Development of an Efficient Regeneration Protocol for Sesame (*Sesamum indicum* L. var. BINATIL-02) through Optimized *In Vitro* Culture Techniques.

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A thesis submitted to the Department of Biotechnology, School of Life Sciences (SLS) in partial fulfillment of the requirements for the degree of
Bachelor of Science in Biotechnology

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Brac University

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Declaration

It is hereby declared that

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

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Approval

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Ethics Statement

We, Sorna Rani Das, Fariha Bintey Shahid and Sujana Afrin Khan, hereby certify that the following criteria are fulfilled for the manuscript “Development of an Efficient Regeneration Protocol for Sesame (*Sesamum indicum* L. var. BINATIL-02) through Optimized *In Vitro* Culture Techniques”.

1. This content is our own original work and has not been previously published.

2. All sources used are appropriately acknowledged through accurate citations and justified references.

Abstract

Sesame (*Sesamum indicum* L.), one of the earliest cultivated oilseed crops in human civilization, is an important agricultural and economic crop in Bangladesh as the second largest oilseed crop contributing to food security and income for the rural people. While, it has an excellent biochemical profile, with up to 57% oil rich in unsaturated fatty acids and naturally occurring antioxidant lignans, its productivity is still below its potential owing to various biotic stresses, abiotic constraints, and recalcitrance of sesame in conventional breeding and *in vitro* regeneration. The main objective of this study is to develop an efficient *in vitro* regeneration system for *Sesamum indicum var*. of BINATIL-02 which is high-oil yielding gamma-ray mutant produced at the Bangladesh Institute of Nuclear Agriculture (BINA). It will create a platform for subsequent micropropagation and genetic engineering. 16 sterilization combinations of 70% ethanol, 0.1% mercuric chloride and sodium hypochlorite (Clorox) with Tween-20 to sterilize the seed surface were evaluated. Among them, one of the combinations, 50% Clorox and Tween-20 for 30 minutes, yielded the best result with 87% germination and 0% contamination as confirmed with a one-way ANOVA test. The best germination medium was Water Agar supplemented with 3% sucrose with 90% germination and 95.7% seedling survival rate. Cotyledonary leaves of 14-day old seedlings had 25 times more regeneration potential than hypocotyl explants. Explants were cultured on MS media containing BAP, IAA, AgNO3 and GA3. The optimal shoot regeneration frequency (71.1%) was achieved with the hormone combination of 2.5 mg/l BAP and 0.75 mg/l IAA, which was further increased to 82% with $AgNO_3$ supplementation, at the same time reducing necrosis and vitrification. The use of activated charcoal caused of total collapse of shoot survival and was excluded, while GA_3 resolved cytokinin-induced shoot stunting, resulting in 89% shoot elongation frequency. Rooting was induced on a ½ strength MS medium supplemented with 1 mg/l IAA and 1 mg/l AgNO3. After 30 days, 80% of shoots formed healthy roots and plants were successfully transferred to sterilized soils. The development of an efficient, rapid and reproducible regeneration system is an important step towards the improvement of sesame crop in Bangladesh.

Keywords: *Sesamum indicum*; BINATIL-02; tissue culture; sterilization; shoot regeneration; silver nitrate; cotyledon explant; rooting; Bangladesh.

Dedication

We dedicate this thesis to our beloved parents, whose unconditional love, endless sacrifices, and unwavering encouragement have been the foundation of every step in our journey. Your faith in us has been our greatest strength, and this achievement is as much yours as it is ours.

We are deeply grateful to our siblings for their constant support, understanding, and love. Your quiet sacrifices and steadfast belief in us made this work possible, and we remain forever indebted to you.

We would also like to express our sincere appreciation to our supervisor, and all mentors who guided us with patience, wisdom, and encouragement throughout this academic journey. Your invaluable support has left a lasting impact on our learning and growth.

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With heartfelt gratitude,
We dedicate this work to all of you.

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We sincerely hope that we will be able to uphold your expectations in the future and apply the knowledge and experience gained from this work toward meaningful contributions in our careers and for the betterment of society.

Sorna Rani Das, Fariha Bintey Shahid and Sujana Afrin Khan
May 2026

Table of content

List of Acronyms

The following abbreviations have been used throughout the text:

L	Liter
mg	Milligram
ml	Milliliter
dH_2O	Distilled Water
SD	Standard Deviation
NAA	Naphthalene Acetic Acid
IAA	Indole-3-Acetic Acid
GA_3	Gibberellic Acid 3
AC	Activated Charcoal
ANOVA	Analysis of Variance
BAP	6-Benzylaminopurine
BINA	BINA Bangladesh Institute of Nuclear Agriculture
MS	Murashige and Skoog (1962) medium

Chapter 01

Introduction

Oilseed crops occupy a central place in global agriculture, not only as a primary source of dietary fat but also as raw materials for a wide range of industrial products. Their importance has grown considerably in recent decades, driven by expanding populations, rising economy, and the diversification of their applications into sectors, such as bioenergy, pharmaceuticals, and the oleochemical industry (Gupta, 2016). Sesame (*Sesamum indicum* L.) is one of the important oilseed crops grown throughout South and Southeast Asia that is valued for its stable and nutritious nature (Sanni *et al.*, 2024). Sesame, or til (তিল) as it is traditionally known in Bangladesh, is already an integral part of the regional cuisine. Sesame oil is commonly used for cooking as a flavoring in traditional dishes, and as an ingredient in seasonings and pickles. Sesame oil is also widely used in traditional medicine, cosmetics, and rituals of the subcontinent (Bedigian, 2004). Distinct from many other oilseed crops, sesame oil is known for its excellent biochemical stability against rancidity due to the presence of naturally occurring antioxidants: sesamin, sesamolin and sesamol, which make it preservative free. This, together with its good fatty acid profile and high protein content in the seed meal, makes sesame an important nutritional and commercial crop (Wei *et al*., 2022).

1.1 Significance of Sesame Production for Bangladesh's Economy

Bangladesh remains a net importer of edible oil, as domestic output is insufficient to meet national consumption requirements. According to the United States Department of Agriculture Foreign Agricultural Service (USDA-FAS, 2024), estimates that domestic oilseeds can only meet 15-20% of total demand for edible oils, with the rest imported as crude oil or oilseeds, amounting to roughly two billion US dollars each year (Dhakatribune, 2021). Although sesame has been grown in Bangladesh for centuries, it contributes modestly to the overall oilseed production. Sesame is the 2nd most important oilseed crop in Bangladesh, which is cultivated in almost all districts, especially in Khulna, Faridpur, Pabna, Barisal, Rajshahi and Mymensingh (Hossain *et al*., 2006). The gap in supply and demand is compensated by imports of palm oil and soybean oil. A significant amount of foreign currency is used each year for importing edible oils and oilseeds to satisfy the demand (Islam *et al*., 2021). Lower farm-gate prices of imported oils have not encouraged the smallholder farmers to grow sesame, as it is less economically viable than

other crops (Hossain *et al*., 2006). However, both the government and agricultural research institutions have shown a long-term interest in sesame, given its capacity to reduce import dependence while improving rural incomes. Sesame is also well-adapted to marginal and drought-prone lands where the main food grains are not grown optimally, so its cultivation does not necessarily come at the cost of crop farming space (Akondo *et al.*, 2022). Therefore, it is believed to be crucial for Bangladesh's oilseed industry to increase the yield and stress tolerance of sesame cultivars using current agriculture and biotechnological methods.

