OPTIMIZATION OF *IN VITRO* REGENERATION, CALCIUM BIO-FORTIFICATION AND MYCORRHIZAL BIOTIZATION OF SELECTED SEED POTATO FOR ENHANCED PERFORMANCE AND BACTERIAL WILT MANAGEMENT

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Optimization of *In Vitro* Regeneration, Calcium Bio-fortification and Mycorrhizal Biotization of Selected Seed Potato for Enhanced Performance and Bacterial Wilt Management

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Horticulture of the Jomo Kenyatta University of Agriculture and Technology

2024

DECLARATION

This thesis is my original work and has not been presented for a degree in any other university

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DEDICATION

To my dearest mother Sekunda, and brother Jeremy, for their relentless support, prayers, and encouragement. This was possible largely because of you.

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LIST OF ABBREVIATIONS AND ACRONYMS

AMF	Arbuscular Mycorrhizal Fungi
BA	6-Benzyladenine
BAP	6-Benzylaminopurine
BW	Bacterial Wilt
CFU	Colony Forming Units
CPG	Casamino Acid-Peptone-Glucose
CSP	Certified Seed Potato
DAC	Days After Culture
DAP	Days After Planting
DoC	Days of Culture
FW	Fresh weight
GA	Gibberellic acid
GAP	Good Agricultural Practices
KALRO	Kenya Agricultural & Livestock Research Organization
KEPHIS	Kenya Plant Health Inspectorate Service
M. A. S. L	Meters above sea level
MS	Murashige and Skoog

NPCK	National Potato Council of Kenya
PGR	Plant Growth Regulator
RSSC	*Ralstonia solanacearum* species complex
SSA	Sub-Saharan Africa
TC	Tissue culture
VC's	Value Chain

ABSTRACT

Potato is an important tuber crop that plays a key role in achieving food security and alleviating poverty in sub-Saharan Africa. The Irish potato is a significant source of dietary energy, and is rich in essential vitamins and nutrients. In Kenya, the potato is the second most important food crop after maize, holding greater economic importance for smallholder cash crop farmers. Despite the increased demand for potato, production is hampered by constraints such as inadequate supply of clean and high quality seed, low soil fertility, pests and diseases such as Bacterial Wilt. The availability of clean and viable seed is the most constraining challenge necessitating the development of new production strategies that foster the production of high quality seed. Agronomic fortification with calcium during tissue culture and mycorrhizal inoculation are ways of stimulating rapid regeneration, while fostering plantlet growth under greenhouse conditions. Two laboratory experiments and a greenhouse experiment were set up to investigate the effect of calcium (Ca) fortification and AMF mycorrhizal inoculation on production of three potato varieties (*Shangi*, *Unica* and *Dutch Robjyn*). The first experiment involved sub-culturing single node cuttings of three varieties onto modified Murashige and Skoog (MS) media with five levels of Ca, from the macronutrient $CaCl_2$; 8.8g/L (conventional MS medium), 10.4g/L, 12g/L, 13.6g/L and 15.2g/L Ca. In the second laboratory experiment, micro-tubers were initiated on MS media modified with the five Ca levels, 60g/L sucrose and 6mg/L 6-Benzylaminopurine. The experiments were set up in a completely randomized design and replicated five times. A pot experiment was established using non-Ca bio-fortified and Ca-bio-fortified plantlets (plantlets with highest mineral Ca content in root-zone section) from the first experiment. Calcium bio-fortified and non-Ca fortified plantlets were inoculated with a commercially available mycorrhizal inoculant (Rhizatech™) in a completely randomized design with six replications. The plantlets were then infected with *Ralstonia solanacearum* after one month of establishment. Performance determination was based on *In vitro* regeneration capacity, micro-tuber number, days to micro-tuber formation, micro-tuber fresh weight, root-zone mineral contents, plantlet height and leaf number, percent mycorrhizal colonization and Bacterial Wilt disease severity. Analyses of variance proved a significant and highly significant effect *($P<0.01$)* on tested factors and their interactions on shoot and root development of the three varieties. MS medium containing 10.4g-13.6g/L Ca levels significantly *($P<0.05$)* increased shoot and root number in the three varieties. Conventional MS medium resulted in a loss of apical dominance in *Unica* shoots. Each variety across all Ca treatments produced at most one micro-tuber. MS media containing 10.4g-13.6g/L Ca significantly (P=<0.0001) reduced the number of days it took to micro-tuber formation for *Unica*, *Shangi*, and *Dutch Robjyn* compared to the conventional MS medium containing 8.8g/L $CaCl_2$. MS media containing 10.4g/L Ca completely inhibited micro-tuber formation in *Unica*, instead promoting the growth of etiolated shoots. MS media containing 13.6g/L Ca resulted in plantlets with the highest Ca content in the mid-stem and root-zone sections of *Shangi, Dutch Robjyn* and *Unica*. Analyses of variance proved a significant and highly significant effect ($P<0.01$) on tested factors and their interactions on the plantlet height and leaf number of the three varieties. Agronomic calcium bio-fortification combined with mycorrhizal inoculation resulted in 9% and 11% increase in *Shangi* and *Dutch*

