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IN VITRO REGENERATION OF *Brassica spp*, A LOCAL LANDRACE *SIMUARE*

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GETE FIKADU WOLDESENBET

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In vitro* Regeneration of *Brassica spp*, a Local Landrace *Simuare

A Thesis Submitted to School of Graduate Studies, in Partial Fulfillment of the Requirements for the Degree of Master of Science in Horticulture

Gete Fikadu Woldesenbet

Major Advisor: Addisalem Mebratu (Ph.D.)
Co-advisor: Gizachew Woldesenbet (Ph.D.)
Co-advisor: Ibsa Fite (MSc.)

December, 2024
Wolkite, Ethiopia

Wolkite University

School of Graduate Studies

We hereby certify that we have read and evaluated this Thesis tilted "***In vitro* Regeneration of *Brassica spp*, a Local Landrace *Simuare*"** prepared under our guidance by Gete Fikadu. We recommend that the Thesis shall be submitted as fulfilling the requirements for the award of a MSc. degree in Horticulture.

Addisalem Mebratu (Ph.D.)	__________	________________
Major advisor	Signature	Date
Gizachew Weldesenbet (Ph.D.)	__________	________________
Co-advisor	Signature	Date
Ibsa Fite (MSc.)	__________	________________
Co-advisor	Signature	Date

As members of the Borad of Examiners of the Master of Science Thesis open defense examination, we have read and evaluated this Thesis prepared by Gete Fikadu Woldesenbet and examined the candidate. We hereby certify that; the Thesis is accepted for fulfilling the requirements for the degree of Master of Science (M.Sc.) in Horticulture.

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Name: Gete Fikadu Signature: ___________________ Date: _____________

Department: Horticulture

BIOGRAPHIC SKETCH

The author was born on January 5, 1995, in Enore District, Gurage Zone, South Ethiopia. She began her formal education at Gunchire Primary and Junior School, where she studied from 2001 to 2010. Following this, she attended Gunchire Senior Secondary School, completing her high school education between 2011 and 2012. In 2013, she enrolled at Samara University, joining the College of Agriculture and Natural Resources. She pursued her undergraduate studies in Horticulture, graduating with a Bachelor of Science (BSc) degree on June 11, 2015.

After obtaining her undergraduate degree, she joined the Ethiopian Institute of Agricultural Research at the Holeta Agricultural Research Center. Here, she contributed to research and development in the agricultural sector, applying her academic knowledge to practical challenges in the field of horticulture.

In the 2021/22 academic year, she advanced her education by enrolling in the graduate program at Wolkite University, within the College of Agriculture and Natural Resources. She is currently pursuing her Master of Science (MSc) degree with a specialization in Horticulture. Her academic journey and professional experience underscore her dedication to agricultural science and her commitment to advancing horticultural research and practice in Ethiopia.

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ABBREVIATIONS AND ACRONYMS

BAP	6-Benzyl Amino-purine
DDW	double distilled water
EDTA	Ethylenediamine tetraacetic acid
GA3	Gibberellic acid
GSLs	Glucosinolates
IBA	Indol-Butyric Acid
ITCs	Isothiocyanates
KIN	Kinetin
LRP	Lateral root primordia
NAA	Naphthalene Acetic Acid
PGR	Plant growth regulators
RFLP	Restriction fragment length polymorphisms
SSA	Sub-Saharan Africa
TDZ	Thidiazuron
WRP	Water Resources Policy

ABSTRACT

Simuare (Brassica spp), a leafy vegetable resembling kale, is widely grown and consumed in Ethiopia's Gurage Zone. Due to its inability to flower and set seed, Simuare is propagated solely through cuttings, a method that is inefficient for producing sufficient planting material. This study was conducted to develop an efficient and cost-effective in vitro micro-propagation protocol to address these limitations. Explants were surface sterilized using ethanol (70% for 30 seconds), mancozeb (2.5mg/l), and bleach (2% for 20 minutes), achieving a survival rate of 99.6%. Murashige and Skoog (MS) medium supplemented with various concentrations of auxins and cytokinins was used for culture initiation, shoot multiplication, and root induction. Optimal shoot initiation was observed with 1 mg/L BAP and 1 mg/L kinetin, while the highest shoot multiplication occurred on MS medium containing 3 mg/L BAP and 0.1 mg/L GA3, producing 6.2 ± 1.68 leaves, 4.33 ± 1.87 shoots per explant, and a shoot height of 4.44 ± 0.83 cm. Root induction was most effective with 0.3 mg/L IBA and 0.2 mg/L NAA, yielding 5.04 ± 0.07 roots per plantlet and a root length of 3.06 ± 0.47 cm. The developed protocol successfully overcame challenges of conventional propagation, achieving high explant survival, efficient shoot multiplication, and effective root induction. These findings provide a reliable method for large-scale Simuare propagation, and the protocol is recommended for producing high-quality planting materials to meet growing demand.

Keywords: ***Simuare, MS Medium, Explant, Initiation, Multiplication, Growth Regulators, Rooting, Shooting***

1. INTRODUCTION

1.1. Background

The global population is anticipated to reach 9.7 billion by 2050 (O'Sullivan, 2020), with sub-Saharan Africa (SSA) accounting for half of this growth (WHO, 2019). This population increase is likely to place greater strain on natural resources, such as land and water, and will significantly affect food security, dietary practices, waste management, and employment opportunities (Fernández-Suárez, 2021). To mitigate these challenges, innovative farming technologies that make efficient use of limited space are essential, enabling urban households to grow crops and improve food and nutrition security (O'Sullivan et al., 2019). Vegetable crop production offers a practical solution, as it can be successfully carried out on small plots in urban, peri-urban, and rural areas. This sector is pivotal for enhancing food security, generating employment, and supporting economic development (FAO, 2021).

Brassica carinata, also known as Ethiopian mustard, is an important leafy vegetable and oilseed crop grown in various regions worldwide. It plays a central role in Ethiopian agriculture, providing essential nutrients that contribute significantly to traditional diets. This versatile vegetable is used in a variety of dishes, with products such as dried leaves, extracted juice, leaf protein concentrate, leaf powder, and fried snacks, commonly used to enhance flavor or as ingredients in snack formulations (Getinet et al., 1997). *B. carinata* is rich in vitamins, minerals, trace elements, dietary fiber, and protein (Genet et al., 2005), and also contains antioxidants, carotenoids, and enzymes such as catalase, superoxide dismutase (SOD), and peroxidase (Manchali et al., 2012; Singh et al., 2010). Regular consumption of *B. carinata* has been associated with a reduced risk of several diseases, including cancer (van Poppel et al., 1999), cardiovascular conditions (Higdon et al., 2007), neurode generation (Fimognari et al., 2012), and diabetes (Yokozawa et al., 2002; Cartea et al., 2010). These health benefits are attributed to its ability to detoxify carcinogens, alleviate oxidative stress and inflammation, scavenge free radicals, enhance immune function, and lower serum glucose levels.

Simuare, a kale-like vegetable similar to *Brassica spp* is a popular leafy vegetable commonly grown in Ethiopia's Gurage Zone, Gummer, Geta, parts of the Ezha district, and the Silte Zone of the Southern Nations, Nationalities, and Peoples' Region (Kassa, 2016). Almost every part of the plant, including the leaves, axillary buds, and stems, is utilized. Among the Gurage people, *Simuare* holds cultural significance and is traditionally served at weddings and other ceremonies. Key dishes made from *Simuare* include Gomen Kitfo, Zemojat, and Gomen Wat. In many areas, traditional meals often feature *Simuare* in various forms. The crop has a strong presence in the Addis Ababa market, where its price is two to three times higher than that of other cabbage varieties, driven by demand for ceremonies, even in large hotels and restaurants. Despite this high demand, its market supply remains limited (Kassa, 2016).

The production and productivity of *B. carinata* as a vegetable in Ethiopia are significantly limited (Abdeta Mitiku, 2022). A primary constraint is that rural farmers, driven by market demand and food security concerns, tend to prioritize growing cereals and pulses over vegetables. Additional challenges include low production levels, insufficient pest control, poor soil fertility management practices, and a general neglect of vegetable crops (Hunde, 2017). Moreover, the genetic improvement and conservation of *B. carinata* have been hindered, mainly due to the lack of advanced biotechnological methods (Singh and Tomar, 2018).

