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# MICROPROPAGATION RESPONSE AND PLANTLET QUALITY OF CACAO (*Theobroma cacao* L.) VAR. UF 18 USING MERISTEM TIP CULTURE AS INFLUENCED BY DIFFERENT LEVELS OF PLANT GROWTH REGULATORS

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**ABSTRACT**

Cacao (*Theobroma cacao* L.) is increasingly recognized as a major contributor to economic development in many countries. The Philippines, however, still exports at low levels despite its competitive advantages, largely because high mortality of plant material limits the supply of quality cacao planting materials. This study aimed to establish a sterilization protocol using two-step sodium hypochlorite (NaOCl) treatment, to evaluate the response of terminal and axillary meristems of cacao var. UF18 to different levels of plant growth regulators (PGRs), and to determine the concentration and combination of PGRs that give a higher rate of plantlet regeneration. Meristem tips from a pathogen-screened donor plant were sterilized with 2% NaOCl (20 min) followed by 1% NaOCl (10 min) and cultured for three months on Murashige and Skoog (MS) medium alone or supplemented with 0.5 mg $L^{-1}$ $GA_3$ and increasing levels of kinetin (0.5–2.0 mg $L^{-1}$) and naphthaleneacetic acid (NAA; 0.1–0.7 mg $L^{-1}$). The experiment was a two-factor factorial in a Completely Randomized Design with three replications of 10 explants each; data were analyzed by ANOVA and Tukey's HSD at the 5% level. The two-step sterilization gave a survival rate of 78.33% (contamination, 21.67%) in $T_4$ (MS + 0.5 mg $L^{-1}$ $GA_3$ + 1.5 mg $L^{-1}$ kinetin + 0.5 mg $L^{-1}$ NAA), although differences among treatments were not significant. Terminal meristems consistently produced taller plantlets than axillary meristems (11.50 vs. 8.38 mm after three months) and thicker stems at the third month (5.63 vs. 5.02 mm). Among the PGR-supplemented treatments, $T_4$ gave the earliest leaf formation in both terminal (4.53) and axillary (4.60) meristems and $T_2$ the earliest shoot formation (6.05 and 6.06, respectively). MS alone produced no leaves or shoots, whereas all PGR-supplemented treatments did. Axillary meristems formed leaves and shoots slightly earlier and produced numerically more shoots (1.27 vs. 1.20 per explant) and longer shoots (2.16 vs. 1.99 mm) at three months, suggesting that axillary meristems are the more suitable explant source for micropropagation of *Theobroma cacao*, with moderate kinetin and NAA levels ($T_3$ and $T_4$) giving the best overall response.

**Keywords:** *cacao (Theobroma cacao L.), plant growth regulators, sterilization protocol, terminal meristems, axillary meristem, micropropagation*

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## INTRODUCTION

### Cacao and Its Economic Importance

Cacao (*Theobroma cacao* L.) is a neotropical plant that originated in Central and South America. Its scientific name is commonly translated from the Greek as "food of the gods." Of the 26 species in the genus, it is the only one widely grown to produce chocolate (Richardson et al., 2015). Cocoa also contains phenols and flavonoids, and its antioxidant activity is greater than that of red wine, green tea, or black tea (Subhashini et al., 2010).

In most countries where it is grown, cacao is emerging as a significant driver of economic development. Because the gap between the supply of and demand for cocoa beans has widened in recent years, cacao cultivation has gained momentum in both domestic and foreign markets (Bureau of Plant Industry 2017). In the Philippines, growing cacao has substantial economic benefits, especially for rural development, agricultural diversification, and participation in international trade. Rising worldwide demand for cocoa and chocolate products gives the country the potential to emerge as a competitive supplier in both domestic and export markets (World Cocoa Foundation 2021).

Global demand for cocoa was projected to reach 4.7–5 million metric tons by 2020, while a global shortage of around 1 million metric tons was anticipated in the same year, according to the Department of Agriculture. In the Philippines, annual cocoa consumption stands at approximately 50,000 metric tons, whereas local production supplies only about 10,000 metric tons. To help address this shortfall, the Philippines committed to increasing cocoa production by 40% annually, with the aim of producing 100,000 metric tons of fermented beans for domestic use and export by 2020 (DTI 2019).

Globally, cacao farmers produce about five million tons of cocoa beans each year. Around 70% comes from West Africa, 19% from Asia and Oceania, and the remaining 11% from the Americas. Côte d'Ivoire (Ivory Coast) and Ghana, the top cocoa-producing countries, together account for more than half of the world's cocoa supply, and over a third of all cocoa beans are processed in Europe (Swiss Platform for Sustainable Cacao 2021). According to the World Cocoa Foundation (WCF), there are 5–6 million cocoa farmers worldwide, and roughly 40–50 million people depend on cacao for their livelihoods.

### Status and Constraints of the Philippine Cacao Industry

The Philippine cacao sector is dominated by small farmers who mainly sell dry beans to processors and manufacturers. Cocoa and cocoa products are both exported and imported; in 2018, for instance, the Philippines produced 7,983.20 metric tons of cacao and exported 2,766.2 metric tons of cacao beans. Despite the many competitive advantages it enjoys because of its geography and climate and its proximity to emerging consumer markets, the country still exports at low levels: it ranks 72nd in exports and contributes less than 0.01% of the global market (DTI 2017). This low productivity is linked to high mortality of the plant material, which results in insufficient quality cacao planting materials, and to the limited knowledge of cacao growers.

Since 2006, however, the Philippine cocoa industry has begun to recover and now produces higher volumes. To guarantee the industry's success, the government implemented policy measures through R.A. 7900, also known as the High Value Crop Development Program Act (HVCDPA), which identifies cocoa production and intensification as a priority. Farmers in rural areas have become increasingly interested in cocoa cultivation as a consequence of rising demand in national and international markets (Peace and Equity Foundation 2016). Cacao has also been identified as a critical crop in the Industry Strategic Science and Technology Plans of the Philippine Council for Agriculture, Aquatic, and Natural Resources Research and Development (PCAARRD), which aim to develop the cocoa industry through scientific and innovative approaches.

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Despite these efforts, several challenges continue to threaten the sustainability and growth of the industry. Global climate change, insect and disease outbreaks, and productivity declines brought about by aging crops have all contributed to the instability of the world's food supply. The continual regeneration of cacao is crucial for maintaining export consistency and ensuring the ongoing supply of industrial inputs for the expanding chocolate market. Although cacao production offers a wide variety of options and opportunities, demand for cacao-based products in domestic and international markets is increasing while supply remains insufficient (Bureau of Plant Industry 2017).

Diseases and pests have frequently hampered cacao production in various regions of the world. The earliest Spaniards already noted difficulties in cacao cultivation, including black pod rot (caused by *Phytophthora* spp.) under excessive shade. Diseases that co-evolved with cacao, such as frosty pod rot (*Moniliophthora roreri*) and witches' broom (*Moniliophthora perniciosa*), have not yet reached the newer cacao-growing continents. On the other hand, new diseases that the cacao tree had not previously encountered have emerged, such as cacao swollen shoot virus and *Phytophthora megakarya* in Africa and vascular streak dieback in Asia. The diseases that have adapted to cacao indicate the special susceptibility of this species. In comparison, the rubber tree (*Hevea brasiliensis*), which originates from the same region, is less affected by new diseases, although such projections should be made with caution, especially given the effect of climate change on epidemic risk (Pautasso et al, 2012).

Cacao growing in many geographic areas has also been disrupted by numerous pests, especially insects. Some, such as the pod borer *Carmenta theobromae*, occur in the region where the cacao tree originated (Morillo et al., 2009). The cacao pod borer (CPB) has impeded the expansion of cacao cultivation across most Asian countries; Malaysia, which supplied up to 247,000 t in 1990, now generates only 1,000 t, largely because of this pest (Arshad et al., 2015).

**Meristem Culture and Factors Affecting In Vitro Response**

To address these challenges and ensure the sustainable production of high-quality planting materials, innovative scientific approaches, including tissue culture, are being explored. Among these, meristem culture stands out as a reliable method for virus elimination (Knap et al., 1998; Maliogka et al., 2009). Because meristems lack specialized conducting tissues, they are typically free of systemic viruses, and meristem culture has therefore been widely used to eradicate viral infections from horticultural plants. According to Grout and Brian (1999), meristem tip culture is a successful method for regeneration, virus eradication from infected plants, and production of virus-free plants, and it can eliminate virus particles from explants in a relatively brief period (Hendre et al., 1987).

The meristem culture procedure is generally the same as the other techniques used for *in vitro* culture. To isolate only the apical meristem, rather than permanent cells that may contain viruses, the most important requirement is that the initial explants be as small as possible (Allen, 1975). Cha-um et al. (2006) noted several barriers to producing virus-free plants through meristem culture, including meristem size, meristem position, the detection protocol used, endogenous infections, and recovery time. Because some meristematic tissues are free of pathogens, *in vitro* meristem culture allows uninfected plants to be recovered and grown into healthy plants.

*In vitro* regeneration is more efficient than conventional propagation methods because it yields disease-free plants with high multiplication rates and established plantlets, as well as healthier plants with a shorter production cycle and higher yields (Darvari et al., 2012). However, establishing a highly refined procedure requires appropriate explants, plant growth regulators (PGRs), medium composition, and suitable physical and chemical conditions (Abiri et al., 2015). Using meristem tissue as an explant and optimizing the PGRs in the culture medium are two of the most promising ways to develop an *in vitro* regeneration technique that is efficient, economical, and time-saving (George et al., 2007). In addition, organogenesis can be achieved directly from meristem tissue because of its totipotency, plasticity, and high sustainability (Alam et al., 2010).

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Delage et al. (1999) showed that this approach surpasses earlier reports of bud and shoot tip culture, in which the use of virus-infected tissues resulted in low yields and virus-infected plants. Verma et al. (2009) likewise found that larger meristems regenerate a greater number of plants, whereas the number of virus-free plants obtained is inversely associated with meristem size.

The size of the explant is a major factor in organogenesis. Larger explants, composed of parenchyma, vascular tissue, and cambium, are more regenerative than smaller ones, whereas small groups of uniform tissue formed from the epidermal and subepidermal layers can develop directly into complex organs such as flowers, buds, and roots. Some new plants have even been produced from tissue pieces obtained by aseptic homogenization of the plant in blenders (Srinivasan et al., 2014).

The physiological age of the plant also frequently plays a substantial role in organogenetic events. In *Nicotiana* sp., adventitious shoot buds regenerate only when leaf explants are harvested during the vegetative phase, before flowering. In leaf explants of *Echeveria* sp., only roots are produced when young leaves are harvested, older leaves develop only shoot buds, and medium-aged leaves generate both shoots and roots (Srinivasan et al., 2014).