Robjyn plantlet height while simultaneously resulting in 40% reduction in *Unica* plantlet height compared to control plantlets. Mycorrhizal inoculation resulted in 21% and 25% decrease in *Shangi* and *Unica* plantlet height respectively. There were significant differences ($P<0.05$) in *Shangi* and *Dutch Robjyn* leaf numbers between treatments. Mycorrhizal inoculation using 2g/pot Rhizatech™ resulted in higher plantlet leaf numbers in the three varieties compared to the non-biotized treatments. Agronomic Ca bio-fortification combined with mycorrhizal inoculation resulted in an overall 35% increase in total chlorophyll content in *Dutch Robjyn*. Mycorrhizal inoculation using 2g/pot Rhizatech™ resulted in 12%, 16% 22% increase in total chlorophyll content in *Shangi*, *Dutch Robjyn* and *Unica* respectively, compared to non-biotized plantlets. All biotized plantlets showed 100% mycorrhizal colonization and there were strongly significant differences ($P<0.01$) in the number of mycorrhizal propagules found in 1cm root fragments of the three varieties. Agronomic Ca fortification combined with mycorrhizal inoculation decreased the number of mycorrhizal propagules in *Shangi* and *Unica* by 9% and 90% respectively and increased the number of fungal propagules by 21% in *Dutch Robjyn*. All plantlets across all treatments for the three varieties exhibited bacterial wilt disease symptoms. There were significant differences ($P\leq0.05$) in bacterial wilt severity scores between treatments within the three varieties. Agronomic *In vitro* Ca bio-fortification combined with AMF biotization resulted in a 18.4% and 16.5% reduction in disease expression in *Shangi* and *Dutch Robjyn* while increasing severity in *Unica* by 43% compared to their control treatments. The obtained results confirm the benefits of *In vitro* agronomic Calcium fortification using 10.4-13.6g/L $CaCl_2$, instead of conventional 8.8g/L $CaCl_2$ on promoting shoot and root development, micro-tuber regeneration in the three varieties. Agronomic Ca bio-fortification combined with mycorrhizal inoculation positively influenced *Shangi* and *Dutch Robjyn* TC plantlet development and reduced bacterial wilt disease severity. The study findings point to the benefit of agronomic calcium bio-fortification during tissue culture, and when combined with mycorrhizal inoculation, improves seed potato growth, yield and disease suppression. Interactions between *In vitro* calcium bio-fortification combined with biotization should be investigated in the long term.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Potato is the fourth most important food crop worldwide and most important root and tuber crop (Zhang *et al.*, 2017; Jennings *et al.*, 2020). It is the third most important food crop in Sub-Saharan Africa (SSA), after rice and maize. Production across Africa is varied, with Northern African countries such as Morocco, Egypt and Algeria being the major potato producers on the continent. In SSA, most potatoes are primarily produced in Eastern Africa (71%), compared to Western Africa's (8%) and Southern Africa's (21%). Yields in SSA range between 6-10 tonnes/hectare, compared to attainable yields of 25-35 tonnes/hectare (International Potato Center, 2017; Muthoni & Shimelis, 2023). Potato production in SSA has increased due to an increase in cropping area, with little change in tuber yields (Harahagazwe, 2018). Over the next decades, cereal yields are likely to level off prompting the push for potatoes in SSA. The Irish potato is expected to play a key role in achieving Sustainable Development Goal 2 of achieving Zero Hunger (Licker et al., 2007). However, a myriad of challenges facing potato production in SSA including insufficient clean seed, biotic and abiotic stresses have caused dramatic yield gaps (Harahagazwe., 2018). The potato industry plays a key role in the realization of Kenya's Vision 2030, under the economic pillar due to its contribution to food availability (GoK, 2021).

Kenya currently produces between 2-3M tonnes, valued at KES 40-50B in revenue, against a projected estimate of up to 8M tonnes (Mbego, 2019). The crop is locally produced by over 800, 000 farmers, employing 3.5M people along the value chain, thus contributing to poverty alleviation (Muthoni *et al.*, 2013b; Sanginga, 2015). Annual production in Kenya has increased by 40%, from 1.5 million tonnes in 2017 to 2.1 million tonnes in 2021(Wakaba et al., 2022). Despite increased total production, there has been a marked decrease in output per unit area attributed to production constraints that include,

but is not limited to, the use and over-reliance on farmer-saved seed that is often low quality, and the lack of sufficient clean (disease-free) seed from formal seed systems (Okello *et al.*, 2016; Harahagazwe *et al.*, 2018; Ogola & Ouko, 2021). Major potato varieties in Kenya include *Shangi*, *Dutch Robjyn* and *Unica* with Shangi, a farmer variety, being the most popular potato variety grown in the country (Harahagazwe et al., 2018)(Muthoni et al., 2010). Nonetheless, there have been few reports on the development of efficient *In vitro* nutrient protocols for the three Kenyan potato varieties.

Shortage of high-quality seed potato is recognized as being one of the most important factors limiting potato production in Kenya (Wasilewska-Nascimento *et al.*, 2020). Over the years, there have been concerted efforts towards the development and adoption of new techniques aimed at improving clean seed production (Demo *et al.*, 2015). The introduction and development of Rapid Multiplication Technologies (RMTs) is expected to fast track the production time of disease-free seed. Outputs from RMTs include Tissue Culture (TC) plantlets from micro-propagation, micro-tubers, mini-tubers and rooted apical cuttings from greenhouse establishment (Abebe *et al.*, 2014).