In vitro regeneration presents a promising approach for improving and conserving *Simuare*. Previous research has highlighted the effectiveness of in vitro methods in producing high-quality plantlets, which can be used in breeding programs to develop improved varieties with desirable characteristics such as higher yield, disease resistance, and better nutritional content (Cardoza & Stewart, 2004). The adoption of these techniques in Ethiopia is particularly crucial, considering the country's vulnerability to climate change and the pressing need for resilient crops that can endure environmental stresses (Megersa Chaltu, 2021). Additionally, the genetic diversity within *B. carinata* offers a valuable opportunity to create new cultivars that can enhance food security in Ethiopia. By utilizing in vitro regeneration, it is possible to preserve the genetic integrity of local landraces while introducing genetic improvements that boost their agronomic performance (Tesfaye Misteru et al., 2023).

1. 2. Statement of the Problem

Simuare, a local landrace cabbage is vital to the livelihoods of communities in the Gurage Zone, particularly in the districts of Ezha, Gumer, Geta, and Mihur, as well as in various highland areas of the Silte Zone. The market demand for *Simuare* is strong, extending from local markets to the capital city, which drives high market prices. However, production is limited by a major constraint: the crop’s inability to produce seeds. As a result, farmers must rely exclusively on vegetative propagation through stem cuttings, a practice that poses several challenges. To sustain this valuable crop, farmers preserve planting material in home gardens during the off-season and propagate it for larger-scale planting during the rainy season. However, the limited availability of planting material during the off-season constrains production and hampers the crop's potential for expansion.

Stem cutting propagation is labor-intensive and limited in scale, which hinders widespread planting. Moreover, repeatedly using stem cuttings for propagation can result in the buildup of viral, bacterial, and fungal diseases, ultimately decreasing crop productivity and resilience (Nassar and Ortiz, 2007). This traditional method is inadequate for meeting consumer demand in both rural and urban areas, limiting the potential for *Simuare* cabbage to expand beyond its current production zones. Additionally, the lack of seed propagation hampers the crop's ability to spread to other regions of the country, further restricting its economic opportunities. The local landrace *Simuare* is a valuable yet underutilized leafy vegetable crop that has been largely overlooked in research aimed at improving its propagation, yield, and nutritional value (Hagos Abraha *et al*., 2024).

Plant biotechnology, specifically tissue culture and micro propagation techniques, offers a promising solution to these limitations. Micro propagation can enable the large-scale production of disease-free, clonal plantlets in a short period under aseptic conditions, providing a reliable and uniform supply of planting material free from numerous pathogens. This approach not only minimizes disease risks but also allows for efficient scaling to meet market demands (Rani and Raina, 2000; Cardoso *et al*., 2018). For crops like *Simuare*, where propagation material is limited and no seed is available, micro propagation techniques can

provide a sustainable pathway to meeting market demand while enhancing crop quality and availability. Despite the proven benefits of micro propagation in other crops, no research has yet been conducted to establish an effective protocol for *Simuare* landrace cabbage propagation.

Creating a tailored micro-propagation protocol for *Simuare* could help overcome current propagation challenges and support sustainable production to meet both local and national demand, while also offering valuable insights to the scientific community. The potential for in vitro plant regeneration is often influenced by factors such as genotype, the composition of the culture medium particularly plant growth regulators (PGRs) and environmental conditions (Marinangeli, 2012). Therefore, this research aims to develop a micro-propagation protocol specifically designed for the local *Simuare* landrace, addressing genotype-specific needs and optimizing culture medium conditions to promote the crop's future development and improvement.

1.3. General Objective

- To develop an in vitro regeneration protocol for the local landrace, *Simuare*
-

1.3.1. Specific objectives

- To develop an effective sterilization protocol for the in vitro culture of the local landrace *Simuare*.
- To identify the optimal combination and concentration of BAP and Kinetin for shoot initiation.
- To determine the suitable hormone type and concentration for promoting shoot multiplication.
- To establish the optimal concentration of IBA and NAA combinations for root induction.

2. LITERATURE REVIEW

2.1. Origin and Domestication of *Brassica carinata*

Brassica carinata, originating in Ethiopia's highlands and East Africa, is an amphidiploid species (BBCC, 2n = 34) derived from *Brassica nigra* (BB, 2n = 16) and *Brassica oleracea* (CC, 2n = 18) (Shyam et al., 2012). This versatile crop is grown as an oilseed and leafy vegetable, providing essential micronutrients for human and animal diets. Historically cultivated in Ethiopia since 4000 BC, its exact domestication timeline remains debated (Alemayehu Nigussie and Heiko Becker, 2002; Shyam et al., 2012). Today, Ethiopian kale thrives in semi-arid regions globally, including Europe and South Asia (Mulvaney et al., 2019). Chloroplast DNA analysis confirms its cytoplasm originates from *B. nigra* (Palmer, 1985).

2.2. Taxonomic Description

Brassica carinata is a determinate plant reaching about 1.4 meters tall, with multiple branches and taproots extending up to 1 meter (Zanetti et al., 2013). Its smooth, waxy stems can reach 2 cm in diameter, while its leaves, with short petioles, simple trichomes, and waxy surfaces, are alternate and glabrous to slightly hairy (Al-Shehbaz, 2012; Mnzava and Schippers, 2004). Lower leaves are ovate to oblong with 1–3 deep lobes, measuring up to 20 × 10 cm, green on top, and paler underneath with purple or light green veins. Upper leaves are smaller, narrower, lighter, with fewer lobes and less waxy surfaces (Mnzava and Schippers, 2004).

2.3. Morphological Description

Brassica carinata shows determinate growth, typically reaching a height of 1.4 meters with multiple branches and taproots that can extend up to 1 meter (Zanetti et al., 2013). The stems are smooth, waxy, and can grow up to 2 cm in diameter, while the leaves feature short petioles, simple trichomes, and alternate, glabrous to slightly hairy, waxy surfaces (Al-

Shehbaz, 2012; Mnzava and Schippers, 2004). The lower leaf blades are ovate to oblong, with one to three deep lobes, measuring up to 20 cm long and 10 cm wide. The upper side of the lower leaves is green, while the underside is paler, with purple or light green veins. The upper leaves are smaller, narrower, and lighter in color, have fewer lobes, and are less waxy (Mnzava and Schippers, 2004).

Figure 1: Common *Brassica carinata* vegetable

The local Ethiopian cabbage *Simuare* is one of the most commonly used *B. carinata* leafy vegetables in the Gurage Zone, and it is currently gaining popularity in other parts of the country as well. *Simuare* resembles kale, with wide and long leaves that are lighter in color compared to other kale varieties. The stems are dark red and have a glossy appearance.

2.4. Production and Distribution

The Food and Agriculture Organization (FAO, 2021) recognizes Cole crops as a major global food source, commonly consumed cooked, stir-fried, or fresh in dishes like coleslaw and mixed salads. In 2018, global production of cabbages and other brassicas reached approximately 69 million metric tons, with East Africa producing 14.8 million tons on around 92.5 thousand hectares. *Brassica carinata* (Ethiopian mustard) is believed to have originated in Northeastern Africa around 4,000–5,000 years ago, spreading to East, West, and Southern Africa, as well as parts of Southwest Asia. Initially cultivated for leafy vegetables, fodder, and oilseed, it was introduced to North America in 1957 and is now grown globally for its potential as an alternative energy crop and low-land-use alternative to *Brassica napus* (Stephens, 1994; Seepaul et al., 2021; Bozzini et al., 2007).

2.5. Composition and Food Value of *Brassica carinata*

Brassica carinata has gained prominence as a valuable food crop due to its ability to store substantial nutritional reserves in its leaves during winter. It is rich in essential nutrients such as dietary fiber, vitamins C, B6, and K, along with minerals like calcium, potassium, magnesium, manganese, sulfur, and iron. Additionally, it contains health-promoting phytochemicals, including glucosinolates, flavonoids, anthocyanins, and terpenes, which may collectively help reduce the risk of chronic diseases, cancer, cardiovascular disorders, and immune dysfunction (Francisco et al., 2017).

2.6. Production Status of *Simuare* in Ethiopia

Simuare is predominantly grown in the Gurage Zone, Central Ethiopia Region, particularly in the highland and midland areas. It has been cultivated for generations, with farmers harvesting its leaves both for sale and personal consumption, mostly during the rainy season. However, there are no officially released *Simuare* varieties from the national research system, meaning its production relies on local cultivars and the indigenous knowledge of farmers regarding genetic resource conservation, selection, and cultivation. Since *Simuare* does not naturally produce seeds, farmers use vegetative propagation techniques for its cultivation. Research interventions are crucial to address the production and productivity constraints confronted by farmers in the production area.

2.7. Opportunities and Challengs of *Simuare* Production in Ethiopia

The production of *Simuare* in Ethiopia presents both challenges and opportunities. While the country boasts several public organizations, such as the Ethiopian Horticulture Development Agency and regional bureaus of agriculture, which support horticultural development, the production system for *Simuare* remains predominantly traditional. Many farmers lack essential skills in variety selection, fertilizer use, and modern crop management practices, leading to reduced productivity and suboptimal product quality. Traditional methods for sorting, grading, packing, and transportation further contribute to these issues. Additionally, the absence of

scientific recommendations for improving production practices hampers the potential for scaling up *Simuare* cultivation.

