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MICROPROPAGATION OF TWO BANANAS (MUSA SPP.) VARITIES USING SHOOT EXPLANT FROM GURAGE ZONE, ETHIOPIA

MSc. THESIS

BY

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School of Graduate Studies

Micropropagation of Two Bananas (Musa spp.) Varities Using Shoot Explant from Gurage Zone, Ethiopia

A Thesis Submitted to the School of Graduate Studies, in Partial Fulfillment of the Requirement for the Degree of Masters of Science in Plant Biotechnology.

By

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Final approval and acceptance of the Thesis is contingent upon the submission of the final copy to the Council of Graduate Studies through the School Graduate Committee of the Candidates major Department.

DEDICATION

This thesis is dedicated to my beloved families for their encouragement, and unforgettable support in the success of my academic career.

DECLARATION

By my signature below, I hereby declare that the work described in this thesis is entirely original to me and would not have been previously used in a thesis or dissertation submitted to this or any other institution to earn a degree, diploma, or other credentials.

This thesis is submitted in partial fulfillment of the requirements for an M.Sc. degree at Wolkite University. The thesis is deposited in the Wolkite University Library and is made available to borrowers under the rules of the library. I have followed the University's current research ethics guidelines, and accept responsibility for the conduct of the procedures. I have attempted to identify all the risks related to this research that may arise in conducting this research, obtained the relevant ethical or safety approval was applicable, and acknowledged my obligations and the rights of the participants. Brief quotations from this thesis are allowable without special permission provided that accurate acknowledgment of the source is made.

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BIOGRAPHY

The author Miss. Genet Darge was born from her father Darge Andu, and her mother W/Mariam Haile September on, 1997 G.C in Southern Nations, Nationalities and Peoples` Region (SNNPR) of Ethiopia, at Gurage Zone. She attended her elementary and junior school at yesere and followed secondary and preparatory school at Cheza and Hawariyat.

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ABBREVIATIONS AND ACRONYM

AA	Ascorbic acid
AC	Activated charcoal
BAP	6- Benzyl Amino Purine
CRD	Completely randomized design
IAA	Indole-3- Acetic Acid
IBA	Indole butyric acid
KN	Kinetin
Kpa	kilopascal
LSD	Least Significant Difference
MS	Murashige and Skoog
N	Normality
NAA	Naphthalene acetic acid
PGR	Plant Growth Regulators
SNNPR	Southern Nation Nationalities and People Region State
TDZ	Thidiazuron

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ABSTRACT

The banana (*Musa spp*.) is one of the most important fruits for production and consumption due to its great nutritional importance. Nearly all banana genotypes are being threatened by bacterial wilt disease due to conventional propagation. Banana propagation rates achieved by tissue culture techniques are much higher than those reported by conventional methods. The objectives of this research were to develop a mass in vitro regeneration protocol for two elite banana dwarf Cavendish and ducase varieties from shoot tip explants. The experiment was laid out in CRD with three replications in factorial arrangement. Sodium hypochlorite (NaOCl) in three concentration levels (1, 2, and 5%) with 70% ethanol was used for surface sterilization. For controlling browning on explants at shoot initiation, explants were aseptically cultured on MS basal medium supplemented with 25mg, 50mg, ascorbic acid, and 1g and 2g of activated charcoal. Then the initiated shoots were transferred to MS basal media supplemented with 2, 2.5, or 3 mg/l of BAP alone and in combination with 0.5 mg/l of Kn. Plantlets were planted on different mixes of soil in (2:1:1). The sterilization experiment showed that 2% NaOCl was found to be effective. The results showed that from levels of antioxidant treatments up to 85%, initiated shoots survived medium containing 2 g/l activated charcoal. The use of 2g AC and 50mg AA as anti-browning agents in the medium for banana explants was suitable for shoot tip culture. On the shoot multiplication, maximum shoots per ex-plant (8.33) were obtained on a medium containing 3 mg/l BAP and 0.5 mg/L KN. The highest increase in root length was observed with the 80 g/l bulla, resulting in 6.90cm on the ducase variety. The acclimatization experiment showed 100% plantlet survival in all media mixtures. In this study, among the two cultivars tested, dwarf Cavendish was found to be more responsive to in vitro propagation techniques.

Key words: Antioxidant, Banana, *In vitro* Regeneration, Plant growth regulator, Shoot tip

1. INTRODUCTION

1.1. Background

The edible banana (banana and plantain, *Musa spp*.) is an important perennial herbaceous plant grown in tropical and sub-tropical countries around the world, mainly for its seedless fruits (Brown *et al*., 2017). Some species can grow to be as tall as 15 meters. The banana plant is a monocotyledon that can reproduce vegetative through its underground true stem, which can produce suckers, and its false stem, which is made up of leaf sheaths. It belongs to the *Musaceae* family and is among the oldest fruits in the world (Prasad K, *et al*).

Bananas originated in Southeast Asia, where a region considered the primary center of diversification and the earliest domestication occurred (Drenth and Kema, 2021). The low land areas of West Africa also contain the largest range of genetic diversity of plantains (Musa AAB). In East Africa, bananas are highly evolved into an important zone of secondary genetic diversity for the East African highland bananas (Musa AAA) (Akankwasa *et al*., 2021). Nowadays, bananas have grown in more than 130 countries throughout the tropics and subtropics (Oselebe *et al*., 2018), both as a staple food and an export commodity.

It is the fourth most important food crop after rice, wheat, and maize in many developing countries (Kulimbe *et al.*, 2021). Banana is a principal source of food, employment, and income in its major production areas .The global socioeconomic significance of the banana crop is immense (Gregory, 2021). According to data from the FAO (2020), the world economy receives around $12 billion annually from exports of bananas, and in 2017, 116 billion kg of bananas were produced globally. Many tropical and subtropical countries use this significant fruit as a major export as well as a staple cuisine (Drenth and Kema, 2021). Bananas are currently farmed on 4.84 million hectars of land in 150 countries throughout the world, yielding 95.6 million tons (Buvaneswari and Kannahi 2018). Commercial bananas, including hybrid varieties and commercial varieties of *M. acuminate* (carrying genome A) and

M. balbisiana (carrying genome B), are among the most significant banana species. Of these cultivars, the dwarf Cavendish cultivar is a member of the Cavendish group, which is the most widely grown due to its resistance to Panama disease and superior cold climate adaptation. With roughly 47% of the world's production going to the Cavendish variety, it is the most commercially available variety (Dale *et al*., 2017).

Ethiopia is one of the tropical nations with abundant arable land that is excellent for growing bananas. In terms of production and area covered (67,387 ha), bananas are mostly grown in traditional agricultural systems for domestic use and to supply local markets. In addition, bananas play a vital socioeconomic role in the country's rural communities to make money, guarantee access to food, and also provide work opportunities. Ethiopian farmers had long-standing experience planting and producing this fruit. But they didn't obtain plant materials that were free from contaminants (Asmare Dagnew *et al.*, 2012).

Bananas are typically propagated by suckers; however, this approach carries the risk of nematode, fungal and viral disease transmission to the regenerated plants (Bhaya and Salim, 2019). Asexual propagation techniques are the main way that bananas are produced because domesticated or cultivated banana varieties and hybrids are highly sterile and polyploid (Vendrame *et al*., 2022). Nonetheless, a number of disease , including banana bunchy top virus, burrowing nematodes, black sigatoka, fusarium wilt, and banana weevil borer, are linked to conventional propagation methods.

Plant tissue culture techniques have made significant progress in the last 20 years in producing large numbers of virus-free plantlets quickly and easily transportable between laboratories and fields (Bairwa *et al*., 2015). Thus, giving farmers with the desired planting materials is important through tissue culture for selected cultivars. Tissue culture, unlike sucker-derived planting material, has various advantages. These include producing uniform planting materials, clean, disease-free materials, and many plants in a small space. It offers mass propagation and clean planting material (Sujin *et al.*, 2020). They are cheaper to transport than conventional suckers, and the coupling with virus indexing allows for safe movement, exchange, and conservation of germplasm (Adugna Mosissa, 2021).

The use of biotechnological approaches, in particular tissue culture techniques, is the best option in order to tackle all the problems mentioned above with regard to banana crops. *In vitro* culture provides a means of rapidly producing large quantities of healthy, identical propagates for large-scale cultivation, eliminating diseases using shoot tip culture technology Adelegn Bogale, (2014). It is also allowing for large-scale production of crop plantlets within a short period of time and with limited space.

Shoot tip culture has several major applications that are appropriate for both germplasm conservation and the large-scale production of homogeneous; rapidly growing propagates for field establishment. Plant growth regulators are essential for the *in-vitro* regeneration of crops in any artificial medium. Auxins assist proliferating shoots in re-rooting, while cytokines are responsible for the regeneration of new shoots. The establishment, proliferation, and regeneration of shoots and roots in plants under in vitro conditions can vary significantly based on various factors such as genotype, explant type, composition of culture media, presence of plant growth regulators (PGR), and culture environment, even though there have been numerous reports on banana micro propagation through shoot tip culture (Justine *et al*., 2022).

Therefore, the purpose of this study is to enhance the protocol development and *in vitro* micropropagation process for two varieties of bananas, dwarf Cavendish and ducase. Based on their high production potential, these varieties were certified, registered, and released nationally by the Wabe Agricultural Research Centre (Zinabu Ambisa *et al*., 2019). Because the traditional method of propagating bananas takes a long time to produce a corm suitable for sucker multiplication, an in vitro propagation protocol must be developed for the quick and mass regeneration of this significant agronomic trait. In addition, it spreads disease easily and yields a small number of suckers per corm over an extended period of time (Kapadia and Patel 2021). Consequently, the goal of this experiment was to create the best possible protocol for the in vitro propagation of two varieties of banana in sterilization, media preparation, and concentration of plant growth hormone.

1.2. Statement of the Problem

Traditional vegetative methods of propagating bananas have a number of drawbacks, such as poor preservation of the original plant genetic material, low production, and a very slow rate of sucker multiplication. By propagating bananas through in vitro propagation (tissue culture), which provides clean planting material through mass propagation, the drawbacks associated with conventional propagation can be eliminated. The best option for producing a large quantity of banana planting material in a shorter amount of time, using less space, producing disease-free plants, and adequately preserving germplasm has been shown to be in vitro culture technology. Auxins and cytokines are important in *in vitro* culture because primarily it needed for plantlet growth and shoot formation, also concerned with root formation. Cytokinins, including BAP (benzyl aminopurine) and kinetin are known to reduce the shoot tip dominance and induce both auxiliary and adventitious shoot formation from meristematic explants in banana (Mohapatra and Deo, 2019). The goal of the current study was to optimize the protocols for the fast multiplication of Musa spp. in Murashige and Skoog medium supplemented with various plant growth regulators. Adsorbent and antioxidant were also utilized to prevent lethal browning. More investigation is needed into the in vitro regeneration of bananas using plant growth hormones in order to produce large quantities. Although there are numerous reports on in vitro propagation, the procedures are intricate, and numerous researchers have documented the regeneration of Musa spp. through micro propagation. In this study reported a very simple economical, rapidly multiplying and highly reproducible protocol for large scale micro propagation.

1.3. Objectives

1.3.1. General objectives

To develop *in vitro* micropropagation protocol of two bananas (musa spp.) using shoot-tip culture.

1.3.2. Specific objectives

- ✓ To determine the optimal concentration of sodium hypochlorite for sterilizing shoot tip explants.
- ✓ To determine the effects of different concentrations of antioxidants on initiated shoots.
- ✓ To determine the optimal concentration and combination of plant growth regulators for shoot multiplication.
- ✓ To evaluate the effectiveness of cost-effective, locally available solidifying agent, bulla, compared to agar.
- ✓ To optimize the acclimatization soil mixture to assess the survival rates of plantlets in the greenhouse.

1.4. Significance of the study

The study is also being valuable as baseline data for further research in the area of *in vitro* propagation using various tools. It has also economic, time-consuming, disease-free resource management and quality food value for the study area. In order to maximize production of banana *in- vitro* regeneration of using shoot tip culture is more efficient rather than using the conventional method of propagation through sucker of banana (Babu, 2019). Applying other efficient tools like, sterilization, least coast effective methods in media preparation and using antioxidant and adsorbents also used to standardize the quality production of Banana. The Banana variety dwarf Cavendish and ducase expect for disease free plants demanded development of an efficient protocol for *in- vitro* regeneration. In the present investigation, ascorbic acid used effective in inhibiting browning of shoot tip explant due to phenolic compounds. Each of the chemical sterility effective in reducing microbial contamination Addition of BAP was valuable to maximum number of shoots during multiplication. The survival of maximum plantlets also success in green house and promotes production independent of seasonal restriction for the farmers.

2. LITERATURE REVIEW

2.1. Origin, Taxonomy and Distribution of Banana

The domestication journey of Musa spp. was a complex process unfolding over millennia, marked by various stages occurring across different geographical locations and time periods. De Langhe's account in 1995 provides a succinct overview. Bananas were reportedly mentioned in Sanskrit texts as early as 500 BCE. It is believed that traders from Arabia, Persia, India, and Indonesia disseminated banana suckers along the coastal areas of the Indian Ocean, excluding Australia, between the 5th and 15th centuries.