1.2 Challenges with Traditional Sesame Farming in Bangladesh

Despite its adaptability, sesame production in Bangladesh is constrained by various factors that limit production to the crop's full yield potential. On the biotic side, fungal diseases, such as *Phytophthora blight* and *Alternaria* leaf spot, along with bacterial blight and infestations by sucking insects and leaf-eating caterpillars, routinely cause significant pre-harvest losses. Sesame is also prone to charcoal rot during a drought-stressed period that can wipe out an entire field during the pod-filling stage. According to Rajput *et al.* (2023), stress factors that limit sesame productivity globally include temperature sensitivity, early senescence, pest attacks, waterlogging, and disease infestations. Current commercial varieties, although they have been improved, may not be resistant to these stresses, forcing farmers to apply high doses of pesticides that not only increase costs, but also cause pollution (Hossain *et al*., 2006).

On the abiotic side, sesame production in Bangladesh is affected by inconsistent monsoon rains, mid-season droughts and heat waves, becoming more frequent due to climate change. Sesame's intolerance of waterlogging, even a couple of days of waterlogging can destroy the crop, and high water-table in many regions render it susceptible to water stress (Hafeez *et al*., 2021). Sesame has moderate amounts of phytic acid and oxalic acid, which can affect mineral intake and the open-capsule types of sesame are prone to shattering at maturity and cause significant post-harvest losses (Angamuthu *et al*., 2025). The traditional breeding methods that have been used to overcome these limitations are time-consuming and require several rounds of selection and evaluation for variety development. Furthermore, the production of new types using tissue culture and traditional breeding methods is severely limited by sesame's resistant nature, sexual incompatibility, and post-fertilization incompatibilities (Rajput *et al*., 2023). These collective limitations have created a

compelling case for deploying biotechnological tools, beginning with reliable *in vitro* regeneration systems as a foundation for more precise genetic improvement.

1.3 Origin of *Sesamum indicum*

Sesamum indicum L. is among the early domesticated oilseed plants known to humanity. Archaeological remains of carbonized sesame belonging to about 3500-3050 BC indicate that sesame was domesticated in the Indian subcontinent at least 5,500 years ago, and trading of sesame between Mesopotamia and the Indian subcontinent is documented to have occurred by 2000 BC. The genus *Sesamum* is large and predominantly African in origin, comprising around 36 recognized species, the majority of which grow wild across sub-Saharan Africa (Angamuthu *et al*., 2025). The cultivated species, *S. indicum,* is a diploid organism with a chromosome number of 2n=26, and is the sole member of the genus that has been brought into widespread agricultural cultivation (Sanni *et al.*, 2024). The precise geographic origin of *S. indicum* as a domesticated crop has been the subject of ongoing scholarly debate. The Indian origin of sesame is strongly supported by the presence of its wild ancestor, *Sesamum indicum* subsp. *malabaricum* (Burm.), in India as demonstrated by morphological, cytogenetic, and molecular data, as well as the presence of lignans sesamin and sesamolin in both the seeds and the wild ancestor (Angamuthu *et al*., 2025). People spread out sesame both eastward and westward, reaching China and Japan, which later developed into secondary distribution hubs. This wide geographic diffusion has resulted in considerable diversity in seed color, plant architecture, oil composition, and maturity duration, a diversity that plant breeders and biotechnologists continue to explore and exploit for crop improvement (Bedigian, 2004).

1.4 Taxonomy

Scientific Name:

Sesamum indicum L.

Taxonomic Classification:

Kingdom	**Plantae**
Phylum	**Tracheophyta**
Class	**Magnoliopsida**
Order	**Lamiales**
Family	**Pedaliaceae**
Genus	***Sesamum***
Species	***Sesamum indicum* L.**

(Sanni *et al.*, 2024)

1.5 Reproductive Biology of *Sesamum indicum*

Sesamum indicum is an erect, annual herb whose vegetative and reproductive development unfold in a relatively compact growing period of 75-120 days depending on the variety and environmental conditions (Angamuthu *et al.*, 2025). The plant begins its life cycle with the emergence of simple cotyledons, followed by the development of an upright stem that can reach 50 to 150 cm in height at maturity. Leaves are variable in shape along the stem broader and often opposite near the base, becoming narrower and alternate toward the apex. Flowering commences from the lower nodes and proceeds upward, with individual flowers appearing in the leaf axils. The flowers are tubular, bisexual, and slightly zygomorphic, with a five-lobed corolla that ranges from white to pale lavender or pink, often bearing purple nectar guide markings on the inner surface. Each plant produces 50 to 70 fruits, each of which has four or more chambers and may contain 55 to 70 seeds (Hossain *et al*., 2006). Pollination is predominantly achieved through

self-fertilization, as the anthers dehisce and deposit pollen directly onto the stigma before the flower fully opens. Sesame is a diploid, self-pollinated oilseed crop, though insect-mediated cross-pollination by bees does occur at rates that vary by location and pollinator abundance (Wei *et al*., 2022). In wild and many traditional varieties, the capsule dehisces explosively at maturity, scattering seeds and causing harvest losses, a trait that breeders have worked to suppress through selection for indehiscent or semi-indehiscent capsule types (Angamuthu *et al*., 2025).

1.6 Significance of *Sesamum indicum*

Few oilseed crops match sesame in the breadth of its utility or the depth of its integration into human civilization. From a nutritional standpoint, sesame seed is an exceptional food source. Since the earliest times, sesame has been referred to as the "Queen of Oil" because it has the highest oil content of any major oil crop—up to 45–57%. Its oil is said to contain 80% unsaturated fatty acids (Wei *et al*., 2022). The oil is predominantly composed of oleic and linoleic acids, giving it a balanced fatty acid ratio beneficial for cardiovascular health. The natural antioxidant lignans sesamin, sesamol, and sesamolin, along with tocopherols and phytosterols, contribute to both the superior oxidative stability of the oil as well as its possible preventive benefits against coronary, metabolic, and cardiovascular illnesses (Oboulbiga *et al*., 2023). In Bangladesh's agronomic context, sesame is additionally valued for its drought tolerance, its capacity to grow in sandy loam and well-drained soils of limited fertility, and its relatively modest water requirements compared to major cereal crops (Akondo *et al*., 2022). Sesame seeds and their derivatives are widely used in the manufacturing of biodiesel and animal feed, as well as in the medical, cosmetic, and industrial sectors after harvest (Khan *et al*., 2025). In broader biotechnological contexts, sesame has attracted attention as a model system for studying lipid biosynthesis and the molecular regulation of antioxidant compound production, further underscoring its academic and applied research significance (Gayatri & Basu, 2020).

1.7 Types/Varieties of *Sesamum indicum* in Bangladesh

Organized sesame variety development in Bangladesh has been led primarily by the Bangladesh Agricultural Research Institute (BARI) and the Bangladesh Institute of Nuclear Agriculture (BINA). Through conventional hybridization and mutation breeding programs, both institutions have released improved varieties with better yield potential, improved oil content, and greater

tolerance to major biotic and abiotic stresses. The currently recommended varieties for cultivation in Bangladesh include (Akondo *et al*., 2022):

1. BARI Til-2
2. BARI Til-3
3. BARI Til-4
4. BINATIL-1
5. **BINATIL-2**
6. BINATIL-3
7. BINATIL-4

BINATIL-02 was chosen for this study because it is simply the best-performing sesame variety in Bangladesh and no tissue culture protocol had ever been developed specifically for this variety. Since sesame regeneration responses differ significantly from one variety to another, protocols from other cultivars cannot be assumed to work for BINATIL-02 (Gayatri & Basu, 2020). This gap made developing a dedicated regeneration system not just useful, but necessary.