However, Ethiopia's favorable agro-climatic conditions and abundant water resources offer significant opportunities for improving *Simuare* production. The country's diverse altitudes, temperatures, and soil types create an ideal environment for growing a variety of crops, including *Simuare*. Despite having vast potential for groundwater and surface water resources, only a small fraction is currently utilized. Ethiopia's extensive drainage network, spanning both sides of the Rift Valley, presents opportunities for expanded irrigation systems that could boost vegetable production. Moreover, institutional challenges, such as limited access to improved production technologies, lack of proper storage and processing facilities, and weak market linkages, need to be addressed. By strengthening farmers' access to these resources, improving agricultural practices, and enhancing market connections, *Simuare* production could be significantly increased, supporting both local consumption and broader market demands.

2.8. Application of Plant Tissue Culture

Plant biotechnology has replaced traditional propagation methods by addressing their limitations (Debnath et al., 2006). Tissue culture, a key technique, ensures reliable biomass production and high-quality extracts for nutraceutical plants (Shibli et al., 2006). It involves cultivating small tissue samples (explants) on artificial media under sterile conditions to regenerate complete plants (Chandran et al., 2020). Explants are sterilized, often with alcohol, though mercuric chloride, commonly used, poses hazards and disposal challenges (Singh et al., 2011).

2.8.1. Micro propagation

Plant micro-propagation is an in vitro method for mass-producing genetically identical plants by culturing sterilized plant tissues in a controlled environment (Altman, 2000; Omar and Aouine, 2007). It is essential for propagating seedless or recalcitrant plants, preserving genetic resources, and advancing breeding techniques (Maara et al., 2006; Dodds, 2012). Significant

progress has been made in regenerating *Brassica* species, benefiting traditional breeding and genetic transformation methods. Advances in organogenesis have enabled uniform plant production (Vinterhalter et al., 2007; Cardoza and Stewart, 2003; Ravanfar et al., 2009).

2.8.1.1. Key stages of micro propagation

Plant micro propagation is a multi-stage process used to produce genetically identical plant clones. Stage 0 involves maintaining mother plants in a disease-free, controlled environment (Cassells and Doyle, 2006). Stage 1 focuses on sterilizing and establishing explants (Husain and Anis, 2009). Stage 2 is key for multiplication, using methods like axillary branching and callusing (Saini and Jaiwal, 2002). Stage 3 transfers the plantlets to a rooting medium, often using half-strength MS medium (Hussain et al., 2012). Finally, Stage 4, acclimatization, adapts the plantlets to field conditions (Bhojwani et al., 2013).

2.8.1.2. Benefits and Drawbacks of Micro-propagation

Micro-propagation is a tissue culture technique that enables rapid and efficient production of large numbers of plants from small explants, allowing thousands of genetically identical plants to be produced in a year without seasonal interruptions. This technique offers several advantages, including rapid propagation, clonal reproduction, germplasm conservation, genetic transformation, and the production of disease-free, high-quality plants (Hussain et al., 2012; Feyissa et al., 2005).

However, micro-propagation also presents challenges. It can be costly, with labor accounting for a significant portion of expenses. The process creates monocultures, which are more susceptible to disease outbreaks, as all plants may share the same vulnerabilities. If the initial plant is infected, the entire progeny can be affected, and some plants are difficult to disinfect from fungal contamination (Rani et al., 2023). Additionally, the aseptic nature of the process requires specialized skills and equipment and issues such as slow growth in perennial plants and the release of phenolic compounds can complicate tissue culture procedures (Lemma Dadi et al., 2020; McCown, 2000).

2.8.2. Explants, sources and aseptic techniques

In Brassica species, commonly used explants for tissue culture include shoot tips, nodal buds, and root tips. The success of tissue culture depends on factors such as the age, type, and position of the explants, as different plant cells have varying capacities for tot potency. Larger explants are more prone to contamination, while smaller explants, like meristems, may show slower growth (Sasikumar et al., 2009). The source of the explant, whether from in vitro or in vivo material, also affects plant regeneration. In vitro explants typically exhibit better organogenesis potential due to higher levels of endogenous hormones, making seedling explants more responsive than those from mature plants (Feyissa Tileye et al., 2005; Ochatt et al., 2018).

Surface sterilization of explants is crucial to prevent contamination, as plant material, especially from axillary nodes, can harbor microorganisms. Explants are treated with chemical disinfectants such as ethanol, sodium hypochlorite, hydrogen peroxide, or mercuric chloride, followed by thorough washing to remove any residues. After sterilization, the explants are placed on a culture medium containing inorganic salts, organic nutrients, vitamins, and plant hormones, often solidified with agar. Fungicides like Mancozeb and sodium hypochlorite are also used to control microbial contamination and promote successful in vitro propagation (García-González et al., 2010; Davidson et al., 2004; Nirwan et al., 2016; Ayele

2.8.3. Composition of culture medium

Different plant tissues require specific conditions for optimal growth in vitro. The culture medium contains macronutrients, micronutrients, vitamins, organic compounds, plant growth regulators, a carbon source, and sometimes gelling agents. The composition varies based on plant species, explant type, and experiment objectives, with adjustments often made for specific genotypes or developmental stages (Al-Limoun et al., 2020; Chimdessa Emiru, 2020).

Macronutrients like nitrogen, potassium, calcium, phosphorus, magnesium, and sulfur are essential for plant growth, while micronutrients such as iron, nickel, manganese, and zinc support metabolic processes. The MS medium provides these nutrients, along with vitamins like thiamine and nicotinic acid, which are vital for enzymatic reactions. Carbohydrates, primarily sucrose, serve as the main energy source for in vitro growth, though other sugars like glucose and fructose can also be used (De Fossard, 1976; Filek et al., 2010; Ślesak et al., 2004; Bhojwani and Dantu, 2013; Ruby et al., 2007).

2.8.4. Plant growth regulators

Plant growth regulators (PGRs) are crucial for optimizing micro propagation, influencing processes like cell elongation, division, and tissue differentiation. Selecting the right explants, growth regulators, and medium is key for successful regeneration. PGRs, used in small quantities, have species-specific effects, so testing different concentrations and combinations is essential when developing protocols for new species (Bairu et al., 2007).

2.9. Effect of PGR on Shoot initiation

The plant shoot system originates from the shoot apical meristem, which generates leaves and stems. Explants with younger tissue generally have better regeneration potential. In Brassica species, optimal regeneration occurs from 3-4-day-old seedlings, though 5-10-day-old seedlings are typically used for practical purposes (Cardoza and Stewart, 2004; Rafat et al., 2010). Shoot regeneration can be achieved from tissues like shoot tips, cotyledons, and roots, with shoot tips being preferred for clonal multiplication and virus elimination (Ravanfar et al., 2009). Successful shoot initiation is often achieved using hormones like BAP and kinetin (Khan et al., 2009; Mollika et al., 2011; Shah and Munir, 2019; Alam et al., 2013; Murata and Orton, 1987).

2.10. Effect of PGR on Multiplication

Shoot branching in plants is regulated by axillary meristems, primarily controlled by cytokinins, with auxins playing an indirect role. The success of micro propagation in Brassica

species depends on factors like genotype, media composition, and environmental conditions. For optimal shoot regeneration, BAP, often combined with GA3, has been shown to be effective (Jin et al., 2000; Sharma et al., 2000; Kaushal et al., 2005; Munshi et al., 2007; Maheshwari et al., 2011). The addition of BAP, especially in B. oleracea, significantly boosts shoot production, with concentrations typically ranging from 0.5 to 3 mg/L (Cheng et al., 2001).

2.11. Effect of PGR on Rooting

Roots are crucial for plant development, and understanding their function requires examining root systems. In micro propagation, rooting of in vitro propagated shoots, or micro cuttings, follows shoot multiplication. Shoots can be rooted on solid or liquid media, often with supporting materials like cellulose plugs (De Klerk, 2002). Indole-3-butyric acid (IBA), an auxin, is commonly used to promote root formation and enhance flower and fruit development. When combined with cytokinins like kinetin, IBA can induce callus formation, which differentiates into roots through indirect organogenesis. Direct organogenesis occurs when roots form directly from the explant (Bridgen et al., 1994). NAA, another auxin, is used as a rooting agent but can be toxic at high concentrations (Morikawa and Takahashi, 2004).

