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Establishment of Micropropagation System for Mulberry (*Morus alba* L.)

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Abstract

Mulberry (*Morus alba* L.) is an economically and ecologically significant woody perennial widely cultivated for sericulture, pharmaceuticals, and nutraceutical applications. However, conventional propagation methods are limited by low multiplication rates, genetic heterogeneity, and vulnerability to pests and diseases. This study aimed to develop a standardised and efficient micropropagation protocol for *M. alba* to support large-scale, uniform plant production. Explants (nodes, stems, and leaves) from healthy three-year-old mother plants were subjected to ten surface sterilisation treatments using combinations of Clorox (10%), $AgNO_3$ (0.1 g/L), Tween 20, fungicides, and antibiotics. The optimised sterilisation method achieved a contamination rate of <5% and a survival rate >90%. Murashige and Skoog (MS) medium supplemented with 5.0 mg/L 6-Benzylaminopurine (BAP) and 0.2 mg/L Naphthalene Acetic Acid (NAA) yielded the highest shoot induction and shoot biomass across nodal and leaf explants. Significant callus formation was observed in nodal and stem explants, while browning -particularly in leaf cultures was mitigated using Woody Plant Medium and citric acid.

Keywords: BAP, mulberry, micropropagation, NAA, surface sterilisation

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Introduction

Mulberry (*Morus alba* L.) is a deciduous tree from the Moraceae family that holds economic importance, particularly in sericulture, because its leaves are the main food for silkworms (*Bombyx mori*)(Acharya, 2024). Besides sericulture, mulberry is valued for its nutritional, medicinal, and industrial benefits. Its fruits are rich in antioxidants and bioactive compounds (Taha et al., 2020). However, traditional propagation methods face problems like disease transmission and inconsistent plant quality. Micropropagation provides a practical solution by allowing the large-scale production of genetically uniform, disease-free plants in controlled settings. This method includes key steps such as selecting explants, sterilisation, culture initiation, shoot multiplication, rooting, and acclimatization (Thomas, 2002). The use of Murashige and Skoog (MS) medium, along with cytokinins like BAP and auxins such as NAA, is essential for successful growth and development. A proper balance of growth regulators, careful explant selection, and optimized culture conditions help ensure genetic stability and reduce somaclonal variation (Neysa, 2024). This study aims to create a reliable and efficient micropropagation protocol for *Morus alba* by improving sterilisation methods and growth regulator concentrations. Various explants were tested under different sterilisation conditions and cultured on MS medium with BAP (3.0–5.0 mg/L) and 0.2 mg/L NAA. The protocol was assessed for contamination rates, survival, and shoot proliferation.

The primary objective of this research is to establish an efficient micropropagation system for mulberry (*Morus alba*) by optimising culture media and sterilisation protocols. Specifically, the study aims to test various combinations of plant growth regulators to induce effective culture initiation, shoot formation, evaluate different surface sterilisation methods to reduce contamination, and identify the most suitable explant type, such as nodes, stems, or leaves, for initiating successful tissue cultures.

Materials and Methods

Mother plant selection and maintenance

Healthy, three-year-old *Morus alba* L. plants from the Kurunegala District, Sri Lanka, were selected as mother plants for micropropagation. *Morus alba* L. can be identified by naked eye inspection from its distinct morphological features. Leaves are broad, bright green, and serrated, with both lobed and unlobed shapes often found on the same tree. Fruits tend to be white to pink when mature, which differentiates it from the rest of the species of mulberry (Rana et al., 2022). These plants grew well under optimal greenhouse conditions with balanced fertilisation and regular pruning. They provided strong, disease-free shoots for explants. Nodal segments, stems, and leaves were collected early in the morning, trimmed to 1 to 2 cm, and kept in distilled water to stay viable (Attia et al., 2014).

Media preparation

Culture media were prepared using Murashige and Skoog (MS) basal medium with 3% sucrose and different concentrations of BAP ranging from 3.0 to 5.0 mg/L, combined with 0.2 mg/L NAA. The media were adjusted to a pH of 5.7 to 5.8 and solidified with agar (Anis et al., 2003). Sterilised media were placed into culture vessels and autoclaved. Autoclaving was done to destroy bacteria, fungi, spores, and other microorganisms that could contaminate the culture.