1.8 BINATIL-02

BINATIL-02 is the variety selected for investigation in the present study. Seeds for this research were procured from the Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh. Gamma ray-induced mutation techniques were implemented to create BINATIL-02, which has been used to improve sesame varieties in Bangladesh and around the world (Hafeez *et al*., 2021). This variety exhibits a distinctive combination of high yield potential and improved agronomic traits suited to the agro-climatic conditions of Bangladesh.

BINATIL-02 has been observed to produce the highest seed yield among the BINA-developed sesame varieties, with the most branches per plant, making it one of the greatest potential varieties to grow during the kharif season (June to October/November) (Akondo *et al*., 2022). Plants grow to a moderate height with an erect growth habit and bear capsules with a relatively high seed count per capsule. After 90 days of sowing, farmers harvest the high-yielding BINATIL-02 sesame, and approximately 45% oil content is found in this variety, which also possesses numerous medicinal properties. BINATIL-02 performs well under rain-fed conditions during the pre-kharif season and shows reasonable tolerance to waterlogging stress, as

demonstrated under controlled experimental conditions (Hafeez *et al*., 2021). Its combination of early maturity, high oil yield, and adaptability to diverse agro-ecological zones has made it a variety of choice both for farmers and for researchers interested in sesame improvement. Its relevance is further underscored by Bangladesh's pressing need to increase domestic oilseed production and reduce dependence on costly edible oil imports (USDA-FAS, 2024).

1.9 Significance of this Study

BINATIL-02 has earned considerable recognition among sesame farmers in Bangladesh, yet its full genetic potential remains constrained by the limitations of conventional improvement strategies. Precise, targeted improvement whether for resistance to a specific pathogen, modification of the fatty acid profile, or elimination of anti-nutritional factors calls for biotechnological approaches that go beyond what crossing and selection programs can efficiently deliver. According to Sajjanar & G, 2025, cross-pollination ranges from 0.5% to 65%, depending on insect activity, the environment, and the presence of other vegetation in sesame plants. . Genetic transformation and CRISPR-based genome editing hold considerable promise in this regard, but both depend entirely on the prior availability of a robust, genotype-specific *in vitro* plant regeneration system. There is essentially no repeatable regeneration and transformation procedure for producing separate primary transformed plant lines of *S. indicum* on a big scale (Gayatri & Basu, 2020), making the establishment of such a protocol an urgent research priority. Sesame has a highly genotype-dependent callus induction and regeneration frequency **(**Rajput *et al*., 2023), meaning that protocols developed for one cultivar cannot be assumed to be applicable to another. The use of contemporary biotechnological tools for genetic advancement investigations in sesame is hampered by the absence of reports on tissue culture techniques including regeneration through the callus phase, which are genotype dependent and have been documented with low regeneration frequency (Anandan *et al*., 2018). Furthermore, the variety BINA TIL-02 may have undergone shifts in its regeneration competence due to continued open-field cultivation and natural genetic drift since its original release. Under these circumstances, there is a clear need to establish a robust, reproducible *in vitro* regeneration system for BINATIL-02 by evaluating all relevant regeneration parameters.

With this background, the present study was undertaken to establish a comprehensive and reproducible tissue culture system for *Sesamum indicum* var. BINATIL-02, with the following specific objectives:

1. To standardize the seed sterilization procedure,
2. To determine the optimal explant age for *in vitro* regeneration,
3. To optimize hormone concentrations for shoot induction and multiplication,
4. To establish an effective rooting protocol for regenerated shoots, and
5. To successfully acclimatize and transplant *in vitro*-raised plantlets to soil conditions.

Chapter 02

Materials and Methods

2.1 Materials

2.1.1 Plant Material (Seeds)

Seeds of *Sesamum indicum* L. variety BINATIL-02 were used as the plant material for all experiments conducted in this study.

2.1.2 Sterilization Agents

The following sterilization agents were used for surface sterilization of seeds: 70% ethanol, commercial bleach (Clorox, 100%,50% & 20%), Tween-20, mercuric chloride ($HgCl_2$), distilled water, and tap water. These agents were used individually or in combination across the sterilization treatments evaluated in this study.

2.1.3 Hormones and Plant Growth Regulators

For sesame some plant growth regulators and hormones were used in this study included 6-Benzylaminopurine (BAP), Indole-3-acetic acid (IAA), Naphthaleneacetic acid (NAA), Gibberellic acid (GA_3), and Silver nitrate ($AgNO_3$). Stock solutions of BAP and IAA were prepared at a concentration of 1 mg/mL prior to use.

2.1.4 Reagents and Chemicals for Media Preparation

Murashige and Skoog (MS) basal salts, sucrose, agar, Gelrite, activated charcoal (AC), hydrochloric acid (HCl), sodium hydroxide (NaOH), and distilled water were used for the preparation of all media in this study.

2.2 Preparation of Stock Solutions

2.2.1 Preparation of 1 mg/mL BAP Stock Solution

I. Gloves were worn during the preparation.
II. 0.03 g of BAP was measured on foil paper or a Falcon tube cap.
III. 1 mL (or less) of NaOH was measured and added for dissolution.

IV. The NaOH and BAP were mixed by repeated pipetting until dissolved.
V. The solution was transferred into the Falcon tube, ensuring complete transfer.
VI. dH_2O was added to volume up the solution to 30 mL.
VII. The solution was vortexed to ensure proper mixing.
VIII. Care was taken to ensure that all droplets were collected at the bottom of the Falcon tube.
IX. The solution was stored in a Falcon tube wrapped with foil.
X. The solution was labeled and stored for future use.

2.2.2 Preparation of 1 mg/mL IAA Stock Solution

I. Gloves were worn during the preparation.
II. 0.03 g of IAA was measured on foil paper to avoid surface reaction and exposure.
III. 1 mL (or less) of ethanol was measured and added for dissolution.
IV. The ethanol and IAA were mixed by repeated pipetting until dissolved.
V. The solution was transferred into the Falcon tube, ensuring complete transfer.
VI. dH_2O was added to make the volume up to 15 mL.
VII. The solution was vortexed to ensure proper mixing.
VIII. Care was taken to ensure that all droplets were collected at the bottom of the Falcon tube.
IX. The volume was further adjusted to 30 mL with distilled water.
X. The solution was stored in a Falcon tube wrapped with foil.
XI. The solution was labeled and stored for future use.

2.2.3 Preparation of $AgNO_3$ Stock Solution

I. A lab coat, gloves, mask, and eye protection were worn because $AgNO_3$ is hazardous (corrosive, light- and heat-sensitive).
II. A Falcon tube was taken for preparation and storage of the solution.
III. 0.1 g of $AgNO_3$ powder was weighed on aluminum foil to avoid surface reaction and exposure.
IV. The $AgNO_3$ powder was transferred into the Falcon tube and was dissolved by repeated pipetting.
V. 10 mL of dH_2O was added into the Falcon tube using a pipette or measuring device.
VI. The solution was mixed using a vortex machine to dissolve the powder properly.

VII. Additional dH_2O was added to make the final volume up to 20 mL.

VIII. The solution was vortexed again to ensure complete mixing.

IX. The solution was filtered under laminar airflow using a syringe and a 0.22 µm cellulose nitrate filter.

X. Filtration was performed to remove bacteria and microparticles; cellulose nitrate was used due to its suitability for aqueous solutions and fast flow rate.

XI. The filtered solution was stored in 5 microcentrifuge tubes (1 mL each) and wrapped with foil to protect from light. The remaining solution was kept in the Falcon tube, sealed with foil and wrapped with parafilm to prevent contamination and light exposure. The $AgNO_3$ solution was thus rendered sterile and ready for use.