2.12. *In vitro* Regeneration of Some *Brassica carinata* Vegetables

Getachew et al. (2014) studied in vitro propagation of two B. carinata varieties, Holeta-1 and Yellow Dodola, using MS medium with varying concentrations of NAA, 2,4-D, and BAP. Cotyledons showed the best callus induction, with rapid growth. The highest callus growth rates (80.7% and 95%) were observed in hypocotyl and cotyledon explants on MS medium with 0.5 mg/L 2, 4-D in Yellow Dodola. Shoot induction was successful with 2 mg/L BAP. In a separate study, Shyam et al. (2021) developed a reliable regeneration protocol for Brassica juncea using mature cotyledons, seeds, and embryogenic cell suspension cultures. The best callus induction occurred with 3.0 mg/L 2, 4-D, and successful regeneration through embryogenesis and organogenesis was achieved with 0.5 mg/L BAP and 0.5-1.0 mg/L 2, 4-D. Both studies highlighted the influence of genotype, explant type, and growth regulator

concentrations on callus formation and regeneration efficiency (Getachew et al., 2014; Shyam et al., 2021).

3. MATERIALS AND METHODS

3.1. Site Description

This study was carried out at the Plant Tissue Culture Laboratory of the Holeta National Agricultural Biotechnology Research Center (NABRC), located 29 km west of Addis Ababa, at coordinates 9º 00' N and 38º 30' E, with an elevation of 2400 meters above sea level.

3.2. Establishing Stock Plants

A cabbage landrace, locally called *Simuare* (*Brassica carinata*), was used in this experiment. Cuttings were obtained from farmers' backyard in Gumer district of Gurage zone and planted in a field at Holeta HARC. The mother plants were watered twice a week, followed by proper weeding and other agronomic management practices.

Figure 1: *Ex vitro* multiplication of *Simuare* cabbage landrace at Holeta HARC

3.3. Preparation of Stock Solution and Culture Media

3.3.1. Stock solution preparation

The basal medium used throughout the study was Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). Separate full-strength stock solutions of macronutrients, micronutrients, Fe-Na-EDTA, FeSO4 mixtures, and vitamins were prepared. Each nutrient was weighed according to the Murashige and Skoog (1962) dissolved consecutively in double-distilled water, ensuring each component was completely dissolved using a magnetic stirrer. The prepared stock solutions were then transferred into bottles and stored at +4°C for up to four weeks. The macronutrient solution was concentrated 10 times, with 100 ml used per liter of medium, while the micronutrient, iron-EDTA, and vitamin solutions were 100 times concentrated, with 10 ml of each used per liter of medium.

In a similar process, stock solutions for various plant growth regulators (PGRs) including 6-benzylaminopurine (BAP), kinetin (KIN), α-naphthalene acetic acid (NAA), indole-3-butyric acid (IBA), and Gibberellin acid (GA3) were prepared. The PGRs were weighed and dissolved in the appropriate solvent at a ratio of 1 mg per 1 ml of solution. To assist with the dissolution, 3-4 drops of either 1N NaOH or 1N HCl were added, depending on the specific PGR. The final volume was adjusted with double-distilled water, and the solution was stirred with a magnetic stirrer. The PGR stock solutions were stored in labeled bottles in a refrigerator at +4°C for short-term use.

3.3.2. Preparation of culture medium

The preparation of the culture medium was standardized across all experiments. First, a stock solution was prepared by combining the full composition of the MS nutrient medium, including macro- and micronutrients, vitamins, and iron sources. To prepare the medium, 50

ml of MS1 stock solution was mixed with 5 ml each of MS2, MS3, MS4, MS5, and MS6 stocks in a 1L beaker (refer to Appendix Table 5). Next, 30 g/L of sucrose was measured and dissolved in double-distilled water. The solution was stirred with a magnetic stirrer until the sucrose dissolved completely, and the volume was adjusted with additional double-distilled water. The pH was then carefully adjusted to 5.7–5.8 using HCl and NaOH solutions. After pH adjustment, 6 g/L of agar powder was added to solidify the medium. The final MS basal medium was labeled and stored at +4°C in a refrigerator for short-term use.

3.4. Explant Sterilization

Immature shoot tips, free of disease, were collected from the top branches of each mother plant to serve as explants. These explants were initially cleaned using a commercial detergent, followed by rinsing with tap water and distilled water. Surface sterilization was then performed to ensure aseptic conditions and prevent contamination. To begin the sterilization process, the explants were transferred into a washing bottle and treated under a laminar flow hood. They were first washed with 70% ethanol for varying exposure times of 10, 20, 30, and 40 seconds. Following this, the explants were exposed to a mancozeb solution at concentrations of 1.5, 2, 2.5, and 3 mg/L for 5, 10, 20, and 40 minutes.

The final sterilization step involved immersion in chlorine bleach at concentrations of 1%, 1.5%, 2%, and 2.5% for exposure times of 5, 10, 20, and 40 minutes, with the addition of three drops of polyethylene glycol sorbitol mono laurate (Tween 20). After each treatment, the explants were rinsed thoroughly with double distilled water (at least three changes) to remove any residual sterility and then dried using sterile blotting paper. Any dead tissues were carefully removed from the explants before they were cultured. The cleaned explants were then placed into jars containing hormone-free full-strength MS medium, supplemented with 30g/L sucrose and solidified with 6g/L agar The cleaned explants were then placed into jars containing hormone-free full-strength MS medium, supplemented with 30g/L sucrose and solidified with 6g/L agar.

3.5. Culture Initiation

MS basal medium was prepared by supplementing it with various concentrations of BAP (0.5, 1, 1.5, and 2 mg/L) and KIN (0.5, 1, 1.5, and 2 mg/L), as outlined by Abrha Tafere *et al*. (2014) and Cristea *et al*. (2007). Aseptic shoot tip cultures were then placed on the MS medium, both individually for each hormone, in combination, and without hormones (a total of 25 treatments). The growth of the shoot tip cultures was regularly monitored

3.6. Shoot Multiplication

MS medium was prepared by supplementing it with varying concentrations of BAP (0.5, 1, 1.5, 2, and 3 mg/L) and GA3 (0.1, 0.2, and 0.3 mg/L) as described by Getachew *et a*l. (2014). Well-initiated shoots, which had been cultured on the initiation medium for three weeks, were then transferred to fresh MS basal medium supplemented with different concentrations of BAP and GA3 for shoot multiplication, resulting in a total of 24 treatments.

3.7. Rooting

Half-strength MS basal medium was prepared by supplementing it with varying concentrations of IBA (0.1, 0.2, 0.3, 0.5, and 1 mg/L) as per Naz et al. (2018) and NAA (0.1, 0.2, 0.5, and 1 mg/L) according to Khan et al. (2010) and Abrha Tafere et al. (2014). Shoots were transferred to a total of 25 treatments, which included combinations of four concentrations of IBA and four concentrations of NAA, as well as four concentrations of each hormone alone, with an additional control group without any rooting hormone.

Afterward, the prepared medium was dispensed into culture jars, which were sealed with caps and autoclaved at 121°C and 105 kPa for 15 minutes. The medium was then stored in the culture room until it was needed. Shoots were transferred onto shooting, multiplication and rooting medium, which was then sealed, labeled, and placed in a growth room. The growth room was maintained under a 16/8-hour (day/night) photoperiod, with a light intensity of 2500 lux provided by cool white fluorescent lamps, and a temperature of 25 ± 2°C.

3.8. Data collection and Experimental Designs

Data on various stages including surface sterilization, initiation, shoot multiplication, and rooting was collected. This encompassed the percentage of contamination-free cultures, contamination percentage, tissue death rate, days to shoot initiation, shoot height, number of shoots per plant, number of leaves per plant, root induction percentage, root length, and number of roots.

All experiments were laid in a Completely Randomized Design (CRD) with factorial treatment combinations, having three replications per treatment, three explants per jar under each replicate. Before laying the multiplication and rooting experiments, sufficient explants were made to multiply till the desired numbers of explants are gained at all times. Explants were cultured on a PGR-free medium before their use for an experiment; to avoid any sort of carry-over effects from the previous culture medium they were retained. Controls were set for each experiment with zero concentration of growth regulators.

3.9. Statistical Analysis

The collected data organized in a Microsoft Excel spreadsheet, then imported into SAS software version 9.3 (SAS, 2011) for statistical analysis. Analysis of variance (ANOVA) was performed to assess presence of significant differences among the treatments. Significant treatment means were compared using DMRT test

4. RESULT AND DISCUSSION

4.1. Culture Initiation

4.1.1. Explant surface sterilization

The analysis of variance indicated that the combined effects of sterilizing agents (mancozeb, bleach (Berekina), and ethanol), exposure time to these agents, and the interaction between concentration and exposure time had a highly significant impact ($P < 0.0001$) on the survival and contamination-free culture of shoot tip explants. Additionally, varying concentrations of mancozeb, bleach, and ethanol significantly affected ($P < 0.0001$) the levels of contamination and explant mortality (Appendix Table 1).