During the 16th to 19th centuries, Portuguese and Spanish traders facilitated the exchange of banana suckers in tropical America, leading to their establishment in Latin America and the Caribbean through global trade. Presently, banana cultivation is widespread across tropical and subtropical regions in Asia, America, Africa, and Australia. Southeast Asia serves as the primary center of origin and diversity for bananas (Musa spp. Musaceae), while Africa harbors a secondary center (Hapsari *et al*., 2022). Bananas rank as the fourth most significant global crop, playing crucial roles in socio-economic development, health, and food security, particularly in developing nations (Molla, 2017). Their domestication stems from the intricate evolutionary history of wild bananas, notably originating from two wild diploids, *M. acuminata* (AA) and *M. balbisiana* (BB) (Singh *et al*., 2021). Among the various banana cultivars, the widely recognized and cherished Cavendish Banana stands out as the most prevalent worldwide, originating in Southeast Asia. Chances are the bananas you regularly purchase or include in your morning smoothies are the Cavendish variety. Despite their ubiquitous availability today, their roots trace back to Southeast Asia.

Plantains are a type of fruit that remains starchy even when fully mature. Bananas are categorized scientifically based on genome groups, which are determined by the combination of genes from two wild diploid species, as well as by ploidy. One genome, designated as A, originates from *Musa acuminata* Colla, native to western Melanesia and Southeast Asia, while the other, designated as B, comes from *Musa balbisiana* Colla, native to Southeast Asia and China (Hamilton *et al*., 2016).

The Cavendish Banana (Dwarf Cavendish AAA) is among the most popular sweet eating bananas globally. It features bright yellow skin, a smooth texture, and a flavorful taste. This variety typically starts fruiting within 9-15 months. Providing bananas with sufficient compost, fertilizer, and moisture is crucial for optimal growth. While much is known about the Cavendish Banana, including its origins and cultivation methods, there remain some uncertainties, such as its spread across five continents. Recent interdisciplinary studies combining archaeological, ethnographic, linguistic, and genetic data shed light on the domestication and dispersal histories of various banana cultivar groups (Lorenzo, 2022).

2.2.Botanical Description of Banana

Bananas and plantains are derived from giant herbaceous monocotyledonous plants belonging to the genus Musa. These plants typically grow up to 3 meters tall without any secondary thickening of the stems or lignification of the leaves (Kishor, 2017). The above-ground part of the plant is referred to as the pseudo stem, while the middle portions of the rhizome contain buds, also known as eyes. The base of the leaf sheaths tends to be somewhat enlarged (Subagyo and Chafidz, 2018). The dwarf Banana, also called ducase or Sugar Banana, is a smaller-fruited variety known for its exceptional taste and texture, boasting a sweet, aromatic flavor. When ripe, they turn bright yellow and become creamy in texture. These trees are well-suited for backyard cultivation, reaching a modest height of 2-3 meters. They also exhibit some resilience to cooler climates, although they should be protected from frost. Bananas have the capacity to flower and fruit throughout the year, contributing a tropical ambiance to any garden setting.

2.3. The Economic Value of Banana

With an annual production of over 115 million tons, bananas are among the most consumed foods in the world, only surpassed by wheat, maize, and rice (FAO, 2020). As a staple food for underprivileged communities in areas where cultivation is feasible, a significant amount of banana production is not included in official statistics. Bananas have been the focus of numerous studies, including botanical, genetic, historical, and economic ones, due to their large production and consumption (Wahome *et al*., 2021). Bananas are a major cash crop and contribute significantly to increased global food security (Esan *et al*., 2022). Bananas, which are grown all over the world and are estimated to produce 153 million tons annually, are the most traded fruit globally. They are members of the Musaceae family. Bananas are cultivated worldwide, with an estimated annual production of 153 million tones, making them the most traded fruit internationally (Voora *et al*., 2020). Major banana producers include India, China, Indonesia, Brazil, Ecuador, the Philippines, Guatemala, Colombia, and Angola, with India leading the pack with a production of 30.808 million metric tons (FAO, 2020). Particularly in developing nations, banana fruit is highly favored due to its affordability and high nutritional value (Justine *et al*., 2022). Food insecurity is a serious global problem that is, however, more challenging to address in Africa where several million people suffer from the burden of hunger and malnutrition (Olufemi, 2024).

Africa has one of the fastest-growing populations globally, which consequently puts much pressure on its food supply. The estimated cultivable land mass in Africa for possible banana production is estimated to be about 50 million hectares, with the majority of this land located in West and Central Africa. However only a small portion of this land is currently being utilized for banana cultivation, with the majority of bananas being produced on smallholder farms. Yet the banana sector in Africa remains chronically static, compared to other crops in Africa and compared to banana enterprises in other continents. The bulk of production is carried out by smallholders, who lack the means to cope with challenges such as declining soil fertility, pests and diseases (Akankwasa *et al,. 2020*).

Bananas are consistently available for sale throughout the year in both major and minor cities and towns across Ethiopia. The majority of marketed bananas originate from the southwestern region of the country, particularly in the Southern Nations, Nationalities, and Peoples' (SNNP) region. In other regions like Amhara, Oromiya, Tigray, Benishangul-Gumuz, and Gambella, banana cultivation primarily serves domestic consumption needs, often within traditional garden systems (Zinabu Ambisa *et al*., 2019). Nationally, about 14% of farming households engage in banana production, either for household consumption or for sale.

The highest concentration of banana-growing households is observed in the SNNP region, followed by Oromiya, Amhara, and Benishangul-Gumuz. Over the years, there has been a gradual increase in the land area allocated for banana cultivation, with an estimated national allocation of 28,102.5 hectares in 2006 (Asmare Dagnew *et al*., 2021). However, in major banana production areas, small-scale farmers often do not adhere to recommended research practices (Kubiriba *et al*., 2016). Utilizing tissue culture technology in banana production has shown significant efficiency, allowing for the timely production of clean planting materials within limited space. Consequently, tissue culture technology holds promise for enhancing banana yields (Wahome *et al*., 2021).

2.4. Nutritional Value of Banana

Vegetables and fruits play a vital role in maintaining a balanced diet, with fruits such as bananas offering numerous health benefits. Bananas contribute to the retention of essential nutrients like calcium, nitrogen, and phosphorus, which are crucial for the development and regeneration of strong tissues (Kuma *et al*., 2012). One notable advantage of bananas is their effectiveness in managing ulcerative colitis. They serve as one of the few fruits that ulcer sufferers can safely consume, as they help coat the stomach lining and reduce inflammation by balancing gastric acidity (Hailu, 2017). Additionally, bananas aid in the treatment and healing of painful intestinal ulcers and other conditions (Michael, 2017). Moreover, bananas are utilized for treating burns and wounds; applying mashed ripe banana pulp to affected areas can provide instant pain relief.

Despite the presence of potassium in various fruits, vegetables, and meats, a single banana provides approximately 23% of the daily potassium requirement. Potassium plays a crucial role in ensuring proper muscle function and preventing spasms, making it beneficial for overall muscle health. Furthermore, several studies suggest potential additional health benefits associated with potassium intake (Robert Bell, 2023).

Bananas are consumed in various forms, either raw as a dessert or as a staple food through cooking or processing into products like juice, puree, chips, drinks, sausages, and flour (Singh *et al.*, 2016). Different parts of the banana plant, including the rhizome, pseudo stem, leaf, flower, and fruit, have been utilized in traditional medicine systems for treating a wide range of ailments such as ulcers, stomach disorders, burns, wounds, diarrhea, arthritis, anemia, kidney stones, migraine headaches, hemorrhages, epilepsy, and neurodegenerative diseases (Ranjha *et al.*, 2020). Banana pulp contains various bioactive compounds, including phenolic acids and flavonoids, known for their high antioxidant properties, anti-tumor activity, anti-depressant effects, antibacterial properties, anti-hypertensive effects, anti-ulcer effects, prevention of kidney stones, laxative effects, and anti-helminthic properties.

2.5. Plant Tissue Culture

Plant tissue culture is a method wherein plant tissue fragments, known as explants, are cultivated in a controlled environment on a sterile nutrient medium, allowing for cell multiplication and plant regeneration (Sudheer *et al.*, 2022; Hasnain *et al.*, 2022). This technique, also referred to as in vitro culture, is rooted in the cell theory proposed by Schwann and Schleiden in 1838, as well as the concepts introduced by Gottlieb Haberlandt in the early 20th century (Tang et al., 2021).

In vitro plant tissue culture exploits the concept of totipotency which refers to the potential of any plant cell to undergo dedifferentiation and re-differentiation, ultimately leading to the formation of organized tissues, structures, and complete organisms (Fehér , 2019). The in vitro culture of plants under controlled conditions offers the advantage of efficient screening for desirable characteristics, as external environmental influences can be minimized (Yapa and Bandaralage, 2023). Various tissue culture techniques have been developed for bananas, including shoot and meristem culture, callus culture, somatic embryogenesis, cell suspension, and protoplast cultures. While commercially produced tissue-cultured banana seedlings may not always be readily available, larger-scale banana producers may opt to establish their own tissue-culture facilities to ensure the availability of disease-free seedlings for replanting (destroying).

Micropropagation typically involves five stages: preparation of mother stock, initiation of cultures, shoot multiplication, rooting of in-vitro grown shoots, and acclimatization. Explanation of actively growing shoots is a common practice for large-scale multiplication, with shoot initiation and multiplication being crucial stages facilitated by cytokinins, a type of plant growth regulator. Subsequent rooting of elongated shoots can occurred either ex-vitro or in-vitro, using auxins. Acclimatizing in-vitro grown plants is a crucial step in the micropropagation process (Murthy, 2023). In Ethiopia, both private and public sectors have limited protocols for plant micropropagation to produce disease-free plants (Yemisrach Melkie *et al*., 2021). Case studies suggest there are approximately fifty in vitro protocols utilized across all plant micropropagation laboratories in the country. However, these protocols require further optimization to improve efficiency and reduce associated costs, particularly concerning the hardening of in vitro derived plantlets. Achieving this optimization demands well-equipped laboratories staffed with competent personnel.

2.6. Micro propagation Method of Banana

Plantains and bananas are mostly propagated by vegetative division; seeds are hardly ever used and are usually saved for wild species, ornamental varieties, and banana breeding (Nelson *et al*., 2017). Sword suckers are used in vegetative propagation to produce about 90% of the total output. Peepers, sword suckers, maiden suckers, water suckers, and bull head corms are some of the developmental stages these suckers go through and can all be used as planting materials. However, because they develop more slowly than other varieties, peepers are not advised for starting banana plantations (Suryanarayana *et al*., 2018). According to Josiane and Karemera (2015), there are three primary techniques for propagation: tissue culture propagation, macro propagation of plantlets, and traditional propagation with suckers. Even though tissue culture can yield a large number of uniform, disease-free plantlets, its high capital cost, need for skilled labor, and overall expense often make it unfeasible for smallholder growers in Africa (Opata, 2020). The adoption rates of advanced propagation methods in banana production are still low, despite the significance of improving agricultural productivity and attaining sustainability (Wahome et al., 2021). Because of this, planting materials for smallholder farms and small-to medium-sized businesses in Ethiopia and other developing nations are frequently obtained through the natural regeneration of banana suckers. But this process is laborious and frequently results in insufficient amounts of high-quality, nutritious planting material (Pyabalo *et al*., 2021). Because macro propagation is easy to multiply, has low production costs, and can yield 50–60 shoots per corm in 4-5 months, it is a more practical method for creating affordable, high-quality planting materials. It provides a straightforward, affordable, and comparatively quick method for Musa species vegetative multiplication; it is especially appropriate for small- and medium-sized farmers who are less skilled and have lower incomes (Hussen Muhie and Seid Teshome, 2023).

2.7. Application of Plant Tissue Culture

In the case of plants, it is a combination of reintroduction and *in vitro* propagation, which are currently considered as the most effective methods for preserving the genetic resources of species threatened by extinction (Parzymies *et al*, .2023). Micro-reproduction allows one to obtain new, healthy plants that origin from the natural resources of the species, even if they are already on the verge of dying out (Schäfer *et al*, .2020) .Ex situ conservation with the use of biotechnological techniques requires one to establish methods for explants disinfection, tissue culture initiation, multiplication, rooting, acclimatization of the plant species, and evaluation of its genetic stability (Coelho *et al*, .2020). The tissue culture method breaks tradition, in that, there is no soil or sunshine needed to initiate production and growth of the cells. In the wild and in agriculture, there are many confounding variables that can impact plant growth and compound production. (Thacker *et al*, .2018).

Plant tissue culture is one method to ensure the least amount of confounding variables for plant growth. In addition, this in vitro approach is the quickest and most reliable way to preserve a germ line that has been bred for the production of specific compounds. The process involves aseptic cultures, exemplified through thorough sterilization of plant tissue, a nutrient medium containing mineral salts, sugars and hormones for the explant tissue to thrive, and the production of callus as the explant utilizes the nutrients in the medium(Abdalla *et al*, .2022).