2.2.4 Preparation of 1 mg/mL GA3 Stock Solution

I. Gloves were worn during the preparation.

II. 0.03 g of GA3 was measured on foil paper to avoid surface reaction and exposure.

III. 1ml (or less) of 1N of NaOH was measured and added for dissolution.

IV. The NaOH and GA3 were mixed by repeated pipetting until dissolved.

V. The solution was transferred into the Falcon tube, ensuring complete transfer.

VI. dH_2O was added to make the volume up to 15ml.

VII. The solution was vortexed to ensure proper mixing.

VIII. Care was taken to ensure that all droplets were collected at the bottom of the Falcon tube.

IX. The volume was further adjusted to 30ml with distilled water.

X. The solution was stored in a Falcon tube wrapped with foil.

XI. The solution was labeled and stored for future use.

2.3 Media Preparation

2.3.1 Germination Media

2.3.1.1 Preparation of Water Agar (WA) Germination Media

To prepare 1 liter of Water Agar germination media, the following steps were performed:

I. Distilled water, less than the desired final volume, was taken in a beaker and placed on a magnetic stirrer.
II. After accurately measuring 30g of sucrose with an electronic balance, it was added to the water and stirred until it was completely dissolved.
III. After adding distilled water to a measuring cylinder to raise the capacity to 1000 ml, the solution was then transferred to the beaker.
IV. The pH of the solution was measured and adjusted to 5.8 ± 0.2. If pH exceeded 5.8, HCl was added dropwise; if pH was below 5.8, NaOH was added.
V. An electronic balance was used to measure 8.5g of agar powder, which was then added to the mixture while stirring.
VI. The agar mixture was melted in a microwave for 4 minutes, with stirring at the 1-minute mark to remove any clumps.
VII. The melted agar was poured into conical flasks, sealed with aluminum foil, and labeled with media name, date, and group name.
VIII. Before being used, the flasks were kept at 25 ± 2°C after being autoclaved for 1.5 hours at 121°C and 15 psi pressure.

2.3.1.2 Preparation of Full-Strength MS Germination Media

I. Measure MS 4.404 g, dissolve in dH2O.
II. Distilled water should be less than the desired amount to dissolve sugar & MS, add a magnetic stirrer.
III. Measure 30g sugar, add to water to dissolve.
IV. After dissolve, volume up to desired amount.
V. Measure pH 5.8, If pH>5.8, add HCl/ pH<5.8, add NaOH
VI. Measure 8.5g agar, add to the mixture, stir
VII. Melt for 4 minutes, after 1 min stirrer the mixture to remove clump
VIII. Agar must be plated. Sealed with foil, labeled as MS> Date>Name of group
IX. Autoclave for 1.5 hours.

2.3.1.3 Preparation of Half-Strength MS Germination Media

I. 2.202 g of MS salts (half-strength) was measured and dissolved in distilled water.

II. Distilled water should be less than the desired amount to dissolve sugar & MS, add a magnetic stirrer.
III. Measure 30g sugar, add to water to dissolve.
IV. After dissolve, volume up to desired amount.
V. Measure pH 5.8, If pH>5.8, add HCl, if pH<5.8, add NaOH
VI. Measure 8.5g agar, add to the mixture, stir
VII. Melt for 4 minutes, after 1 min stirrer the mixture to remove clump
VIII. Agar must be plated. Sealed with foil, labeled as MS> Date>Name of group
IX. Autoclave for 1.5 hours.

2.3.1.4 Preparation of Cotton Bed Germination Media

I. A conical flask was sterilized prior to use.
II. Sterilized forceps were used to introduce sterile cotton into the flask.
III. The cotton was distributed evenly across the bottom of the flask to form a uniform bed.
IV. An appropriate volume of distilled water was added to moisten the cotton bed sufficiently.
V. The flask was sealed with aluminum foil.
VI. The flask was labeled with the media name, date, and group name.
VII. The cotton bed was now ready for seed inoculation under sterile conditions.

2.3.2 Shoot Regeneration Media

2.3.2.1 Preparation of MS Shoot Regeneration Media with $AgNO_3$

I. 4.404 g of MS medium was measured and dissolved in distilled water to prepare 1 L of solution.
II. A smaller volume of distilled water than required was initially taken, and a magnetic stirrer was used to facilitate dissolution.
III. 30 g of sugar was measured and added to the solution and was dissolved completely.
IV. After complete dissolution, hormones (BAP 4 mL and IAA 1 mL) were added, and the volume was adjusted to 1 L.
V. The pH was measured and adjusted to 5.8 (HCl was added if pH was greater than 5.8, and NaOH was added if pH was less than 5.8).

VI. 8.5 g of agar was measured, added to the mixture, and stirred.

VII. The mixture was heated in a microwave for 4 minutes, with intermittent stirring after 1 minute to remove clumps.

VIII. The medium was distributed into two Duran bottles (500 mL each) to prevent bubbling and loss during heating.

IX. The bottles were autoclaved.

X. The medium was allowed to cool to a warm state.

XI. The bottles were transferred to a laminar airflow cabinet.

XII. 0.5 mL of $AgNO_3$ solution was added to each 500 mL Duran bottle.

XIII. The solution was gently swirled to mix without forming foam.

XIV. The medium was poured into sterile conical flasks.

XV. The flasks were sealed with aluminum foil.

XVI. The flasks were labeled and stored.

2.3.2.2 Preparation of Shoot Regeneration Media with Activated Charcoal (AC)

The following procedures were followed to prepare one litre of shoot regeneration media that included MS, sucrose, hormones, and agar:

I. An electronic scale was used to weigh 4.404g of MS powder, which was then dissolved in distilled water in a beaker set on a magnetic stirrer-less than the final volume that was intended.

II. 30g of sucrose was measured and added to the solution, stirring until fully dissolved.

III. The appropriate volume of hormones : BAP and IAA from stock solutions of 1 mg/ml were added to the media solution using sterile pipettes and 1g of Activated Charcoal grind was added.

IV. After adding dH_2O to a measuring cylinder to raise the capacity to 1000 ml, the solution was returned to the beaker.

V. The pH was measured and adjusted to 5.8 ± 0.2. If pH exceeded 5.8, HCl was added dropwise; if pH was below 5.8, NaOH was added.

VI. 8.5g of agar powder was measured and added to the solution with stirring.The agar mixture was melted in a microwave for 4 minutes, with stirring at the 1-minute mark to remove any clumps.

VII. The melted agar was poured into conical flasks, sealed with aluminum foil, and labeled with media name, date, and group name.

VIII. Before being used, the flasks were kept at 25 ± 2°C after being autoclaved for 1.5 hours at 121°C and 15 psi pressure.

2.3.2.3 Preparation of Shoot Regeneration Media with Gibberellic Acid (GA_3)

The following procedures were carried out in order to prepare one litre of shoot regeneration media that included MS, sucrose, hormones, and agar:

I. An electronic scale was used to weigh 4.404 g of MS powder, which was then dissolved in distilled water (less than the required final volume) in a beaker set on a magnetic stirrer.

II. 30 g of sucrose was measured and added to the solution, and the mixture was stirred until fully dissolved.

III. The appropriate volume of hormones, BAP (2.5 mL) and IAA (0.75 mL) from stock solutions (1 mg/mL), was added to the media solution using sterile pipettes.

IV. After adding dH_2O to a measuring cylinder to raise the capacity to 1000 mL, the solution was returned to the beaker.

V. The pH was measured and adjusted to 5.8 ± 0.2. If the pH exceeded 5.8, HCl was added dropwise; if the pH was below 5.8, NaOH was added.