The lowest contamination and highest survival rates were observed in in vitro cultures treated with 70% ethanol, 2.5 mg/L mancozeb, and 2% bleach, with exposure times of 30 seconds, 20 minutes, and 20 minutes, respectively. This resulted in 99.2% survival of clean cultures on hormone-free medium (Figure 2 A). On the other hand, higher concentrations of mancozeb (3 mg/L), bleach (2.5%), and ethanol (70%) with longer exposure times (40 minutes for mancozeb and bleach, and 40 seconds for ethanol) almost eliminated all contaminants but led to a higher percentage of shoot tip death 58%).

Although mancozeb at 2.5 mg/L, bleach at 2 %, and 70% ethanol at 20 seconds of exposure resulted in a survival rate of 84.44%, it also caused a higher contamination level (14.81%) compared to the optimal concentrations with 70% ethanol, 2.5 mg/L mancozeb, and 2% bleach, with exposure times of 30 seconds, 20 minutes, and 20 minutes,. This highlights the importance of determining the optimal sterilant concentration and exposure time for the successful micro-propagation of *Simuare* (Figure 1).

Table 1: Effect of concentration and duration of explant exposure time on the percentage alive culture (PAC), percentage of contaminated culture (PCC) and percentage of death culture (PDC) from shoot tip explants of *Simuare*

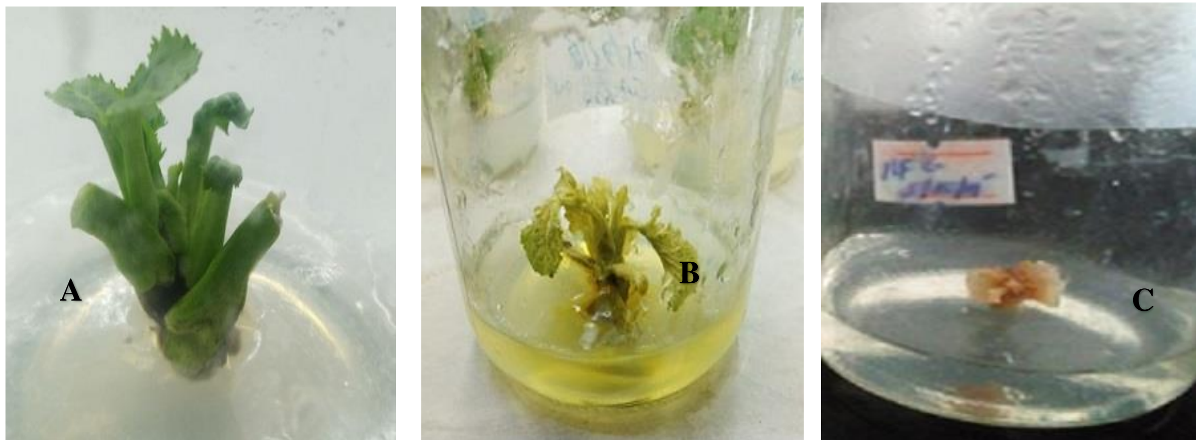
Sterility Concentration			Exposure Duration					
Ethanol (%)	Mancozeb(mg/L)	Bleach (%)	Ethanol (sec)	Mancozeb (min)	Bleach (min)	PAC (%)	PCC (%)	PDC (%)
0	0	0	0	0	0	0	100	0
70	1.5	1	10	5	5	22.22	77.78	0
70	1.5	1	20	10	10	25.92	74.07	0
70	1.5	1	30	20	20	34.81	65.19	0
70	1.5	1	40	40	40	62.22	37.78	0
70	2	1.5	10	5	5	51.11	48.89	0
70	2	1.5	20	10	10	56.29	43.7	0
70	2	1.5	30	20	20	58.52	41.48	0
70	2	1.5	40	40	40	46.66	53.33	0
70	2.5	2	10	5	5	74.81	24.44	0.74
70	2.5	2	20	10	10	84.44	14.81	0.74
70	2.5	2	30	20	20	99.26	0.74	0
70	2.5	2	40	40	40	87.41	0.74	10.37
70	3	2.5	10	5	5	85.92	0	14.07
70	3	2.5	20	10	10	76.29	0	23.7
70	3	2.5	30	20	20	72.59	0	27.41
70	3	2.5	40	40	40	41.48	0	58.52

Explants treated with increasing concentrations of mancozeb, bleach, and 70% ethanol for extended periods exhibited high sterilization efficiency. However, beyond certain optimal concentrations and exposure durations, the sterilizing agents caused injury and subsequent death of the explants. The highest mortality rate (58.52%) was observed with 3% mancozeb and 2.5% bleach after 40 minutes of exposure, as well as with 70% ethanol after 40 seconds of exposure (Figure 2 C). This elevated mortality was attributed to oxidative damage caused by these chemicals, resulting in lethal DNA damage to the plant tissues.

The results further indicated that contamination levels significantly decreased as concentration level increased, for each sterilizing agent concentration. The highest contamination rate

(77.78%) was observed with mancozeb at 1.5 mg/L for 5 minutes, bleach at 1% for 5 minutes, and 70% ethanol for 10 seconds (Tabel 2 B). This high contamination was likely due to insufficient concentration of sterilizing agents and inadequate exposure time, which failed to sufficiently remove or kill contaminants, primarily fungi and bacteria. Alam et al. (2016) noted that sterilization requirements can vary depending on the tissue type and the explants used in micro propagation.

Sterilizing explants effectively is a critical step in removing microbial contaminants and ensuring successful in vitro establishment of plant tissue cultures. Explants collected from open fields or greenhouses are often contaminated with fungi and bacteria (Daud et al., 2012). As a result, achieving clean cultures in such cases can be particularly challenging (Cassells, 2012). In this study, surface sterilization of *Simuare* shoot-tip explants using 70% ethanol, 2.5 mg/L mancozeb, and 2% bleach with exposure times of 30 seconds for ethanol and 20 minutes for mancozeb and bleach, respectively, proved to be optimal for effectively disinfecting microbial contaminants.



A= clean alive culture, B= contaminated culture C= dead culture

Figure 2: Effect of sterilization time, concentration and type of chemicals on culture survival

4.1.2. Shoot Initiation

The analysis of variance revealed that the interaction between BAP and kinetin (BAP*Kin) had a highly significant effect ($P < 0.0001$) on both the time to shoot initiation and shoot height. Shoot tip explants cultured on MS medium supplemented with BAP alone also showed

a significant effect ($P < 0.0001$) on both parameters. Additionally, different concentrations of kinetin significantly influenced shoot initiation time and shoot length ($P < 0.0001$).

Shoot development occurred across all concentrations of BAP and kinetin, both individually and in combination, to varying degrees. The highest shoot induction (100%) was achieved with MS medium supplemented with 1 mg/L BAP and 1 mg/L kinetin, followed by 93.33% with 1 mg/L BAP alone. This aligns with findings from Abrha Tafere et al. (2014) and Vanitha et al. (2017), who reported 90% and 91% shoot induction in B. carinata and B. oleracea, respectively. The difference in BAP concentration used may be related to the explant type and the source of induction. The effect of BAP on shoot initiation also varies among Brassica varieties (Gerszberg et al., 2015; Kumar et al., 2015).

Shoot induction was 83.33% on MS medium with 1 mg/L kinetin alone. Mollika et al. (2011) also reported 83.33% induction in B. juncea with MS medium containing 2 mg/L BAP and 0.5 mg/L kinetin. The higher induction percentage in this study, despite lower hormone concentrations, may be due to the type of explant, as shoot tips generally require lower hormone levels compared to seed explants. The lowest induction (46.67%) occurred with 2 mg/L BAP and 2 mg/L kinetin, while the control medium (no hormones) exhibited the lowest shoot induction (38.33%).

The study also showed that the combination of 1 mg/L BAP and 1 mg/L kinetin resulted in the shortest shoot initiation time, with an average of 7.09 ± 0.65 days. In contrast, 1 mg/L BAP or 1 mg/L kinetin alone had slightly longer initiation times (6.89 ± 0.00 days and 7.08 ± 1.08 days, respectively), but lower induction percentages. This finding is consistent with Cristea et al. (2009), who reported a 6-day initiation period.

Of all the treatments, MS medium with 1 mg/L BAP and 1 mg/L kinetin resulted in the longest shoot length (3.98 ± 0.43 cm). The second and third longest shoots were observed with other combinations of BAP and kinetin (3.95 ± 0.56 cm and 3.23 ± 0.83 cm, respectively). Hence, MS medium with 1 mg/L BAP and 1 mg/L kinetin is considered the optimal concentration for shoot initiation, providing the highest shoot induction percentage, the shortest initiation time, and the longest shoot height.