Among the variables that affect organogenesis *in vitro*, phytohormones appear to be the single most important factor. In their classic work with tobacco stem pith tissue, Skoog and Miller (1957) established that modifying the concentrations of auxins and cytokinins in the culture medium leads to different forms of organogenesis: shoots are stimulated when cytokinin concentrations are high relative to auxin, roots when cytokinin concentrations are low relative to auxin, and unorganized tissue develops at intermediate concentrations. Other plant hormones, notably abscisic acid and gibberellins, also have a significant impact on organogenesis *in vitro* (Skoog & Miller, 1957).

According to Srinivasan et al. (2014), the macro (major) and micro (minor) salts are key components of plant cell culture media. Vitamins, amino acids, carbohydrates, and other nutrients are also necessary for plant cell growth and development *in vitro*. Inorganic nitrogen is especially important in plant cells because it forms part of the protein molecule; nitrogen is also found in a variety of other important compounds such as purines, pyrimidines, and coenzymes.

**Plant Growth Regulators**

Plant growth regulators include natural plant hormones—such as indoleacetic acid (IAA), gibberellins, zeatin, abscisic acid (ABA), and ethylene—as well as a variety of synthetic compounds that influence and regulate plant growth and development. Each type of growth regulator has a number of physiological effects on different plant types, and these effects depend on its concentration, its type, the addition of other PGRs, and the biological characteristics of the target tissue. Different growth regulators or combinations of growth regulators may also be needed to obtain the same physiological response in some tissues. Synergies and quantitative interactions between two or more growth regulators are common, and a growth regulator that stimulates a favorable response in the tissue is likely to inhibit the same response when used at higher concentrations (Minocha, 1987).

Auxin is normally needed to initiate callus from most types of tissue, with the exception of cambial tissues, which can develop callus on their own unless an external auxin supply is present. The most effective and widely used auxin is 2,4-dichlorophenoxyacetic acid (2,4-D). At much higher concentrations, auxins cause root regeneration in some plants, and they are frequently used in combination with other growth regulators (Minocha, 1987).

Cytokinins are normally used together with auxin to initiate and maintain callus cultures. They are often involved in, or supportive of, multiple shoot formation when micropropagation starts from embryos, apices, and axillary buds, in both angiosperms and gymnosperms, and they can often be used to produce adventitious buds from cotyledons, leaves, and internodal segments. When combined with auxins at appropriate concentrations, cytokinins may stimulate shoot

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regeneration from callus in many kinds of woody angiosperms. The main cytokinins are benzyladenine and kinetin, although zeatin has been shown to be more efficient in some cases (Arnold & Eriksson, 1979).

Abscisic acid, which is typically considered a growth inhibitor, speeds up callus production and embryo development in numerous plant species, including forest plants (Altman & Goren, 1971). An antagonistic or synergistic effect is sometimes seen when GA and ABA are combined, and the presence of auxin or cytokinins in the medium may alter the effects of ABA. Ethylene is the least explored growth regulator with respect to its effects on cell culture and morphogenesis in forest plants; in cell cultures, it is generated in measurable quantities and inhibits cell division and morphogenesis while promoting senescence (Wochok & Wetherell, 1972).

**Pathogen Diagnosis and Healthy Planting Materials**

It is timely for nursery operators to work along highly technical and scientific lines so that cacao growers are provided with disease-free, certified plants. The main area to be addressed is the identification of viruses and virus-like diseases, which is a prerequisite for establishing disease-free nurseries. To detect and index cacao viruses and other plant diseases, traditional and modern high-throughput biological, serological, and molecular indexing techniques such as ELISA, RT-PCR, or PAGE should be applied. In addition, cacao nurseries should be registered and the indiscriminate multiplication and sale of uncertified cacao plants prohibited.

Rapid, sensitive, and accurate detection and diagnostic techniques can now be developed despite significant advancements in remote sensing and molecular biology technologies. Certain issues remain to be resolved, however, such as confirming that the organisms detected in the plant microbiota are active and able to cause disease when molecular tests are used. Plant diseases threaten the world's food supply and are among the primary issues restricting agricultural production (Jeger et al., 2023). Attempts to reduce the losses caused by pests and diseases have been made throughout history; disease control measures include preventing a pathogen from entering particular areas and managing or eliminating a pathogen already detected in an area (Spadaro & Gullino, 2019). Precise detection and identification of a pathogen are also crucial because they provide critical information about its ecology, the potential threats it poses to crops, and the implementation of appropriate management practices to reduce its deleterious impacts (Koebnik et al., 2023).

Methods for detecting plant pathogens include immunological or serological tests based on specific antibodies. Microorganisms produce a large diversity of antigenic molecules that can be used for detection (Alvarez, 2004), and in such assays the antibodies bind to specific epitopes of these antigens (Fang & Ramasamy, 2015; López et al., 2003). Binding to the antigens can be detected using specific antibodies conjugated with an enzyme, a fluorophore, or a nanoparticle, so the presence of pathogens can be measured indirectly (Fang & Ramasamy, 2015).

To support effective eradication of viruses and an increased rate of plant regeneration, meristem tips therefore have to be excised and tested. This is an essential prerequisite for establishing healthy cacao plants that can be distributed to farmers, and ensuring the availability of virus-free planting materials is crucial. Against this background, this study examined the response of terminal and axillary meristems of cacao var. UF18 to different levels of plant growth regulators, together with a two-step sterilization protocol and pathogen diagnosis of the donor plant.

**Objectives of the Study**

This study specifically aimed to:

1. Establish a sterilization protocol using two-step sterilization at different concentrations of sodium hypochlorite;

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2. Evaluate the response of meristems as influenced by different levels of plant growth regulator treatments; and
3. Determine the appropriate concentration and combination of plant growth regulators for a higher rate of plantlet regeneration.

**Scope and Limitation of the Study**

This study was limited to the growth and development of *Theobroma cacao* L. var. UF18 using meristem tip culture as influenced by different levels of plant growth regulators over a three-month period.

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## MATERIALS AND METHODS

### Study Site and Duration

The study was conducted from September to December 2024 at the Tissue Culture Laboratory of the Department of Horticulture, Visayas State University, Visca, Baybay City, Leyte. Shoot-tip meristems of cacao (*Theobroma cacao* L.) were subjected to different levels of plant growth regulators.

### Pathogen Diagnosis of the Donor Plant

Pods and leaves of a donor plant were brought to the Plant Disease and Diagnostic Laboratory, Visayas State University, Visca, Baybay City, Leyte, where visual assessment and microscopic examination were done to determine the presence of pathogens. Pods were inspected externally for indicators of pest infestation, including entry holes, discoloration, and premature opening, and were then dissected to check for larvae and the corresponding damage. Leaves were visually evaluated for chlorosis, necrotic lesions, discoloration, marginal burn, desiccation, and other physical damage. For microscopic assessment, leaf segments were thin-sectioned and stained with plain lactophenol and blue lactophenol to enhance the contrast of cellular structures, and epidermal peels were prepared to facilitate observation of surface features. All specimens were examined using a compound light microscope at magnifications of up to 400×.

### Collection and Selection of Shoot Tips

Shoot tips were randomly selected from terminal and axillary buds of healthy cacao trees and collected at around 7:00–9:00 AM from the Cacao Techno-Demo and Learning Farm of the Department of Horticulture, Visayas State University, Baybay City, Leyte. The trees were sprayed with fungicides three days before the explants were collected. The shoot tips were kept in sealed polythene bags and immediately brought to the laboratory for pretreatment and sterilization.

### Pretreatment and Sterilization

In the laboratory, the shoot tips were subjected to two-step sterilization. They were first immersed in 2% sodium hypochlorite (NaOCl) for 20 min and rinsed four times with sterile distilled water, then sterilized a second time in 1% NaOCl for 10 min and again rinsed four times. To further control microbial contamination, streptomycin (2 g $L^{-1}$) was added to the medium. Under a binocular dissecting microscope, a sterile hypodermic needle and scalpel were used to isolate meristem tips consisting of the apical dome with one to two leaf primordia.

### Media Formulation and Culture Conditions

The explants were cultured on Murashige and Skoog (MS) medium, whose components are listed in Table 1. The pH of the medium was adjusted to 5.8 using 0.1 M HCl or 0.1 M NaOH, and the medium was gelled with 0.5% (w/v) agar before autoclaving at 121 °C for 20 min. After sterilization, the explants were cultured on MS medium containing three plant growth regulators—kinetin, $GA_3$, and NAA—at the levels described below.

**Table 1. Murashige and Skoog medium components used as stock solutions for plant tissue culture experiments**

| Macronutrients | | |
|---|---|---|
| Components | Chemical formula | Weight ($mgL^{-1}$) |
| Ammonium Nitrate | $NH_4NO_3$ | 1650 |

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| | | |
|---|---|---|
| Potassium Nitrate | $KNO_3$ | 1900 |
| Calcium Chloride Anhydrate | $CaCl_2 2H_2O$ | 440 |
| Magnesium Sulfate Anhydrate | $MgSO_4 7H_2O$ | 370 |
| Potassium Phosphate Monobasic | $KH_2.PO_4$ | 170 |
| **Micronutrients** | | |
| Boric Acid | $H_3BO_3$ | 6.2 |
| Potassium Iodide | Kl | 0.83 |
| Manganese Sulphate. 4H2O | $MnSO_4.4H_2O$ | 22.3 |
| Zinc Sulphate. 7H2O | $ZNSO_4.7H_2O$ | 8.6 |
| Molybdic Acid (Sodium salt). 2H2O | $Na_2MoO_4 2H_2O$ | 0.25 |
| Cupric Sulphate. 5H2O | $CuSO_4 5H_2O$ | 0.025 |
| Cobalt Chloride. 6H2O | $CoCl_2.6H_2O$ | 0.025 |
| **Chelated Iron** | | |
| Sodium Ethylene Diamine Tetraacetate | $Na_2$- EDTA | 33.6 |
| Ferrous Sulphate. 7H2O | $FeSO_4 7H_2O$ | 27.8 |
| **Vitamins** | | |
| Thiamine. HCL | $C1_2H_{17}CIN_4OS.HCL$ | 0.1 |
| Nicotinic Acid (Free Acid) | $C_8H_{11}NO_3HCL$ | 0.5 |
| Pyrodoxine. HCL | $C_6H_5NO_2$ | 0.5 |

The explants were cultured on the media under evaluation and incubated in an incubation room maintained at 25 ± 2 °C. After four weeks, the cultures were transferred to fresh medium and subcultured at 4-week intervals for 3 months. The regenerated plantlets were grown in a growth room kept at 25 ± 2 °C under cool white fluorescent light of about 2000 lux with an 8 h/16 h photoperiod (Reinart & Bajaj, 1977).