Table 1: *Different Surface Sterilization methods for the 10 treatments*

Surface sterilisation method	**IPA (70%) Time duration / RPM**	**Soap + Distilled water RPM**	**Clorox Time duration / RPM**	**Fungicide Time duration / RPM**	**Tween twenty Time / RPM**	**AgNo3 Time duration**	**Fungicides Time duration**	**Antibiotic Time duration**
01	Wipe the explants	10 min 180 rpm	10% 10 min 180rpm	0.018% v/v per 5 min 180rpm	0.018% v/v per 5 min 180rpm	-		30µL 15min
02	Wipe the explants	10 min 180 rpm	10% (Nodes) 10 min 180 rpm 5% (leaves) 5min180rpm	0.018% v/v per 15 min 180rpm	0.018% v/v per 15 min 180rpm	30mg/300ml 5 min		1.2ml/L 30min
03	Wipe the explants + 2 mins 180 rpm	10 min 100 rpm 5min 180rpm	10% (Nodes) 10 min 180 rpm 5% (leaves) 5min 180rpm			0.1g/L 10min	0.01% v/v per 10min	1.2ml/L 30min
04	Wipe the explants	10 min 100 rpm 5min 180rpm	10% (Nodes) 10 min 180 rpm 5% (leaves)5min 180rpm	0.02% v/v per 15 min 180 rpm	0.02% v/v per 15 min 180 rpm	0.1g/l 5min	0.01% v/v per 5min	10ml/L 30min (Nodes) 10 min(leaves)

05	-	10 min 100 rpm 5min 180rpm	10% 10 min 180 rpm	0.02% v/v per 15 min 180 rpm	0.02% v/v per 15 min 180 rpm		0.01% v/v per 5min	10ml/L 30min (Nodes) 10 min(leaves)
06	Wipe the explants	10 min 100 rpm 5min 180rpm	10% (Nodes) 10 min 180 rpm 5% (leaves)5min 180rpm	0.02% v/v per 15 min 180 rpm	0.02% v/v per 15 min 180 rpm	0.1g/L 5min	0.01% v/v per 5min	10ml/L 30min (Nodes) 10 min(leaves)
07	Wipe the explants	10 min 100 rpm 5min 180rpm	10% (Nodes) 10 min 180 rpm 5% (leaves)5min 180rpm	0.02% v/v per 15 min 180 rpm	0.02% v/v per 15 min 180 rpm	0.1g/L 5min	0.01% v/v per 5min	10ml/L 30mins (Nodes) 10 mins(leaves)
08	Same steps as 7th treatment							
09	Same steps as 7th treatment							
10	Same steps as 7th treatment							

Explant selection and surface Sterilisation

Explant sterilisation involved a step-by-step process. This included treatments with liquid soap, 70% isopropyl alcohol, 5% to 10% Clorox, silver nitrate, fungicides, and antibiotics under controlled agitation. A standardised surface sterilisation protocol was developed to ensure high explant viability and minimal contamination during the micropropagation of *Morus alba*. Using a 500 mL Csup solution (10 mL Csup + 90 mL distilled water), glass jars were first sterilised. Each jar was then cleaned in three sets, clockwise and counterclockwise (Peiris et al., 2012). To lower the surface microbial burden, the explants were first washed for ten to fifteen minutes under running tap water (Bhau & Wakhlu, 2003). Several changes were tested to improve sterilisation, especially for leaves, to reduce contamination and boost explant survival. Seventh trial was identified as the most effective surface sterilisation method for *Morus alba* explants, ensuring high survival rates and minimal contamination. Initially, explants were rinsed under running tap water for 10–15 min to remove surface dust and debris. This was followed by wiping the explants with 70% isopropyl alcohol (IPA) for 10 min at 100 rpm to eliminate surface microbes. Next, the explants were washed in a soap

