# Optimization of *In-vitro Callus* Induction and Cell Suspension Cultures of *Gyrinops walla* for Commercialization

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## Abstract

*Gyrinops walla* (*G.walla*), often referred to as 'Walla Patta' in Sinhala, is an indigenous, economically important plant renowned for its production of agarwood, which is a highly valuable resin having high economical, religious and traditional values. *G. walla* trees take 5-7 years on average to grow naturally before being inoculated to produce resin. In-vitro callus culture approach will shorten the agarwood resin production process significantly and be important for the industry. However, tissue culture methods are challenging due to explant contamination and low rate of callus production. Therefore, this study aims to optimize the conditions for surface sterilization and enhance in-vitro callus induction from leaf explants, with the objective of advancing the development of cell suspension cultures for commercialization. The experiment for surface sterilization and callus induction was conducted using leaf explants obtained from two *G.walla* mother plants, a home garden plant and a wild plant. The results suggested that 100 mg/L silver nitrate ($AgNO_3$) and Dettol provide a better surface sterilization for callus production, especially in explants from a home garden mother plant exhibiting a low contamination rate (31%) compared to explants from wild plants (80%). Also, explants from home garden mother plant possessed better callus induction (65%) compared to explants from wild mother plants (13%). Furthermore, this study suggests that $AgNO_3$ can be used as an alternative for hazardous chemicals such as mercuric chloride ($HgCl_2$), which is commonly applied in surface sterilization and, introducing ground callus to suspension cultures will yield an improved callus proliferation in suspension cultures.

***Keywords:*** G.walla; In-vitro callus induction; cell suspension cultures; $AgNO_3$; commercial use

## Introduction

*Gyrinops walla*, a renowned member of the Thymelaeaceae family, can also be referred to as Walla Patta or Sri Lankan Agarwood. This plant is native to Sri Lanka's wet and intermediate zones, where it is most common in lowland rainforests and gardens in homes (Jayaweera, 1982). The Thymelaeaceae family, particularly those in the genera *Aquilaria* and *Gyrinops*, is economically significant due to its ability to produce agarwood, a fragrant wood used in religious rites, traditional medicine, and perfumes, due to its protective mechanism against harm during plant injuries or infections. *G.walla* is uniquely positioned as the only agarwood-producing species

in Sri Lanka, even though Aquilaria species are more commonly known for their production throughout Southeast Asia (Subasinghe S. M. C. U. P, 2013).

Physical harm or pathogenic infection frequently causes *G.walla* to produce more agarwood by causing dark, resinous patches to grow inside the heartwood. These patches are widely valued for their unique perfume and therapeutic qualities. They are rich in sesquiterpenoids and chromone derivatives (Takemoto H., 2008). The *G. walla*, a species protected under the Sri Lankan Flora and Fauna Protection Ordinance since 2004, is facing significant strain due to increased demand for agarwood and its essential oil. Illicit harvesting and trade have led to a decline in wild populations. Despite being included in CITES, unlawful exploitation continues due to the species' financial value (CITES, 2005). *G.walla*, a plant species with a prolonged lifespan and limited seed viability, is being researched for sustainable propagation using novel strategies like plant tissue culture. This method can also produce agarwood chemicals in vitro, potentially alleviating strain on wild populations (Walker K., 2002). The generation of undifferentiated plant cells in response to chemical or physical stimuli under controlled hormonal circumstances is a crucial step in tissue culture known as "in vitro callus formation." In addition to making large-scale plant growth easier, this method generates useful secondary metabolites like agarwood resin. Artificial neural network (ANN) techniques have been utilized to forecast the ideal conditions for callus induction, enabling more productive and economical cultivation procedures (Odutayo O. I., 2007).

This research focuses on improving *G.walla* tissue culture techniques to maximize callus production and ensure sustainable commercial exploitation. This creates a sustainable callus formation strategy and synthesize secondary metabolites from cell suspension cultures to simplify the manufacturing process and reduce costs by using explants from various settings and to enhance *G.walla*'s usefulness in biotechnological and commercial situations.

## Materials and Methods

### Leaf Explant Preparation

Immature leaves were obtained from two *G.walla* plants; one which has been grown in the wild and the other one grown in a home garden, without any special growth conditions. The process of explant preparation was followed according to *Selvaskanthan et al.* (Selvaskanthan S, 2018). Fresh, immature leaves were collected and washed thoroughly under slow running tap water for 10 minutes prior to washing with Vim solution (which contained 5 drops of Vim liquid - sodium laureth sulfate and sodium dodecyl benzene sulfonate) for 1 minute and then rinsed thoroughly with running tap water for another 10 minutes.

### Surface Sterilization

An optimized procedure of *Selvaskanthan et al.* (Selvaskanthan S, 2018) was conducted. The leaf explants were surface sterilized by gently stirring manually with 10% (v/v) of Clorox and 0.6 ml of Tween-20 (Polyethelene (20) Sorbitan Monolaurate) for 10 minutes. This procedure was repeated with distilled water containing 3 drops of Dettol instead of Clorox (sodium hypochlorite) and Tween-20. Subsequently, the leaves were rinsed 3 times inside the laminar flow cabinet (each rinse lasted for approximately 1 minute) with autoclaved distilled water. Thereafter, instead of the use of $HgCl_2$, the leaves were dipped in a silver nitrate solution ($AgNO_3$, 100 mg/L) for a 1 minute.