**Experimental Design, Treatments, and Data Analysis**

The experiment was laid out as a two-factor experiment arranged in a Completely Randomized Design (CRD). Factor A consisted of two sources of meristem tips, and Factor B consisted of five treatments with the same level of $GA_3$ and varying levels of kinetin and NAA. There were five treatments with three replications per treatment, and each replication consisted of 10 explant samples. The treatments were as follows:

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**Factor A:** Source of meristem

$MT_1$ – terminal meristem tip

$MT_2$ – axillary meristem tip

**Factor B:** Levels of PGRs

$T_1$ – MS alone

$T_2$ – MS + 0.5 mg $L^{-1}$ $GA_3$ + 0.5 mg $L^{-1}$ kinetin + 0.1 mg $L^{-1}$ NAA

$T_3$ – MS + 0.5 mg $L^{-1}$ $GA_3$ + 1.0 mg $L^{-1}$ kinetin + 0.3 mg $L^{-1}$ NAA

$T_4$ – MS + 0.5 mg $L^{-1}$ $GA_3$ + 1.5 mg $L^{-1}$ kinetin + 0.5 mg $L^{-1}$ NAA

$T_5$ – MS + 0.5 mg $L^{-1}$ $GA_3$ + 2.0 mg $L^{-1}$ kinetin + 0.7 mg $L^{-1}$ NAA

The data were analyzed by analysis of variance (ANOVA) using John's Macintosh Project (JMP) software, and Tukey's Honestly Significant Difference (HSD) test was used to compare treatment means at the 5% level of significance. The original data and the corresponding ANOVA tables are presented in the Appendices.

**Data Gathered**

***Pathogen diagnosis of the donor plant***

The presence of pathogens and pests in the pods and leaves of the donor plant was determined by visual assessment and microscopic examination as described above.

***Contamination assessment***

*Types of contamination.* These were determined and identified through visual signs and symptoms: fungal contamination shows thin filamentous mycelia, sometimes as denser clumps of spores, while bacterial contamination shows cloudiness in the medium and sometimes a slight whitish film on the surface of the plates.

*Percent contamination.* This was determined by dividing the number of contaminated explants by the total number of explants and multiplying by 100.

$$\text{Percent contamination} = \frac{\text{number of contaminated explants}}{\text{Total number of explants}}\ x\ 100$$

*Survival rate.* This was determined by dividing the number of surviving explants by the total number of explants cultured and multiplying by 100.

$$\text{Survival rate} = \frac{\text{number of explants survived}}{\text{total number of explants cultured}}\ x\ 100$$

***Morphological and growth parameters***

*Plant height (mm).* Ten sample plants per replication were measured from the base of the plant to the tip of the longest leaf after three months of inoculation, and the average value was computed.

*Stem diameter (mm).* The base diameter of the plantlets was measured after three months of inoculation. The average stem girth of 10 sample plants in each replication was obtained.

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*Number of days from inoculation to the formation of leaves.* This was obtained by counting the number of days from inoculation until the first leaf appeared in each explant, using 10 sample plants per replication. The average value was computed.

*Number of leaves per plantlet.* All fully opened leaves of the 10 sample plants per replication were counted once a month, and the mean was computed.

*Number of days to shoot formation.* This was determined by counting the number of days from inoculation until the formation of the first shoot, using 10 sample plants per replication. The mean was then computed.

*Number of shoots per plantlet.* The number of shoots formed per plantlet was counted once a month using 10 sample plants per replication, and the mean was computed.

*Shoot length (mm) per plantlet.* This was obtained by measuring the height of the shoot from the base to the tip of the longest leaf. The mean of the sample plantlets in every replication was obtained once a month.

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## RESULTS

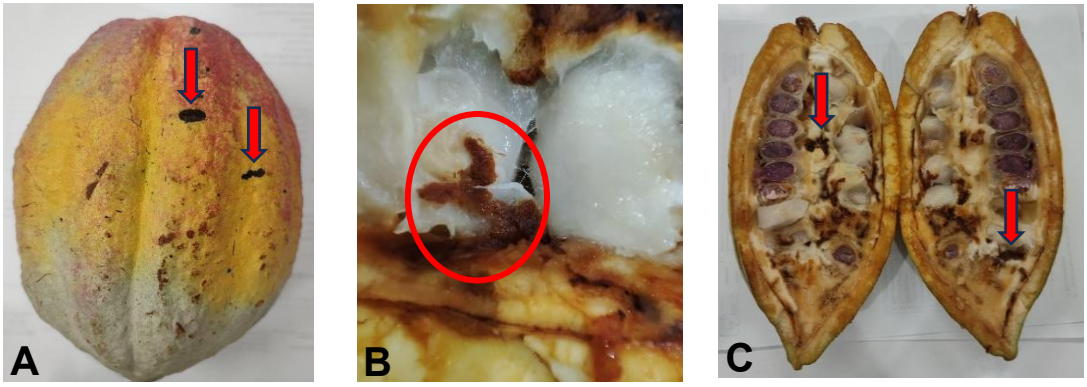
Results are presented for the pathogen diagnosis of the donor plant, the contamination and survival of the explants, and the morphological and growth parameters recorded over three months of culture. Treatments $T_1$–$T_5$ are as defined in the Materials and Methods, and the original data and ANOVA tables are given in Appendix Tables 1–17 and Appendix Figures 1–10.

### Pathogen Diagnosis of the Donor Plant

#### *Cacao pod*

Visual inspection of the cacao pod revealed symptoms of pest infestation both externally and internally (Fig. 1). Externally, tiny, circular entry holes were found on the pod surface, indicating larval tunneling activity (Fig. 1A). Uneven ripening, shown by yellow and green patches on mature pods, was also observed. Internally, larval feeding had produced tunnels and scars in the pod tissue (Fig. 1C). Frass deposits were found in the placenta (Fig. 1B), and the surrounding tissues showed necrosis and decomposition. Together, these symptoms indicate cocoa pod borer infestation.

**Figure 1. Visual symptoms observed in cacao pod: (A) external exit and entry point of larvae, (B) presence of frass on the seeds, (C) tunnels and scars on the placenta section**



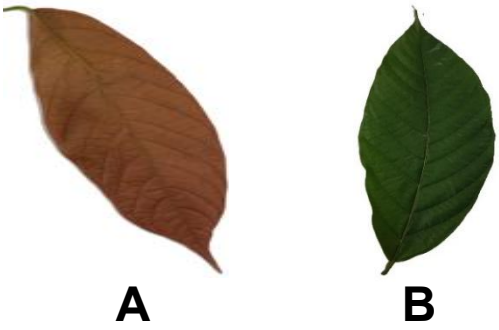

#### *Cacao leaves*

Visual evaluation of the cacao leaves (Fig. 2) showed no chlorosis, necrotic lesions, or discoloration on the leaf surface. The leaf edges were intact, with no physical damage, marginal burn, or desiccation. Some young leaves exhibited minor drooping, which is regarded as a typical physiological reaction rather than a sign of pathogenic activity. There were no visible signs of disease or pest infestation, indicating that the leaves were generally healthy.

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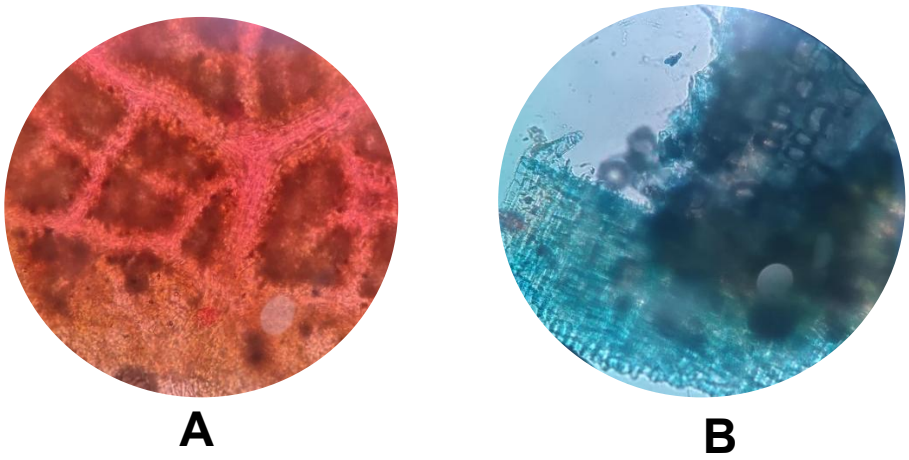
**Figure 2. Visual observation of cacao leaves: (A) young leaf and (B) mature leaf.**




***Microscopic assessment***

Microscopic examination of the leaf samples at up to 400× revealed no fungal hyphae, spores, or bacterial colonies on the leaf surface (Fig. 3). No abnormal cell enlargement, discoloration, or tissue disruption was observed, and there were no intracellular inclusions indicative of pathogens. The cellular architecture was typical of healthy cacao leaf tissue, with all observed structures consistent with normal morphology. Overall, no fungal, bacterial, or other pathological abnormalities were detected.

**Figure 3. Microscopic examination (400x) in cacao leaves: (A) young leaf and (B) mature leaf**




**Contamination and Survival of Explants**

Fungi and bacteria were the major types of contamination recorded (Table 2). Axillary meristems had a slightly higher survival rate (74.66%) and slightly lower contamination (25.34%) than terminal meristems (72.67% and 27.33%, respectively). Fungal contamination was higher in axillary explants (16.67%) than in terminal explants (13.33%), whereas bacterial contamination was lower (8.67% vs. 14.00%). Among the PGR treatments, $T_4$ (MS + 0.5 mg $L^{-1}$ $GA_3$ + 1.5 mg $L^{-1}$ kinetin + 0.5 mg $L^{-1}$ NAA) gave the highest survival rate (78.33%) and the lowest contamination (21.67%), followed by $T_3$ (76.67% and 23.33%). $T_2$ had the lowest survival (70.00%) and the highest contamination

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(30.00%), with fungal contamination also highest in $T_2$ (20.00%) and $T_1$ (18.33%) and bacterial contamination highest in $T_3$ and $T_5$ (15.00% each). None of these differences were statistically significant.

**Table 2. Survival rate and contamination assessment of *Theobroma cacao* L. as influenced by the different levels of plant growth regulators**

| SOURCES OF MERISTEM | SURVIVAL RATE (%) | PERCENT CONTAMINATION (%) | TYPES OF CONTAMINATION | |
|---|---|---|---|---|
| | | | FUNGI | BACTERIA |
| Terminal | 72.67 | 27.33 | 13.33 | 14.00 |
| Axillary | 74.66 | 25.34 | 16.67 | 8.67 |
| Levels of PGRs | | | | |
| $T_1$= MS alone | 73.33 | 26.67 | 18.33 | 8.33 |
| $T_2$= MS + 0.5 $mgL^{-1}$ GA3 + 0.5 $mgL^{-1}$ Kinetin + 0.1 $mgL^{-1}$ NAA | 70.00 | 30.00 | 20.00 | 10.00 |
| $T_3$= MS + 0.5 $mgL^{-1}$ GA3 + 1.0 $mgL^{-1}$ Kinetin + 0.3 $mgL^{-1}$ NAA | 76.67 | 23.33 | 8.33 | 15.00 |
| $T_4$= MS + 0.5 mgL-$^{1}$ GA3 + 1.5 $mgL^{-1}$ Kinetin + 0.5 $mgL^{-1}$ NAA | 78.33 | 21.67 | 13.33 | 8.33 |
| $T_5$= MS + 0.5 $mgL^{-1}$ GA3 + 2.0 $mgL^{-1}$ Kinetin + 0.7 $mgL^{-1}$ NAA | 71.67 | 28.33 | 15.33 | 15.00 |
| CV (a) | 16.39 | 16.65 | 19.77 | 19.77 |
| CV (b) | 16.59 | 17.21 | 18.64 | 15.27 |
| Means within the same column followed by a common letter and/or no letter designation are not significantly different from each other using Tukey's honestly significant difference (HSD) at the 5% level of significance | | | | |

## General Observations

Browning of a few explants was noticeable about two weeks after the explants were inoculated into the culture media. The browned explants subsequently died and failed to regenerate.