Micro-propagation, an RMT technique, is utilized for the production of disease free *In vitro* mother plants for the production of mini-tubers (Prakash, 2008; Shiji *et al.*, 2014). Micro-propagation is also essential for producing genetically uniform plants that are disease-free from highly heterozygous species such as potato (Hajare *et al.*, 2021). Potatoes can be micro-propagated rapidly by shoot-tip cultures, meristem cultures, *In vitro*-cultured nodal cuttings. Microtuberization, an alternative to conventional tuber production, is a method used to increase potato under *In vitro* conditions. Micro-tubers are important for long-term storage of virus-free seed potato, *In vivo* plantlet transportation and healthy germplasm exchange (Mohamed & Girgis, 2023).

Micro-propagation techniques involve the transfer of cell/plant tissue under aseptic conditions into sterilized containers containing growth medium enriched with macro- and micro-nutrients essential for plant development, as well as vitamins, organic acids and a carbohydrate source (Alexopoulos & Petropoulos, 2021). The efficiency of micro-

propagation can be enhanced greatly through the modification of the culture media by enhancing mineral nutrient content that promotes plant regeneration and vigor while shortening the regeneration period; and confer additional protection against biotic or abiotic stressors to the TC plantlets under greenhouse or field conditions (Hunková et al., 2020). Successful acclimatization ensures optimal conditions for increased survival rates, impacting subsequent growth and development of tissue culture plantlets.

Agronomic bio-fortification involves the development of nutrient-dense staple crops through the application of optimal conventional breeding methodologies and contemporary biotechnological approaches (Nestel *et al.*, 2006). Agronomic bio-fortification strategies aim to increase the availability and accumulation of nutrients with the goal of increasing the nutrient status of a crop during plant growth, without sacrificing agronomic performance and important consumer-preferred traits (Bhardwaj *et al.*, 2022). Staple cereals, including maize, rice, wheat, and pearl millet, have been engineered to enhance micronutrient content of Zinc, Selenium and Iron (Govindaraj et al., 2016; de J. Mangueze *et al.*, 2018). Puranik *et al.* (2017) suggests the inclusion of finger millet as a model for calcium bio-fortification due to its inherent Ca-rich nutritional attribute among all cereals.

Calcium (Ca) is an essential macronutrient for plants. Ca is usually acquired from form the soil solution in the form of Ca^{2+} and delivered to the shoot through the xylem. However, Ca^{2+} mobility is limited and can often lead to local deficiencies, despite an adequate supply of Ca (Knez & Stangoulis, 2021). Calcium is involved in a wide range of physiological processes such as plant defense, by promoting disease resistance to pathogens through the strengthening of cell wall structures, maintaining membrane stability, influencing growth pattern, anatomy and chemical composition of a plant, and as a secondary messenger (Zhang *et al.*, 2014; Gilroy *et al.*, 2016). Calcium, by maintaining the rigidity of the cell wall structure and changing the mechanical properties of pectin, strengthens potato stems to maceration by bacterial pectin-degrading enzymes (Ngadze, 2018; Kemat & Krens, 2019).

“Biotization” is the process of inoculating tissue culture plantlets with beneficial microorganisms such as mycorrhizal fungi to deter competition from other potentially pathogenic soil microbes during acclimatization *in vivo* (del Rosario Espinoza-Mellado et al., 2021). During the biotization process, non-pathogenic organisms such as Plant Growth Promoting Bacteria (PGPR) and beneficial fungi induce stress-resistance responses in host plants, potentially occupying host plant microsites, making them unavailable to pathogens. Castro-Restrepo *et al.* (2022) demonstrated that *In vitro* co-cultivation of spearmint TC plantlets (*Mentha spicata* L.) with *Trichoderma asperellum* and *Bacillus subtilis* during the rooting phase positively impacted plantlet performace during acclimatization. Campos *et al.* (2017) found that AMF inoculation of *Meloidogyne* spp.-infected *Alpinia purpurata* during acclimatization lowered the number of galls per gram of root than the non-AMF inoculated treatment. Tahat *et al.* (2012) reported that *Ralstonia solanacearum*-infected tomato plants inoculated with *Glomus mosseae* exhibited no disease symptoms over 20 days of infection. These beneficial organisms enhance plant performance through improved nutrient and water uptake and soil physico-chemical properties (Jefwa *et al.*, 2010).

Bacterial wilt is a disease of economic importance with no single, economically feasible method offering complete protection (Njau *et al.*, 2021). *Ralstonia solanacearum,* the causal agent of Bacterial Wilt, invades the roots of its plant hosts, spreading rapidly throughout the host, releasing pectin-degrading enzymes which result in the death of the plant (He *et al.*, 2017). The pathogen has a wide host range and a high survival longevity in the soil, rendering most control methods employed by small-scale farmers in Kenya as ineffective, once the pathogen is established (Yuliar *et al.*, 2015; Njau *et al.*, 2021). Nonetheless, the application of calcium and use of biological control products for the management of bacterial wilt has shown promising results. Jiang *et al*. (2013) reported that increased plant tissue calcium, through the addition of calcium in the growth medium, mitigated bacterial wilt disease development by slowing down infection in tomato plants grown in 25mmolL^{-1} Ca^{2+}. Hashem *et al.* (2019) demonstrated that combined application of Ca and AMF inoculation ameliorated the effects of salt stress in *Bassia indica* Wight.

There is a need to develop and optimize TC-nutrient protocols to level up the availability and production of potato seed. This study evaluated the effects of *In vitro* Ca enhancement on plantlet regeneration, microtuberization, bio-fortification. The study also investigated the effects of Ca fortification and mycorrhizal inoculation on plantlet performance and Bacterial Wilt disease severity during greenhouse establishment of three selected potato varieties.