### Establishment of Leaf Cultures

Culture establishment was conducted under aseptic conditions inside the laminar flow cabinet. Without rinsing the excess silver nitrate, the leaves were cut into approximately 1 cm x 1 cm pieces containing the midrib and cultured into culture tubes, 1 piece per tube. Culture tubes contained 10 ml of culture medium which consisted of MS medium, 3% (w/v) sucrose, 1% (w/v) Agar as the solidifying agent, 0.5 mg/L of 6-Benzylaminopurine (BAP) and 3 mg/L of

1-Naphthaleneacetic acid (NAA) were used as plant growth regulators (PGRs) at pH 5.8. The BAP and NAA concentrations were selected referring *Munasinghe S.P et al* (Munasinghe S.P, 2022). The culture tubes containing leaf explants were then incubated in a dark cabinet at 25 ± 2 °C. Established cultures were monitored every 2 – 3 days and data on contaminated samples and callus growth was collected. After 2 months, the formed callus was sub-cultured by carefully separating from leaf explants, and they were cut into pieces about 0.5 cm before inoculating into culture jars with 30 ml of culture medium that contained MS medium, 3% (w/v) sucrose, 1% (w/v) Agar, 3 mg/L NAA and 0.5 mg/L BAP at 5.8 pH, each jar containing 4 – 5 callus.

**Suspension Cultures**

Sub-cultured callus (5 weeks old) was selected for subculturing. An amount of 1.2 ± 0.2 g callus was weighed from each petri dish. Inside the laminar flow cabinet and under aseptic conditions, one weighed sample of 1.4 g of callus was chopped into pieces about 0.2 cm and the other weighed sample of 1 g of callus was ground using mortar and pestle, before transferring them separately into media bottles, each containing a 100 ml of a liquid medium consisted of MS medium, 3% (w/v) sucrose, 3 mg/L NAA and 0.5 mg/L BAP at pH 5.8. Then the media bottles were covered using Aluminium foil to provide a dark condition and placed on the orbital shaker (130 rpm) for constant shaking. The suspension cultures were monitored every 2 weeks for growth and contaminations. They were subculture after 7 weeks.

**Results**

1. Leaf cultures and callus growth: to date, two trials of leaf cultures have been established.

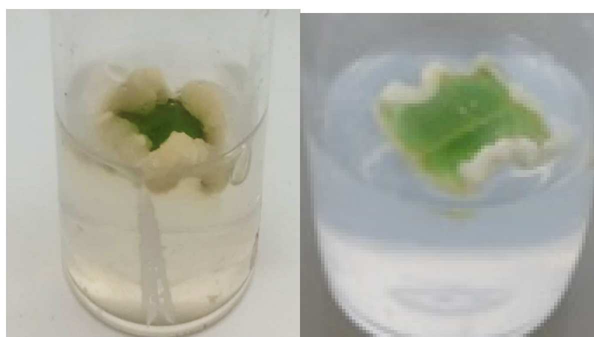

**Figure 1.** Callus growth in leaf explants. a) Callus growth after 6 weeks (leaf explants obtained from home garden *G. walla* plant). b) Callus growth of a leaf explant after 6 weeks (explant obtained from wild *G.walla* plant).

***Table 01.*** Comparison of Tissu*e* culture parameters of *G.walla*.

| Leaf explants | **Plant obtained from home garden** | **Wild plant** |
|---|---|---|
| Total number of samples | 55 | 55 |
| Total number of contaminated samples | 17 | 44 |
| Rate of contaminations | 31% | 80% |
| Total number of samples that developed callus | 36 | 7 |
| Rate of callus induction | 65% | 13% |
| Time for callus induction | 16 days | 28 days |

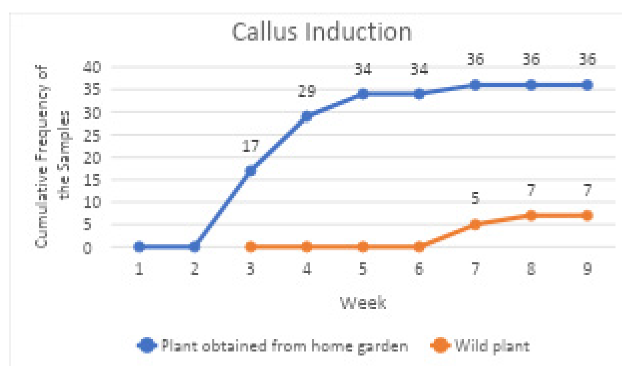


**Figure 2.** Graphical interpretation of the cumulative rate of callus growth of *G.walla* leaf cultures (culture initiation of explants obtained from the wild plant was conducted 17 days after the culture initiation of explants obtained from home garden plant).