## Morphological and Growth Parameters

### *Plant height*

Plant height was recorded over the three-month culture period (Table 3). Terminal meristems produced significantly taller plantlets than axillary meristems in every month; after three months, terminal plantlets reached 11.50 mm compared with 8.38 mm for axillary plantlets. Among the PGR treatments, a significant difference was observed only in the first month, when $T_3$ (MS + 0.5 mg $L^{-1}$ $GA_3$ + 1.0 mg $L^{-1}$ kinetin + 0.3 mg $L^{-1}$ NAA) gave the tallest plantlets

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(7.66 mm), significantly taller than $T_4$ (6.85 mm), while $T_1$, $T_2$, and $T_5$ (7.01–7.32 mm) did not differ significantly from either. No significant differences among PGR levels were detected in the second and third months; nevertheless, plantlets in $T_3$ (10.56 mm) and $T_4$ (10.3 mm), the treatments with 0.3 and 0.5 mg $L^{-1}$ NAA, were numerically the tallest at three months, followed by $T_5$ (9.99 mm), $T_2$ (9.95 mm), and $T_1$ (8.89 mm).

**Table 3. Plant height of *Theobroma cacao* L. as influenced by the different levels of plant growth regulators after 3 months of incubation**

| SOURCES OF MERISTEM | PLANT HEIGHT (mm) | | |
|---|---|---|---|
| | 1ST MONTH | 2ND MONTH | 3RD MONTH |
| Terminal | 8.42 a | 9.89 a | 11.50 a |
| Axillary | 5.97 b | 6.78 b | 8.38 b |
| Levels of PGRs | | | |
| $T_1$= MS alone | 7.01 ab | 7.96 | 8.89 |
| $T_2$= MS + 0.5 $mgL^{-1}$ GA3 + 0.5 $mgL^{-1}$ Kinetin + 0.1 $mgL^{-1}$ NAA | 7.32 ab | 8.38 | 9.95 |
| $T_3$= MS + 0.5 $mgL^{-1}$ GA3 + 1.0 $mgL^{-1}$ Kinetin + 0.3 $mgL^{-1}$ NAA | 7.66 a | 8.13 | 10.56 |
| $T_4$= MS + 0.5 $mgL^{-1}$ GA3 + 1.5 $mgL^{-1}$ Kinetin + 0.5 $mgL^{-1}$ NAA | 6.85 b | 8.69 | 10.3 |
| $T_5$= MS + 0.5 $mgL^{-1}$ GA3 + 2.0 $mgL^{-1}$ Kinetin + 0.7 $mgL^{-1}$ NAA | 7.13 ab | 8.51 | 9.99 |
| CV (a) | 7.65 | 7.38 | 7.62 |
| CV (b) | 19.87 | 11.57 | 17.88 |
| Means within the same column followed by a common letter and/or no letter designation are not significantly different from each other using Tukey's honestly significant difference (HSD) at 5% level of significance | | | |

A significant interaction between meristem source and PGR treatment was found for plant height after one month of incubation (Table 4), indicating that the effect of the PGRs depended on whether the explant was a terminal or an axillary meristem. Among terminal meristems, plant height ranged from 7.95 mm in $T_1$ to 9.00 mm in $T_2$ (MS + 0.5 mg $L^{-1}$ $GA_3$ + 0.5 mg $L^{-1}$ kinetin + 0.1 mg $L^{-1}$ NAA); $T_2$ to $T_5$ (8.07–9.00 mm) were statistically similar, and $T_1$ did not differ significantly from them. Axillary meristems showed minimal response to all treatments, with plant height ranging from 5.63 to 6.78 mm; the highest value, in $T_3$ (6.78 mm), was not significantly different from the control or the other treatments. Terminal meristems in $T_2$ to $T_5$ were significantly taller than all axillary meristem treatments.

**Table 4. Interaction effect on plant height of *Theobroma cacao* L. as influenced by the different levels of plant growth regulators after 1 month of incubation**

| FACTOR B | FACTOR A | |
|---|---|---|
| | TERMINAL | AXILLARY |
| Levels of PGRs: | | |
| $T_1$= MS alone | 7.95 ab | 6.06 c |
| $T_2$= MS + 0.5 $mgL^{-1}$ GA3 + 0.5 $mgL^{-1}$ Kinetin + 0.1 $mgL^{-1}$ NAA | 9.00 a | 5.65 c |

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| | | |
|---|---|---|
| $T_3$= MS + 0.5 $mgL^{-1}$ GA3 + 1.0 $mgL^{-1}$ Kinetin + 0.3 $mgL^{-1}$ NAA | 8.53 a | 6.78 bc |
| $T_4$= MS + 0.5 $mgL^{-1}$ GA3 + 1.5 $mgL^{-1}$ Kinetin + 0.5 $mgL^{-1}$ NAA | 8.07 a | 5.63 c |
| $T_5$= MS + 0.5 $mgL^{-1}$ GA3 + 2.0 $mgL^{-1}$ Kinetin + 0.7 $mgL^{-1}$ NAA | 8.53 a | 5.73 c |
| Means within the same column followed by a common letter and/or no letter designation not significantly different from each other using Tukey's honestly significant difference (HSD) at 5% level of significance | | |

***Stem diameter***

Stem diameter was assessed monthly over the three-month culture period (Table 5). The two meristem sources did not differ significantly in the first (4.45 vs. 4.58 mm) and second (5.01 vs. 4.77 mm) months, but in the third month terminal meristems produced significantly thicker stems than axillary meristems (5.63 vs. 5.02 mm) at the 5% level. PGR levels significantly affected stem diameter in the first two months. $T_3$ (MS + 0.5 mg $L^{-1}$ $GA_3$ + 1.0 mg $L^{-1}$ kinetin + 0.3 mg $L^{-1}$ NAA) gave the greatest stem diameter in all three months (4.81, 5.29, and 5.58 mm); in the first month, $T_3$ and $T_4$ (4.78 mm) were significantly thicker than $T_1$ (4.17 mm) and $T_2$ (4.30 mm), and in the second month $T_3$ and $T_4$ (4.93 mm) were significantly thicker than $T_1$ (4.70 mm) and $T_5$ (4.54 mm). No significant differences among PGR levels were observed in the third month, although $T_3$ (5.58 mm), $T_4$ (5.51 mm), and $T_2$ (5.36 mm) remained highest and $T_1$ (4.99 mm) and $T_5$ (5.18 mm) lowest.

**Table 5. Stem diameter of *Theobroma cacao* L. as influenced by the different levels of plant growth regulators after 3 months of incubation**

| SOURCES OF MERISTEM | STEM DIAMETER (mm) | | |
|---|---|---|---|
| | 1ST MONTH | 2ND MONTH | 3RD MONTH |
| Terminal | 4.45 | 5.01 | 5.63 a |
| Axillary | 4.58 | 4.77 | 5.02 b |
| Levels of PGRs | | | |
| $T_1$= MS alone | 4.17 b | 4.7 b | 4.99 |
| $T_2$= MS + 0.5 $mgL^{-1}$ GA3 + 0.5 $mgL^{-1}$ Kinetin + 0.1 $mgL^{-1}$ NAA | 4.3 b | 4.98 ab | 5.36 |
| $T_3$= MS + 0.5 $mgL^{-1}$ GA3 + 1.0 $mgL^{-1}$ Kinetin + 0.3 $mgL^{-1}$ NAA | 4.81 a | 5.29 a | 5.58 |
| $T_4$= MS + 0.5 $mgL^{-1}$ GA3 + 1.5 $mgL^{-1}$ Kinetin + 0.5 $mgL^{-1}$ NAA | 4.78 a | 4.93 a | 5.51 |
| $T_5$= MS + 0.5 $mgL^{-1}$ GA3 + 2.0 $mgL^{-1}$ Kinetin + 0.7 $mgL^{-1}$ NAA | 4.51 ab | 4.54 b | 5.18 |
| CV (a) | 9.36 | 10.29 | 8.3 |
| CV (b) | 7.89 | 9.64 | 9.88 |
| Means within the same column followed by a common letter and/or no letter designation not significantly different from each other using Tukey's honestly significant difference (HSD) at 5% level of significance | | | |

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The interaction between meristem source and PGR level was significant in the first two months (Table 6). In the first month, the greatest stem diameters were observed in axillary meristems in $T_4$ (5.03 mm) and $T_3$ (4.93 mm), which were statistically similar and significantly higher than axillary $T_1$ (3.92 mm). These treatments contained moderate to high levels of kinetin (1.0–1.5 mg $L^{-1}$) with 0.5 mg $L^{-1}$ $GA_3$ and low to moderate levels of NAA (0.3–0.5 mg $L^{-1}$). In the second month, terminal meristems in $T_2$ and axillary meristems in $T_3$ recorded the highest stem diameters (5.39 mm each), significantly higher than axillary $T_1$ (4.22 mm); the other combinations were intermediate. Overall, PGR application generally improved stem thickening relative to the MS control, particularly in axillary meristems.