2.8. Tissue Culture Medium Preparation

The growth, development, and response of an explant in culture are influenced by its genetic makeup, environmental conditions, and the composition of the culture medium. The selection of the appropriate culture medium is crucial for the success of a plant tissue culture experiment. A culture medium consists of a comprehensive blend of nutrients and growth regulators (Chimdessa, 2020). The composition of the culture medium significantly impacts the growth and morphogenesis of plant tissues. Essential components of most plant tissue culture media include mineral salts, sugars as a carbon source, and water. Additionally, organic supplements, growth regulators, and a gelling agent may be included (Sudheer *et al*., 2022). As per the International Association for Plant Physiology, elements present in concentrations exceeding 0.5 mM.l-1 are categorized as macroelements, while those required in concentrations below 0.5 mM.l-1 are considered microelements (Georg and Manue, 2013). It's important to note that the optimal concentration of each nutrient varies among species to achieve maximum growth rates.

2.9. Surface Sterilization of Explants

Sterilization is a crucial process to rid explants of contaminants before establishing in vitro cultures. Various sterilizing agents are employed for this purpose, but they can also be harmful to plant tissues. Thus, it's essential to standardize the concentration, exposure duration, and sequence of sterilants to minimize explant injury and enhance survival rates (Goswami, 2013).

Traditionally, banana plants were propagated via suckers, limited to 5-15 per plant throughout their lifespan. This practice often resulted in yield fluctuations, virus susceptibility, inherent pathogens, and somaclonal variations, leading to decreased revenue for farmers (Kapadia and Patel, 2021). A critical step in aseptic inoculation is optimizing decontamination treatments tailored to different plant species due to their diverse cell wall compositions. Sensitivity to sterilants, exposure time, and concentration significantly influence the success of decontamination treatments. Despite efforts worldwide to eliminate contamination through various chemical treatments, micro propagation still experiences 10-15% plant losses after each subculture cycle. ..Calcium and sodium hypochlorite are two frequently used surface sterilants in plant micropropagation; calcium is kinder to tissues than sodium (Eriksson, 2022). However, the reaction of calcium hypochlorite with atmospheric carbon dioxide makes it unstable. Generally, an emulsifier like Tween-20 is added to a diluted solution of sodium hypochlorite (0.25–2.63%) for optimal results. Ethanol and sodium hypochlorite are commonly used as sterilizing agents in Ethiopian plant studies. While these parameters are not consistently correlated, sodium hypochlorite concentrations range from 1 to 20% w/v and exposure times from 5 to 20 minutes. It is essential to completely remove hypochlorite from tissues in order to avoid interfering with the absorption and metabolism of amino acids. However, there's a lack of reports testing for residual hypochlorite after water washing, underscoring the necessity for guidelines on residual sterilant levels before explant inoculation.

2.10. Effects of Adsorbent and Antioxidant on Explant

Antioxidants are chemical compounds that can delay or slow down lipid oxidation in food systems, counteracting oxidation reactions initiated by oxygen or peroxides (Gupta, 2015), which are also known as reactive oxygen species (ROS) (Dontha, 2016). Studies comparing callus extract to *in vivo* plant parts have shown varying antioxidant properties and polyphenolic content, indicating that callus extract may exhibit higher or lower antioxidant activity than its source explants (Upadhyay, 2013). The browning of explants, known as phenolic browning, occurs naturally due to enzymatic oxidation of polyphenolic compounds, which can inhibit plant tissue culture (PTC). To promote cell growth and development in tissue culture medium, antioxidants are often used (Rajani *et al*., 2019). These antioxidants play a crucial role in absorbing inhibitory chemicals in the culture medium, reducing the accumulation of harmful metabolites, phenolic exudation, and other exudates (Thomas, 2008). They can also neutralize oxygen radicals generated during tissue damage, protecting cells from oxidative damage. Therefore, when establishing a standard tissue culture procedure for mass reproduction of any plant species, the inclusion of antioxidant growth regulators is essential (Dayarani *et al*., 2013). Activated charcoal is a critical ingredient in plant tissue culture medium, as it adsorbs harmful substances like polyphenols produced by injured tissues, thereby inhibiting tissue and medium browning.

2.11. The Role of Auxins and Cytokines on Plant Tissue Culture

Plant growth regulators (PGRs) are chemical compounds utilized in plant tissue culture mediums for their specific functions (Pacheco *et al*., 2016). These PGRs play a crucial role in organogenesis, with various types available in nature and synthetic ones added to artificial culture mediums for this purpose (Sudheer *et al*., 2022). Major classes of PGRs include auxins, cytokinins, gibberellins, abscisic acid, and ethylene. The specific response regarding organogenesis, unique to each species, is defined by the ratio of auxins to cytokinins incorporated into the medium.

Micropropagation shoot and root proliferation relies on the totipotency potential, yet studies have consistently shown metabolic enhancement with the inclusion of plant growth regulators such as cytokinins and auxins (Kidasi *et al*., 2023). Cytokinins like 6-Benzylaminopurine (BAP) stimulate shoot growth and elongation by breaking axillary and bud dormancy, mechanistically triggering cell division and lateral bud development. Similarly, auxins like 1-Naphthalene acetic acid (NAA) have been demonstrated in many studies to significantly improve rooting initiation, growth, and development (Kang *et al*., 2018).

2.12. Shoot Initiation

Banana shoot cultures typically originate from plant parts containing a shoot meristem, like the parental pseudo stem, small suckers, peepers, and lateral buds (Thriveni et al., 2021). It's essential to choose explant material from mature plants with well-understood responses to environmental factors and identified quality traits influenced by both genetics and environment. Meristem cultures may encounter challenges such as higher mortality rates and slower initial growth. The explant is directly placed on a culture medium suitable for multiplication. For banana micropropagation, MS-based media are commonly used, supplemented with sucrose as a carbon source at concentrations of 30–40 g/l (Murashige & Skoog, 1962) (Sugandh Suman, 2017). Banana tissue cultures often suffer from excessive blackening due to the oxidation of polyphenolic compounds released from wounded tissues. These unwanted exudates form a barrier around the tissue, hindering nutrient uptake and growth. Therefore, during the initial 4–6 weeks, fresh shoot-tips are transferred to new medium every 1–2 weeks. Alternatively, freshly initiated cultures can be kept in complete darkness for one week.

2.13. Multiplication of Shoot-Tip Cultures

Shoot multiplication is a critical stage in developing an effective micro propagation protocol, directly impacting its success (Nowa *et al*., 2022). Multiple shoot and bud formation is encouraged by adding relatively high concentrations of cytokinins to the medium. In banana, BA (6-Benzylaminopurine) is the preferred cytokinin, typically included at concentrations ranging from 0.1 to 20 mg/l (Nadra *et al*., 2015). For propagule multiplication, the same medium used for initiating shoot cultures is employed, typically containing 2.25 mg/l BA and 0.175 mg/l IAA. However, if highly proliferating meristem cultures are required, a tenfold higher concentration of BA is used, such as 22.5 mg/l BA and 0.175 mg/l IAA in the medium (Ngomuo *et al*., 2013). It's important to note that excessively high concentrations of BA can negatively impact both the multiplication rate and the morphology of the culture and should be avoided. The rate of multiplication is affected by both the concentration of cytokines and the genotype. Usually, shoot tips of cultivars with only a genomes generate 2–4 new shoots, whereas those with AA or AB genomes produce clusters of multiple shoots and buds during each subculture cycle. Approximately 6–12 weeks after initiating culture, new axillary and adventitious shoots may arise directly from the shoot-tip explant. These clusters can be divided, pruned, and then subculture at intervals of 4–6 weeks.

2.14. Rooting of Regenerated Plantlets

Auxins, in combination with other growth regulators such as gibberellins, are pivotal in regulating the growth and differentiation of cultured cells and tissues. For instance, the auxin naphthalene acetic acid (NAA) has been noted for its ability to stimulate plant rooting in vitro (Hussein, 2012). When individual shoots or shoot clumps are transferred to a nutrient medium designed to encourage root formation without promoting further shoot proliferation, the choice of auxin becomes critical, especially for woody plants which often struggle with *in vitro* rooting. It's been observed that the optimal auxin for rooting varies depending on the plant species. To facilitate root formation, the concentration of cytokines in the regeneration medium is significantly reduced or even eliminated entirely (Subban *et al*., 2021). Within approximately two weeks, shoot tips develop into unrooted shoots under these conditions.

2.15. Acclimatization

Acclimatization refers to the process of adapting plantlets to a new environment. When plantlets or shoots are initially grown in culture vessels, they experience a micro-environment that differs from their eventual growth conditions. The goal during acclimatization is to customize this micro-environment to minimize stress and provide optimal conditions for growth and multiplication (Donnelly and Tis, 2015). Traditionally, the acclimatization process involves gradually transitioning transplants from the culture environment to ambient relative humidity and light levels outside the laboratory. This transitional period allows the plants to develop anatomical characteristics and physiological functions that are independent of the in vitro culture conditions (Kumar and Rao, 2012). Fully rooted plants from in vitro cultures are selected for planting. Initially, they are placed in a mist chamber to acclimatize to the climate outside the lab. After 2-3 weeks, they are transferred to a greenhouse to further adjust to field conditions (Jaime *et al*., 2017). Eventually, the plants are planted in potting mixture and then transferred to soil within a plastic cover after 5-6 days. After 2-3 months, the plants are ready for field planting (Joy and Jose, 2013). Effective acclimatization of plantlets propagated in vitro is essential for a smooth transition from laboratory conditions to natural settings (Khatik and Joshi, 2016). Several factors influence this process, including the composition of the potting mixture, plant genotype, temperature, humidity, and environmental conditions (Twaij *et al*., 2020). Various soil mixtures incorporating different proportions of vermiculite, peat moss, perlite, sand, and vermicompost are employed to ensure the successful acclimatization of banana cultivars.

3. MATERIAL AND METHODS

3.1. Description of the study area

The study was conducted from April 2023 –January 2024 at Wolkite University; Plant cell and Tissue Culture Laboratory of the Department of Biotechnology. Wolkite University is located in Gubrye town, Wolkite, Ethiopia. Wolkite is located 158km South West of Addis Ababa. It is found at latitude of 8^0 17N37^0 47E, a longitude of 8.283N37.7830E and an elevation of between 1910-1935m above sea level (Wikipedia: Wolkite). The average annual minimum and maximum temperatures and rainfall ranged from 18°C to 39°C and 450 to 820mm, respectively (Climate: Wolkite from: (Climate-data.org).Mother plant dwarf Cavendish and ducase banana varieties taken from wabe research center (Figure 1).

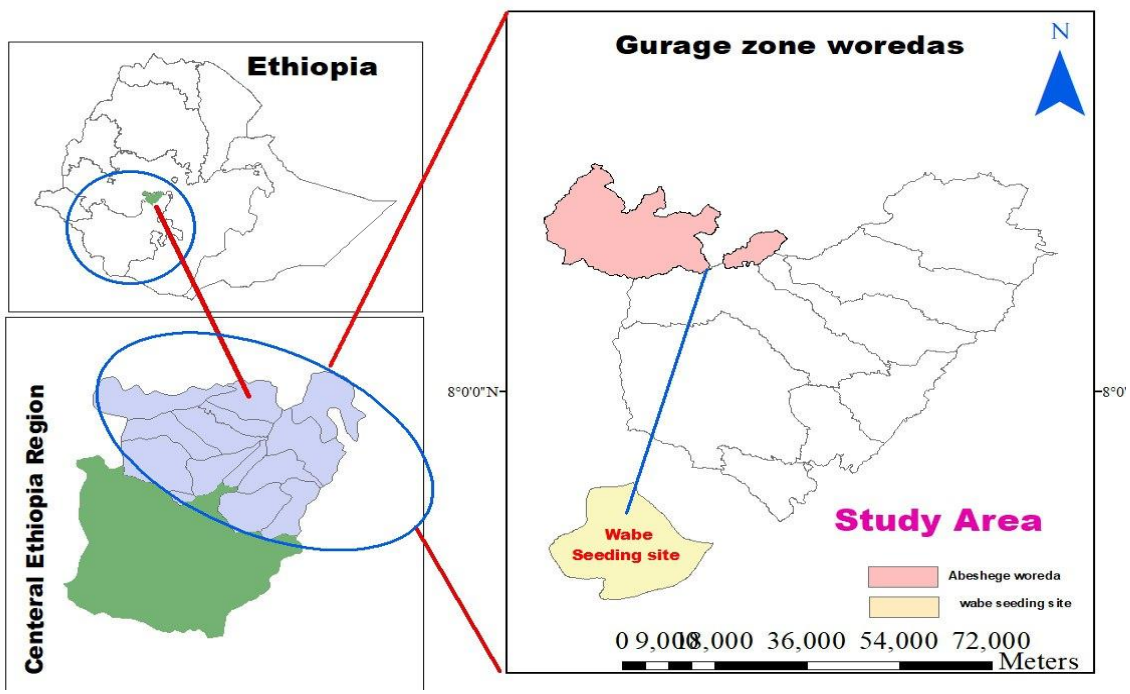

Figure 1. Map of study site

3.2. Plant Material Source

For this study, Banana (*Musa spp*.) was employed as a plant source. A popular banana variety Dwarf Cavendish (high yielding commercial cultivar) and ducase was selected. These banana varieties are improved and released internationally on their production potential, environmental adaptation performance and fruiting ability .For present investigation a 4 month of mother plant were obtained from the Wabe research center. After collecting the mother plant, was brought to greenhouse (Figure 2). After growing a healthy and vigorous plant material in green house about 1 month, the shoot tip of newly sprouted, were used as source of explants (Figure 2c). A micro- propagation protocol optimization study of the banana was carried out in the Wolkite university tissue culture laboratory, Department of Biotechnology.