and hand wash solution for 5 minutes at 180 rpm to further reduce microbial load and improve sterilant penetration. For chemical sterilisation, nodal explants were treated with 10% Clorox for 10 min at 180 rpm, while leaf explants were treated for 5 minutes at the same conditions using 5% Clorox. To prevent fungal contamination, explants were exposed to a fungicide solution (200 µL/L) for 15 minutes at 180 rpm, and then to Tween 20 (200 µL/L) for another 15 min under the same agitation. Explants were then treated with silver nitrate ($AgNO_3$) at 0.1 g/L for 5 min, followed by exposure to an antibiotic solution (100 µL/L) for the next 5 minutes to destroy bacterial contamination. Lastly, a second fungicide application (10 mL/L) was carried out for 30 minutes for nodal explants and 10 minutes for leaf explants. Explants were then washed 3-4 times with sterile distilled water to eliminate chemical residues before culture. After the last rinse with autoclaved distilled water, leaves and other explants were cut into 1-2 cm pieces before culturing. Sterilised explants were inoculated onto prepared media in sterile Petri plates and culture tubes. Cultures were maintained under controlled conditions at 25±2°C, 16-hour photoperiod, and 60-75% relative humidity.

Data Collection and analysis

Data collected consisted of major parameters such as contamination rate, presented as a percentage of contaminated cultures, Callus induction weight, shoot induction, calculated by the percentage of explants that formed shoots; number of shoots per explant. All the data were subjected to statistical analysis using ANOVA software programs.

Results

Identification of the best explant type for Mulberry culturing

The response of leaf, stem, and nodal explants of *Morus alba* L. to different BAP concentrations (3, 4, and 5 mg/L) showed that nodal explants had the best results. They initiated shoots more quickly and had higher shoot induction rates. Nodal explants produced the most shoots on MS medium with 5 mg/L BAP and 0.2 mg/L NAA.

Callus Induction

Table 2: *Number of Calli Observed on Different Media*

Hormone Combination	Number of Callus observed
3mg/L BAP+ 0.2mg/L NAA +full MS+3%sugar	5
4mg/L BAP+ 0.2mg/L NAA +full MS+3%sugar	12
5mg/L BAP+0.2mg/L NAA +full MS+3%sugar	24

According to the table, 5 mg/L BAP was identified as the best medium, based on results from the 1st, 2nd, and 3rd sterilisation treatments. Further culturing was carried out using 5 mg/L BAP with 0.2mg/L NAA.

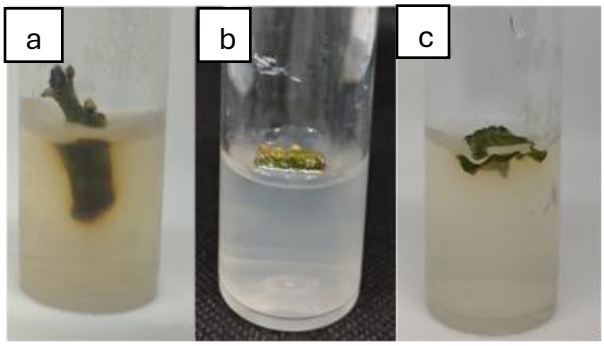

Figure 1: *Observed Calli. a) Nodal explant. b) Stem cut explant. c) curling leaf explant.*

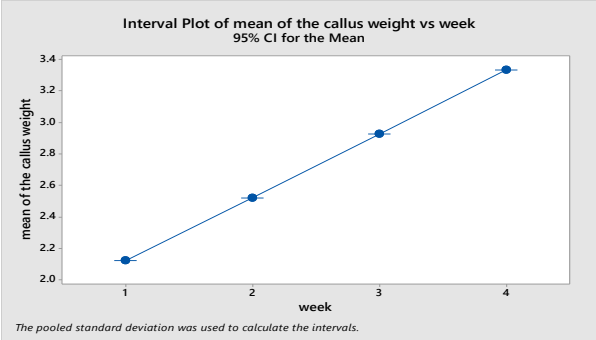

Figure 2*: Mean of the callus weight*

Callus induction in *Morus alba* was evaluated using nodal, stem, and leaf explants grown on full-strength MS medium with 5.0 mg/L BAP and 0.2 mg/L NAA. Callus formation started about four weeks after inoculation, mainly in nodal and stem explants. This produced 20 calluses with different textures and colors, showing active growth. These findings show that using light-cultured nodal and stem explants is effective for callus induction in mulberry.