**Table 6. Interaction effect on stem diameter of *Theobroma cacao* L. as influenced by the different levels of plant growth regulators for 2 months of incubation.**

| FACTOR B | SOURCES OF MERISTEM | | | |
|---|---|---|---|---|
| | 1ST MONTH | | 2ND MONTH | |
| | TERMINAL | AXILLARY | TERMINAL | AXILLARY |
| Levels of PGRs: | | | | |
| $T^1$= MS alone | 4.42 ab | 3.92 b | 5.17 ab | 4.22 b |
| $T^2$= MS + 0.5 $mgL^{-1}$ GA3 + 0.5 $mgL^{-1}$ Kinetin + 0.1 $mgL^{-1}$ NAA | 4.36 ab | 4.23 ab | 5.39 a | 4.57 ab |
| $T^3$= MS + 0.5 $mgL^{-1}$ GA3 + 1.0 $mgL^{-1}$ Kinetin + 0.3 $mgL^{-1}$ NAA | 4.69 ab | 4.93 a | 5.20 ab | 5.39 a |
| $T^4$= MS + 0.5 $mgL^{-1}$ GA3 + 1.5 $mgL^{-1}$ Kinetin + 0.5 $mgL^{-1}$ NAA | 4.53 ab | 5.03 a | 4.94 ab | 4.92 ab |
| $T^5$= MS + 0.5 $mgL^{-1}$ GA3 + 2.0 $mgL^{-1}$ Kinetin + 0.7 $mgL^{-1}$ NAA | 4.25 ab | 4.78 ab | 4.36 ab | 4.73 ab |
| Means within the same column followed by a common letter and/or no letter designation are not significantly different from each other using Tukey's honestly significant difference (HSD) at the 5% level of significance | | | | |

***Days to the formation of leaves and shoots***

The number of days to leaf and shoot formation is shown in Table 7. The values in Tables 7 and 8 are transformed data (see the table notes) rather than original day counts, which are given in the Appendix Tables. Axillary meristems formed leaves and shoots slightly but significantly earlier than terminal meristems (leaves, 4.20 vs. 4.36 days; shoots, 5.39 vs. 5.48 days). The PGR levels also differed significantly. Explants on MS alone ($T_1$) recorded 2.24 for both leaves and shoots; this value should not be read as rapid organogenesis, since $T_1$ produced no leaves or shoots (Tables 9 and 11). Among the PGR-supplemented treatments, leaf formation was earliest in $T_4$ (4.56 days) followed by $T_2$ (4.68 days) and slowest in $T_3$ (4.98 days) and $T_5$ (4.96 days), whereas shoot formation was earliest in $T_2$ (MS + 0.5 mg $L^{-1}$ $GA_3$ + 0.5 mg $L^{-1}$ kinetin + 0.1 mg $L^{-1}$ NAA; 6.05 days), intermediate in $T_3$ (6.23 days) and $T_5$ (6.24 days), and slowest in $T_4$ (6.44 days).

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**Table 7. Days to the formation of leaves and shoots of *Theobroma cacao* L. as influenced by the different levels of plant growth regulators**

| Sources of meristem | Days to the formation of leaves | Days to the formation of shoots |
|---|---|---|
| Terminal | 4.36 a | 5.48 a |
| Axillary | 4.20 b | 5.39 b |
| Levels of PGRs | | |
| $T_1$= MS alone | 2.24 d | 2.24 d |
| $T_2$= MS + 0.5 $mgL^{-1}$ GA3 + 0.5 $mgL^{-1}$ Kinetin + 0.1 $mgL^{-1}$ NAA | 4.68 b | 6.05 c |
| $T_3$= MS + 0.5 $mgL^{-1}$ GA3 + 1.0 $mgL^{-1}$ Kinetin + 0.3 $mgL^{-1}$ NAA | 4.98 a | 6.23 b |
| $T_4$= MS + 0.5 $mgL^{-1}$ GA3 + 1.5 $mgL^{-1}$ Kinetin + 0.5 $mgL^{-1}$ NAA | 4.56 c | 6.44 a |
| $T_5$= MS + 0.5 $mgL^{-1}$ GA3 + 2.0 $mgL^{-1}$ Kinetin + 0.7 $mgL^{-1}$ NAA | 4.96 a | 6.24 b |
| CV (a) | 14.11 | 12.22 |
| CV (b) | 4.59 | 4.57 |
| Means within the same column followed by a common letter and/or no letter designation are not significantly different from each other using Tukey's honestly significant difference (HSD) at 5% level of significance<br>Note: Original data were transformed using log(√x) of each value to stabilize variance and homogeneity | | |

A significant meristem source × PGR interaction was found for both parameters (Table 8). For days to leaf formation, terminal meristems were earliest in $T_4$ (4.53 days), whereas axillary meristems were earliest in $T_2$ and $T_4$ (4.60 days each), with $T_5$ (4.65 days) statistically comparable; terminal meristems in $T_5$ were slowest (5.26 days). For days to shoot formation, $T_2$ gave the earliest response in terminal meristems (6.05 days) and, together with $T_5$, in axillary meristems (6.06 days each), whereas $T_4$ was slowest in both sources (6.43 and 6.46 days), as was $T_5$ in terminal meristems (6.42 days). The development of terminal and axillary plantlets under the different treatments is illustrated in Figs. 4 and 5.

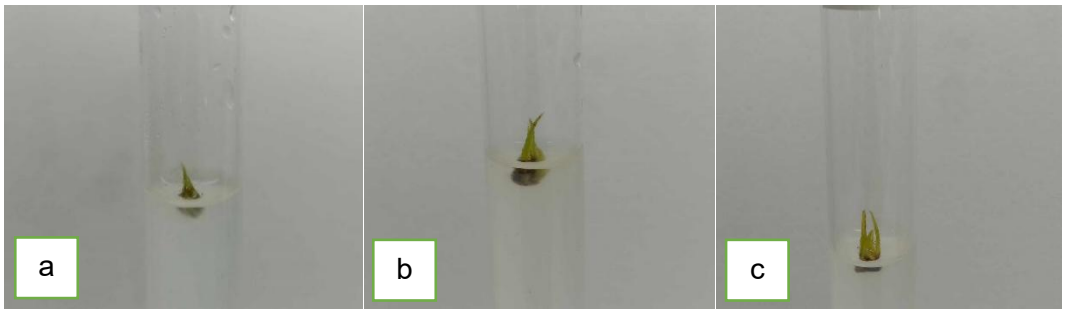
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**Table 8. Interaction effect on days to leaves and shoot induction of *Theobroma cacao* L. as influenced by different levels of plant growth regulators**

| FACTOR B | Days to formation of shoots | | Days to formation of leaves | |
|---|---|---|---|---|
| | TERMINAL | AXILLARY | TERMINAL | AXILLARY |
| Levels of PGRs: | | | | |
| $T_1$= MS alone | 2.2360 e | 2.2360 e | 2.2360 e | 2.2360 e |
| $T_2$= MS + 0.5 $mgL^{-1}$ GA3 + 0.5 $mgL^{-1}$ Kinetin + 0.1 $mgL^{-1}$ NAA | 6.0454 d | 6.0619 d | 4.7637 c | 4.5995d |
| $T_3$= MS + 0.5 $mgL^{-1}$ GA3 + 1.0 $mgL^{-1}$ Kinetin + 0.3 $mgL^{-1}$ NAA | 6.3068 b | 6.1517 c | 5.0176 b | 4.9336 b |
| $T_4$= MS + 0.5 $mgL^{-1}$ GA3 + 1.5 $mgL^{-1}$ Kinetin + 0.5 $mgL^{-1}$ NAA | 6.4331 a | 6.4562 a | 4.5254 d | 4.5980 d |
| $T_5$= MS + 0.5 $mgL^{-1}$ GA3 + 2.0 $mgL^{-1}$ Kinetin + 0.7 $mgL^{-1}$ NAA | 6.4226 a | 6.0566 d | 5.2648 a | 4.6549 cd |
| Means within the same column followed by a common letter and/or no letter designation are not significantly different from each other using Tukey's honestly significant difference (HSD) at the 5% level of significance<br>Note: Original data were transformed using log(√x) of each value to stabilize variance and homogeneity | | | | |

**Figure 4. Morphological and growth development of *Theobroma cacao* L. var. UF18 using terminal meristem as influenced by different levels of plant growth regulators within 2 months of incubation**



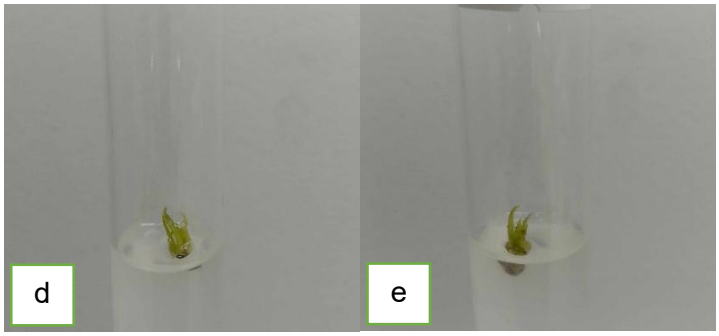

T1= MS alone

T2= MS + 0.5 $mgL^{-1}$ GA3 + 0.5 $mgL^{-1}$ Kinetin + 0.1 $mgL^{-1}$ NAA

T3= MS + 0.5 $mgL^{-1}$ GA3 + 1.0 $mgL^{-1}$ Kinetin + 0.3 $mgL^{-1}$

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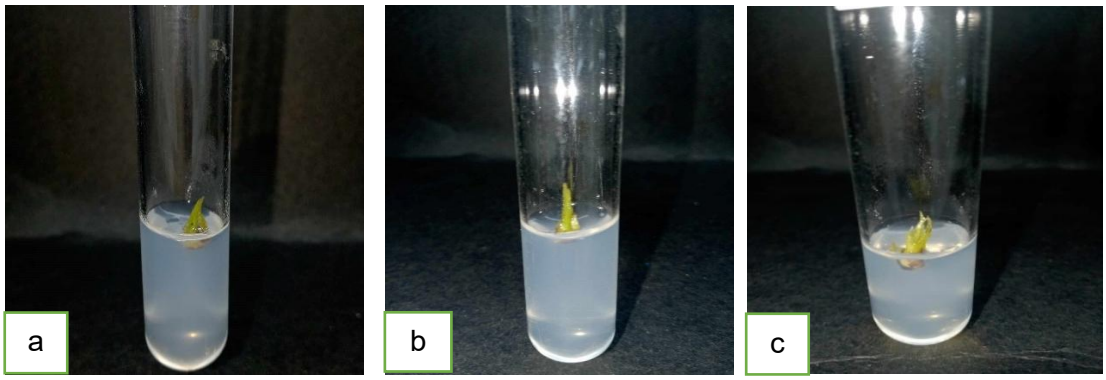
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T4= MS + 0.5 mgL$^{-1}$ GA3 + 1.5 mgL$^{-1}$ Kinetin + 0.5 mgL$^{-1}$ NAA

T5=MS + 0.5 mgL$^{-1}$ GA3 + 2.0 mgL$^{-1}$ Kinetin + 0.7 mgL$^{-1}$ NAA

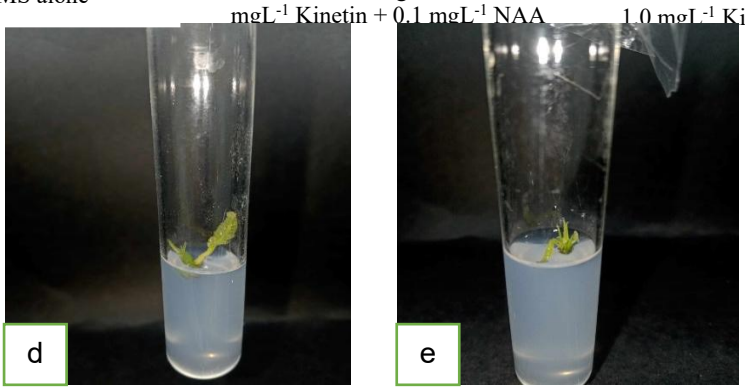
**Figure 5. Morphological and growth development of *Theobroma cacao* L. var. UF18 using axillary meristem as influenced by different levels of plant growth regulators within 2 months of incubation**