Although hormone-free MS medium resulted in shoots with a mean length of 2.1 ± 0.4 cm, it took much longer for shoot initiation (21.87 ± 2.04 days), and the shoots were not vigorous enough for multiplication. Nonetheless, successfully initiated explants were retained for further shoot multiplication.

Table 2: Effect of varying concentrations and combinations of bap and kinetin on shoot initiation from shoot tip explants of *Simuare*

Treatment		Shoot induction	Day to initiation	Shoot height(cm)
mg^{-1} PAP	mg^{-1} Kinetin	(%)	(day) Mean±SD	Mean±SD
00	00	38.33	19.61±3.28[a]	2.04±0.41[b]
0.5	00	65.00	12.89±2.72[d]	2.26±0.76[b]
1.0	00	93.33	6.89±0.00[e]	3.95±0.56[a]
1.5	00	61.67	13.84±2.11[c]	2.00±0.63[b]
2.0	00	48.33	15.31±2.32[b]	1.97±0.5[b]
00	00	38.00	16.91±2.02[a]	2.03±0.53[cd]
00	0.5	56.67	13.35±2.79[c]	1.93±0.49[d]
00	1.0	83.33	7.08±1.08[d]	3.23±0.83[a]
00	1.5	58.30	12.31±2.63[c]	2.55±0.66[b]
00	2.0	48.00	15.75±2.09[b]	2.32±0.74[bc]
00	00	34.16	21.87±2.04[a]	2.1±0.4cd[e]
0.5	0.5	63.33	13.16±2.61[g]	2.07±0.65[cde]
0.5	1	63.33	15.58±2.14[ef]	2.08±0.69[cde]
0.5	1.5	66.67	15.68±2.14[ef]	2.09±0.46[cde]
0.5	2	66.67	18.53±3.48[bc]	1.95±0.07[de]
1	0.5	73.3	13.4±3.15[g]	2.22±0.37[cd]
1	1	100	7.09±0.65[h]	3.98±0.43[a]
1	1.5	86.67	13.15±2.54[g]	2.67±0.27[b]
1	2	46.67	17.00±1.30[cde]	2.15±0.54[cde]
1.5	0.5	60.00	17.83±1.89[bc]	2.08±0.17[cde]
1.5	1	50.00	15.13±1.92[f]	2.08±0.17[cde]
1.5	1.5	63.33	16.11±2.84[def]	2.29±0.24[c]
1.5	2	56.67	17.71±1.53[bcd]	2.05±0.16[cde]
2	0.5	50.00	18.17±2.03[bc]	1.99±0.02[cde]
2	1	60.00	18.28±2.22[bc]	2.06±0.26[cde]
2	1.5	50.00	19.13±2.57[b]	1.85±0.2[e]
2	2	46.67	19.22±3.84[b]	2.33±0.48[c]
CV		3.47	8.85	8.5
LSD			9.55	1.5

Shoot tips cultured on hormone-free MS medium took the longest duration for shoot initiation, averaging 21.87 ± 2.04 days (Table 2). Similar results were reported by Bano et al. (2010) (22 days), Goswami et al. (2020) (19 days), and Cristea et al. (2007) (18 days). Kamal et al.

(2007) found an even longer initiation period of 28.30 days in B. juncea. The extended initiation time observed in these studies may be due to differences in the shoot initiation method, with initiation from callus typically taking longer than from shoot tips.

Among all media combinations of BAP and kinetin, the highest mean shoot length was observed on MS medium supplemented with 1 mg/L BAP and 1 mg/L kinetin, with an average shoot length of 3.98 ± 0.43 cm. The second and third longest shoots were observed with MS media containing 1 mg/L BAP and 1 mg/L kinetin, with average lengths of 3.95 ± 0.56 cm and 3.23 ± 0.83 cm, respectively. Therefore, the combination of 1 mg/L BAP and 1 mg/L kinetin can be considered the optimal concentration for shoot initiation in this variety, as it provides the highest shoot induction percentage, shortest initiation time, and longest shoot height (Figure 3). Although the hormone-free MS medium produced a mean shoot length of 2.1 ± 0.4 cm, it required a longer initiation period and was not vigorous enough for multiplication (Table 2). The well-initiated explants were retained for further shoot multiplication.

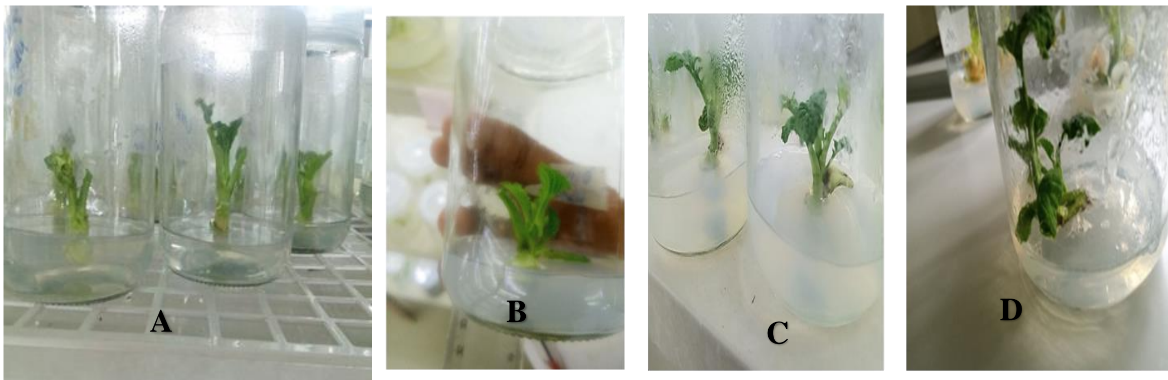


Figure 3: *In vitro* shoot initiation of *B. carinata* a local landrace *Simuare* on A is MS 1mg^{-1}BAP and 1mg^{-1}KN B is 2mg/l BAP and KIN C 1.5MG/L BAP D is HF from shoot tip

4.2. Shoot Multiplications

The analysis of variance revealed that the combination of different concentrations of BAP and GA3 significantly influenced both the number of leaves and the number of shoots per explant ($P < 0.0001$) (Appendix Table 3). This finding supports the observations of Mantell and Hugo (1989), who reported that the nature and concentration of cytokines significantly affect shoot multiplication. The induction of shoots in all treatments cultured on MS media supplemented

with varying concentrations of BAP and GA3 in the present study aligns with the results reported by Goswami et al. (2020).

In the present study, media supplemented with plant growth hormones yielded better results in terms of the number of leaves per plant this in line with (Naz et al., 2018), as well as the number of shoots per plant and shoot height also similar result (Jia et al., 2019). Among all the combinations tested, the best multiplication response was observed on MS medium supplemented with 3 mg/L BAP and 0.1 mg/L GA3, which resulted in a mean number of leaves per plant of 6.2 ± 1.68.The second highest number of leaves (4.46 ± 1.3) was observed on MS medium containing 3 mg/L BAP and 0.2 mg/L GA3. In contrast, the control treatment, which lacked BAP and GA3, resulted in the fewest leaves (0.32 ± 0.69) and shoots (0.2 ± 0.4) per plant.

The highest mean number of shoots per explant (4.33 ± 1.87) was recorded on MS medium containing 3 mg/L BAP and 0.1 mg/L GA3 (Figure 4). This was followed by the combination of 3 mg/L BAP and 0.2 mg/L GA3, which produced 2.43 ± 0.43 shoots per explant. This result is consistent with the findings of (Dina et al. (2019), who reported 3.13 shoots per explant. However, increasing the concentrations of BAP and GA3 beyond this level resulted in a decrease in the number of shoots, with some explants showing necrosis and shoot fasciation (Table 3). This finding contrasts with the results of Goswami et al. (2020), who reported higher shoot numbers (8.2 and 7.00 shoots per explant) for B. campestris var. Agrani and B. campestris var. BINA Sarisha-10, respectively. The discrepancy may be due to differences in hormone concentrations, explant types, media, and varieties. The lowest shoot number per plant (1.1 ± 0.16) was recorded on MS medium supplemented with 0.5 mg/L BAP and 0.3 mg/L GA3, which is consistent with the findings of Dina et al. (2019), who reported 1.66 shoots per plant The longest shoots (4.44 ± 0.83 cm) were observed on the multiplication medium supplemented with 3 mg/L BAP and 0.1 mg/L GA3, while the second longest shoots (3.63 ± 0.98 cm) were recorded on MS medium containing 3 mg/L BAP and 0.2 mg/L GA3. Therefore, the combination of 3 mg/L BAP and 0.1 mg/L GA3 can be considered the optimal concentration for shoot multiplication in this variety, as it provided the highest values for the number of shoots, shoot length, and leaf number (Figure 4).