1.2 Statement of Problem

Inadequate supply of clean and certified seed has been identified as one of the major challenges facing potato production in Kenya (Muthoni *et al.*, 2012; Harahagazwe *et al.*, 2018) forcing most potato farmers to use farmer-saved seed (Kwambai *et al.,* 2023) of which 95% of the total seed produced is of poor quality (Okello *et al.*, 2016). Farmers follow no systematic seed selection of left-over tubers (after consumption or sale), unconsciously selecting small-sized tubers with negative characteristics leading to lower potato yields (Kaguongo *et al.,* 2014). This problem is further compounded by years of degeneration from farmers recycling seed that has been exhausted over generations of multiplication. Tubers are often laden with viral diseases and other soil-borne pathogens (Aheisibwe *et al.*, 2016). These seed quality-related constraints consequently lead to low on-farm potato yields. In Kenya, potato yields are reported to average 8.6 tonnes/hectare compared to the world's average of 20.7 tonnes/hectare (CABI, 2022; Adekanmbi *et al.*, 2023).

Formal potato seed systems, consisting of governmental regulatory agencies such as Kenya Plant Health Inspectorate Service (KEPHIS), are responsible for the release and certification, seed multiplication production, distribution and marketing system of disease-free seed (Wachira Kaguongo et al., 2014). However, seed potato from the formal seed sector accounts for only 7,000 tonnes of clean seed produced, compared to an annual demand of 30,000 tonnes, which does not satisfy farmer demands (National Irrigation Authority, 2022). Tissue culture plantlets produced under aseptic *In vitro* conditions and controlled environments during culture, tend to be "fragile" when exposed to much wider

and rapid swings in environmental conditions during transfer and acclimatization, predisposing them to high and rapid mortality rates (Grob, 2019).

The high prevalence of disease such as bacterial wilt in potato producing regions has also constrained the performance of clean seed potato, which includes TC seed material, especially under field conditions (Muthoni *et al.*, 2014; Birech *et al*., 2016). Additionally, the high costs of seed potato and poor agronomic practices, among other production constrains also contribute to abysmally low yields in the country (Muthoni et al., 2017).

1.3 Justification

Outputs from Rapid multiplication Technologies (RMT's) enhance the production of disease-free, high quality seed (Haapala, 2013). Rapid production of disease-free seed could enhance seed availability addressing production shortages and ultimately leading to an increase in yields (Wachira Kaguongo et al., 2014). In Kenya, private sector seed potato producers and multipliers play a major role in production of *In vitro* plantlets for seed potato, -producing up to 300,000 plantlets per season. Government-controlled institutions such as Tigoni Potato Research Centre at KALRO (Kenya Agricultural & Livestock Research Organization) contributes about 20,000 plantlets (per production season), however, Kenya's tissue culture production capacity is still limited by financial, technical and administrative constraints thus limiting potato production (Wandui *et al.*, 2013; Harahagazwe *et al.*, 2018). Micro-tubers possess benefits such as ease of storage and transport compared to conventional tubers, benefits not fully exploited in Kenya (Harahagazwe *et al.*, 2018).

Investments and partnerships between the public and private sector in potato production, through the establishment of the National Potato Council of Kenya (NPCK) and the launch of the National Potato Strategy 2021-2025, point to the need to address systemic challenges plaguing potato production in the country (Wakaba *et al.*, 2022). Several key Government policies such as The Agricultural Sector Transformation and Growth Strategy (ASTGS) prioritized 13 value chains (VCs) including potato with the potential

to raise smallholder farmer incomes and offer dietary diversity (Government of Kenya (GoK), 2021). There exists relatively strong regulatory framework for (certified) seed in Kenya. These include the National Seed Policy 2010, the National Food and Nutrition Security Policy, and the National Root and Tuber Crops Strategy 2019-2022. Despite the existence of such strong legal frameworks, challenges still exist with respect to the regulation of potato seed (Kuhlmann *et al.*, 2022)

Calcium (Ca) is an essential macronutrient for plant growth and development, functioning as a structural component of cell walls and membranes (He *et al.*, 2018). Calcium is also recognized for its capacity to mitigate disease occurrence (Ngadze, 2018). Calcium application improves the yield of potato, under *In vitro* and field conditions (Chomdao *et al.*, 2011). Tissue culture plantlets are characterized by weak and inefficient roots that hinder nutrient acquisition (Vettori *et al.*, 2010). However calcium application could improve root growth, impacting nutrient acquisition (He *et al.*, 2018).The use of pesticides and chemical fertilizers, as a control against high bacterial wilt incidences and low soil fertility, is detrimental to tissue culture plantlets and to environmental health. Environmentally-friendly alternatives such as the use of beneficial control agents which includes mycorrhizal inoculants, are thus required to address these challenges (González-Rodríguez *et al*., 2013; Ehirim *et al.*, 2014). Biological control agents provide ecologically-sustainable disease mitigation diminishing the reliance on non-renewable resources (Yuliar *et al.*, 2015).