An interval plot illustrating 95% confidence intervals demonstrated a steady increase in mean callus weight over a four-week culture period, indicating successful callus induction and proliferation. The cultures were maintained on MS medium supplemented with 5 mg/L BAP, 0.2 mg/L NAA, and 3% sugar. Mean callus weight increased from 2.125 g for Week 1 to 3.335 g for Week 4. Pooled standard deviation was applied in confidence intervals since 20 calli were measured for every week to offer biological replication. The findings confirm the validity of the selected culture conditions to be capable of maintaining normal biomass accumulation and callus development.

Shoot initiation response with treatment of BAP (5 mg/L) with NAA(0.2mg/L) combination

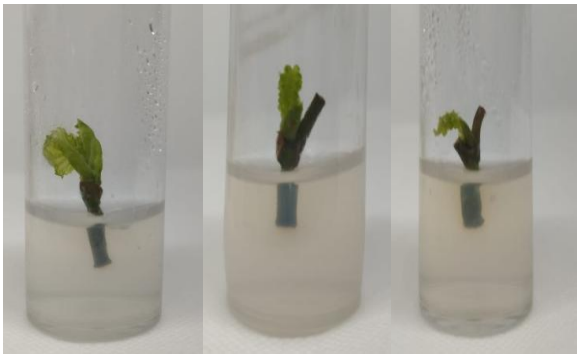

Figure 3: *Shoots induction*

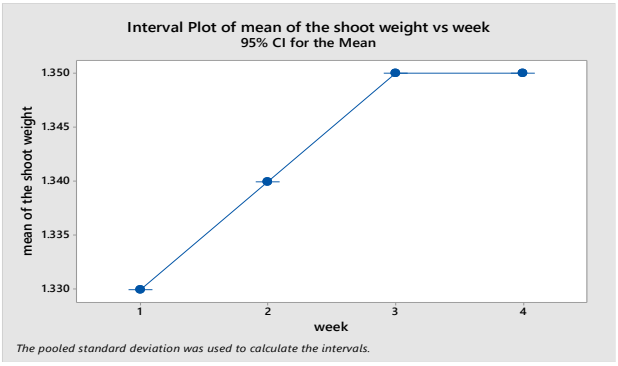

Figure 4: *Mean of the shoot weight*

An interval plot illustrating 95% confidence intervals tracked mean shoot weight changes over a four-week period, highlighting early shoot development in *Morus alba*. The average weight increased slightly from 1.330 g in Week 1 to 1.350 g by Week 3, then plateaued through Week 4. Mean values were based on measurements from five individual shoots, with pooled standard deviation used for statistical accuracy. The initial weight gain reflects early shoot growth, while the later stabilization suggests a slowdown in biomass accumulation. This trend implies that adjustments to environmental conditions, hormonal balance, or nutrient availability may be required to support continued shoot development in extended cultures.

Contaminations

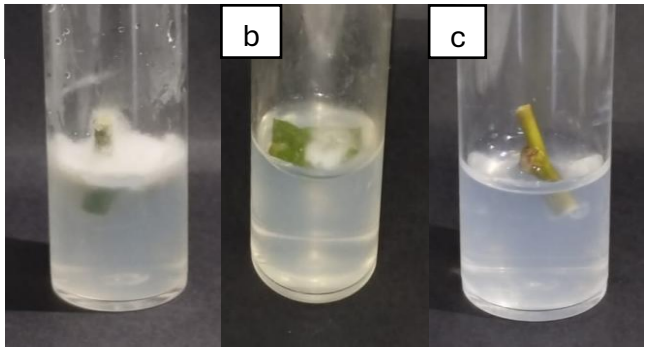

Figure 5: *a)Nodal explant(Fungal Contamination / Observation after 5 days). b)Leaf explant (Fungal Contamination /Observation after 8 days). c)Nodal explant (Bacteria Contmination / Observation after 4 days).*