2. Cell suspension cultures

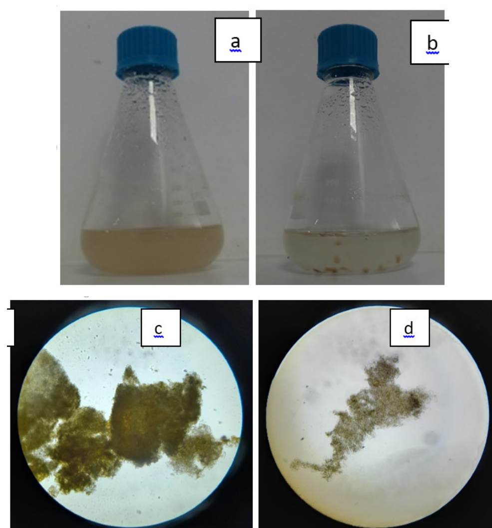


**Figure 3.** Cell suspension cultures of *G.walla*. a) Cell suspension culture containing ground callus. b) Cell suspension culture containing chopped callus. c) Microscopic view (40x) of callus obtained from the cell suspension culture containing ground callus. d) Microscopic view (40x) of callus obtained from the cell suspension culture containing chopped callus.

**Discussion**

Two different environmental conditions were tested when selecting the mother plants for tissue culture. One is from a home garden without any special care or conditions (1-2 year old plant), and the other one was from wild conditions where the plants were surrounded by a variety of other plant species (more than 2 years old plant). Previous research studies have frequently focused on callus induction using mother plants cultivated in greenhouses and even plants cultivated from *G. walla* seeds intended to produce callus (Senaratne W. A., 2017). Furthermore, it has been the common practice to produce callus using immature explants and young mother plants, which are typically 1 to 2 years old (Wickramasinghe D., 2018). This research demonstrates that explants from mother plants can be used to produce calluses, regardless of the age of the mother plant but with the immature leaf explants. Mature leaf explants give low rate of the callus production and higher contaminations. This strategy emphasizes adaptability and practicality, making it applicable to real-world situations where *G. walla* plants are not always available in optimal conditions.

In leaf cultures (Figure 1), callus was mainly observed around the edges. After 6 weeks, explants that developed callus were observed to be completely covered by callus, with the height of about 1 cm. In this study, callus induction was detected 16 days after the culture establishment of explants obtained from home garden plant. According to (Munasinghe S.P, 2022) it took 19 days for the callus induction. Therefore, the PGR combination (3 mg/L NAA and 0.5 mg/L BAP) can be taken as an effective combination to develop callus. Explants of the home garden mother plant were observed to have the highest callus induction rate which was 65% while the explants obtained from the wild mother plant had callus induction rate of 13%.

The surface sterilization process is very important because the microbial contaminants that are present in the surface of the explants can be eliminated in this process. While mercury (II) chloride ($HgCl_2$) is commonly used in surface sterilization research, we chose to use silver nitrate ($AgNO_3$) instead. Because of its great potential for poisoning, mercury chloride ($HgCl_2$) is extremely toxic, carrying serious health concerns and necessitating careful handling and disposal protocols (WHO, 2017). $AgNO_3$ is safer and less hazardous than $HgCl_2$, a persistent environmental contaminant. Its silver ions have broad-spectrum antibacterial action, making it less harmful to the environment and less harmful to the food chain. Effective antiseptic and disinfectant properties of Dettol which has chloroxylenol as the active ingredient against microorganisms, Clorox which is consisted of sodium hypochlorite used as a broad-spectrum disinfectant and Tween - 20 that enhances the efficiency of other sterilant can be identified as a good combination for surface sterilization to be used along with $AgNO_3$. However, there are some other aspects such as plant species, physiological stage of the plant and wild plant's exposure to a wide range of neighboring flora and environmental conditions, which naturally enhance the risk of contamination. Although home garden plants do not require special

growth conditions or care, they tend to be somewhat healthier than wild plants. This is due to the reduced environmental interruptions affecting their growth. Additionally, wild plants are more influenced by other plant species compared to cultivated plants and home garden plants and they are more susceptible to contamination. Consequently, leaf cultures derived from wild mother plant are likely to exhibit a higher contamination rate.

Regarding the cell suspension cultures (Figure 3), ground callus shows better cell suspension growth compared with the chopped callus cell suspension culture. The suspension culture prepared with chopped callus exhibited less growth and less cell separation compared to the growth observed in the ground callus. When grinding, cells were separated well, and the surface area increased for nutrient absorption. Therefore, it is beneficial for callus induction as more surface area is affected by the culture medium and a higher growth can be expected, on the contrary when chopped, larger fragments provide less surface area for nutrient absorption.

**Conclusion**

Our results suggested that the immature leaf explants and home-grown mother plants give less contamination and high callus production in *G. walla*. A combination of silver nitrate ($AgNO_3$) and Dettol show better reduction in surface contamination in the leaf explants. Much better cell suspension cultures can be obtained grinding the callus rather than chopping them.

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