No single strategy has shown 100% efficiency in the control of Bacterial Wilt due to its ability to grow endophytically, survive in soil and have a wide host range (Kago *et al.*, 2016). Calcium application has been reported to reduce Bacterial Wilt incidence (Petrov & Van Breusegem, 2012; Sasanuma & Suzuki, 2016) with mycorrhizal inoculants also acting as bio-control agents against Bacterial Wilt (Aguk, 2013). Mycorrhizal inoculants are also known to improve plantlet performance and promote plant tolerance to various biotic stresses such as Bacterial Wilt during *In vitro* culture and acclimatization under

greenhouse and field conditions (Kavoo-Mwangi, 2012; Yadav *et al.*, 2013; Li *et al.*, 2016; Bahadur *et al.*, 2019).

The use of mycorrhizal inoculants has shown to have an enhancive effect on production in crops such as tomato and eggplant, both species in the Solanaceae family (Elahi *et al.*, 2010; Sellitto *et al.*, 2019). Calcium application has been shown to alleviate *In vitro* disorders and promote plantlet growth and performance (Amalia *et al*., 2014; Machado *et al.*, 2014). Commercial Arbuscular Mycorrhiza (AMF) formulations promoting plant growth are already produced, sold and utilized in Kenya (Mukhongo *et al*., 2016). Little is reported on the use of these commercial products on TC potato plantlets especially when combined with *In vitro* calcium fortification for the reduction of Bacterial Wilt incidence and severity during greenhouse establishment. This establishes the need to evaluate the potential of the combined use of *In vitro* Calcium fortification and commercial mycorrhizal inoculants for the benefit of seed potato producers and the entire potato value chain.

1.4 Objectives

1.4.1 Main Objective

1. To enhance growth of *Shangi, Dutch Robjyn* and *Unica* potato varieties using agronomic calcium bio-fortification and mycorrhizal biotization to promote TC plantlet performance, micro-tuberization, seedling performance and bacterial wilt management

1.4.2 Specific Objectives

1. To investigate the optimal calcium level for *In vitro* plantlet regeneration and micro-tuberization in three selected potato varieties
2. To evaluate the efficacy of *In vitro* Calcium bio-fortification and AMF biotization on plantlet performance and management of Bacterial Wilt in three selected potato varieties

1.4.3 Null Hypotheses

1. *In vitro* plantlet and micro-tuber regeneration and Calcium bio-fortification does not depend on the level of Calcium fortification
2. *In vitro* Calcium bio-fortification and biotization has no effect on potato seedling performance and Bacterial Wilt management

CHAPTER TWO

REVIEW OF LITERATURE

2.1 Origin and Distribution of Irish potato

Irish potato (*Solanum tuberosum* L.) is a perennial plant belonging to the Solanaceae family and a native to Latin America in the regions close to the border of modern day Bolivia and Peru. Hunter-gatherers in the Andes mountain range domesticated the potato 7,000-8,000 years ago. The dissemination of the potato to Europe was facilitated by Spanish *conquistadores*, while sailors played a pivotal role in its global dissemination to regions including India, China, and Japan (Prakash, 2008). The name "Irish potato" comes from the Irish, who largely adapted the potato and introduced it to North America. British colonialists brought the potato to East Africa, particularly the Kenyan Highlands, in the 19th century (Sinelle, 2018).

2.1.1 Botany and Taxonomy of Irish potato

The potato is a tuber vegetable with an edible starchy tuber. Since it is a short season crop maturing in 3-4 months, the potato is cultivated during the coolest months in hot tropical regions. *Solanum tuberosum* L., the most widely and commonly grown species, is a tetraploid (4n=48)(Rosa et al., 2010). The species bears flowers in a variety of colours in clusters or cymes which when fertilized produce globular berries bearing toxic compounds known as alkaloids (Uluwaduge, 2018). Potato stems are angular and branched bearing compound, alternate leaves up to 30cm, supported by fibrous roots from where tubers arise (Ghebreslassie, 2017). The tubers act as storage and reproductive organs for the potato, reserving carbohydrates, proteins, vitamins and mineral elements for plant growth after propagation (Subramanian *et al.*, 2011). Propagation is either sexual or asexual through the production of True Potato Seed (TPS) or tubers respectively (Bradshaw, 2022)

2.2 Current trends of potato production worldwide and in sub-Saharan Africa

In the year 2050, a burgeoning global populace of 9.7 billion individuals is anticipated to necessitate a 70% increase in food production relative to current consumption levels (Devaux *et al.*, 2020). Present-day food systems fail to adequately deliver nutritive sustenance to the global population in an ecologically sustainable manner (Wu *et al.*, 2018). Ensuring the nutritious and sustainable sustenance of the burgeoning population necessitates considerable improvements to the global food system. This necessitates the establishment of a system that not only sustains the livelihoods of farmers but also delivers nourishing commodities to consumers, all the while mitigating today's environmental impact (VanderZaag & Devaux, 2023).

The potato is a staple food consumed daily around the world, contributing to the four pillars of food security "access, availability, stability, and utilization," in alignment with the objectives of Sustainable Development Goal 2, specifically focusing on the eradication of hunger (SDG 2-Zero Hunger) (Adekanmbi *et al.*, 2023). The potato has been identified as being among the most preferred crop for food sustainability in sub-Saharan Africa, due to the potato's price stability influenced by demand and supply factors in the local market. The crop also has a lot of production and utilization potential that could potentially address food insecurity in Sub-Saharan Africa (Chemutai, 2021).