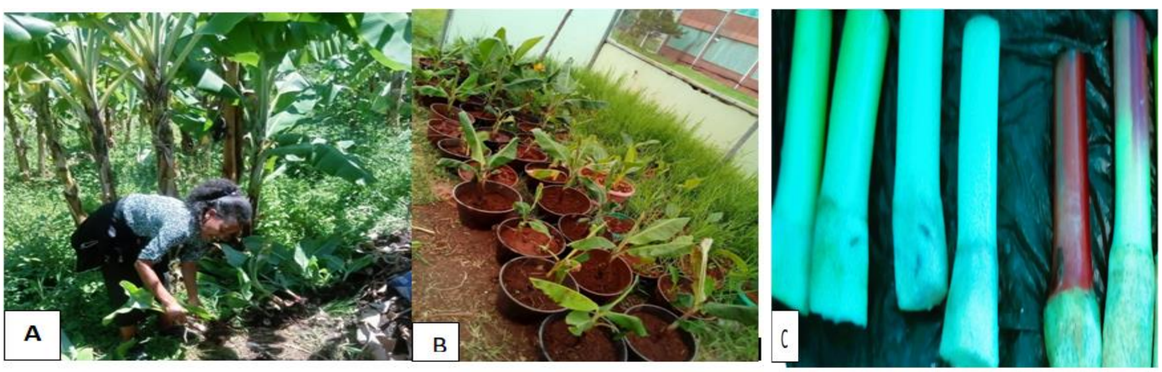

Figure 2. Mother plant source: (A) mother plant collection in the research center, (B) mother plant in greenhouse, and (C) prepared shoot tips of the two banana varieties.

3.3. Stock Solutions and Media Preparation

3.3.1. Preparation of MS stock solutions

For this study, Murashige and Skoog (1962) MS media were used along with the proper type and concentration of plant growth regulators. A stock solution for each of the MS components was weighed in recommended amounts and completely dissolved in distilled water. The stock solutions of MS nutrients, vitamins, and amino acids were prepared fresh every month. For the preparation of the iron stock solution, FeSO4 .7H2O and Na2.EDTA were dissolved in hot distilled water separately. After dissolving, the iron sulfate solution was added to the Na2.EDTA solution in a half-liter beaker. To avoid the problem of precipitation, the solution was protected from light by storing it in bottles wrapped with aluminum foil. Then, the stock solutions were labeled with the date of preparation, name of the stock solutions; quantities used, and stored in a refrigerator at 4 ºC temperatures.

Plant growth regulators preparation stock solutions, used in this experiment such as 6-benzyl amino-purine (BAP), kinetin (KN), α-Naphthalene acetic acid (NAA) were prepared separately. The powder of these hormones was weighed based on the quantity required and dissolved in 1N NaOH and/or 1N HCl. Then, the hormones were dissolved in distilled water at a concentration of 1mg/ml. The volume was adjusted by adding distilled water. These growth regulators stock solutions were labeled with the date of preparation and name of plant growth hormones and stored in a refrigerator at 4 ºC until use.

3.3.2. Medium Preparation

After preparing the MS and plant growth regulators (PGRs) stock solutions, the media were supplemented with sucrose and a gelling agent, and the concentration of ascorbic acid was 25g/l and 50g/l, while activated charcoal was added at 1 g/l and 2g/l solutions as required by the procedure According to (Akhtar *et al*., 2016) ascorbic acid and activated charcoal were added independently to the media. The volume was then adjusted by adding distilled water. The pH of the culture medium was adjusted to 5.8 using 1N NaOH and/or 1N HCl before adding agar. For preparing solid medium, 8g/l of agar and 80g/l of local bulla were added as optional additives to agar, reaching the final volumes, and then the medium was heated in a microwave oven to melt the agar and bulla. The mixture of ‘bulla’ with medium was made carefully since it precipitates at the bottom and forms aggregates. So, ‘bulla’ powder was first mixed with the medium and heated on hotplate with continuous agitation followed by dispensing of the media in culture jars; and after melting the agar, 60 ml of hot medium was poured into pre-cleaned glass culture bottles. The culture bottles with nutrient media were autoclaved at a pressure of 15 lbs/sq. inch (121ºC) for 20 min. After sterilization, the medium was kept in the media storeroom for 4-5 days to ensure that it was free from contamination.

3.4. Explants Preparation and Sterilization

In this study a Simple step of aseptic inoculation were used for sterilizing of banana explant, due to optimal protocol development for effective sterilization for this food source plants. The explant was thoroughly washed under tap water to remove any impurities. Using a sharp knife, the roots and outer tissues of the shoot tip were carefully removed. After removing the outer sheaths of the suckers, the explants were cut into pieces measuring 3 to 5cm in length. These suckers were then washed with a mixture of running tap water and common liquid soap for 30 minutes treated with 3 drops of Tween-20 (a surfactant) .The explants were then rinsed with 70% ethanol for 1 minute, followed by immersion in a 5% sodium hypochlorite solution for 5 minutes. They were thoroughly washed with distilled water five times to ensure complete removal of any residual chemicals. Then, they were kept in the laminar flow chamber.

The next sterilization step was carried out in the laminar flow hood using a 70% ethanol solution for 5 minutes, followed by rinsing three times with sterile distilled water. The explants were further sterilized using varying concentrations (1%, 2%, and 5%) of sodium hypochlorite solutions containing 2-3 drops of Tween 20 (a wetting agent) for 20 minutes (Kahia *et al*., 2015). Afterward, the explants were rinsed in four changes of sterile distilled water to remove any traces of the sterilizing agent. Following these treatments, the explants were allowed to dry for five minutes, and the outer surface of the explant that had been exposed to the sterilizing agent was removed and trimmed to dimension of 3cm in length and 2cm in width using sterilized scapel and forceps. Care was taken to ensure that the tissue enclosing the meristem was not completely severed. Finally, the explants were cultured on the media under evaluation and effects of contamination were assessed.

3.5. Shoot Initiation

After the explants were sterilized, they were cut vertically into two pieces and planted on shoot initiation medium. The shoot initiation medium consisted of MS basal medium supplemented with 30 g/l sucrose, 1 g/l and 2g/active charcoal, 8 g/l agar, 1.5mg/l of BAP, 0.5mg/l of NAA, 25 mg/l and 50 mg/l of ascorbic acid and medium contained growth hormone and without antioxidant was used as to control. The experiment was conducted with three replications. The jars were sealed with cling film and incubated at room temperature in a dark growth room for two successive weeks. According to (Koda, 2021) Subsequently, the cultures were transferred to light conditions at 25 ± 2°C with a 16-hour photoperiod of white fluorescent light at an intensity of 20 µmol/m2/s for 2 weeks. The well-initiated and survived explants on artificial nutrient media during the initiation stage were used for this experiment after the residual effect of hormones used during initiation was avoided by growing the initiated shoots on basal medium free from PGRs for 15 days. They were then sub-cultured to achieve more shoot multiplication.

3.6. Shoot Multiplication

The initiated shoot explants were transferred to shoot multiplication media, which consisted of MS basal media including vitamins and amino acids with 30 g/l sucrose, 8 g/l agar, 1 g/l activated charcoal, and 25 mg/l ascorbic acid. This medium was supplemented with 0.0, 2, 2.5, and 3 mg/l of BAP alone or in combination with 0.5 mg/l of kinetin with 3 replication and one initiated shoot per treatment unit. MS media having antioxidant and without plant growth regulators was used as a control in the multiplication experiments. The initiated cultures were then incubated on the multiplication media for four weeks at 25 ± 2°C with a 16-hour photoperiod of white fluorescent light at an intensity of 20 µmol/m2/s. They were randomly arranged in the lab during this period. After four weeks, the multiplied shoots were cultured for two weeks on PGRs-free MS medium before being transferred to root induction and development medium. This step was taken to prevent the carryover effect of shoot multiplication hormones.

3.7. Rooting Induction

For root induction, about 5 to 8 cm of well-initiated shoots were cultured on Half-strength MS medium consisting of 30 g/l sucrose, 1 g/l Activated Charcoal, 8 g/l agar, supplemented with 0.0, 0.5, 1.0 mg/l IAA alone and combined with 0.5 mg/l of IBA. A jar (experimental unit) contained one explants, making a total of 3 explants per treatment. The experiment was laid out in Completely Randomized Design (CRD), with three replications were used .Half-strength MS medium without hormone was used as a control. The cultures were maintained in a growth room for a month at a temperature of 25± 2°C and 16- hour photoperiods provided with the white florescent tube. Each treatment contained six explants within three replication and treatments were randomly arranged in the lab.

3.8. Acclimatization

Well-regenerated plantlets with shoots, roots, and leaves were gently removed from the culture jars, and the roots were washed in tap water to remove traces of agar. The plantlets were then transplanted into cell trays filled with different sterile soil types: red soil compost and sand mixture at a 2:1:1 ratio. Each acclimatization cell tray with a plantlet was covered with white plastic with a hole to maintain humidity and kept in the greenhouse. After 14 days, the plastic cover was removed, and the plantlets were transferred into plastic pots for further hardening. From two varieties, 16 plantlets were transplanted onto each acclimatization medium mixture. After 4 weeks of acclimatization, data were collected on the percentage of survived plantlets, height of the survived plantlets, and number of leaves per plantlet from each pot. The mean values of each parameter were computed.

3.9. Data Collection

Percentage of explants that were contamination-free and survived the percentage of explants that were survived at the initiation stage due to the presence of adsorbents and antioxidants in the media. The number of shoots per explant, and the mean was computed for each replication. The length of shoots, and the number of leaves per shoot, and the mean was computed for each replication. The number of roots per shoot, and the mean was computed for each replication. The length of roots was measured and root length recorded. The total number of acclimatized plantlets was counted and converted into a percentage after four weeks of greenhouse hardening.

3.10. Experimental Design and Data Analysis

The experimental design for this study was completely randomized design (CRD) as factorial arrangement by three replications. Data recorded were subjected to ANOVA and significant differences among treatments were determined at 5% level of significance by using SAS software packages (version 9.3) and significant differences among mean values were compared using Least Significant Difference (LSD) at alpha level of 0.05.for the differentiation of the effect of treatment, Varity and treatment-Varity interaction.

4. RESULTS

4.1. Sterilization of the Explants

The analysis of variance showed that the main effect of the concentration of sodium hypochlorite solution had a very highly significant effect on the culture survival effect ($P \leq 0.0001$). However, the main effect of varieties and the interaction effect of the two factors had no significant effect on the parameters (Appendix Table2).

The interactive effect of banana varieties and different concentrations of NaOCl solutions (1%, 2%, and 5%) on the same time durations of explant exposure to the sterilants was evaluated to determine the most effective treatment for sterilizing the shoot tips of banana varieties. The results obtained showed that the concentration of 2% in 20 minute NaOCl solution and 10 minutes in 70% ethanol exposure duration was suitable for shoot tip culture (Figure 3).

Regarding the level of percentage, the analysis of variance test revealed that the main effect of varieties had a very highly significant ($p<0.0001$) effect on it, whereas it was not significantly affected by the main effect of different concentrations of NaOCl solution. The interaction of varieties with different concentrations of NaOCl solution had a significant ($p<0.05$) effect on this parameter (Appendix Table 2).The maximum production of contamination- free survived culture (85.0±0.0%) was obtained by 2% NaOCl for both the two varieties, followed by 66.60 ± 0.00 and 55.50 ± 19.23 at 5% for dwarf Cavendish cultivars and 1% for ducase cultivars, respectively. The minimum 33.30 ± 0.00 survival rates was obtained at 1% NaOCl concentration compared to control (Table 1).

Table 1: Effect of NaOCl concentration on explants survival percentage

Varieties	Concentration of NaOCl % and 70% ethanol	Explants immersion time(min) in NaOCl and ethanol	Mean No of Live and Clean explants	Percentage of Live and Clean explants (%)(mean±SD
Ducase	0	20 min and 5min	0.00	0.00 ± 0.00^{d}
	1		1.33	55.50 ± 19.23^{b}
	2		2.55	**85.00 ± 0.00^{a}**
	5		2.16	66.60 ± 0.00^{b}
Dwarf Cavendish	0	20min and 5min	0.00	0.00 ± 0.00^{d}
	1		1.33	33.30 ± 0.00^{c}
	2		2.55	**85.00 ± 0.00^{a}**
	5		2.16	66.60 ± 0.00^{b}
DF				23
LSD (5%)				11.78
CV (%)				13.91
ISL				*

CV; Coefficient of variation, SD; Standard Deviation, LSD; Least Significant Difference; ISL= Interaction Significant Levels, DF= Degree of freedom; * = Significant; in a column, means followed by the same letter are not significantly different at the 5% level by Fisher's LSD.