VI. 8.5 g of agar powder was measured, added to the solution, and stirred properly.

VII. The agar mixture was melted in a microwave for 4 minutes, with stirring at the 1-minute mark to remove any clumps.

VIII. The medium was dispensed into Duran bottles or conical flasks, sealed with aluminum foil, and labeled with media name, date, and group name.

IX. The flasks were autoclaved at 15 psi pressure at 121°C for 1.5 hours.

X. After autoclaving, the medium was allowed to cool to a warm state and transferred to a laminar airflow cabinet.

XI. The required amount of GA_3 solution was then added aseptically to the medium under laminar airflow conditions.

XII. The medium was gently swirled to ensure proper mixing.

XIII. The melted agar was poured into conical flasks, sealed with aluminum foil, and labeled with media name, date, and group name.

XIV. The flasks were autoclaved at 15 psi pressure at 121°C for 1.5 hours and stored at 25 ± 2°C until use.

2.3.3 Preparation of Root Induction Media (1000 mL)

I. 800 mL of distilled water was taken.

II. 30 g of sugar was added and dissolved.

III. 2.202 g of MS medium (½ strength) was added.

IV. 1 mL (1000 µL) of IAA hormone was added.

V. The volume was adjusted to 1 L.

VI. The pH was adjusted to 5.8.

VII. 3.5 g of gelrite was added to the mixture.

VIII. The mixture was heated for 4 minutes until the gelrite was completely melted.

IX. Care was taken to ensure that no clumps remained.

X. The medium was autoclaved in Duran bottles, after which $AgNO_3$ was added under laminar airflow, and the medium was transferred into flasks.

2.4 Experimental Methods

2.4.1 Literature Review

Several publications about the tissue culture of Sesamum indicum L. were examined for the study's initial phase. Springer, International Journal of Agricultural Technology, Bangladesh Journals Online, Journal of Emerging Technologies and Innovative Research, Environmental and Experimental Biology, and the Bangladesh Association for Plant Tissue Culture and Biotechnology were among the databases surveyed.

2.4.2 Seed Surface Sterilization

The sterilant combinations and their respective durations are summarised in Table 2.1.

Table 2.1: Summary of sterilization treatments for surface sterilization of *Sesamum indicum* L. (BINATIL-02) seeds

Treatment no.	70% Ethanol (Minute)	Seed soaking in dH_2O (Hours)	$HgCl_2$ (Minute)	70% Ethanol	% Clorox+(3 drops of Tween20)	Clorox treatment duration (Minute)
1	-	24	2	1	-	-
2	1	2	1	-	-	-
3	1	-	1	-	-	-
4	1	-	-	-	100	1
5	1		-	-	100	15
6	1	-	-	-		30
7	1	-	-	-	50	15
8	1	-	-	-		30
9	1	-	-	-	20	15
10	1	-	-	-		30
11		-	-	-	100	15
12		-	-	-		30
13		-	-	-	50	15
14		-	-	-		30
15		-	-	-	20	15
16		-	-	-		30

For all treatments, seeds were taken in conical flasks, initially washed with tap water, and rinsed 5 times. Forceps and petri plates were transferred into the laminar airflow cabinet. Where Clorox was used as the primary sterilant, a 50% Clorox solution with 3 drops of Tween-20 was prepared and the seeds were agitated in the solution for 30 minutes, followed by 5 rinses with sterile distilled water.

2.4.3 Steps of Surface Sterilization

I. Seeds were rinsed with distilled water (dH2O) 3 to 5 times.
II. Seeds were immersed in 50% Clorox solution supplemented with 3 drops of Tween20 per 100 mL.
III. Seeds were agitated gently on a rotary shaker for 30 minutes.
IV. Clorox solution was discarded under laminar airflow conditions.
V. Seeds were rinsed 5 times with sterile distilled water (dH2O), 3 minutes each.
VI. Excess water was blotted using sterile filter paper.
VII. Seeds were transferred to sterilized culture media immediately.

2.4.4 Seed Germination

1. Approximately 10 surface-sterilized seeds were inoculated per conical flask using sterilized forceps inside a laminar airflow cabinet.
2. The flasks were filled with autoclaved water agar or MS germination media, depending on the treatment.
3. The inoculated flasks were sealed properly.
4. The flasks were kept in a dark chamber for 5–7 days to allow germination.
5. After germination, the flasks were transferred to light conditions.
6. After 7 days, the flasks were incubated at 25±2℃ under a photoperiod of 6 days or more, until the seedlings were suitable for explant excision.

2.4.5 Explant Selection and Inoculation

1. Cotyledonary leaves from 14-day-old *in vitro* seedlings were selected as explants.
2. The cotyledons were carefully excised from the apical shoot meristem using sterilized forceps and a scalpel inside a laminar airflow cabinet.

3. 6 explants were placed in each flask containing MS media supplemented with specific hormone combinations.
4. The cotyledonary leaves were positioned in an upward orientation on the media surface.
5. The culture vessels were sealed with parafilm.
6. The flasks were labeled using a permanent marker to indicate the respective treatments.
7. The cultures were incubated at 25 ± 2°C under illumination provided by 144 W white fluorescent lamps.

2.4.6 Shoot Regeneration and Subculture

1. *In vitro* regenerated shoots were subcultured at regular intervals of 14 days.
2. The shoots were transferred into fresh media containing the same hormonal combinations used for shoot induction.
3. The cultures were incubated at 25 ± 2°C under 144 W white fluorescent lamps throughout the subculture period.

2.4.7 Non-Invasive Shoot Length Measurement

To keep the cultures sterile, we measured shoot length from outside the glass rather than opening the flasks by treating the Erlenmeyer flask as a perfect inverted cone. Using the geometric relationship between height and volume in a conical vessel, the height (h) at any given point was derived from the volume of liquid accumulated (V(h)). Following the calculus principles for volume by integration (Stewart, 2015), we used the following formula to find the height (h) at any point:

$$\mathbf{h = H \times [\, 1 - \sqrt[3]{(1 - V(h) / V)}\,]}$$

Where,

h→ any point height

H→ Total height of flask

V(h) → Volume at any height

V→ Total volume of flask

In this equation, we calculated the height of the shoot's base (h_{base}) and its top (h_{top}) using their respective volume levels. The final shoot length was then calculated as the difference between these two vertical points ($L = h_{top} - h_{base}$). This non-invasive method allowed for precise data collection without compromising the aseptic conditions of the culture.

2.4.8 Rooting

1. Mature shoots were divided from other shoots by cutting them above the nodes.
2. A sterile surgical blade was used to remove vitrified tissues.
3. After that, the individual shoots were moved to the rooting media containing ½ MS and a hormone combination of 1 mg/L IAA and 1 mg/L AgNO3.

2.4.9 Acclimatization of Rooted Plantlets

1. Shoots with strong and mature roots were carefully washed under tap water to remove the media.
2. Sterilized (autoclaved) soil was prepared and moistened with dH2O.
3. The plantlets were planted in the prepared soil.
4. The planted plantlets were covered with plastic bags to maintain humidity.
5. Water was sprayed inside the plastic covering to create a humid environment.
6. The plantlets were kept under fluorescent light conditions.
7. The plantlets were supplemented daily with MS solution containing 3% sucrose and one-tenth strength MS solution.

2.5 Statistical Analysis

IBM SPSS Statistics version 27 was used for the statistical analysis. To ascertain whether the difference is the result of treatment differences or random chance, a one-way analysis of variance (ANOVA) test was conducted at the significance level of $p<0.05$. The outcome was considered statistically significant if the p value was less than 0.05. Based on the results of homogeneity of variance, the data were categorised. The results that were most important were highlighted in bold.