Globally, the potato is the third most dominant food crop for human consumption (Boschi *et al.,* 2017). Çalışkan *et al.* (2023) reports total production reached 359.1 million tonnes in the year 2022, with China's 93,000,000 tonnes contributing the lion's share (Demand, 2023). In Africa, potato production has experienced growth in leaps and bounds with a threefold increase from 30 million tonnes to 165 million tonnes, with the increase attributed to the expansion of cropping area to 1.5-2 million hectares (Atakos, 2018; Shawiza, 2018). Additionally, potato yields in Africa ranged between 12-15 tonnes/hectare while average potato yields around the globe for example, in North America and Western Europe, often reach 40 tonnes/ha while production in developing

countries especially in SSA is often below 20 tonnes/ha (Andati *et al*., 2023; Muthoni & Shimelis, 2023).

2.2.1 Potato Production in Kenya

The potato is considered as the second most important staple food crop after maize, most especially in urban centers, where the crop plays a great role in improving food security and in poverty alleviation (Waaswa *et al.*, 2022). This is attributed to the dual roles the potato plays, as a cash and subsistence crop (Kiama *et al*., 2006). This is because the potato has a shorter production time of about 60 days after the onset of the rainy season by which tubers can be harvested as compared to cereals that require 6-9 months to harvest (Cromme *et al*., 2010).

The potato is grown in 20 of the country's 47 counties where production is concentrated in the highland regions surrounding Mt. Kenya, the Aberdare range, the edges of Rift Valley, the Mau escarpment and Mt. Elgon with small acreages in Kisii, Kericho and Taita Hills (Figure 1). There are 13 major potato-producing counties which are: Meru, Nyeri, Nyandarua, Kiambu, Taita-Taveta, Nakuru, Narok, Bomet, Elgeyo-Marakwet, Trans-Nzoia, Bungoma, Uasin-Gishu and West Pokot (Kaguora *et al.*, 2019). These regions are characterized by deep fertile volcanic soils, 1100-2700mm rainfall per year and are located in altitudes between 1500-3000 meters above sea level (M.A.S.L), enabling potato production through rain-fed farming following a bi-modal rainfall pattern (Okello *et al*., 2016; Muthoni *et al.*, 2017). Other potato producing counties include Kisii, Nyamira, Kirinyaga, Muranga, Baringo, Nandi, Laikipia and Kericho. Nyandarua county is the main potato growing county in Kenya, contributing about 29.8% of the country's total potato production, followed by 18.9% from Nakuru county and 16.2% from Elgeyo Marakwet county (Beatrice *et al.*, 2023).

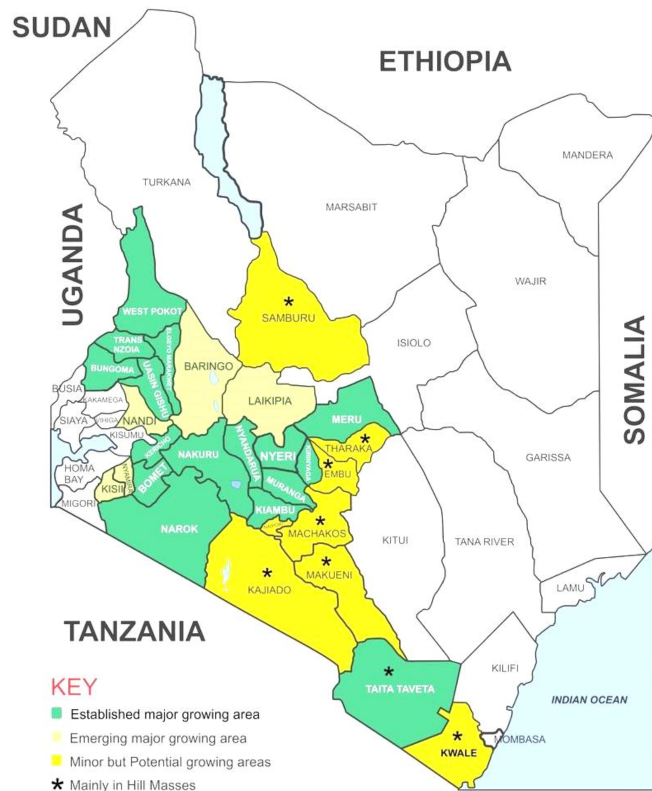


Figure 2.1: Potato Growing Regions in Kenya (Muthoni *et al.,* 2017)

Potatoes have a high climatic adaptability due to their ability to grow in high-altitude areas where maize, which is Kenya's main staple food does not perform well (Taiy *et al.*, 2017). Currently, potato production output in Kenya is estimated to fluctuating between 3-9.5 tonnes/ha with production done over 108-120,000 hectares of land in the country (Mbego, 2019). Potatoes are grown and eaten locally, with little significant international trade compared to cereals, making them particularly valuable as food in Kenya (Janssens *et al.*, 2013). The potato is highly nutritive due to its richness in essential minerals and possesses a high energy content, contributing to about one-third of overall diet energy consumption (Devaux *et al.*, 2014; National Potato Council of Kenya, 2020). Potatoes are often eaten with beans in most poor rural households during the 'hunger period' just before the maize crop matures during the long rains season (Muthoni & Nyamongo, 2009). Change of eating habits, particularly in urban areas, has led to increased demand and consumption of the potato, where preference for potato French fries (chips) and crisps has increased

over the years (Maina *et al.*, 2019). The crop also has higher annual productivity ratio compared to other main staple cereal, roots, and tuber crops and has good industrial qualities for processing and industrial use (Ayieko *et al*., 2006; Prokop & Albert, 2008).