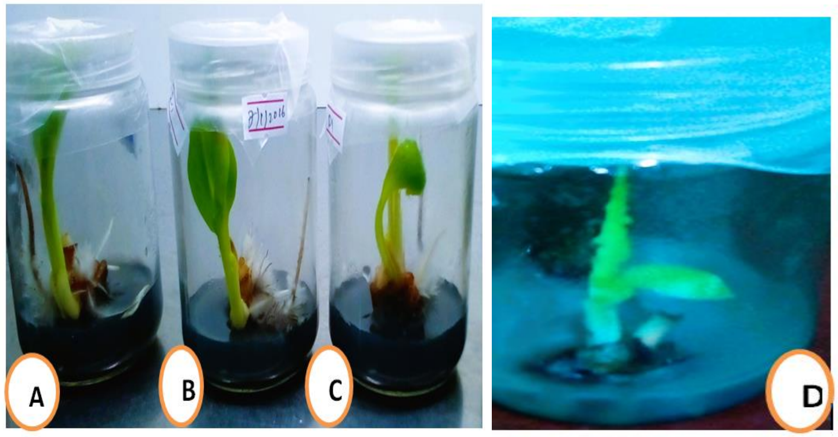

Figure 3. Response of survived culture with different concentration of NaOCl: (A) 1% sodium hypochlorite (B) 2% and (C) 5% with the same exposure time (D) contaminated explant with control.

4.2. Shoot Initiation

Data regarding plant shoot length of the two banana varieties (Appendix Table 3) exhibited that all antioxidant had a significant positive effect ($P \leq 0.0001$) on the shoot height compared to the control. However, interaction effects of varieties and antioxidant on shoot length had no significant effect ($p > 0.05$) on the parameter. The optimum concentration of antioxidants was used for control of the browning of the explant four different concentrations of antioxidants (25mg ascorbic acid, 50mg ascorbic acid, 1 g activated charcoal, 2 g activated charcoal) in the culture medium to obtain maximum survival production. Data regarding the plant shoot length of bananas (Table 3) exhibited that all antioxidant concentrations had a positive effect on the shoot height. Among the treatments, the control group resulted in the maximum increase in shoot height, reaching 3.75 ± 0.54. Additionally, the antioxidants 50 mg/l AA and 25 mg/l AA exhibited significant increases in shoot height, with values of 3.02 ± 0.45cm and 2.77 ± 0.28 cm, respectively. Notably, the 2 g/l AC treatment resulted in a decrease in shoot height, with a 2.47 ± 0.45cm shoot length (Table 2).

Table 2: Effect of concentrations of antioxidants on shoot length.

Antioxidants	Conc. of Hormones		SL (Mean ± SD)
	IBA (mg/l)	NAA (mg/l)	
Control (AA&AC free)	1.5	0.5	**3.75 ± 0.54[a]**
25 mg/l AA	1.5	0.5	2.77 ± 0.28[b]
50 mg/l AA	1.5	0.5	**3.02 ± 0.45[b]**
1 g/l AC	1.5	0.5	2.65 ± 0.40[b]
2 g/l AC	1.5	0.5	2.47 ± 0.45[b]
DF			29
LSD (5%)			0.55
CV (%)			15.68
Significant level			**

CV; Coefficient of variation, SD; Standard Deviation, LSD; Least Significant Difference; DF= Degree of freedom; ISL= Interaction Significant Levels, ** = Highly significant; In a column, means followed by the same letter are not significantly different at the 5% level by Fisher's LSD.

The data revealed that antioxidants showed a significant increase in plant survival percentage compared to their respective controls (figure 4). The maximum production was observed after 4 weeks of survival: 85% by the 2 g AC at the ducase cultivar, followed by 80% by dwarf Cavendish at the same treatments, and also the 1g AC and 50mg AA, which produced a higher survival rate of 55% and 50%, respectively. On the other hand, 1g AC, 50mg AA, and 25mg AA obtained a higher survival rate of 65%, 65%, and 50% at dwarf Cavendish cultivars. The minimum value of 40% was obtained on the ducase from the treatment of 25mg AA compared to the control. The use of 2g AC and 50mg AA as anti-browning agents in the medium for banana explants was suitable for shoot tip culture (figure 5).

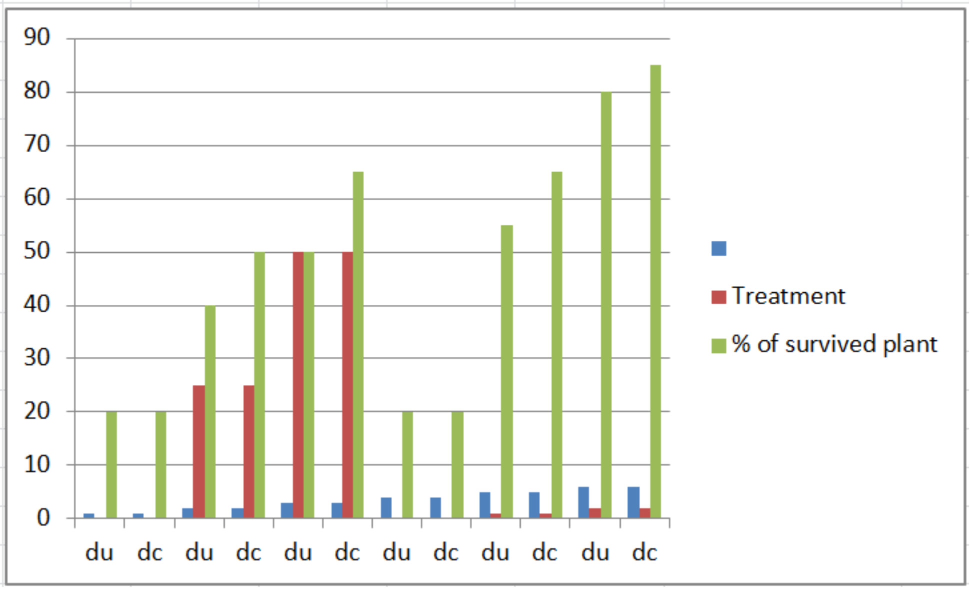

Figure 4. The effectiveness of ascorbic acid and activated charcoal in reducing lethal browning on degree of explant survived percentage

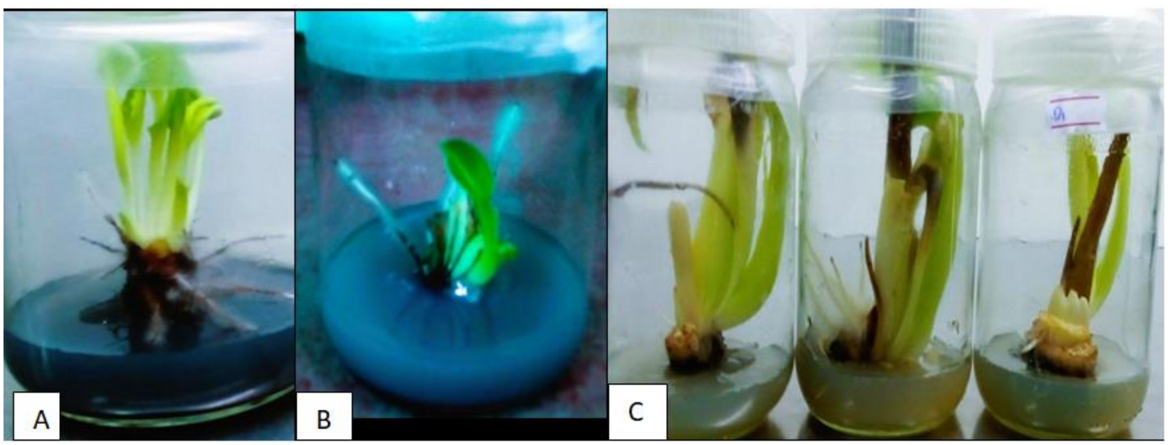

Figure 5. The influence of ascorbic acid and activated charcoal on morphological features of banana in tissue culture: (A) survived explant due to application of 50mg AA (B) survived explant with 2g AC (C) blanking and browning of plant and medium with control group.

4.3. Effect of BAP Alone and Combination with Kin on Shoot Multiplication

In this study, the significance of BAP and its interaction with kin were considered. The ANOVA showed that the concentration of BAP both alone and in combination with kin had a highly significant effect ($P < 0.0001$) on shoot multiplication rate (Appendix Table 4). Analysis of variance showed that hormones, varieties, and their interactions had a highly significant ($p< 0.0001$) effect on the number of shoot, shoots length, and number of leaves.

The study findings indicated that there was a notable variance in the mean values of all parameters across the various treatments for both cultivars. (Table 3). At the ducase cultivar, the maximum number of shoots (8.00 ± 0.00) was obtained on MS medium containing 2.5 mg/l BAP alone, and the minimum number of shoots (5.00 ± 2.00) at 3 mg/l BAP,and 2 mg/l BAP was obtained as compared to the control (2.33 ± 0.57) (Table 3).However, at the dwarf Cavendish cultivar, the maximum number of shoots was 8.33 ± 0.57 on MS medium containing 3 mg/l BAP and 0.5 kin, and the minimum number of shoots was 2.67 ± 0.57 at 0.5 kin alone. Another value also revealed 7.33 ± 0.57 at 3.0 mg/L BAP and 0.5 mg/l kin at ducase cultivar and 7.00 ± 1.00 at 2.5 mg/l BAP alone at dwarf Cavendish cultivar. They were higher than the control but lower than the maximum value.

The maximum mean number of leaves per shoot (9.67 ± 0.57) was obtained on the medium containing 2.5 mg/L BAP alone (Figure 6D), and the minimum value was 3.67 ± 1.52 on the medium containing MS medium with 0.5 mg/l kin alone compared to the other value 2.33 ± 0.57 at the ducase cultivar. A maximum value of 9.67 ± 0.57 (9.33 ± 0.57) was obtained on the medium containing 3.0 mg/L BAP and 0.5 mg/l kin, and a minimum 5.00 ± 1.00 was recorded on the MS medium containing 2.5 BAP and 0.5 kin alone at the dwarf Cavendish cultivar. Other results that were highest (8.00 ± 2.00) and 9.00 ± 1.00 at 3 mg/l BAP at the ducase cultivar and 2.5 BAP and 0.5 kin at the dwarf Cavendish cultivar, respectively, but lower than the maximum, were also recorded.

Table 3: Effects of concentrations and combinations of BAP and kin on shoot multiplication parameters.

Varieties	Hormones (mg/l)		Shoot number (Mean ± SD)	Leaf number (Mean ± SD)
	BAP	**kin**		
Ducase	-	-	1.67 ± 0.57^{g}	2.33 ± 0.57^{f}
	2	-	5.00 ± 2.00^{de}	3.67 ± 0.57^{ef}
	2.5	-	**8.00 ± 0.00^{a}**	**9.33 ± 0.57^{ab}**
	3	-	5.00 ± 2.00^{de}	8.00 ± 2.00^{abc}
	0	0.5	5.67 ± 1.52^{bcde}	3.67 ± 1.52^{ef}
	2	0.5	4.00 ± 1.00^{ef}	5.00 ± 1.00^{de}
	2.5	0.5	5.33 ± 0.57^{cde}	8.00 ± 1.00^{abc}
	3	0.5	7.33 ± 0.57^{ab}	6.00 ± 1.00^{cde}
Dwarf Cavendish	-	-	2.67 ± 0.57^{fg}	4.00 ± 1.00^{ef}
	2	-	5.33 ± 1.52^{cde}	5.00 ± 1.00^{de}
	2.5	-	7.00 ± 1.00^{abc}	5.00 ± 2.65^{de}
	3	-	4.00 ± 1.00^{ef}	8.67 ± 3.51^{ab}
	-	0.5	2.67 ± 0.57^{fg}	5.00 ± 1.00^{de}
	2	0.5	4.33 ± 0.57^{def}	7.00 ± 1.00^{bcd}
	2.5	0.5	6.00 ± 1.00^{bcd}	9.00 ± 1.00^{ab}
	3	0.5	**8.33 ± 0.57^{a}**	**9.67 ± 0.57^{a}**
DF			47	47
LSD (5%)			1.8124	2.46
CV (%)			21.17684	23.82
ISL			*	**

CV; Coefficient of variation, SD; Standard Deviation, LSD; Least Significant Difference; DF= Degree of freedom; ISL= Interaction Significant Levels, *** = Very highly significant; in a column, means followed by the same letter are not significantly different at the 5% level by Fisher's LSD.

Data regarding the plant shoot length of both banana varieties exhibited that all plant growth hormones had a significant positive effect on the shoot height compared to the control (Table 4). Among the growth hormones, the treatment of MS medium containing 3.0 mg/L BAP and 0.5 mg/l kin resulted in the maximum increase in shoot length, reaching 4.02 ± 0.32, which corresponds to an increase compared to the control (Figure 6C). Additionally, the growth hormones 2.5mg/l BAP and 3mg/l BAP exhibited significant increases in shoot length, with values of 3.95 ± 0.52 and 3.88 ± 0.45, respectively. While the other treatment also led to significant increases in plant shoot height compared to the control, their potential was relatively lower compared to the treatment mentioned earlier. Notably, the medium containing 2mg/l BAP and 0.5 kin resulted in a decrease in shoot length (2.83 ± 0.68) compared to the control.