A1= MS alone

A2= MS + 0.5 mgL$^{-1}$ GA3 + 0.5 mgL$^{-1}$ Kinetin + 0.1 mgL$^{-1}$ NAA

A3= MS + 0.5 mgL$^{-1}$ GA3 + 1.0 mgL$^{-1}$ Kinetin + 0.3 mgL$^{-1}$




T4= MS + 0.5 mgL$^{-1}$ GA3 + 1.5 mgL$^{-1}$ Kinetin + 0.5 mgL$^{-1}$ NAA

T5=MS + 0.5 mgL$^{-1}$ GA3 + 2.0 mgL$^{-1}$ Kinetin + 0.7 mgL$^{-1}$ NAA

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***Number of leaves***

The number of leaves per plantlet is shown in Table 9. Axillary and terminal meristems did not differ significantly in the first and second months, and in the third month, axillary meristems had a statistically higher leaf count than terminal meristems, although the absolute difference was negligible (1.93 vs. 1.92 leaves). MS alone ($T_1$) produced no leaves throughout the culture period, whereas all PGR-supplemented treatments ($T_2$–$T_5$) produced significantly more leaves than $T_1$ as early as the first month, with a continuous increase over time. By the third month, $T_5$ yielded the highest mean leaf number (2.53), but $T_2$ to $T_5$ were statistically similar, so increasing the levels of kinetin and NAA in combination with 0.5 mg $L^{-1}$ $GA_3$ consistently promoted leaf formation without significant differences among the PGR-supplemented treatments.

**Table 9. Number of Leaves of *Theobroma cacao* L. as influenced by the different levels of plant growth regulators after 3 months of incubation**

| SOURCES OF MERISTEM | NUMBER OF LEAVES | | |
|---|---|---|---|
| | 1ST MONTH | 2ND MONTH | 3RD MONTH |
| Terminal | 1.15 | 1.47 | 1.92 b |
| Axillary | 1.21 | 1.5 | 1.93 a |
| Levels of PGRs | | | |
| $T_1$= MS alone | 0.00 b | 0.00 b | 0.00 b |
| $T_2$= MS + 0.5 $mgL^{-1}$ GA3 + 0.5 $mgL^{-1}$ Kinetin + 0.1 $mgL^{-1}$ NAA | 1.35 a | 1.64 a | 2.22 a |
| $T_3$= MS + 0.5 $mgL^{-1}$ GA3 + 1.0 $mgL^{-1}$ Kinetin + 0.3 $mgL^{-1}$ NAA | 1.55 a | 1.90 a | 2.38 a |
| $T_4$= MS + 0.5 $mgL^{-1}$ GA3 + 1.5 $mgL^{-1}$ Kinetin + 0.5 $mgL^{-1}$ NAA | 1.56 a | 1.89 a | 2.48 a |
| $T_5$= MS + 0.5 $mgL^{-1}$ GA3 + 2.0 $mgL^{-1}$ Kinetin + 0.7 $mgL^{-1}$ NAA | 1.43 a | 1.98 a | 2.53 a |
| CV (a) | 14.09 | 17.49 | 16.09 |
| CV (b) | 15.14 | 15.17 | 12.10 |
| Means within the same column followed by a common letter and/or no letter designation are not significantly different from each other using Tukey's honestly significant difference (HSD) at the 5% level of significance | | | |

The interaction effect on leaf number is shown in Table 10. Plantlets in the PGR-supplemented treatments showed a progressive increase in leaf production over time, with the highest counts in the third month. Significant differences among the PGR-supplemented combinations occurred only in the first two months, when terminal meristems in $T_2$

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had fewer leaves (1.17 and 1.19) than axillary meristems in $T_2$ in the second month (2.09) and terminal meristems in $T_5$ (2.30); by the third month, all PGR-supplemented combinations were statistically similar. The highest leaf count after three months of incubation was recorded in terminal meristems in $T_5$ (MS + 0.5 mg $L^{-1}$ $GA_3$ + 2.0 mg $L^{-1}$ kinetin + 0.7 mg $L^{-1}$ NAA; 2.88 leaves), the treatment with the highest PGR concentration, while axillary meristems in $T_2$ (MS + 0.5 mg $L^{-1}$ $GA_3$ + 0.5 mg $L^{-1}$ kinetin + 0.1 mg $L^{-1}$ NAA) responded better to a moderate PGR concentration and also produced a high leaf count (2.62 leaves).

**Table 10. Interaction effect on the number of leaves of *Theobroma cacao* L. as influenced by different levels of plant growth regulators for three months of incubation**

| FACTOR B | SOURCES OF MERISTEM | | | | | |
|---|---|---|---|---|---|---|
| | 1ST MONTH | | 2ND MONTH | | 3RD MONTH | |
| | TERMINAL | AXILLARY | TERMINAL | AXILLARY | TERMINAL | AXILLARY |
| Levels of PGRs: | | | | | | |
| $T_1$= MS alone | 0.00 c | 0.00 c | 0.00 c | 0.00 c | 0.00 b | 0.00 b |
| $T_2$= MS + 0.5 mgL$^{-1}$ GA3 + 0.5 mgL$^{-1}$ Kinetin + 0.1 mgL$^{-1}$ NAA | 1.17 b | 1.54 ab | 1.19 b | 2.09 a | 1.83 a | 2.62 a |
| $T_3$= MS + 0.5 mgL$^{-1}$ GA3 + 1.0 mgL$^{-1}$ Kinetin + 0.3 mgL$^{-1}$ NAA | 1.48 ab | 1.63 a | 1.94 ab | 1.86 ab | 2.36 a | 2.41 a |
| $T_4$= MS + 0.5 mgL$^{-1}$ GA3 + 1.5 mgL$^{-1}$ Kinetin + 0.5 mgL$^{-1}$ NAA | 1.57 ab | 1.55 ab | 1.90 ab | 1.88 ab | 2.51 a | 2.45 a |
| $T_5$= MS + 0.5 mgL$^{-1}$ GA3 + 2.0 mgL$^{-1}$ Kinetin + 0.7 mgL$^{-1}$ NAA | 1.56 ab | 1.30 ab | 2.30 a | 1.66 ab | 2.88 a | 2.17 a |
| Means within the same column followed by a common letter and/or no letter designation not significantly different from each other using Tukey's honestly significant difference (HSD) at 5% level of significance | | | | | | |

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***Number of shoots***

The number of shoots per plantlet is shown in Table 11. Explants cultured on MS alone ($T_1$) produced no shoots, whereas all treatments containing $GA_3$, kinetin, and NAA ($T_2$–$T_5$) produced significantly more shoots than $T_1$, with no significant differences among them. The average number of shoots per explant increased from the second to the third month in all PGR-supplemented treatments, with $T_5$ (MS + 0.5 mg $L^{-1}$ $GA_3$ + 2.0 mg $L^{-1}$ kinetin + 0.7 mg $L^{-1}$ NAA) yielding the highest shoot number in the third month (1.66). Axillary meristems produced slightly more shoots than terminal meristems in the second (1.03 vs. 0.92) and third (1.27 vs. 1.20) months, but this difference was small and not statistically significant.

**Table 11. Number of shoots of *Theobroma cacao* L. as influenced by the different levels of plant growth regulators after 3 months of incubation**

| SOURCES OF MERISTEM | NUMBER OF SHOOTS | |
|---|---|---|
| | 2ND MONTH | 3RD MONTH |
| Terminal | 0.92 | 1.20 |
| Axillary | 1.03 | 1.27 |
| Levels of PGRs | | |
| $T_1$= MS alone | 0.00 b | 0.00 b |
| $T_2$= MS + 0.5 $mgL^{-1}$ GA3 + 0.5 $mgL^{-1}$ Kinetin + 0.1 $mgL^{-1}$ NAA | 1.21 a | 1.47 a |
| $T_3$= MS + 0.5 $mgL^{-1}$ GA3 + 1.0 $mgL^{-1}$ Kinetin + 0.3 $mgL^{-1}$ NAA | 1.29 a | 1.52 a |
| $T_4$= MS + 0.5 $mgL^{-1}$ GA3 + 1.5 $mgL^{-1}$ Kinetin + 0.5 $mgL^{-1}$ NAA | 1.20 a | 1.54 a |
| $T_5$= MS + 0.5 $mgL^{-1}$ GA3 + 2.0 $mgL^{-1}$ Kinetin + 0.7 $mgL^{-1}$ NAA | 1.18 a | 1.66 a |
| CV (a) | 14.64 | 13.57 |
| CV (b) | 19.12 | 13.86 |
| Means within the same column followed by a common letter and/or no letter designation not significantly different from each other using Tukey's honestly significant difference (HSD) at 5% level of significance | | |

***Shoot length***

Shoot length after two and three months of incubation is shown in Table 12. MS alone ($T_1$) failed to induce shoot elongation in both months. At the third month, axillary meristems produced slightly longer shoots (2.16 mm) than

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terminal meristems (1.99 mm), although this difference was not statistically significant. All treatments with combinations of $GA_3$, kinetin, and NAA promoted shoot elongation, with values ranging from 2.22 to 2.81 mm at the third month. $T_3$ (MS + 0.5 mg $L^{-1}$ $GA_3$ + 1.0 mg $L^{-1}$ kinetin + 0.3 mg $L^{-1}$ NAA) yielded the longest shoots (2.81 mm), significantly longer than those in $T_5$ (2.22 mm) but statistically comparable to $T_2$ (2.61 mm) and $T_4$ (2.72 mm).