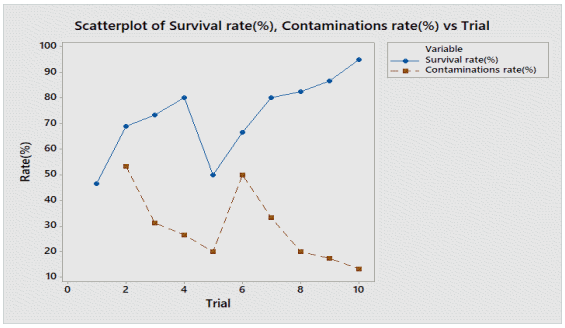

Figure 6: *Survival rate (%) vs Contamination* rate (%)

The scatterplot displays the variation in survival rate (%) and contamination rate (%) of cultured explants over ten sequential trials. The data suggests a dynamic process of optimisation, where improvements were likely implemented over time to enhance culture success. Two contrasting trends are clearly visible: an upward trend in survival rate and a downward trend in contamination rate.

According to the results I obtained for the establishment of a micropropagation system for mulberry (*Morus alba* L.), I referred to the previous study conducted by MICROPROPAGATION OF MULBERRY (MORUS ALBA L.) CV. AL-TAIFY Attia O. Attia, Eldessoky S. Dessoky, Ehab I. El-Hallous, and Hanan F. Shaaban.

Discussion

This study focused on developing an efficient micropropagation protocol for *Morus alba* by evaluating multiple culture parameters, including explant type, hormonal treatments, media composition, surface sterilisation techniques, and culture conditions. The combination of 5 mg/L BAP and 0.2 mg/L NAA in full-strength MS medium with 3% sucrose was shown to be the most efficacious for shoot initiation and multiplication among the several media compositions evaluated. Direct organogenesis was encouraged by this ideal cytokinin-to-auxin ratio, which resulted in callus-free, robust shoot development. These results corroborate earlier research on BAP's function in promoting woody perennial branch multiplication.

Explant type played a significant role in culture responsiveness. Nodal explants exhibited the highest shoot induction rates, faster response times, and better morphological quality compared to leaf and stem segments. However, nodal explants responded well to the same hormone combination, producing multiple shoots with visible internodes. Callus induction was successfully achieved using 5 mg/L BAP and 0.2 mg/L NAA in full MS medium, particularly from nodal and stem explants. Within four weeks, calli formed, displaying a variety of hues and textures that demonstrated tissue differentiation and active cellular proliferation. The fact that various leaf explants reacted differently to the same treatment indicates that distinct hormonal approaches are required for dedifferentiation. The significance of light for morphogenesis in mulberry tissue cultures was highlighted by the notable failure of explants grown in total darkness to produce viable calli. Contamination is a critical issue in the development of a micropropagation system for mulberry that limits success. The crucial ones among them are bacterial and fungal contamination from explant surface, media, or laboratory environment, and endophytic microbes hidden inside plant tissues overcoming sterilisation and manifesting later in culture. Chemical residues, inadequate sterilisation of media, and cross-contamination among cultures also cause failures. Human error in handling and climatic seasons also increase the risk of contamination(Abdalla et al., 2022). To reduce contamination and increase culture survival, sterilisation techniques were essential. Over the course of multiple treatments, contamination rates were considerably decreased by a multistep sterilisation procedure that included IPA, Clorox, fungicides, $AgNO_3$, antibiotics, and Tween 20. Strong sterilising procedures are necessary for the successful creation of *in vitro* cultures, as evidenced by the fact that survival rates rose from 45% in early treatments to over 90% in subsequent experiments.

Conclusion

In this study, an efficient micropropagation protocol for *Morus alba* was developed, with nodal segments identified as the best explant for culture initiation. The optimal media combination was MS medium supplemented with 5 mg/l BAP and 0.2 mg/l NAA, which effectively promoted shoot induction. This protocol enables the rapid clonal production of disease-free, genetically uniform plants, supporting large-scale propagation. The findings have significant implications for sericulture, genetic conservation, and sustainable agriculture.

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