Table 3: Effects of various concentrations and combinations of BAP and GA3 on shoot multiplication from shoot tip explants *Simuare*

Treatment		No. of leaf per shoot (Mean±SD)	No. of shoot per explant (Mean ± SD)	Shoot height(cm) (Mean±SD)
mg^{-1} BAP	mg^{-1} GA3			
00	00	0.32±0.69^{i}	0.2±0.4^{h}	3.53±1.1^{j}
0.5	0.1	2.2±0.32^{fgh}	1.2±0.23^{fg}	2.73±0.39^{efgh}
0.5	0.2	3.23±1.69^{cde}	1.17±0.28^{g}	2.39±0.2^{fi}
0.5	0.3	2.4±0.21^{fgh}	1.1±0.16^{g}	1.97±0.15^{i}
1	0.1	2.43±0.53^{fgh}	1.2±0.32^{fg}	2.98±0.5^{cdefg}
1	0.2	2.57±0.47^{efgh}	1.23±0.35^{efg}	2.68±0.67^{fgh}
1	0.3	2.00±00^{h}	1.3±0.33^{efg}	2.59±0.44^{gh}
1.5	0.1	2.87±0.32^{def}	1.43±0.27^{defg}	3.1±0.49^{dcdefg}
1.5	0.2	2.77±0.41^{efg}	1.13±0.23^{g}	2.52±0.39^{gh}
1.5	0.3	2.1±0.2^{gh}	1.67±0.23^{g}	2.89±0.39^{defgh}
2	0.1	3.5±0.72^{cd}	1.77±0.39^{cd}	3.43±0.77^{bc}
2	0.2	2.72±0.37^{efg}	1.6±0.54^{de}	3.36±1.0^{bcd}
2	0.3	2.27±0.31^{efg}	2.0±0.35^{c}	3.17±1.1^{bcdef}
3	0.1	6.2±1.68^{a}	4.33±1.87^{a}	4.44±0.83^{a}
3	0.2	4.46±1.3^{b}	2.43±0.73^{b}	3.63±0.98^{b}
3	0.3	3.57±0.67^{c}	1.57±0.44^{c}	3.22±0.66^{bcde}
CV		3.27	4.36	2.16
LSD		30.6	3.18	1.23

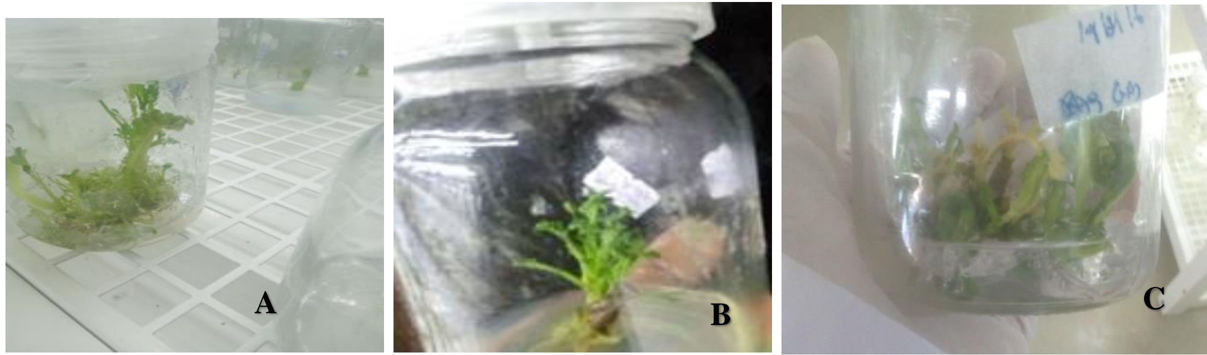


Figure 4: *In vitro* shoot multiplication of *B. carinata* a local landrace *Simuare* on MS A 3mg^{-1}BAP and 0.1mg^{-1} GA3 B is 0.1GA3 and 2 BAP C is 0.1GA3 and 3BAP from shoot tip

4.3. Rooting of Micro Shoots

The analysis of variance indicated that the different concentrations and combinations of IBA and NAA significantly affected root induction (%), root length (cm), and the number of roots ($P<0.0001$) (Appendix Table 4). The highest rooting percentage (77%) was observed in MS medium supplemented with 0.3 mg/l IBA in combination with 0.2 mg/l NAA. The application of 0.3 mg/l IBA alone resulted in a 55% rooting response, while 0.2 mg/l NAA alone induced 52% rooting. This finding aligns with Bhuiyan et al. (2009), who also reported a high frequency of rooting in B. juncea on MS media containing 0.2 mg/l NAA. On the other hand, the highest concentration of IBA and NAA (1 mg/l) resulted in the lowest rooting response (22%). The control treatment, with no rooting hormones, showed poor rooting characterized by few roots per shoot and short root lengths (Table 4).

The best rooting performance, including the highest mean number of roots (5.04±0.07), was observed on MS medium supplemented with 0.3 mg/l IBA and 0.2 mg/l NAA. This result supports the findings of Khan et al. (2010) and Mollika et al. (2011), who also found that a combination of IBA and NAA promotes better root production. The second highest mean number of roots (2.28±0.57) was recorded with 0.3 mg/l IBA, followed by 0.2 mg/l NAA, which resulted in a mean number of 2.22±0.62 roots per explant (Figure 5).

Moreover, the longest roots were obtained with the combination of 0.3 mg/l IBA and 0.2 mg/l NAA, with a mean root length of 3.06±0.47 cm. The second longest roots were recorded in media containing 0.2 mg/l NAA, which produced roots measuring 2.47±0.41 cm. In contrast, the highest concentrations of both IBA and NAA (1 mg/l each) led to the shortest root lengths, with a mean of 1.33±0.29 cm (Table 4).

In this experiment, the best rooting response was observed on MS medium supplemented with 0.3 mg/l IBA in combination with 0.2 mg/l NAA, yielding a mean of 5.04±0.07 roots per plant. This finding is supported by previous studies, where the combination of IBA and NAA was shown to enhance root production (Khan et al., 2010; Mollika et al., 2011). The second-highest mean number of roots (2.28±0.57) was recorded from MS medium with 0.3 mg/l IBA, followed by 0.2 mg/l NAA, which resulted in 2.22±0.62 roots (Figure 5).

Additionally, the longest roots were obtained from the combination of 0.3 mg/l IBA and 0.2 mg/l NAA, with a mean root length of 3.06±0.47 cm per plantlet. The second-longest roots were produced by MS medium containing 0.2 mg/l NAA, with a mean length of 2.47±0.41 cm. In contrast, the highest concentrations of both NAA and IBA (1 mg/l each) resulted in the shortest root lengths, with a mean of 1.33±0.29 cm (Table 4).

Forsyth and Van Staden (1981) reported the best rooting percentage on MS medium supplemented with 5 mg/l IBA, which contradicts the current findings. In this study, low concentrations of IBA and NAA were found to promote root induction and elongation, while extremely high concentrations hindered rooting. These results align with the findings of Mao et al. (2009), who observed that optimal concentrations of IBA and NAA produced better root formation than lower concentrations. The activation of various processes, such as protein and nucleic acid synthesis, cell division, and expansion, likely contributed to the observed rooting response. These processes enhance apical dominance, a natural side effect of auxin-induced growth and cellular changes in plants.In the present experiment, the highest rooting percentage (77%) was achieved with a combination of 0.3 mg/l IBA and 0.2 mg/l NAA, which is consistent with the idea that moderate concentrations of auxins are most effective for promoting root formation. This result further supports the notion that auxins, at optimal concentrations, stimulate root induction and elongation, while higher concentrations, as seen

in the 1 mg/l IBA and NAA treatments, resulted in poor rooting and root elongation. Therefore, the results of this study align with the general principle that low to moderate levels of IBA and NAA are most effective for root induction in *B. carinata*.