Chapter 04

Discussion

4.1 Sterilization

The optimisation of the surface sterilisation technique is an essential prerequisite for the effective installation of any in vitro growth system. As the contamination and the viability of the sterilized material directly determine the quality and availability of explants for succeeding culture stages. In the present study, a total of 16 treatment combinations involving 70% ethanol, 0.1% $HgCl_2$, and Clorox (NaOCl) with Tween 20 were evaluated to identify the most effective sterilization protocol for *Sesamum indicum* L. (BINATIL-02).

The present study showed that $HgCl_2$-based treatments (Treatments 1-3) completely eliminated germination (0%), regardless of the time and chemicals used because of the strong embryotoxic effect of $HgCl_2$ on sesame seeds. The phytotoxic effects of $HgCl_2$ on sesame seeds have also been reported by Taskin and Turgut (1997), who found that increasing concentrations and time of exposure to $HgCl_2$ damaged seed germination during the development of an *in vitro* regeneration protocol for *Sesamum indicum.* Chattopadhyay *et al*. (2010) also stated that $HgCl_2$ treatment for sesame seed sterilization needed to be carefully calibrated to avoid damage to the embryo, and even short time can significantly decrease germination in some sesame types. This fact aligns with the results of the current study in which all the treatments that involved $HgCl_2$ completely inhibited germination in BINATIL-02, thus confirming the sensitivity of this variety to $HgCl_2$.

Treatments involving 70% ethanol with Clorox (Treatments 4-10) resulted in germination rates of (0-71)%; however, these treatments were phytotoxic, and were not able to simultaneously produce contaminant-free and healthy explants. This observation correlates with Sen *et al* (2013) results, who reported that ethanol, a robust sterilant with strong phytotoxic properties, is highly damaging to plant tissues, even at short exposure times, especially when used in combination with other chemical sterilants. Similarly, Taskin and Turgut (1997) reported that the sequential treatment with ethanol and sodium hypochlorite for surface sterilization, resulted in tissue damage if the exposure time is not optimised. In this study, the combination of ethanol and Clorox at any concentration did not result in

healthy explants, which could be the reason for the toxic effect of two chemicals used as sterilants on the thinner coated seed coat of *Sesamum indicum.*

Among all the different treatment combinations, Treatment 14, which used 50% Clorox in combination with Tween 20 for 30 minutes without prior ethanol or $HgCl_2$ treatment, resulted in the desired effect of 0% contamination and 87% germination. This finding is similar to the Chattopadhyay *et al.* (2010) who reported that clorox (NaOCl) with a surfactant was highly efficient in achieving complete sterilization of sesame seeds while maintaining seed viability. The addition of Tween 20, a wetting agent, in the present study probably increased the penetration effect of the hypochlorite solution into the seed coat surface, which plays a role in the complete elimination of surface contaminants. The optimum concentration and duration of Clorox treatment in the present study and other protocols for sesame seed sterilization vary because of the difference in seed coat thickness. This may also be due to variation in the initial contamination levels in sesame varieties.

4.2 Optimization of Seed Germination Media

The selection of an appropriate germination medium is critical in tissue culture research, as it directly determines the quality, consistency, and availability of explants for succeeding regeneration experiments. In the present study, 4 types of media were evaluated for *in vitro* germination of *Sesamum indicum* L. (BINATIL-02): Water Agar supplemented with Sucrose, full-strength MS medium, half-strength (½) MS medium, and Cotton Bed. Water Agar + Sucrose was found to be the most effective medium, producing the highest germination rate of 90.0 ± 2.5% with an early initiation at day 5 and high seedling viability of 95.7 ± 1.2%, allowing explant collection by day 14.

Most previous studies on *in vitro* sesame regeneration have employed MS medium as the standard germination medium. Taskin and Turgut (1997) germinated sesame seeds on MS medium and obtained satisfactory germination; however, they noted that seedlings required an extended period before reaching the collectable explant stage. In the present study, MS medium produced only 70.0 ± 4.1% germination, with a delayed initiation period of 11 days and a prolonged explant collection stage requiring more than 21 days. Compared to Water Agar + Sucrose, the salts and vitamins in MS medium did not improve early germination, indicating that a simpler carbon-supplemented agar is sufficient. Similar observations have been reported by Li

et al. (2018), sesame is sensitive to salt stress, particularly at the germination stage, salinity strongly inhibits seedling emergence. In our study, sesame seeds had more efficient growth in media without any MS added. This explains the efficient germination in water agar sucrose media.

The performance of ½ MS medium was even lower, recording only 50.0 ± 5.8% germination with a collection period of 18-25 days, while Cotton Bed was the least effective medium with only 37.0 ± 7.2% germination and an unpredictable collection stage. The poor performance of Cotton Bed is consistent with the general observation that non-sterile or semi-sterile physical substrates provide inconsistent moisture distribution and inadequate nutrient supply, resulting in variable seedling development and low explant quality. Based on these results, Water Agar + Sucrose was selected as the standard germination medium for all following experiments in this study due to its superior germination rate, early initiation, high seedling viability, and the predictable 14-day collection schedule it offered.

4.3 Explant Type Selection

Explant selection is critical for predicting the success of *in vitro* plant regeneration because different tissue types have different regeneration potential depending on the explant's developmental stage, cell differentiation status, and hormone responsiveness. In this study, cotyledon and hypocotyl explants of *Sesamum indicum* L. (BINATIL-02) were compared for their shoot regeneration capacity on the optimal hormonal combination of 2.5 mg/l BAP + 0.75 mg/l IAA.

The cotyledon explants were more successful in terms of shoot regeneration (71.1%) with shoots initiated after 7-9 days. On the other hand, hypocotyl explants showed a very low percentage of shoot regeneration (0.93%) with shoot initiation after more than 20 days and shoot development after 35-40 days. These findings are consistent with the results reported by Taskin and Turgut (1997) in their study of *in vitro* regeneration of *Sesamum indicum,* where cotyledon explants consistently proved to be superior to hypocotyls for shoot regeneration, in the terms of both shoot frequency and the rate of organogenesis. They connected this difference to the greater

meristematic potential and response of cotyledonary tissue to cytokinin treatment compared to hypocotyl tissue.

Similarly, Chattopadhyay *et al.* (2010) compared the effect of explant types and found that cotyledons produced a much higher frequency of regeneration than hypocotyls at the same medium. They found sesame hypocotyls are more lignified and less sensitive to cytokinin-induced shoot formation, a natural trait which hampers its use in sesame tissue culture. This finding aligns with the current study, which found that hypocotyl explants of BINATIL-02 were highly recalcitrant and did not regenerate in 4 out of 5 hormonal combinations. The high difference in efficiency of regeneration (25 times more efficient in cotyledons than hypocotyls) indicates cotyledons to be the preferred explant for *Sesamum indicum* L. (BINATIL-02). The observed variation in performance of this study and previous studies could be assigned to genetic differences between BINATIL-02 sesame and the sesame varieties used by other authors.

4.4 Comparative Effect of Auxin Type on Shoot Regeneration

The type of auxin added into the shoot induction medium plays a different role in determining the nature of the organogenic response, as different auxins vary considerably in their modes of uptake, metabolism, and biological activity within plant tissue. Following the findings of (Al-Shafeay *et al.*, 2011), which identified a 4:1 BAP:IAA ratio as optimal for shoot induction, this study utilized combinations of 4.0 mg/l BAP + 1.0 mg/l IAA or 8.0 mg/l BAP + 2.0 mg/l IAA to maximize regeneration efficiency in the BINATIL-02 variety. In the present study, the effect of two auxins- naphthaleneacetic acid (NAA) and indole-3-acetic acid (IAA)- was evaluated alongside 4 mg/l BAP in cotyledon explants of *Sesamum indicum* L. (BINATIL-02).

