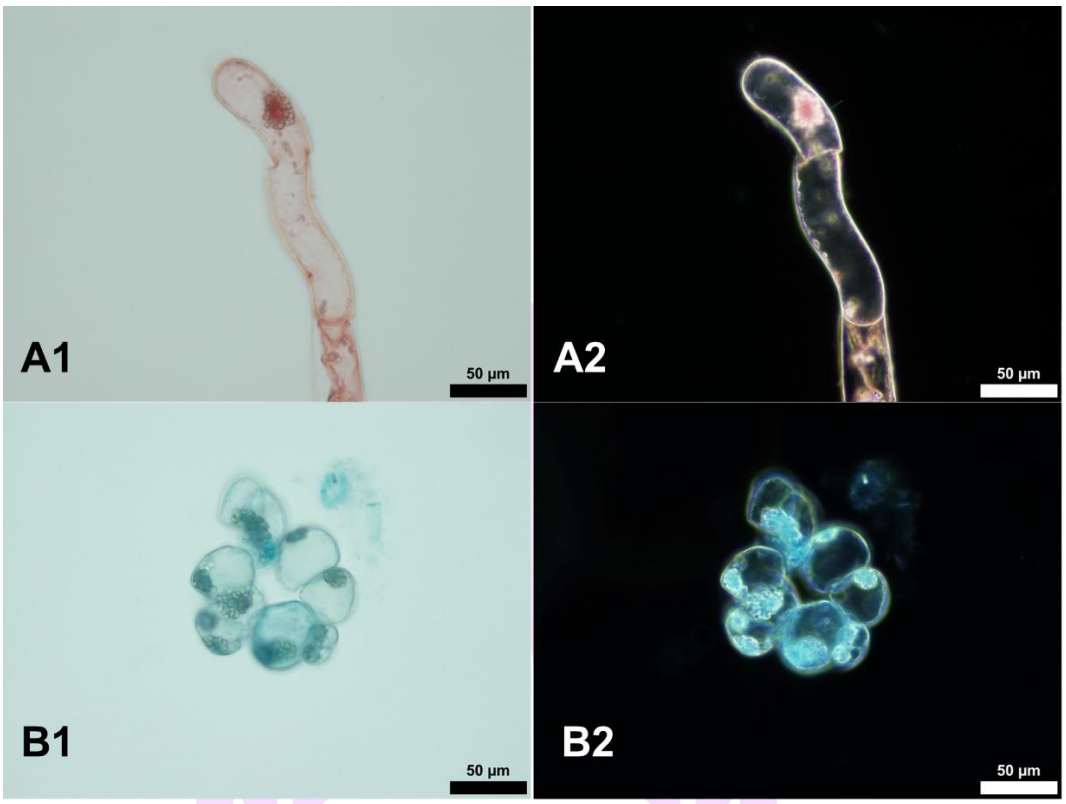

Figure 17 Suspension Cells Structure of *G. sootepensis* at Room Temperature

Note: Cells stained with safranin and observed with bright field (A1) and dark field (A2). Cells stained with coomassie blue under bright field (B1) and dark field (B2).