Table 4: Effects of concentrations of BAP alone or in combination with kin on shoot length.

Conc. of Hormones (mg/l)		**SL (Mean ± SD)**
BAP	**Kin**	
0	-	2.91 ± 0.76^{bc}
2	-	3.41 ± 0.39^{abc}
2.5	-	3.95 ± 0.52^{a}
3	-	3.88 ± 0.45^{a}
0	0.5	3.50 ± 0.67^{ab}
2	0.5	2.83 ± 0.68^{c}
2.5	0.5	3.75 ± 0.76^{a}
3	0.5	4.02 ± 0.32^{a}
LSD (5%)		0.65
CV (%)		15.73
Significant level		**

CV; Coefficient of variation, SD; Standard Deviation, LSD; Least Significant Difference; ISL= Interaction Significant Levels, ** = highly significant; in a column, means followed by the same letter are not significantly different at the 5% level by Fisher's LSD.

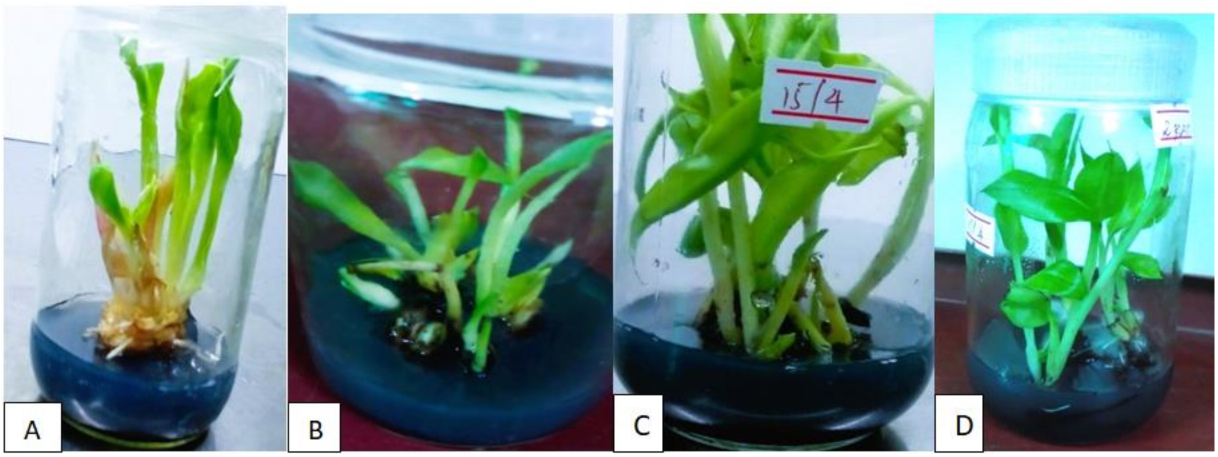

Figure 6.In vitro shoot multiplication of banana, Shoots multiplied on: (A) control (B) 3 mg/l of BAP, (C) 3 mg/BAP and 0.5kin (D) 2mg/l BAP

4.4. Effects of Auxin Combination on Root Induction of Banana

The results of analysis of variance showed that IAA and IBA had significant ($P<0.05$) effect on root number per shoot, root length and days induced root (Appendix 6).

A maximum mean number of roots per shoot (6.83 ± 1.16) were scored on MS medium supplemented with 1mg/l IAA (Table 5). This was followed by 4.67 ± 0.81mean number of roots per shoot on MS medium containing 0.5 IAA + 0.5 IBA.The low mean number of roots per shoot 3.33 ± 0.82 was scored on MS medium containing concentration of 1 IAA + 0.5 IBA and 0.5 IBA compared to the control (Table 5). The highest mean root length (8.5 ± 1.37cm) was achieved on MS media fortified by 0.5 IBA hormones, followed by 5.67 ± 1.37 cm MS medium containing 0.5 IAA + 0.5 IBA (figure 7). The shortest roots length (4.31 ± 0.74 cm) was achieved on medium supplemented with1 IAA + 0.5 IBA. Maximum time duration (15.1667 ± 1.47days) was observed in MS medium supplemented with only 1 IAA (figure 7). Followed by 15.00 ± 2.09 time duration observed at the control group where as minimum time (**9.67 ± 1.03** days) was observed in MS medium supplemented with 1 IAA + 0.5 IBA.

Table 5: Mean effect of different concentrations of growth hormones on RN, RL, and DIR.

Hormone Concentration (ml)	RN (Mean ± SD)	RL (Mean ± SD)	DIR (Mean ± SD)
IAA (0.0)	2.6667 ± 0.82^{d}	4.7500 ± 1.40^{bc}	15.00 ± 2.09^{ab}
IAA (0.5)	4.000 ± 0.89^{bc}	4.5000 ± 1.00^{bc}	10.00 ± 1.41^{c}
IAA (1.0)	**6.8333 ± 1.16^{a}**	4.4833 ± 0.91^{bc}	15.1667 ± 1.47^{a}
0.0 IAA + 0.5 IBA	3.3333 ± 0.82^{cd}	**8.5000 ± 1.37^{a}**	13.3333 ± 1.21^{b}
0.5 IAA + 0.5 IBA	4.6667 ± 0.81^{b}	5.6667 ± 1.37^{b}	14.6667 ± 2.25^{ab}
1 IAA + 0.5 IBA	3.3333 ± 0.81^{cd}	4.3167 ± 0.74^{c}	**9.6667 ± 1.03^{c}**
DF	35	35	35
LSD (5%)	1.1409	1.2973	1.8093
CV (%)	23.13247	20.27593	11.70505

RN = Root number; RL = Root length; DIR = Days for Induce Root; CV; Coefficient of variation, SD; Standard Deviation, LSD; Least Significant Difference. Means within columns having different letters in superscript are significantly different at $p<0.05$ by LSD test.

Figure 7. Well rooted plantlets: (A) Ducase at medium supplemented, with 0.5mg/l IAA (B) dwarf Cavendish cultivar (1mg/l IAA), and (C) Ducase at medium supplemented with 0.5mg/l IBA

4.5. Effect of Bulla as Gelling Agent on Shoot and Root Induction

The results of the analysis of variance showed that gelling agents had a significant (P<0.05) effect on the number of shoots per ex-plant, shoot length, root number, and root length. As regarding the ANOVA, shoot number, shoot length, and root number had a non-significant (p >0.05) effect on both variables and their interaction effects (Apendex Table 6) (Figure 8). However, root length had a significant (P<0.05) effect on varieties, gelling agents, and their interaction effects. In this study, the two solidified agents had a significant positive effect on the root length of the banana cultivars (Table 7). The highest increase in root length was observed with the 80g/l bulla, resulting in 6.90 ± 0.56cm and the minimum value of 4.14 ± 0.34cm with 8g/l agar on the ducase variety. On the other hand, the dwarf Cavendish cultivar resulted in a maximum of 4.70 ± 0.33cm with 80g bulla in root length and a minimum value of 3.17 ± 0.16 with 8g agar.

Table 6. Mean effects of gelling agents on root length of banana varieties.

Varieties	Gelling agents	Concentration	RL (Mean ± SD) in cm
Ducase	agar	8g/l	4.14 ± 0.34^{b}
	bulla	80g/l	$\mathbf{6.90 \pm 0.56^{a}}$
Dwarf Cavendish	agar	8g/l	3.17 ± 0.16^{c}
	bulla	80 g/l	4.70 ± 0.33^{b}
LSD (5%)		0.7053	
CV (%)		7.920818	
ISL		*	

CV; Coefficient of variation, SD; Standard Deviation, LSD; Least Significant Difference; ISL= Interaction Significant Levels, * = Significant; in a column, means followed by the same letter are not significantly different at the 5% level by Fisher's LSD.

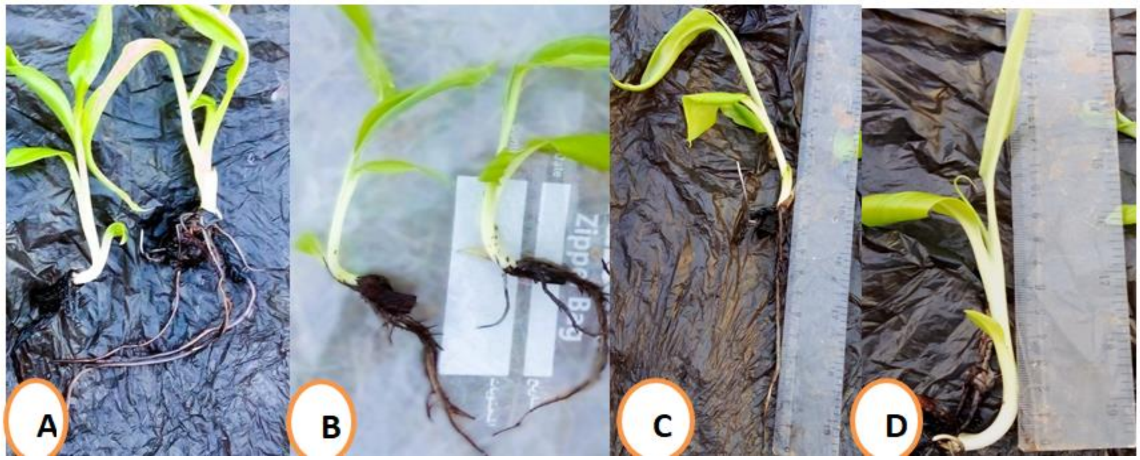

Figure 8. Effects of gelling agents on shoot length and root length: (A) rotting on bulla (B) rooting on agar (C) measuring root length (D) measuring shoot length.

This study revealed that two gelling agents, namely agar and bulla, showed a significant increase in shoot number, shoot length, and root number (Table 7). The maximum increase in shoot number was observed with 8 g/l agar, while the minimum increase was seen with 80 g/l bulla. Significant increases in shoot length were observed with the two gelling agents. Bulla showed the highest increase at 8.01 ± 0.78 and the minimum result of 5.82 ± 0.84 at 8 g/l of agar. Additionally, the gelling agents agar and bulla exhibited significant increases in root numbers, with values of 5.00 ± 0.89 and 3.67 ± 0.82, respectively. This study demonstrated that the two gelling agents tested significantly enhanced the shoot length, shoot number, and root number.

Table 7: Effect of gelling agents on shoot and root induction

Gelling Agents	SN (Mean ± SD)	SL (Mean ± SD)	RN (Mean ± SD)
8g/l Agar	**11.33 ±2.16^{a}**	5.8267 ± 0.84^{b}	**5.00** ± 0.89^{a}
80g/l bulla	6.17 ± 1.16^{b}	**8.0167 ± 0.78^{a}**	3.67 ± 0.82^{b}
LSD (5%)	2.0697	1.0483	1.2154
CV (%)	17.76644	11.37558	21.06625
SL	***	**	*

CV= Coefficient of variation; SD= Standard Deviation; LSD= Least Significant Difference; SL = Significant level; * = significant; ** = highly significant; *** = Very highly significant. In a column, means followed by the same letter are not significantly different at the 5% level by Fisher's LSD.

4.6. Effect of Acclimatization Media Mixture on Banana Verities Plantlet Survival in Greenhouse

In the greenhouse experiment, after 45 days of acclimatization on a potting medium prepared from red soil, sand, and compost in a 2:1:1 ratio, plant survival rates of 100% in the media mixture of red soil and sand soil in the ratio 2:1 and 100% in red soil, and sand soil, and compost in the ratio 2:1:1 were recorded (Figure 10). The results of the analysis of variance showed that the main effect of the acclimatization media mixture had a significant ($p<0.05$) effect on plant height. However, the main effect of varieties and the interaction effect of the two factors had no significant $p>0.05$ effect on this parameter (Appendix Table 7). The highest plant height (22.94cm) was scored on a medium containing red soil and sand soil (2:1), whereas the minimum value of plant height (19.75 cm) was obtained with the treatment of red soil, sand soil, and compost (2:1:1) (Table 8) .

Table 8: Mean effect of different acclimatization soil mixtures treatments on plant height of Banana plantlets.

Acclimatization media mixture (Ratio)	Plant height (cm) (Mean ± SD)
Red soil: Sand soil: Compost (2:1:1).	19.75 ± 3.42^{b}
Red soil: Sand soil (2:1)	**22.94 ± 3.35^{a}**
LSD (5%)	2.5248
CV (%)	16.33387

RS = Red soil; SS = Sand soil; CT = Compost; CV; Coefficient of variation, SD; Standard Deviation, LSD; Least Significant Difference. Means within columns having different letters in superscript are significantly different at $p<0.05$ by LSD test.

Figure 9.Acclimatized Plantlets in the green house: (A) Plantlets acclimatized in first week, (B) Plantlets acclimatized after 15 days, and (C) Acclimatized Plantlets after 30 days.