Potato production in Kenya has increased from 1.5 million tons in 2017 to 2.1 million tons in 2021 with national per capita potato consumption estimated at 25 Kilograms a year (Government of Kenya (GoK), 2021). While total production has increased over the years, the output per unit area has decreased by six to ten metric tons per hectare in recent years (Kaguora *et al.*, 2019). Similarly, notwithstanding a rise in the overall potato yield within Kenya, there exists a disparity between the supply and demand of potatoes, specifically intended for processing purposes (Maina *et al*., 2019). The domestic demand within the national context of Kenya is approximately 3.1 million tons, while the available quantity for utilization is recorded at 2.8 million tons (Kenya National Bureau of Statistics, 2020). While there is high variability in yields, Kenya has the capacity to produce up to 40 tonnes/hectare under recommended agronomic practices (Zhou & Kretschmer, 2023). This disparity is attributed to a number of challenges including the inadequate access, supply and use of good quality seed potato of preferred varieties and other production inputs/chemicals, low soil fertility in potato producing regions, high postharvest losses and challenges associated with climate change and the poor dissemination of new varieties (Muthoni *et al*., 2012; Harahagazwe *et al*., 2018; National Potato Council of Kenya, 2020; Government of Kenya (GoK), 2021).

Potato production is characterized by rapid and significant fluctuations in supply and demand due to the sector's dependence on rain-fed production (Taiy *et al.*, 2017). Most potato growing zones in Kenya have a bi-modal rainfall pattern, with the long rains falling between March and June and the short rains from September to November. Potato supply is contingent upon the bi-modal rainfall pattern, thereby influencing potato prices by virtue of the heightened perishability of the potato and the inadequacy of on-farm and market-based storage infrastructure (Muthoni *et al.*, 2017; Maina *et al.*, 2019). Additionally, projections of extreme weather events indicate an increase in the duration

or frequency of hazardous climactic events which ultimately will hamper potato production (Mwongera *et al.*, 2019).

Potato cultivation is linked to temperature conditions as well (Kanters, 2018). The cultivation of cool-season crops such as potatoes in warm tropical regions is anticipated to pose a challenge in light of the escalating global temperatures (Muthoni *et al.*, 2017). Elevated temperatures have the potential to defer or outright hinder the initiation of tubers and curtail the duration of bulking, thereby possibly resulting in diminished yields and compromised tuber quality (Handayani *et al.*, 2019). Given the reliance of potato cultivation in Kenya on rainfall and temperature patterns, fluctuations in climatic conditions could trigger the onset of novel potato pests and diseases, diminishing tuber quality and amplifying post-harvest losses (National Potato Council of Kenya, 2020).

Mulema *et al.* (2021) highlight that pest infestations represent the primary source of diminished agricultural yields within the potato industry in Kenya, having been estimated to precipitate losses ranging from 30% to 40%, with the potential for escalation beyond 80%. Increased incidences of economically important pests including bacteria (*R. solanacearum* causing bacterial wilt), fungi and oomycete (*Phytophthora infestans* causing Potato blight), nemtaodes (*Meloidogyne incognita,* that increases bacterial wilt infection when there is co-infection), and viruses such as Potato Leaf Roll Virus and Potato Virus Y, result in losses in quantity and quality of yield, constraining production (Aguk *et al.*, 2016). Seed-borne and soil-borne pests are mainly spread through using infested planting materials, with the majority of Kenyan potato farmers relying on their own visually selected and saved seed form previous seasons' planting, due to the limited access and availability of certified seed (Chemutai, 2021).

The majority of farmers from five potato-producing counties in Kenya (Elgeyo Marakwet, Meru, Nakuru, Narok, Nyandarua, Trans Nzoia) reported that they largely obtained planting materials (seed potato) from their own-saved planting materials and from other farmers (Mulema *et al.*, 2021). Kwambai *et al.* (2023) reported that the lack of quality seed and diseases (specifically bacterial wilt and late blight) were among the most

important constraints limiting optimal potato production in Bungoma, Trans Nzoia and Elgeyo Marakwet counties in Kenya.

van der Waals & Krüger (2020) reports that latently infected farm-saved seed (especially with *R. solanacearum*) are the primary source of *R. solanacearum* inoculum and pathway of dissemination in sub-Saharan Africa. Bacterial wilt accumulation in seed potato used by the majority of small-scale farmers is associated with the observed disparity in potato yield between farmers in the African tropical highlands and their counterparts in more economically advanced nations (Okello *et al.*, 2020). Other factors that contribute to disease spread include continuous cultivation in previously infected soil due to the potato's importance in traditional production areas, low usage of agro-inputs (biological, organic and inorganic pest control and pesticides) and poor agricultural practices (limited use of certified seed potato, abuse of crop rotation regimes, inadequate capacity to undertake potato pest diagnosis). Sub-optimal yields occasioned by the above named factors confine the small scale farmers' capabilities in terms of development, commercialization and production (Barrett, 2007).

2.3 Seed Potato Production in Kenya

In Kenya, potato production is mainly undertaken by small scale farmers, representing 90% of the major players in potato production having more than 80% of potato acreage under production (Masinjila, 2022). The propagation of potatoes can be conducted both sexually and asexually/vegetative. Under sexual reproduction, botanical seed is produced as starter planting material (Muthoni *et al.,* 2013). Vegetative propagation of potatoes is through tubers -shortened and thickened underground stems developing at the end of a stolon that serve as storage organs (Prakash, 2008). A seed potato is defined as a tuber and potato micropropagative material of cultivated tuber-forming *Solanum* spp. for planting which is certified by a governmental body and found to meet the specified rquirements (UNECE, 2013). For high yields and high quality of potato production, high quality seed is a prerequisite to achieving desirable results.