Table 4: The effects of various concentrations and combinations of IBA and NAA on rooting from shoot tip explants *Simuare*

Treatment		Root induction	Number of root	Root length(cm)
Gm^{-1} IBA	Mg^{-1} NAA	(%)	Mean±SD	Mean±SD
00	00	20	0.65±0.58[c]	0.73±0.65[c]
0.1	00	32	1.4±0.46[b]	1.33±0.44[b]
0.3	00	55	2.28±0.57[a]	2.22±0.65[a]
0.5	00	52	1.42±0.77[b]	1.56±0.57[b]
1	00	40	1.15±0.53[bc]	1.33±0.25[b]
00	00	12	0.5±0.53[b]	0.78±0.83[b]
00	0.1	30	0.8±0.59[b]	1.08±0.79b
00	0.2	52	2.22±0.62[a]	2.47±0.41[a]
00	0.5	50	0.89±0.5[b]	1.16±0.67[c]
00	1	47	1.73±1.55[a]	1.33±0.25[d]
00	00	16	0.34±0.53[d]	1.18±0.32[e]
0.1	0.1	30	1.05±0.5[cd]	1.56±0.25[cd]
0.1	0.2	32	1.3±0.63[cd]	1.54±0.37[cde]
0.1	0.5	35	1.2±0.35[cd]	1.57±0.29[cde]
0.1	1	42	1.00±0.24[d]	1.49±0.37[cde]
0.3	0.1	45	1.47±0.4[cd]	1.73±0.31[bcd]
0.3	0.2	77	5.04±0.07[a]	3.06±0.47[a]
0.3	0.5	55	1.33±0.41[cd]	1.18±0.34[bc]
0.3	1	40	2.3±1.27[b]	1.66±0.88[bcd]
0.5	0.1	35	1.3±0.42[cd]	1.65±0.43[bcd]
0.5	0.2	47	1.65±0.34[c]	1.65±0.3[cd]
0.5	0.5	40	1.35±0.62[cd]	1.72±0.22[cde]
0.5	1	27	0.95±0.37[d]	1.35±0.62[de]
1	0.1	42	1.1±0.31[cd]	1.38±0.31[cde]
1	0.2	47	1.3±0.42[cd]	1.75±0.22[bcd]
1	0.5	30	1.1±0.57[cd]	1.41±0.31[cde]
1	1	22	0.8±0.42[d]	1.33±0.29[de]
CV		5.24	4.48	2.46
LSD		34.2	2.925	1.12

A

Figure 5: *In vitro* rooting of *Simuare,* on MS medium supplemented with A 0.3mg^{-1}IBA and 0.2mg^{-1}NAA cultured from the shoot tip

5. CONCLUSION AND RECOMMENDATIONS

This study successfully developed an optimized micro-propagation protocol for *Simuare* (*Brassica spciace*), addressing key aspects such as sterilization, shoot initiation, multiplication, and root induction. The findings highlighted the importance of selecting appropriate sterilization agents and hormonal combinations for efficient tissue culture. Optimal sterilization was achieved using 2.5 mg/L mancozeb, 2% bleach for 20 minutes, and 70% ethanol for 30 seconds, effectively eliminating microbial contaminants.

For shoot initiation, the highest success rate (100%) was observed with MS medium supplemented with 1 mg/L BAP and 1 mg/L kinetin, while the shortest initiation time (7.09 ± 0.65 days) was also recorded with this formulation. Shoot multiplication was optimized using 3 mg/L BAP combined with 0.1 mg/L GA3, yielding high shoot and leaf production. Root induction was most effective on half-strength MS basal medium with 0.3 mg/L IBA and 0.2 mg/L NAA, achieving a 77% rooting rate and superior root quality.

Based on the findings of this study, the following recommendations and directions for future research and implementation are made to scale up *Simuare* micro-propagation, thereby enhancing its commercial viability and supporting efforts for food nutrition, agricultural sustainability, and crop improvement.

- ✓ Explore alternative explant parts, beyond nodal shoot tips, for *in vitro* propagation of *Simuare* to assess if they yield more efficient responses.
- ✓ - Investigate the impact of different auxin concentrations, rather than BAP and KN, on shoot initiation.
- ✓ - Conduct further studies on the effectiveness of various cytokinins in combination with other hormones (both cytokinins and auxins) for improved shoot multiplication. Also, evaluate the impact of different auxin concentrations, other than IBA and NAA, on root induction.
- ✓ - Investigate the use of alternative techniques, such as culturing explants in liquid medium, for mass propagation.

- ✓ - Focus on applying the developed micro propagation protocol for large-scale production of *Simuare*, ensuring its distribution to farmers and boosting production in Ethiopia.
- ✓ - Prioritize the investigation of the molecular diversity of *Simuare* for its conservation, due to the unique propagation method.
- ✓ This study emphasizes *Simuare*'s potential for micro propagation as a sustainable solution to address plant material shortages and contribute to food nutrition in the Gurage and Silte zones and beyond.
- ✓ conduct effective for acclimatization brassica spices local landrace *Simuare*
- ✓ - Given the limited duration of our study, it is essential to conduct multi-season field performance evaluations across different locations. This will allow for a comprehensive comparison between tissue culture-based and conventionally propagated plant materials, helping to inform farmers about the benefits of using high-quality planting materials over traditional sources.

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7. APPENDICES

Appendix Table 1 Effect of concentration and duration of explant exposure time on the percentage alive culture (PAC), percentage of contaminated culture (PCC) and percentage of death culture (PDC) from shoot tip explants of *Simuare*

Source	Df	PAC	PCC	PDC
Concentration (C)	4	1028.5***	1718.6***	183.1***
Duration (D)	3	41.9***	44.6***	33.4***
C*D	12	398.58***	15.8***	19.49***
CV (%)		2.91	7.55	0.112
R^2		0.99	0.9	0.57
Mean		57.6	55.26	9.15
STD. Error		0.00929	0.0093	0.04724
LSD		28.75	5.72	6.35

*** = highly significant at $P < 0.001$, PAC= percentage alive culture; PCC= percentage of Contaminated culture; PDC= percentage of death culture; CV= coefficient of variation

Appendix Table 2: Effect of BAP and Kinetin on initiation percentage (PI) day to initiation (DI) and shoot height from shoot tip of *Simuare*

Source	Df	PI	DI	SH
BAP	3	7319***	320.0***	3.056***
KIN	3	1583**	115.7**	0.85**
BAP*KIN	9	1367***	38.9***	0.97***
CV (%)		3.47	8.85	8.5
R^2		0.04	0.1	0.01
Mean		55.83	16.63	2.24
STD. Error		0.0203	0.247	0.0293
LSD		56.64	9.55	1.5

*** = highly significant at $P < 0.001$ PI= percentage initiation; DI= day to initiation; SH=shoot height

Appendix Table 3: Effect of BAP and GA3 on number of shoot (NS), shoot height (SH) and number of leaf from shoot tip of *Simuare*

Source	Df	NS	SH	NL
BAP	4	13.96***	8.34***	28.89***
GA3	2	4.531***	4.224***	12.66***
BAP*GA3	8	4.042***	0.611***	3.74***
CV (%)		4.36	2.16	3.27
R^2		0.75	0.42	0.8
Mean		1.28	2.98	2.34
STD. Error		0.0472	0.0617	0.0721
LSD		3.18	1.23	30.6

*** = highly significant at $P < 0.001$; NS= number of shoot; SH= shoot height; NL= number of leaf

Appendix Table 4: Effect of IBA and NAA on rooting percentage from shoot tip of *Simuare*

Source	Df	PR	NR	RH
IBA	3	7067***	25.168***	2.87***
NAA	3	2344**	11.56**	2.22**
CV (%)		5.24	4.48	2.46
R^2		0.4	0.76	0.52
Mean		35.87	1.28	1.67cm
STD. Error		1.641	0.0799	0.0427
LSD		34.2	2.925	1.12

*** = highly significant at $P < 0.001$; PR= percentage rooting; NR= number of root; RH= root height

Appendix Table 5: Stock solution for MS media Source (Murashige and Skoog1962)

Code	Nutrients	Stock solution(gm)		Volume of stock for 1Lfull MS media
MS1	Ammonium nitrate (NH4NO3)	33.0	In 1000 ml	50 ml
	Potassium nitrate (KNO3)	38		
MS2	Magnesium sulphate ($MgSO_4.7H_2O$)	18.07	In 500 ml	5 ml
	Manganese sulphate ($MnSO_4.H_2O$)	1.69		
	Zinc Sulphate ($ZnSO_4.7H_2O$)	0.86		
	Copper Sulphate ($CuSO_4.5H_2O$)	0.0025		
MS3	Calsium Chloride ($CaCl_2.2H_2O$)	33.22	In 500 ml	5 ml
	Potassium Iodide (KI)	0.083		
	Cobalt Chloride ($CoCl.6H_2O$)	0.0025		
MS4	Potassium dibasic phosphate (KH_2PO_4)	17	In 500 ml	5 ml
	Boric acid(H_3BO_3)	0.62		
	Sodium molbdate ($Na_2MoO_4.2H_2O$)	0.025		
MS5	Na EDTA	3.726	In 500 ml	5 ml
	Iron sulphat e ($FeSO_4.7H_2O$)	2.78		
MS6	Myo-inositol	10.0	In 500 ml	5 ml
	Glycine	0.2		
	Thiamine HCl	0.01		
	Pyridoxine HCl	0.05		
	Nicotine acid	0.05		