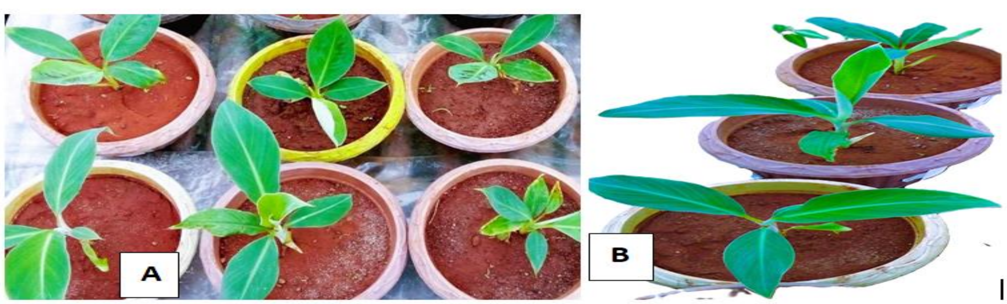

Figure 10. Plantlet survival in greenhouse: (A) platelet survived on red soil and sand soil (B) platelet survived on red soil, sand soil and compost

4.7. Cost Analysis of Gelling Agent

To optimize *in vitro* media and find cost-effective, locally available alternatives for its components, attention is particularly focused on the gelling agent, which is typically agar and is known to be one of the most expensive ingredients. As a substitute, bulla starch has been proposed as an alternative gelling agent. A comparative cost analysis between agar and bulla starch was performed for their application in banana micropropagation. The expenses associated with the gelling agents were evaluated both per liter of medium and per kilogram of the gelling agents. Subsequently, the total cost savings achieved by using bulla starch per liter of media were calculated using a specific mathematical formula. (%) = [100 − (bulla cost/agar cost) ×100]. Components required for in vitro media must be minimized by optimizing and locating affordable, locally obtainable substitutes. Agar, or gelling agent, is widely recognized as the most expensive ingredient in in vitro cultures. Consequently, employing bulla starch as a substitute gelling agent. Two gelling agents used in banana micropropagation were subjected to a cost-benefit analysis (Table 9). The price of gelling agents was determined for each kilogram of gelling agents as well as per litter of medium.

Table 9: Cost analysis of gelling agent

Gelling agent	Conc./L (w/v)(%)	Cost (ETB*)/kg	Cost (ETB)/L	Cost saved (%)
Agar	8g	17200	137.6	-
Bulla	80g	250	20	85

5. DISCUSSION

In this study, among the different concentrations of NaOCl disinfectant treatments, 2% sodium hypochlorite treatment for 20 min with 70% ethanol for 5 min resulted highest percentage of contamination- free explants (85%) for the two cultivars. This result indicates that the concentration of NaOCl solution used for disinfectant was one of the main factors in obtaining contamination-free explants. This finding aligns with previous studies that have found a maximum survival rate of clean and alive plants at the 2% concentration of NaOCl (Dejene Zinabu *et al.*, 2018; Belete Kebede, 2014). The success of *in vitro* regeneration and the production of micro-propagated plants heavily rely on the effectiveness of the sterilization protocol (Da Silva et al. 2016). Achieving successful in vitro regeneration can be accomplished by optimizing both the concentration of the sterilizing agent and the duration of exposure.

However, in contrast with this study, Shukla *et al*. (2019) reported that bananas were surface sterilized with 0.1% HgCl2 for 8 min and 70% ethanol for 20 min was found to be effective sterilization for Musa paradisiac variety "Udhayam. However, high levels of HgCl2 showed a negative impact on explant viability. The use of HgCl2 in the sterilization stage is not recommended due to its neurotoxic and immunotoxin properties, which are highly environmental pollutants (Da Silva *et al.*, 2016; Shukla et al., 2019). Kapadia and Patel (2021) also reported the highest percentage of aseptic culture establishment at the disinfectant: lactic acid (0.15%), Tween-20 (0.1%), and commercial bleach (0.8%) (30 min), followed by sodium chlorite (0.3%) (30 min.). Kidasi *et al*. (2023) found maximum 91% culture survival by using surface sterilization with 10% NaOCl, followed by spraying 70% ethanol for 20s. Hence this decontamination treatment is considered as best to control initial deep seated endophytic contamination for culture initiation in the micro propagation of banana from shoot tips. Sodium hypochlorite contains a single chlorine atom, and its cytotoxic effects arise from the production of oxygen when the salt decomposes, which is responsible for its bactericidal activity.

In this experiment, a low rate of clean and alive culture (33.3%) was recorded these contamination differences between genotypes may be caused by the presence of genotype-dependent endophytic and surface contaminants on the explants. In lower concentrations of NaOCl (1%), significantly highest percentage of contamination was observed, while shoot death is highly low in both cultivars. This finding contradicts with the work of Kahia *et al*. (2015), who discussed that this disinfectant has proven to have a very effective effect against all kinds of contaminants. However, insufficient sterilant concentrations are needed to kill surface contaminants, mainly fungi and bacteria, which are strongly associated with the culture explants.

The use of high-concentration and systemic sterilizers, such as mercuric chloride and systemic fungicides, might harm the explants, and endophytic bacteria are typically difficult to eliminate (El-Banna *et al*., 2021). To get contamination-free and alive cultures, it must optimize concentration and time duration for exposure to the disinfectant. Comparing the effects of chemical concentrations of disinfection, the aim of sterilizing banana cultivars with a 2% NaOCl solution for 20 min and 70% ethanol for 5 min was the most effective sterilization treatment for the two cultivars, which gave the highest culture survival percentage, less no of contamination, and moderately clean explants.

In this experiment, the effects of ascorbic acid and activated charcoal (AC) on the initiation of banana cultivars were investigated. The Various concentrations of antioxidants (25mg ascorbic acid, 50mg ascorbic acid, 1 g activated charcoal, and 2 g activated charcoal combined with 1.5 BAP and 0.5 NAA) were employed, and the results showed significant improvements in both shoot length and shoot survival compared to the control group. These findings demonstrate that the impact of high-concentration antioxidants had no influence on shoot length. This study was similar to the finding of Kariyana and Nisyawati (2013), who reported that the height of the explant on media with ascorbic acid and activated charcoal with all light duration was not influenced by explant browning.

The length of the shoots was observed to fluctuate depending on the concentrations of antioxidants and the control treatments. The minimum shoot length was 2.47 cm, which was observed on MS medium containing 2 g/l activated charcoal. This result showed that shoot length was greatly influenced by type and concentrations of antioxidants. This result was contradicting with Irshad *et al.* (2018*)* study, who reported 5.3cm shoot length at supplementation with 2 g/l activated charcoal. Due to their ability to enhance shoot survival, investigations have revealed that antioxidants were a key factor in controlling the browning and blacking of multiple shoot proliferations. The present investigation also showed that the ability of shoot induction of two banana varieties were highly dependent on the concentration of antioxidants used in this study.

The maximum shoot initiation percentage was 85.0% and 80% at the dwarf Cavendish and ducase cultivars, respectively, which were observed on MS medium containing 2 g/l activated charcoal. Similar investigation was reported by Kim *et al*. (2019), the highest frequency of shoot (91.7%) was obtained on MS medium containing 2 g/L AC. Previous studies on the tissue culture of bananas showed different results regarding the best concentration of antioxidants for the induction of shoots. The positive effect of 100 mg/L AA on the reduction of phenolic compound production was observed by Ngomuo *et al*. (2014) in Musa spp. in vitro. (Anicezio, 2012) reported that the addition of 15 mL Ascorbic acid to the MS culture medium was efficient in preventing oxidation in banana explants (Musa spp.). Assis, *et al*. (2018) reported that PVP, or ascorbic acid, must be use in the process at a concentration of 300 mg L-1, along with 2 g L-1 of activated charcoal. This was helps to minimize phenol oxidation Eugenia pyriformis.

Safwat *et al*., (2015) reported the best results for controlling lethal browning were obtained when explants were cultured with activated charcoal (1.5 g/L) while adding ascorbic acid (150 mg/L), citric acid (150 mg/L), or a combination of both in bananas. The preparation of the explant by immersion for one hour in a 1.2 g/l solution of ascorbic acid was just as effective as the addition of 100 mg/l of this product to the culture medium (Ngomuo *et al*., 2014). A study reported by Shimeles *et al*. (2015 examined that 100% and 80% of explants of the C86-56 and C86-12 genotypes, respectively, were obtained from MS medium supplemented with 0.2 g L and 0.3 g L-1 of PVP. A finding from the present study was agreed with the findings of Pushpraj and Patel, 2015) who have noted that the most effective browning control was observed in activated charcoal 200 mg/L into the medium. In general, from the present investigation low concentration of antioxidant 25mg and 1g activated charcoal and control were effective for shoot length. However, highest plant survival observed at 2g activated charcoal and 50mg ascorbic acid. The prevalence of browning varies among species, cultivars, the physiological state of the plant or tissue, and the size and age of the explants (Ahmad *et al*., 2013). The variety in particular, having a genome AAB, is difficult to regenerate *in vitro* because of the higher exudation of phenolic compounds.

In this study uses of BAP and kin revealed that for the mean of all parameters for both cultivars, there was a significant difference among the treatments; different concentrations of BAP in combination with kin were showed a significant difference among the treatments. For the mean of all parameters for both cultivars. In the current study maximum of 8.33 shoots per explant with a 4.02 cm shoot length on a medium fortified with 3 mg/l BAP and 0.5kin on the dwarf Cavendish cultivars, while the ducase cultivar has a maximum of 8.0 shoots per explant on a medium fortified with 2.5 mg/l BAP alone. These results were in accordance with Sivakumar and Visalakshi (2021), who reported the maximum multiplication of shoots of 8.00 was obtained when explant were cultured on MS medium supplemented with 3 mg of BAP and 0.5 TDZ for banana cv. Poovan. This might be due to the result of BAP at high concentration reduced apical dominance and increased lateral growth of shoot.

However Khatun *et al.* (2018) reported a 3.4 mean shoot number was recorded at a maximum concentration of 5mg BAP on the Banana Variety of Sabri. Genotypes varied in their maximum shoot induction potential and in their requirement for optimum BAP concentration. A lower concentration of kinetin (0.5 mg/l) resulted in a maximum shoot number of 5.67 at ducase varieties. This suggests that multiplication depends on the genotype of plants, the types and the concentration of plant growth regulators. Hence, medium supplemented with both BAP and KN induced the best shoot multiplication, indicating that the effectiveness of each of the growth hormones. In this experiment, the culture medium with 2.5 mg/L BAP showed significantly the highest leaf number (9.33) at ducase cultivars, followed (9.67) at dwarf Cavendish media containing 3.0 mg/L BAP and 0.5 mg/L KN growth hormones. Different kinds of cytokines have been used for the micro propagation of banana cultivars, and the shoot proliferation rate is significantly affected by the type of banana cultivar and their genomic constitution. Similar work were observed by Solomon Nigusu, 2020 who explained that highest leaf number was observed at 3.0 mg/L BAP and 0.5 mg/L KN.

The maximum shoot height of (4.02cm) was recorded on a medium containing 3 mg/l BAP + 0.5 kin and 2.5 BAP alone. This result was achieved on a medium containing a high concentration of BAP with a low concentration of KN. 3 mg of BAP was combined with 0.5 KN and 2.5 BAP alone. This means the optimum concentration of growth regulator for this particular parameter was 2.5 mg/L BAP alone and 3 mg/L BAP + 0.5 KN. The current study was contradict with Singh (2014) who reported that the highest shoot length was observed in 0.5 mg/l BAP and 0.5 mg/l Kinetin. Different researchers optimized different protocols for in vitro propagation of banana shoot tips; this could be due to differences in explant types, genotypes, and media formulation. As per studies accomplished so far, the genotype and concentration of plant growth regulators appear to play a greater role in *in vitro* responses than the composition of culture media (Cavallaro *et al*., 2023). The proliferation of shoots emerges as a crucial stage in developing a successful micropropagation technique, exerting significant influence on the overall efficacy of the procedure.

In this experiment, the effects of IAA and IBA on the root induction of banana cultivars were investigated. The combination of auxin (0.5mg IAA and 1mg IAA with 0.5 IBA) was employed, and the results showed significant improvements in root number and root length compared to the control group. The root length was found to vary with the different concentrations of auxin treatments. The minimum root length was 4.3 cm, which was observed on MS medium containing 1 IAA and 0.5 IBA. This might be due to auxins promote adventitious root with low level of an auxins in most of banana cultivars.

This result showed that root length was greatly influenced by type and concentrations of auxin. This result was contradictory to the Singh *et al*., (2024*)* study, who reported 6.5cm root length at supplementation with 0.5 mg/L IAA for micro propagation of Banana

Cultivar Grand Naine (*Musa spp*.).The maximum root number percentage was 6.83, which was observed on MS medium containing 1 mg/l IAA (Table 9). Other results also revealed 4.67 and 4.0, which were recorded on MS medium containing 0.5 IAA + 0.5 IBA and 0.5 IAA, respectively. A similar investigation was reported by Nakul *et al*. (2019), and the highest frequency of root (6.4) was obtained on MS medium containing 1 mg/l IAA for micropropagation banana (*musa spp*.) cv. grand. In this experiment, days to root induction varied with different concentrations of IAA and IBA. The culture medium with 1 IAA + 0.5 IBA mg/l and 0.5 IAA alone showed minimum time duration of 9.67 and 10 days, respectively. This result was similar to Miilion (2013), who reported a short duration of 0.5 mg/l IAA for 8.9 days for Banana (Musa paradisiaca) Cv.Grand Naine. This might be due to the increase or decrease in concentration of these hormones led to variability of root induction percentage. Induction percentage was less at high concentration; shoots were longer at low concentration; number of roots, was high at optimum low concentration; and root length was also based on the auxins concentrations.