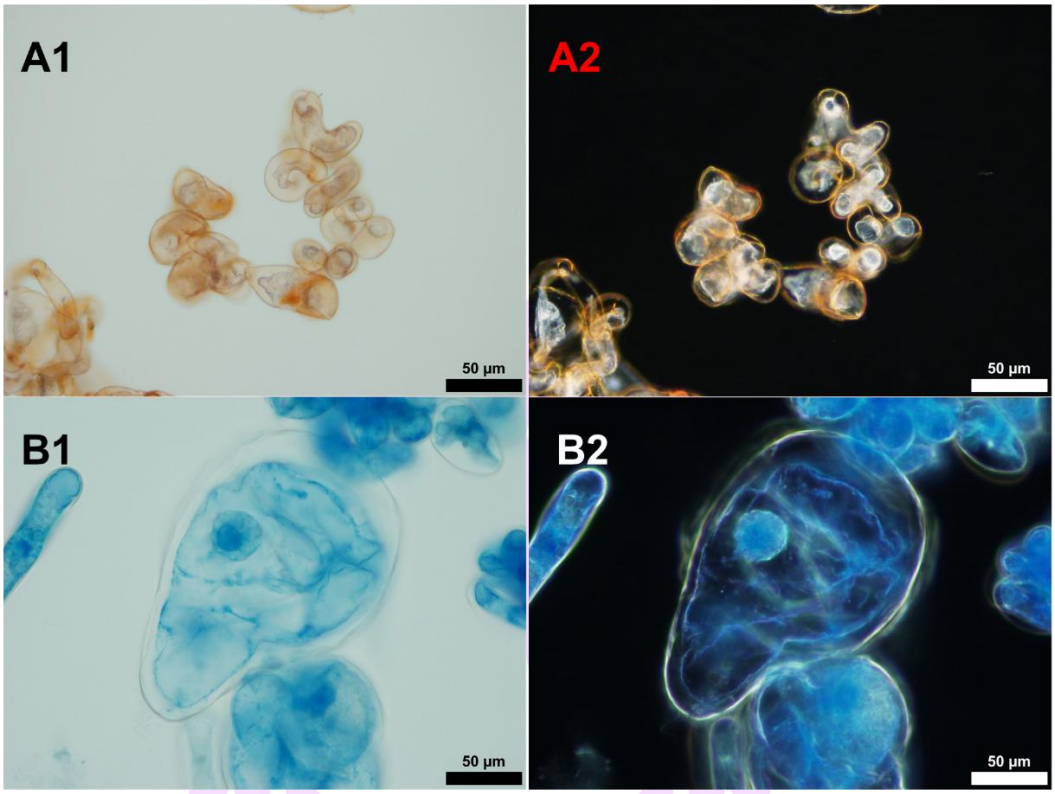

Figure 18 Suspension Cells Structure of *G. sootepensis* after 5-min Heat Shock at 65 °C

Note: Heat shock-treated cells stained with safranin solution observed with bright field (A1) and dark field (A2). Cells stained with coomassie blue observed with bright field (B1) and dark field (B2).

CHAPTER 5

CONCLUSION

Golden Gardenia Callus Induction

This study successfully established a callus induction protocol for golden gardenia (*G. sootepensis* Hutch.) using leaf explants. The findings indicate that the combination of plant growth regulators significantly influences both the rate and characteristics of callus formation. The application of 2,4-D (0.5–2.0 mg/L) in combination with low concentrations of kinetin (0.1 or 0.2 mg/L) was the most effective treatment, consistently achieving a 100% callus induction rate. At 0.5–1.0 mg/L 2,4-D, the resulting callus was uniformly friable and olive-green, whereas higher concentrations (2.0 mg/L) produced brown callus. In contrast, treatments using NAA resulted in variable and generally lower induction rates, underscoring the importance of auxin type in this species. These results suggest that the optimal combination of plant growth regulators for callus induction can vary significantly, even within the same genus, highlighting the need for species-specific optimization.

Golden Gardenia Cell Suspension Culture

This study provides valuable insights into the establishment and optimization of *G. sootepensis* cell suspension cultures. The auxin 2,4-D was found to be a key factor in promoting cell growth and proliferation, with optimal concentrations ranging from 0.5 to 1.0 mg/L in conjunction with 0.1 mg/L Kn. Higher concentrations of 2,4-D (2.0 mg/L) had a negative impact on cell viability. NAA proved to be unsuitable for *G. sootepensis* suspension cultures, as cells cultured with NAA remained non-viable. Interestingly, successive subculturing improved cell growth and viability, suggesting a process of cellular adaptation to the liquid culture

environment and selection for cells with enhanced growth characteristics. These findings underscore the critical role of auxin type and concentration, as well as the benefits of subculturing, in establishing and maintaining viable and productive *G. sootepensis* cell suspension cultures.

Golden Gardenia Heat Shock Response

The heat shock experiment provided critical insights into the temperature sensitivity of *G. sootepensis* suspension cells. The results indicate that these cells can tolerate temperatures up to 55 °C (corresponding to an extracellular temperature of 46.7 ± 0.10 °C) without significant structural damage. However, exposure to temperatures of 65 °C and 75 °C induced marked cellular damage, including the degradation of chloroplasts and disruption of cell membranes, leading to a loss of cell viability. The corrected A480 value sharply decreased at temperatures above 55 °C, confirming this loss of viability. Microscopic observations corroborated these findings, revealing distinct structural alterations in cells subjected to higher temperatures. These results establish a critical temperature threshold of approximately 55 °C for *G. sootepensis* suspension cells.

Further Study

Although the optimal medium formulation for *G. sootepensis* cell suspension culture has been identified, the long-term stability of the culture has not yet been conclusively demonstrated. Therefore, continued subculturing over multiple generations is necessary to validate this hypothesis and confirms the establishment of a stable, new cell line. Additionally, the potential for preserving the genetic integrity of *G. sootepensis* through the suspension culture method remains an important concern. Further research is required to explore the feasibility of

regenerating and differentiation process from suspension cultures to support conservation and propagation efforts.

There remains significant room for improvement in the design and interpretation of heat shock experiments on *G. sootepensis* suspension cells. While extracellular pH showed low sensitivity as an indicator of heat stress in this study, its potential utility may be enhanced by increasing the number of replicates and integrating the data with results from complementary assays. Similarly, the A595 value obtained from the Bradford assay, used to estimate total extracellular protein, showed promise as an indicator of cellular response, despite the lack of clearly statistically significant differences observed in the current experiment. Generating a standard curve to convert A595 values into precise protein concentrations may yield more definitive insights into protein leakage or secretion under stress, thereby improving the reliability of this metric.

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