The combination of 4 mg/l BAP + 1 mg/l IAA achieved a shoot regeneration frequency of 50.93 ± 4.8% with no spontaneous rooting, while the combination of 4 mg/l BAP + 1 mg/l NAA produced only 24.07 ± 3.4% shoot regeneration accompanied by a significantly high spontaneous rooting rate of 68.5%.

The dominant root-promoting effect of NAA observed in the present study is well established in the plant tissue culture literature. NAA is a synthetic auxin that is more stable compared to the

natural hormone IAA, and its stronger auxin activity pushes the plant to grow roots instead of starting new shoots (Bhaskaran and Smith, 1990). In sesame specifically, Taskin and Turgut (1997) reported that NAA in combination with BAP tended to produce callus and root formation rather than clean shoot organogenesis, and recommended IAA as the more appropriate auxin supplement for shoot induction media. The present study confirms these findings, as the high spontaneous rooting rate of 68.5% observed with BAP + NAA effectively made this combination unsuitable for a shoot-specific regeneration protocol.

In contrast, IAA, being a natural auxin, is rapidly metabolized within plant tissue and maintains a relatively low effective concentration over time, which is more compatible with the high cytokinin-to-auxin ratio required for shoot induction (George *et al*., 2008). The combination of 4 mg/l BAP and 1 mg/l IAA is more effective for shoot induction because it promotes faster development (8-9 days to initiation, 16-17 days to development), without the interference of root growth, which clearly demonstrates its superiority over NAA for shoot-specific induction in sesame. Similar conclusions regarding the superior use of IAA over NAA in shoot induction media for various plant species have been reported by several researchers, further validating the finding of the present study.

4.5 Optimization of BAP Concentration for Shoot Regeneration

The cytokinin benzylaminopurine (BAP) is the most widely used and effective cytokinin for *in vitro* shoot induction in a broad range of plant species, including sesame. In the present study, 5 concentrations of BAP in combination with proportionally adjusted IAA were evaluated in cotyledon explants of *Sesamum indicum* L. (BINATIL-02). The combination of 2.5 mg/l BAP + 0.75 mg/l IAA was found to be optimal, yielding the highest shoot regeneration frequency of 71.1% with the earliest shoot initiation (7-9 days). Both lower and higher BAP concentrations produced progressively lower results.

Various previous studies on sesame have reported different optimal concentrations of BAP for shoot development. According to Taskin & Turgut (1997), 1 mg/l BAP was sufficient to induce *in vitro* shoot regeneration from sesame cotyledon explants of the varieties used. But in this present study, 1 mg/l BAP + 0.25 mg/l IAA resulted in a much lower regeneration rate of 52.5%,

suggesting that this amount of BAP was not sufficient for BINATIL-02. This can be explained by genotypic differences as cytokinin responsiveness and optimal BAP concentration for sesame shoot organogenesis are highly genotypic dependent (Chattopadhyay *et al.*, 2010). Also, the concentration of BAP required for sesame shoot regeneration was highly dependent on the sesame variety, despite similar culture conditions for all varieties.

The negative impact of higher BAP concentrations (4, 7 and 10 mg/l) on shoot regeneration observed in the current study is also in accordance with many other sesame studies. In the current study, increasing BAP concentration from 2.5 mg/l to 4, 7, and 10 mg/l resulted in a progressive decline in shoot regeneration frequency from 71.1% to 48.3%, 20.0% and 13.3%, respectively. According to Taskin and Turgut (1997) reporting that higher concentrations of BAP beyond the optimum concentration progressively decreased shoot regeneration. Such inhibition at high concentrations of cytokinins is usually connected to the disruption of normal cytokinin signalling in the tissue, resulting in excessive growth without formation of the new shoots or leaves.

An interesting result of the study was that the highest percentage of regenerations (71.1%) was obtained at a BAP level of 2.5 mg/l, which is intermediate among the 5 concentration experiments. This observation implies that there is a narrow range of optimal cytokinin concentration for shoot induction in sesame, below which the regenerative stimulus is insufficient and above which it becomes inhibitory. The ideal concentration of BAP (2.5 mg/l) found in the current study is different from the 1 mg/l reported by Taskin and Turgut (1997) and the higher concentrations suggested by some other reports, but this variation highlights the need for variety-specific optimisation in sesame tissue culture work.

4.6 Role of Silver Nitrate ($AgNO_3$) in Improving Regeneration Efficiency

Physiological disorders, such as necrosis and vitrification are widely prevalent in plant tissue culture systems, and their incidence can seriously affect the regeneration response and health of the growing culture. In the present study, prolonged culture of *Sesamum indicum* L. (BINATIL-02) cotyledon explants on a shoot induction medium in the absence of silver nitrate ($AgNO_3$) led to high rates of necrosis (18.6%) and vitrification (10.3%) with the best response

for shoot induction (2.5 mg/l BAP + 0.75 mg/l IAA), limiting the maximum frequency of regeneration to 71.1 ± 5.14%. The addition of $AgNO_3$ to this medium enhanced the regeneration response, increasing the frequency to 82.0 ± 2.84%, and decreasing the necrosis (11.4%) and vitrification (6.6%).

The beneficial effect of $AgNO_3$ in plant tissue culture is well established and primarily connected to its role as an ethylene action inhibitor. Silver ions (Ag^+) compete with and displace copper ions (Cu^{2+}) from the ethylene receptor protein, thereby preventing ethylene from using its degeneration-promoting and developmental-inhibiting effects on cultured plant tissues (Beyer, 1979). Ethylene is known to accumulate rapidly within sealed tissue culture vessels, and this accumulation promotes tissue browning, necrosis, and premature senescence, all of which are thought to be a reason in the present study in the absence of $AgNO_3$. Chattopadhyay *et al*. (2010) have reported the effectiveness of $AgNO_3$ in sesame tissue culture, reporting that its addition to the culture medium prevented browning and necrosis in sesame cotyledon explants and improve the shoot regeneration efficiency. Similar necrosis-avoiding and regeneration-enhancing effects of $AgNO_3$ supplementation have been reported in many other plants. In our study, $AgNO_3$ was found to be effective with all 3 hormonal combinations, with the greatest effect seen at the optimum 2.5 mg/l BAP + 0.75 mg/l IAA combination, confirming its general usefulness as a tissue health promoting supplement in sesame shoot induction media.

4.7 Effect of Activated Charcoal (AC) on Shoot Survivability

Activated charcoal (AC) has been widely investigated as a medium additive in plant tissue culture due to its capacity to adsorb inhibitory compounds such as phenolic exudates, ethylene, and growth-inhibiting metabolites from the culture medium. In the present study, the addition of 1 g/l AC to the subculture medium was evaluated for its potential to further reduce vitrification and improve shoot health in *Sesamum indicum* L. (BINATIL-02). Contrary to expectations, AC supplementation resulted in a total collapse of shoot survivability across all 3 hormonal combinations tested, with survival rates dropping to approximately 0% by day 14, while control cultures without AC maintained shoot survival rates of 71.4%, 89.0%, and 80.2% at the corresponding hormonal treatments of (1, 2.5 & 4 mg/l) BAP.

This strongly negative outcome of AC supplementation is most likely due to its non-selective adsorption of the hormones present in the medium. AC is known to adsorb a wide range of organic molecules, including cytokinins, such as BAP and auxins, such as IAA, thereby significantly reducing their effective concentration in the medium and depriving the developing shoot tissue of the essential growth regulators required for continued survival and proliferation. Pan and van Staden (1998) reviewed the behavior of activated charcoal in tissue culture media and highlighted that its adsorption of growth regulators, particularly cytokinins, represents a major limitation of its use in hormone-supplemented media, and can lead to severe growth inhibition or death of cultured tissues. Similar inhibitory outcomes following AC application have been reported in several plant species. Bhuiyan *et al.* (2009) noted that AC supplementation during subculture of *Brassica* species resulted in reduced shoot strength and growth, due to the isolation of BAP from the medium.