There was a significant difference (P >0.05) effect on the number of shoots per explant, shoot length, root number, and root length due to two kinds of gelling agents, and with 2 mg/l BAP and 0.5mg/l NAA. In this study, two gelling agents, agar and bulla, were used for shoot and root regeneration on the banana cultivars. The highest root length was recorded at 6.90 cm and 4.70cm on the ducase and dwarf Cavendish cultivars, respectively at MS medium supplement with bulla as a solidified agent. In addition to replacing traditional agar, bullas have been found to increase root length compared to traditional agar. This may be due to high carbon content (Biruk Ayenew *et al.*, 2012).The lowest root length (3.17) was obtained when these bananas varieties were cultured on MS medium supplemented with 8g of agar. The increased growth of shoots and roots is due to improved water and nutrient availability, which is a result of reduced diffusion resistance and enhanced contact between the explant and the medium. (Cacatian, 2017). Additionally, they discovered that as the agar concentration in the medium increases, nutrient diffusion to the explants may decrease, leading to reduced nutrient availability and diminished growth. Similar to this study, Biruk Ayenew *et al*. (2017) observed that 3.63cm of root length was supplemented with 80 g/l of bulla for cassava *(Manihot Esculenta Crantz*) *in vitro* propagation. A maximum shoot number of 11.33 with a 5.0 root number were revealed on

MS medium supplement with 8 g/l agar, followed by a 6.17 shoot number and 3.67 root numbers at 80 g/l of bulla. Furthermore, high nutrient concentrations in ‘Bulla’ are associated with cell wall development, plant signal transduction, and various enzymes and co-factors, all of which influence the in vitro regeneration of bananas.

In contrast to these experiments, 4.09 shoot numbers at 10% bulla were reported by Ayelign Mengesha *et al*, (2012). According to the results of the present study, bulla had approximately equivalent values to agar in almost all parameters. Due to these reasons, it’s necessary to study its biochemical or hormonal activity across crop genotypes to use ‘Bulla’ as a cheap alternative commercial gelling agent.

In the current study, acclimatization media mixture treatments had a significant $p \leq 0.05$ effects on plant height and a non-significant $p > 0.05$ effect on leaf number and banana cultivars. The highest plant height (22.93cm) was scored on a medium containing red soil and sand soil (2:1) (Figure 9A). The acclimatization of *in vitro*-rooted plantlets was successful; 100% of the plants survived and were all established as healthy plants. Hence, it can be concluded that the composition of red soil, compost, and sand in the mixture might have been well drained with high water holding capacity, good aeration, and nutrients present that contributed to the survival rates. Similar reports by Dejene Zinabu (2017), reported that 100% survival of enset plantlet acclimatization with red soil to sand at 2:1; Mulugeta Chemdisa *et al*. (2017), who found the best survival rate of the Grandniece and Poyo banana varieties with a potting mixture containing red soil, sand, and compost (2:1:1). In generally, those acclimatization media mixtures were best for a higher plant survival percentage for the two cultivars. The study suggested that sand might have helped in giving better grip for roots, ample aeration, and sufficient organic matter for the platelet.

6. CONCLUSION AND RECOMMENDATIONS

6.1. Conclusion

Based on this study banana micropropagation potential depends on genotype, the time duration and concentration of NaOCl for surface sterilization; and concentration of growth regulators. These factors strongly affect survival rate of explants, shoot regeneration, number of produced shoots and root length. The analysis of variance revealed that 2% sodium hypochlorite and 20 min with 70% alcohol for 5 min duration were optimal sterilization for contamination-free and moderate survival for banana shoot tips. The shoots showed an intention response for controlling browning, with survival rates of 85% and 80% obtained from explants cultured on MS medium supplemented with 2g activated charcoal for two banana dwarf Cavendish and ducase, respectively. After growing in vitro cultures at different antioxidant concentrations with hormonal MS medium supplemented with BAP and KIN, The highest number of shoots was 9.67 and 9.33 obtained on MS medium supplemented with 3 mg BAP and 0.5 KIN for dwarf Cavendish and 2.5 mg BAP for dwarf Cavendish and ducase cultivars, respectively. Dwarf Cavendish cultivars were most productive and produced a maximum of 9 shoots at 2.5mg BAP+0.5 KIN, followed by 3 mg BAP. Therefore, 3 mg/l BAP combined with 0.5 mg/l KIN and 2.5 BAP alone were found to be optimal for producing the maximum number of shoots per explant. Application of 80 g/l of bulla was successful and revealed a positive effect on shoot length and root length. 80 g of concentration was optimal as a solidified agent in this experiment. In conclusion, this protocol can be utilized to micro-propagate the two banana cultivars to regenerate good-quality planting material. In this study, efficient explant sterilization, control of explant browning at shoot initiation and multiplication, and use of locally produced bulla were compared to agar for shoot and root induction. For acclimatization, a good soil combination for plantlet adaptation was observed on potting mixture containing red soil, sandy soil, and compost (2:1:1) with 100% of the plantlets that survived. Therefore, the optimized protocol is useful for the in vitro propagation of these specific cultivars. From this protocol, dwarf Cavendish gave the best response in almost all the parameters.

6.2. Recommendations

Based on the findings of this study the following recommendation were forwarded

- ✓ Sterilant substitution with locally available chemicals should be recommended for reduction of coast, and toxic component in the chemicals for the environment and food plants.
- ✓ Further study should be done using other plant growth regulators for shoot multiplication and rooting; and using other types of explants.
- ✓ Further researches needed on effects of all chemicals on seedling age of banana that used in protocol developments.
- ✓ The optimization study should also need to continue with new technologies on the other elite genotype of banana cultivars.

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8. APPENDIX

Appendix table 1 A: Nutrient composition and concentration of full strength MS medium

MS media component	Concentration (g) Of stock solution in g/l	Amount of stock solution taken for one litre medium (ml)
MS1 Ammonium Nitrate (NH4NO3) Potassium Nitrate (KNO3) Magnesium Sulphate (MgSO4.7H2O Potassium dibasic Phosphate (KH2PO4)	33	50
	38	
	7.40	
	3.48	
MS2 Calcium Chloride (CaCl2.2H2O)	8.80	50
MS 3 Boric Acid (H3BO3)	1.24	5
Manganese Sulphate (MnSO4.H2O) Zinc Sulphate (ZnSO4.7H2O) Sodium Molbdate (Na2MoO4.2H2O) Potassium Iodide (KI) Copper Sulphate (CuSO4.5H2O) Cobalt Chloride (CoCl.6H2O)	3.38	
	1.72	
	0.05 1.66	
	0.005	
	0.05	
MS 5 Na2EDTA Iron Sulphate (FeSO4.7H2O)	7.46 5.57	5
MS6 Myoinositol Thiamine HC Pyridoxine HCl Nicotinic acid Glycine	20	5
	0.02	
	0.10	
	0.10	
	0.40	

Appendix table 1B: Other Organic Supplements for media

Organic Supplements	(gm/l) for media
Sucrose	30
Activated Charcoal	1 and 2
Agar	8
bulla	80
Ascorbic acids	25 and 50

Appendix table 1C: Plant growth regulators hormone supplied in the media

Plant growth regulators	Concentration(mg/ml) for stoke solution	Amount dispensed for 1 liter media(mg/l)
BAP	50	1.5, 2,2.5 and,3
NAA	50	0.5
IAA	50	0.5
IBA	50	0.5
Kn	50	0.5

Appendix Table 2 ANOVA Table for the effect of NaOCl on clean and live explants culture

Source of variation	**DF**	**MS**	**F value**	**Pr > F**
Varieties	1	0.04166667	0.50^{NS}	0.4897
ST	3	9.81944444	117.83***	<.0001
V * ST	3	0.26388889	3.17^{NS}	0.0532
Error	16	0.08333333		
Total	23			
V	1	150.00000	4.00^{NS}	0.0628
ST	3	10400.000	277.33***	<.0001
V * ST	3	150.00000	4.00*	0.0266
Error	16	37.50000		
Total	23			

*** Significant at $p \leq 0.001$ by LSD; NS = Not significant at $p > 0.05$; DF= Degree of freedom; MS = Mean square; V = Varieties; ST = sterialant amount.

Appendex table 3: ANOVA for the effects of antioxidants on shoot initiation

Parameter	Source of variation	DF	MS	F value	Pr > F
SL	V	1	0.29008333	1.37^{NS}	0.2550
	AO	4	1.49550000	7.08**	0.0010
	V * AO	4	0.04133333	0.20^{NS}	0.9378
	Error	20	0.21125000		
	Total	29			

** Significant at p ≤ 0.01 by LSD; NS = Not significant at p >0.05; DF= Degree of freedom; MS = Mean square; AO = Antioxidants.

Appendix Table 4. ANOVA table for in vitro shoot multiplication

Parameters	Source of variation	DF	MS	F value	Pr > F
SN	V	1	0.5208333	0.44^{NS}	0.5125
	GHC	7	20.7827381	17.50***	<.0001
	V * GHC	7	2.8541667	2.40*	0.0427
	Error	32	1.1875000		
	Total	47			
SL	V	1	0.19507500	0.63^{NS}	0.4327
	GHC	7	1.25805119	4.07**	0.0027
	V * GHC	7	0.56467024	1.83^{NS}	0.1160
	Error	32	0.30900208		
	Total	47			
LN	V	1	10.0833333	4.61*	0.0395
	GHC	7	25.4166667	11.62***	<.0001
	V * GHC	7	7.9880952	3.65**	0.0052
	Error	32	2.1875000		
	Total	47			

* Significant at p ≤ 0.05 by LSD; ** significant at p ≤ 0.01 by LSD; *** significant at p ≤ 0.001 by LSD; NS = Not significant at p >0.05; DF= Degree of freedom; MS = Mean square; GHC = Growth hormone concentrations.

Appendix Table 5 ANOVA table for effects of gelling agent on shoot and root induction

Parameters	Source of variation	DF	MS	F value	Pr > F
Soot number	V	1	6.75000000	2.79^{NS}	0.1332
	GA	1	80.08333333	33.14***	0.0004
	V * GA	1	4.08333333	1.69^{NS}	0.2298
	Error	8	2.4166667		
	Total	11			
Soot length	V	1	1.16563333	1.88^{NS}	0.2075
	GA	1	14.38830000	23.21**	0.0013
	V * GA	1	0.45630000	0.74^{NS}	0.4159
	Error	8	0.61996667		
	Total	11			
Root number	V	1	0.33333333	0.40^{NS}	0.5447
	GA	1	5.33333333	6.40*	0.0353
	V * GA	1	0.33333333	0.40^{NS}	0.5447
	Error	8	0.83333333		
	Total	11			
Root length	V	1	7.53667500	53.71***	<.0001
	GA	1	13.76020833	98.07***	<.0001
	V * GA	1	1.13467500	8.09*	0.0217
	Error	8	0.14031667		
	Total	11			

* Significant at $p \leq 0.05$ by LSD; ** significant at $p \leq 0.01$ by LSD; *** significant at $p \leq 0.001$ by LSD; NS = Not significant at $p > 0.05$; DF= Degree of freedom; MS = Mean square.

Appendix Table 6: ANOVA for effect of the auxin hormones on rooting (CGH) on RN, RL and DIR.

Parameters	Source of variation	DF	MS	F value	Pr > F
RN	V	1	1.36111111	1.48^{NS}	0.2349
	CGH	5	13.22777778	14.43***	<.0001

	V * CGH	5	0.16111111	0.18^{NS}	0.9691
	Error	24	0.91666667		
	Total	35			
RL	V	1	0.17361111	0.15^{NS}	0.7053
	CGH	5	15.50627778	13.08***	<.0001
	V * CGH	5	2.42094444	2.04^{NS}	0.1086
	Error	24	1.1852778		
	Total	35			
DIR	V	1	8.0277778	3.48^{NS}	0.0743
	CGH	5	38.0277778	16.49***	<.0001
	V * CGH	5	3.4944444	1.52^{NS}	0.2222
	Error	24	2.3055556		
	Total	35			

*** Significant at $p \leq 0.001$ by LSD; NS = Not significant at $p > 0.05$; DF= Degree of freedom; MS = Mean square; V = Varieties; CGH = Concentrations of Growth Hormones.

Appendix Table 7. ANOVA table for effects of acclimatization media mixture

Parameters	**Source of variation**	**DF**	**MS**	**F value**	**Pr > F**
PH	V	1	0.00	0.00^{NS}	1.0000
	AM	1	81.28125	6.69*	0.0152
	V * AM	1	3.125	0.26^{NS}	0.6161
	Error	28	12.1540179		
	Total	31			

LN	V	1	0.000	0.00^{NS}	1.0000
	AM	1	1.125	0.75^{NS}	0.3924
	V * AM	1	0.000	0.00^{NS}	1.0000
	Error	28	1.49107143		
	Total	31			

* Significant at $p \leq 0.05$ by LSD; NS = Not significant at $p > 0.05$; DF= Degree of freedom; MS = Mean square; V = Varieties; AM = Acclimatization media mixture.