**Table 12. Shoot length of *Theobroma cacao* L. as influenced by the different levels of plant growth regulators after 3 months of incubation**

| SOURCES OF MERISTEM | SHOOT LENGTH (mm) | |
|---|---|---|
| | 2ND MONTH | 3RD MONTH |
| Terminal | 1.73 | 1.99 |
| Axillary | 1.69 | 2.16 |
| Levels of PGRs | | |
| $T_1$= MS alone | 0.00 c | 0.00 c |
| $T_2$= MS + 0.5 $mgL^{-1}$ GA3 + 0.5 $mgL^{-1}$ Kinetin + 0.1 $mgL^{-1}$ NAA | 2.26 a | 2.61 ab |
| $T_3$= MS + 0.5 $mgL^{-1}$ GA3 + 1.0 $mgL^{-1}$ Kinetin + 0.3 $mgL^{-1}$ NAA | 2.38 a | 2.81 a |
| $T_4$= MS + 0.5 $mgL^{-1}$ GA3 + 1.5 $mgL^{-1}$ Kinetin + 0.5 $mgL^{-1}$ NAA | 2.08 ab | 2.72 a |
| $T_5$= MS + 0.5 $mgL^{-1}$ GA3 + 2.0 $mgL^{-1}$ Kinetin + 0.7 $mgL^{-1}$ NAA | 1.83 b | 2.22 b |
| CV (a) | 16.01 | 15.73 |
| CV (b) | 19.28 | 19.60 |
| Means within the same column followed by a common letter and/or no letter designation are not significantly different from each other using Tukey's honestly significant difference (HSD) at 5% level of significance | | |

The interaction between PGR level and meristem source significantly affected shoot length (Table 13). The absence of exogenous hormones in $T_1$ resulted in no shoot elongation in either terminal or axillary explants throughout the incubation period. The longest shoots among terminal meristems were recorded in $T_2$ (MS + 0.5 mg $L^{-1}$ $GA_3$ + 0.5 mg $L^{-1}$ kinetin + 0.1 mg $L^{-1}$ NAA) at both the second (2.75 mm) and third (2.97 mm) months, whereas axillary meristems gave their best response in $T_3$ (MS + 0.5 mg $L^{-1}$ $GA_3$ + 1.0 mg $L^{-1}$ kinetin + 0.3 mg $L^{-1}$ NAA), with the greatest elongation at the third month (3.07 mm), which was statistically comparable to that in $T_4$ (2.81 mm) and, in some instances, $T_2$ (2.26 mm) and $T_5$ (2.63 mm).

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**Table 13. Interaction effect on the shoot length of *Theobroma cacao* L. as influenced by different levels of plant growth regulators for three months of incubation**

| FACTOR B | SOURCES OF MERISTEM | | | |
|---|---|---|---|---|
| | 2ND MONTH | | 3RD MONTH | |
| | TERMINAL | AXILLARY | TERMINAL | AXILLARY |
| Levels of PGRs: | | | | |
| $T_1$= MS alone | 0.00 d | 0.00 d | 0.00 c | 0.00 c |
| $T_2$= MS + 0.5 $mgL^{-1}$ GA3 + 0.5 $mgL^{-1}$ Kinetin + 0.1 $mgL^{-1}$ NAA | 2.75 a | 1.76 c | 2.97 a | 2.26 ab |
| $T_3$= MS + 0.5 $mgL^{-1}$ GA3 + 1.0 $mgL^{-1}$ Kinetin + 0.3 $mgL^{-1}$ NAA | 2.26 abc | 2.51 ab | 2.53 ab | 3.07 a |
| $T_4$= MS + 0.5 $mgL^{-1}$ GA3 + 1.5 $mgL^{-1}$ Kinetin + 0.5 $mgL^{-1}$ NAA | 2.00 bc | 2.17 abc | 2.63 ab | 2.81 a |
| $T_5$= MS + 0.5 $mgL^{-1}$ GA3 + 2.0 $mgL^{-1}$ Kinetin + 0.7 $mgL^{-1}$ NAA | 1.65 c | 2.00 bc | 1.80 b | 2.63 ab |
| Means within the same column followed by a common letter and/or no letter designation not significantly different from each other using least significant difference (HSD) at 5% level of significance | | | | |

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## DISCUSSION

### Donor Plant Health and Pathogen Diagnosis

Diagnosing pathogens in the donor plant before tissue culture is important to ensure that only healthy, disease-free material is used for propagation. Infected donor plants can harbor systemic pathogens such as viruses, bacteria, or fungi, which not only compromise the success of *in vitro* culture through contamination and poor growth but also result in the propagation of infected plantlets. Early detection allows clean explants to be selected, particularly for meristem culture, preserves genetic fidelity, and reduces the economic losses caused by culture failure (Wang et al., 2008). Thorough pathogen diagnosis using visual and microscopic inspection is therefore a vital step toward producing high-quality, healthy, and genetically stable plantlets in tissue culture.

In this study, the pods of the donor plant showed the typical external and internal symptoms of cocoa pod borer infestation, an insect pest of major concern in Asian cacao production (Arshad et al., 2015). The leaves, however, showed no visible or microscopic evidence of fungal or bacterial pathogens, and the minor drooping of some young leaves was regarded as a normal physiological reaction. These observations support the use of the donor plant as a source of explants. Visual and microscopic examination cannot, however, detect systemic viruses; serological or molecular indexing such as ELISA or RT-PCR would be needed to certify the virus-free status of the regenerated plantlets.

### Sterilization, Contamination, and Explant Survival

Fungi and bacteria were the major types of contamination in the experiment, which was managed through the fungicide spray applied to the donor trees, the two-step NaOCl sterilization, and the addition of streptomycin to the medium. The success of tissue culture depends on the efficacy of the sterilization techniques applied to the explants before inoculation (Sessou et al., 2020), and all materials must be sterilized with suitable agents to eliminate the microorganisms present. Successful culture initiation and regeneration are critical requirements for establishing and maintaining an *in vitro* crop collection, and several factors influence the response of plant tissue cultures, including genotype, explant source, culture medium, growth regulators, and the culture environment (Loyola-Vargas & Ochoa-Alejo, 2018).

The highest survival rate (78.33%) and lowest contamination (21.67%) were obtained in $T_4$. Because the differences among meristem sources and PGR treatments were small and not statistically significant, however, the apparent effects of growth regulators on contamination dynamics, explant viability, and survival should be interpreted cautiously. The differing proportions of fungal and bacterial contamination between terminal and axillary explants suggest differential microbial susceptibility or microbial load among the explants. Cassells (2012) noted that explants are commonly surface-sterilized to eliminate epiphytic bacteria, whereas certain microorganisms, known as endophytes, live within plant tissues and may contaminate tissue cultures. This problem is particularly crucial for cultures with a low regeneration rate, because endophytic organisms complicate the long-term maintenance of healthy, contamination-free cultures (Nair et al., 2014). Micropropagation enables large-scale plant production, but contamination remains a significant challenge associated with the technique (Altan et al., 2010).

### Browning of Explants

Browning in a few explants led to their death, adversely affected growth and development, and prevented regeneration. The tissues of cacao and other woody plant species generally contain high concentrations of phenolic compounds,

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which may result in browning during tissue culture (Hesami et al., 2020; Gao, 2019). Certain plant species are also more prone to browning in tissue culture because of inherent traits such as high phenolic content, stress sensitivity, or susceptibility to contamination (Amente & Chimdessa, 2021; Onuoha et al., 2011). Assis et al (2018) recommended adding 2 g $L^{-1}$ of activated charcoal to the culture medium to minimize the effects of phenol oxidation in *Eugenia pyriformis* cultured from nodal segments. Likewise, Sharada et al. (2003) and Prajapati et al. (2003) suggested that incorporating activated charcoal into the culture medium mitigated the inhibitory effects of leached phenolics on the regeneration of *Celastrus paniculatus* and *C. orchioides*, respectively. Activated charcoal reduces tissue browning by effectively adsorbing the free phenolic compounds released by explants into the culture medium (Saenz et al., 2010) and could be tested in future cacao cultures.

**Plant Height**

Terminal meristems consistently produced taller plantlets than axillary meristems. Terminal meristems have a greater capacity for growth than axillary meristems, which grow more slowly, probably because of apical dominance and hormonal regulation that make them more sensitive to plant growth regulators (Dun et al., 2006). The difference in responsiveness between terminal and axillary meristems could also be associated with variations in endogenous hormone levels and physiological roles; terminal meristems are inherently designed for elongation and vertical growth, making them more responsive to growth-promoting hormones such as $GA_3$ (Indira & Kavyashree, 2015), and their higher endogenous cytokinin levels may further promote shoot elongation.

The significant interaction after one month (Table 4) shows that the effect of the PGRs on plant height depends on the type of explant. Terminal meristems responded to PGR supplementation, particularly in $T_2$, which gave the numerically tallest plantlets, although $T_2$ was not significantly different from $T_3$–$T_5$. This suggests that the combination of gibberellic acid, kinetin, and a low concentration of NAA provided a favorable hormonal environment for shoot elongation, consistent with the role of $GA_3$ in promoting shoot elongation and cell expansion and of kinetin in stimulating cell division and shoot development when paired with an appropriate level of auxin such as NAA (George et al., 2008; Taiz & Zeiger, 2010). In tissue culture, $GA_3$ can improve the elongation of terminal explants without causing callus formation, which might hinder regeneration (Maximova & Guiltinan, 2002). Terminal meristems are therefore better suited to shoot elongation, especially with PGR combinations such as $T_2$, which provide a favorable cytokinin-to-auxin ratio together with gibberellic acid. That $T_3$ and $T_4$, with higher NAA, remained numerically tallest at three months suggests that moderate NAA levels support sustained elongation.

In contrast, axillary meristems showed little response to any treatment, which may reflect reduced sensitivity to the PGR combinations evaluated or stronger apical dominance that inhibits their elongation (Hartmann et al., 2011). This is consistent with the observation of Thorpe et al. (2008) that lateral meristems frequently require particular preconditioning to overcome dormancy and commence shoot growth. The limited proliferation of axillary meristems highlights the need for alternative propagation procedures if these explants are to be used on a broad scale. Possible approaches include using other hormone combinations, increasing cytokinin concentrations, or applying physical manipulations such as apical removal to release axillary buds from dormancy (Hartmann et al., 2011).

**Stem Diameter**

Terminal meristems produced thicker stems only in the third month, and the two meristem sources were similar earlier. This is consistent with Aloni (2013), who highlighted that vascular cambium activity is more strongly influenced by overall plant physiology and hormonal balance than by the source of the shoot meristem, so that stem thickening is not strongly dependent on the initial meristem source. Moderate levels of $GA_3$, kinetin, and NAA ($T_3$, $T_4$, and $T_2$)

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promoted the thickest stems, whereas MS alone ($T_1$) and the highest hormone concentrations ($T_5$) gave comparatively thinner stems, suggesting that excess growth regulators may be less effective or may even inhibit growth (Aloni, 2013).

The significant interaction in the first two months showed that axillary meristems benefited most from moderate to high kinetin combined with low to moderate NAA, indicating that such combinations enhance early stem thickness *in vitro*. Traore et al. (2003) developed a micropropagation protocol using somatic embryo-derived plants as a source of both nodal (axillary) and apical (terminal) stem explants. They found that nodal explants cultured on thidiazuron medium promoted axillary bud proliferation and shoot development, but with lower efficiency than the rooting of apical microcuttings, and they reported significant differences in stem diameter between plantlets derived from axillary and terminal meristems. Understanding the interaction between explant type and growth regulators is important for selecting the combinations that most effectively enhance stem growth, which is critical for plant vigor and overall culture potential.