It is also possible that AC adsorbed other essential organic components of the MS medium, including vitamins and sucrose-derived metabolites, further contributing to the observed culture collapse. The finding of the present study therefore stands in contrast to reports from other plant systems where AC has been beneficial, and underscores the highly species-specific and context-dependent nature of AC responses in tissue culture. For *Sesamum indicum* L. (BINATIL-02), the present study conclusively demonstrates that activated charcoal is inhibitory rather than beneficial during shoot subculture, and its use should be avoided in this regeneration system.

4.8 Effect of Gibberellic Acid (GA_3) on Shoot Elongation

A common challenge observed in cytokinin-enriched shoot induction media is the production of compact, stunted shoots with compressed internodes that are difficult to separate and transfer during subculturing. In the present study, shoots of *Sesamum indicum* L. (BINATIL-02) produced on the optimized medium of 2.5 mg/l BAP + 0.75 mg/l IAA + 1 mg/l $AgNO_3$ exhibited a dwarf and clustered shoot phenotype, with average shoot lengths of only 1.5 ± 0.1 cm and mean node counts of 2.8 at the time of subculture. The supplementation of gibberellic acid (GA_3) to this medium significantly improved shoot elongation, increasing average shoot length to 4.1 ± 0.4 cm, mean node count to 5.9, and elongation frequency to 89%.

Gibberellic acid (GA3) promotes shoot elongation mainly by inducing cell elongation via the induction of expanding genes that loosen the cell walls to facilitate cell expansion in the longitudinal direction and thus the internode elongation (Hooley, 1994). The stimulatory effect of GA_3 on shoot elongation is commonly observed in tissue culture. During *in vitro* regeneration of sesame, supplementation of subculture medium with GA_3 was crucial to transform the compact, cytokinin-induced shoot clusters into well-elongated, separated shoots that can be suitable for rooting. Such a positive effect of adding GA_3 on shoot elongation has also been reported in other by Pratik *et al.* (2016), who found that the addition of GA_3 at the optimum concentration substantially increased shoot length and node formation, ensuring better shoot handling and transfer in subculture. In this study, 89% shoot elongation at 2.5 mg/l BAP + 0.75 mg/l IAA + 1 mg/l $AgNO_3$ with GA_3 is the highest frequency of shoot elongation observed, and thus demonstrates that GA_3 is a critical supplement for shoot elongation in the BINATIL-02 regeneration protocol.

The stronger effect of GA_3 at 2.5 mg/l BAP + 0.75 mg/l IAA than at 1 mg/l BAP + 0.25 mg/l IAA could be due to the fact that the combination with higher BAP concentration results in more cytokinin-induced compact meristematic nodules, which have a higher suppression of internode elongation and therefore respond more strongly to gibberellin-induced relief. The present observation shows that the addition of GA_3 is not a visual effect but a critical step in the sesame regeneration protocol, as well-formed shoots with distinct internodes are more responsive to successful rooting and subsequent *ex vitro* establishment.

4.9 Root Induction

Root induction from *in vitro*-regenerated shoots is the final and critical step that bridges *in vitro* regeneration with *ex vitro* plant establishment. In the present study, rooting of *Sesamum indicum* L. (BINATIL-02) shoots was attempted on ½-strength MS medium supplemented with 1 mg/l IAA, with and without the addition of 1 mg/l $AgNO_3$. The result was observed like in the complete absence of $AgNO_3$, no root formation was recorded in any of the treated shoots regardless of their shooting hormone background. In contrast, with the inclusion of $AgNO_3$, rooting was successfully induced, with a response rate of 80% and a mean root count of 4.38 ±

1.75 per shoot achieved for the group derived from 2.5 mg/l BAP shooting hormone, with roots reaching an average length of 3.35 cm over 30 days.

The use of ½-strength MS medium for root induction in sesame is consistent with the widely adopted practice in plant tissue culture, where reduced mineral salt concentration is preferred during rhizogenesis to avoid ionic toxicity and to create an osmotic environment that is more advantages to root differentiation (George *et al*., 2008). Taskin and Turgut (1997) employed ½ MS supplemented with IAA for rooting of *in vitro*-regenerated sesame shoots and reported successful root formation. Similarly, Chattopadhyay *et al.* (2010) reported that ½ MS with IAA was more effective than full-strength MS for root induction in sesame, due to the lower ionic strength of the diluted medium. The use of IAA as the rooting hormone in the present study is also consistent with these reports, as IAA has been shown to be more beneficial than NAA for sesame rooting due to its lower phytotoxicity and more targeted auxin activity (Chattopadhyay *et al*., 2010).

The most interesting observation in the rooting experiment was the complete dependence of root induction on $AgNO_3$ supplementation. In the current study, no rooting was found on ½ MS + 1 mg/l IAA without $AgNO_3$ while the same medium supplemented with 1 mg/l $AgNO_3$ produced healthy roots. $AgNO_3$ allowed IAA to effectively utilize its root inducing effect, promoting successful rooting. The role of $AgNO_3$ in promoting root induction has also been reported in sesame by Chattopadhyay et al. (2010), who found an increase in rooting frequency with the addition of $AgNO_3$ to the rooting medium. The variation in rooting response between the two shooting hormone population (80% for 2.5 mg/l BAP vs 35% for 1 mg/l BAP) may be due to difference in the stored carbohydrate, hormonal status, and physiological maturity of the two shoot populations, as shoots developed under a higher productive and vigorous hormonal treatment are expected to have higher stored carbohydrate and stronger rooting potential. The difference in rooting rates between studies in sesame is due to the differences in genotype, IAA levels, and the type of culture vessel used.

Overall, the current study revealed that there is significant variation between BINATIL-02 and other sesame regeneration protocols reported, as well as variation between other sesame species

and varieties. This is a result of several biological factors including the genetic background, physiology, explant type, hormone sensitivity of this particular variety. The optimization of the tissue culture protocol from sterilization to root induction in this study have addressed several key parameters that were either not tested or not optimized in earlier sesame tissue culture studies, to achieve a more specific and efficient protocol for *Sesamum indicum* L. (BINATIL-02) which can be used as a basis for biotechnologic applications such as genetic transformation and genome editing.

Chapter 05

Conclusion

This study successfully addressed one of the most critical bottlenecks in sesame biotechnology- the absence of a reliable, genotype-specific *in vitro* regeneration system for *Sesamum indicum* L. var. BINATIL-02. The work showed that BINATIL-02 can respond well to tissue culture, but only under the suitable conditions. Getting the effective sterilization without making harm to the seeds, to select the correct germination medium, and chosing cotyledon explants instead of hypocotyls were all found to be essential decisions that directly affected the outcome. The plant hormones also played a central role, the right balance between cytokinin and auxin determined whether shoots formed successfully or not.

Importantly, this study shows that the recalcitrance that has been reported for sesame in previous studies is not an intrinsic biological barrier but rather a failure to find suitably optimized conditions for specific sesame genotypes. With appropriate adjustments, BINATIL-02 is capable of producing healthy, rooted, and soil-adapted plantlets through direct organogenesis.

This protocol now offers a reliable and practical platform upon which future genetic improvement strategies - including transformation and genome editing- can be built, bringing the long-term goal of producing stress-resilient sesame cultivation in Bangladesh.

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