**Days to Leaf and Shoot Formation**

A shorter time to leaf and shoot formation indicates a more favorable hormonal balance and greater responsiveness of the explants to *in vitro* conditions. The slightly greater responsiveness of axillary meristems and their faster adaptation to regeneration conditions make them somewhat more efficient for leaf and shoot induction. This agrees with the observation of Thorpe (2012) that different tissues (axillary vs. terminal buds) respond differently to the same hormonal treatments because of variations in endogenous hormone levels, and with George et al. (2008), who examined the effects of different plant growth regulators on cytokinin-induced shoot formation and confirmed that shorter shoot induction times indicate a more efficient response to hormonal treatment. Ng et al. (2021) also explored the tissue culture responses of different cacao cultivars and highlighted that genetic factors can influence the efficiency of shoot induction. These studies support the use of meristem tissue culture as an effective method for improving shoot development and propagation in cacao.

The short time recorded for MS alone ($T_1$) likely reflects unresponsiveness rather than an advantage, as $T_1$ produced no leaves or shoots; plantlets in hormone-free media often show limited elongation or differentiation capacity (Adu-Ampomah et al., 1992). Among the PGR-supplemented treatments, the earliest leaf formation in $T_4$ and $T_2$ and the earliest shoot formation in $T_2$ are consistent with the report of Figueira et al. (1991) that balanced hormone supplementation, especially cytokinins in combination with auxins, enhances shoot development in cacao. Axillary meristems were also more consistent in their response across treatments, which reinforces previous findings that axillary meristems have greater organogenic potential and responsiveness to the levels of PGRs applied (Maximova et al., 2002). Terminal meristems were capable of producing robust shoots but were more sensitive to high PGR levels, as shown by the longest response time in $T_5$ (MS + 0.5 mg $L^{-1}$ $GA_3$ + 2.0 mg $L^{-1}$ kinetin + 0.7 mg $L^{-1}$ NAA), likely because of hormonal imbalances that delay organogenesis (Adu-Ampomah et al., 1992).

Moderate PGR levels, such as those in $T_2$ and $T_4$, therefore promote a more balanced and efficient leaf-initiation process than higher concentrations, allowing rapid yet stable shoot development, which is essential for producing vigorous plantlets suitable for acclimatization and field planting. While quick response times are generally desirable, they must coincide with adequate hormonal stimulation to ensure the development of vigorous and viable shoots. Relying on hormone-free media may speed up the initial response but compromises plantlet quality, which can reduce survival and increase losses in subsequent stages, whereas excessive hormone concentrations can delay shoot formation and waste resources.

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**Number of Leaves**

All PGR-supplemented treatments produced leaves, whereas MS alone did not. Plant growth regulators stimulate cell division, differentiation, and leaf expansion: kinetin promotes shoot initiation, and gibberellins ($GA_3$) enhance leaf elongation (George et al., 2008). Increased leaf production correlates directly with improved photosynthesis, plantlet vigor, and higher survival during acclimatization, which is crucial for cacao tissue culture propagation (Thorpe, 2007). The highest leaf count in terminal meristems under the highest PGR concentration ($T_5$) and in axillary meristems under a moderate concentration ($T_2$) shows that explants have varying hormonal sensitivities and require optimized PGR levels for maximum growth. These findings underscore the importance of optimizing PGR combinations, including $GA_3$ and kinetin, to enhance shoot proliferation and leaf development in meristem tip cultures of Malvaceae species, including cacao.

**Number of Shoots and Shoot Length**

The lack of shoot development in the control treatment shows that endogenous hormones alone were insufficient to trigger shoot induction and highlights the necessity of hormonal supplementation in cacao tissue culture. The successful shoot proliferation observed with all PGR combinations supports the synergistic roles of $GA_3$ in promoting elongation, kinetin in stimulating cell division and bud initiation, and NAA in facilitating cellular differentiation and stability, in agreement with George et al. (2008) and Taiz and Zeiger (2010), who emphasized the importance of balanced PGRs for successful *in vitro* regeneration. The effectiveness of several PGR combinations gives flexibility in optimizing protocols according to laboratory conditions and explant availability, and the ability of axillary meristems to produce a comparable number of shoots opens possibilities for maximizing shoot yields, which is especially valuable in large-scale propagation and germplasm conservation.

The shoot elongation response was not only hormone-dependent but also influenced by the physiological origin of the meristem. Terminal meristems, which are naturally more dominant and prone to apical elongation, responded better under lower kinetin and NAA concentrations ($T_2$), whereas axillary meristems, which are typically dormant or suppressed *in planta*, responded better to moderately higher cytokinin and auxin levels ($T_3$), which may have helped overcome apical dominance (George et al., 2008; Hartmann et al., 2011). Previous studies (George et al., 2008; Hartmann et al., 2011) indicate that axillary buds naturally transition into lateral shoots when stimulated by cytokinin. Kinetin, a cytokinin, promotes meristematic activity and cell division; gibberellic acid probably played a significant role in improving shoot elongation by inducing internode growth; and NAA, an auxin, may have helped with shoot direction and stability, encouraging consistent elongation (Taiz & Zeiger, 2010; Thorpe et al., 2008).

The importance of cytokinins for shoot elongation corroborates the findings of Wang et al. (2021) regarding their influence on cell division and shoot growth. Schaller et al. (2015) investigated the complex interplay between cytokinin and auxin in the regulation of plant development, with a focus on shoot elongation. Their study identified cytokinin as a significant promoter of shoot proliferation, facilitating cell division and expansion in the shoot apical meristem, whereas auxin functions as a suppressor that preserves apical dominance and prevents excessive shoot elongation. The equilibrium between these two hormones influences whether a plant favors shoot or root development, with an elevated cytokinin-to-auxin ratio promoting shoot elongation.

**Implications for Cacao Micropropagation and Limitations**

These findings have several implications for cacao micropropagation and tissue culture practice. First, treatments containing moderate concentrations of PGRs, such as $T_2$, $T_3$, and $T_4$, supported efficient leaf and shoot formation,

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shoot elongation, and stem thickening, whereas MS alone produced no leaves or shoots. Second, the relatively consistent performance of axillary meristems across treatments, together with their slightly earlier leaf and shoot formation and numerically greater shoot number and shoot length, suggests that they are a more reliable explant source than terminal meristems for standard propagation protocols, helping to ensure uniformity and higher success rates. Third, because terminal and axillary meristems responded differently to particular PGR formulations, hormone concentrations should be adjusted to the explant type to optimize growth performance and to improve the scalability and reliability of clonal propagation of elite cacao genotypes. From a practical standpoint, these insights enable researchers and commercial propagators to refine PGR use, improving efficiency while reducing the costs associated with excessive or ineffective PGR application.

Identifying a suitable explant type and optimizing PGR concentrations can enhance the efficiency and success rate of cacao micropropagation, which is essential for mass propagation, conservation of germplasm, and breeding programs, especially the development of high-yielding, disease-resistant cacao varieties through tissue culture. The findings emphasize the importance of understanding explant–PGR interactions, because plant responses are influenced not only by hormone concentrations but also by the inherent characteristics of the tissue type employed (George et al., 2008; Thorpe, 2007). Optimizing the combination of explant source and PGR concentration, specifically using axillary meristems with moderate levels of $GA_3$, kinetin, and NAA, provided the best balance between propagation response and shoot quality. The ability to achieve early and uniform shoot development contributes directly to the sustainability of cacao production systems: with increasing global demand and the need to rehabilitate aging plantations, reliable *in vitro* techniques are crucial, and this approach can support efficient, cost-effective seedling production for smallholder farmers and for conservation programs that aim to maintain the genetic diversity and productivity of cacao.

Several limitations should be considered. The study was restricted to one cacao variety (UF18) and to three months of culture, and it did not cover rooting or acclimatization. The differences in shoot number, shoot length, and contamination between meristem sources were small and not statistically significant, so the superiority of axillary meristems should be regarded as indicative and confirmed in further work. Future research may investigate a wider range of hormonal combinations or pretreatments to enhance axillary meristem performance and to optimize protocols for cacao regeneration.

## CONCLUSIONS AND RECOMMENDATIONS

### Conclusions

Based on the results obtained, the following conclusions can be drawn:

1. The two-step sterilization protocol (2% NaOCl for 20 min followed by 1% NaOCl for 10 min) gave the highest survival rate (78.33%) and the lowest contamination rate (21.67%) in $T_4$, although differences among treatments were not statistically significant.
2. Plant height was significantly greater in terminal meristems (11.50 vs. 8.38 mm after three months), likely because of their greater sensitivity to growth regulators and possibly higher endogenous cytokinin levels, confirming the effectiveness of terminal meristems in enhancing vertical growth in cacao tissue culture.
3. Stem diameter did not differ between meristem sources in the first two months and was only moderately higher in terminal meristems in the third month. Because stem thickening is largely controlled by vascular cambium activity, both terminal and axillary meristems contribute similarly to stem development. $T_3$ consistently gave the greatest stem diameter.

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4. Among the PGR-supplemented treatments, $T_4$ (MS + 0.5 mg $L^{-1}$ $GA_3$ + 1.5 mg $L^{-1}$ kinetin + 0.5 mg $L^{-1}$ NAA) gave the earliest leaf formation in terminal (4.53) and axillary (4.60) meristems, while $T_2$ (MS + 0.5 mg $L^{-1}$ $GA_3$ + 0.5 mg $L^{-1}$ kinetin + 0.1 mg $L^{-1}$ NAA) gave the earliest shoot formation (6.05 and 6.06, respectively), regardless of explant type.
5. MS alone produced no leaves or shoots, whereas all PGR-supplemented treatments did, confirming that exogenous growth regulators are necessary for cacao meristem regeneration. Moderate PGR levels ($T_3$ and $T_4$, with 1.0–1.5 mg $L^{-1}$ kinetin and 0.3–0.5 mg $L^{-1}$ NAA in combination with 0.5 mg $L^{-1}$ $GA_3$) gave the best overall response.
6. Axillary meristems formed leaves and shoots slightly earlier and showed numerically higher shoot numbers and longer shoots, suggesting that they are the more suitable explant source for micropropagation of *Theobroma cacao*; because these differences were small, this conclusion should be confirmed in further studies.

**Recommendations**

To improve and enhance the study, the following are recommended:

1. Explore molecular and hormonal profiling of meristem types. Investigate the endogenous hormone levels (e.g., cytokinins and auxins) in terminal versus axillary meristems to better understand their differential responses to plant growth regulators (PGRs). This could clarify the underlying physiological mechanisms influencing shoot development.
2. Optimize and compare PGR combinations beyond the current treatments. Future research should explore a wider range of PGR types and concentrations, including combinations not used in the present study, to identify more effective formulations for cacao micropropagation.
3. Assess root induction and acclimatization success. Extend the research to include rooting efficiency and survival during acclimatization. This will help determine which meristem type and treatment combination not only promotes growth *in vitro* but also ensures success after transplanting.
4. Apply the techniques to other cacao varieties or related species. Replicate the experiment using different cacao genotypes or related species to test the consistency and adaptability of the optimized protocols, contributing to broader applications in tropical crop propagation.

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