Seed potatoes in Kenya are obtained from a variety of sources that include formal, semi-formal and informal sources (Atieno *et al.*, 2023). The formal system is governed by legal regulations and characterized by research programs for production of high quality seed for multiplication and selection known as basic seed. Basic seed are tubers derived from *In vitro* generated plantlets and minitubers for production of disease free starter plant stock. Public sector seed potato producers and multipliers such as KALRO Tigoni and Agricultural Development Corporation (ADC)-Molo are charged with the production of the research, production and dissemination of high quality basic seed (Government of Kenya (GoK), 2021). Unfortunately, this supplies less than 5% of the seed used by farmers (Atieno *et al.*, 2023). Mureithi (2023) reported that ADC-Molo has the capacity to produce up to 20,000 (50kilogram) bags of seed potato from about 2000 acres of land but only utilizes 500 acres which produces 50,000 bags of clean basic seed. Muthoni *et al.* (2014) and Ndacyayisenga *et al.* (2015) also found that KALRO Tigoni has limited physical and human capacity hence produces only 25-55 tonnes of basic seed per year against farmers high demand.

Stringent and costly inspection procedures by certification bodies have impacted production by priavte seed multipliers, and this challenge is compounded by limited certification capacities by both KEPHIS and the private sector, even with authorization of private seed inspection (Zhou & Kretschmer, 2023). The collapse of the formal distribution systems in the 90's cut large scale production of clean seed potato contributing to insufficient production and distribution of certified seed (Kaguongo *et al*., 2008; Lemaga *et al.*, 2008).

The high cost of seed potato, lack of information about certified seed and limited availabiltiy of seed for preferred varieties also contributes to the low usage of certified seed (Muthoni et al., 2010; Demo et al., 2015; Mulema *et al.*, 2021). Kwambai *et al.* (2023) determined that the majority of potato farmers in North-Western Kenya cited several major reasons for refraining from the utilization of certified seed. The foremost factors included the unavailability of certified seeds within the local vicinity (59.6%), the

perceived high cost associated with these seeds (35.1%), farmers' lack of awareness regarding the sources of certified seed (24.0%), and a notable proportion of farmers being uninformed about the very concept of certified seed (18.7%). The benefits of using quality seed have been highlighted in previous studies (Wang'ombe & van Dijk, 2013; Kaguongo *et al.*, 2014; Okello *et al.*, 2020) however, the dominance of the informal system can lead to situations where certified seed producers are unable to sell their seed (Atieno *et al.*, 2023). This in turn causes farmers to turn to their own saved seed (informal seed); where quality is always low and not easy to ascertain caused by high pests loads and seed degeneration, limiting small scale farmers' development which in turn engrains food insecurity and poverty (Okello *et al.*, 2016).

The informal (conventional) seed system is characterized by small scale farmers obtaining seed from their own farms (farm-saved), neighbours or local markets (Muthoni *et al.*, 2010). The bulk of seed potatoes is from ware potato growers than seed growers/multipliers (Gildemacher *et al.*, 2009). Farmers constantly select and use seed saved from the harvest on their farms and select small tubers with limiting characteristics leading to low potato yields (Kaguongo *et al.*, 2013). Small-sized tubers, which are not preferred by consumers due to due their difficult and time-consuming peeling process, are sold in local markets as seed (Kuhlmann *et al.*, 2022). Seed degeneration marks and identifies informal seed due to pathogen build-up. This often leads to poor quality produce, low market value and contributes to the spread of pests and disease.

Asexually propagated tubers are more prone to harboring larger pathogen loads than sexual propagules. This is due to the constant exposure of the tuber to soil-borne, air-borne and vector-borne pathogens during multiplication (Thomas-Sharma *et al.*, 2016). Moreover, seed renewal by farmers is low with only 41% of farmers renewing their seed after an average of 6 seasons (Gildemacher *et al.*, 2009) further compounding seed degeneration. Moreover, the chronic shortage of seed potato causes multipliers to produce and clone seed 6-7 times leading to even higher degeneration levels despite the recommended two multiplications (FAO, 2008). Currently, there are concerted efforts

towards the development and adoption of multiplication techniques and technologies that improve seed potato quality and availability for sustainable potato production. These techniques include the adoption of Rapid Multiplication Techniques (RMT) and Good Agricultural Practices (GAP) that boost the production of tubers to upto 10-20+ tubers per plant, compared to the low multiplication rates of 8 tubers per plant under the conventional method (Otazu, 2010; Muthoni *et al.*, 2013a). Kaguongo & Jeruto, (2013) and Okello *et al.* (2020) also report that using quality seed is a largely under-used strategy in addressing bacterial wilt.

2.4 Production Highlights of varieties used in study

The collection of Irish potato varieties listed on the national registry encompasses a compilation of 67 different varieties. A number of varieties have multiple uses, which presents a greater commercialization, utilization and marketing opportunity (Kuhlmann *et al.*, 2022). *Shangi*, by far is the most dominant variety due to yield, market demand and seed availability. It has multiple uses and is used both as a table and chips variety, characterized by its sweet flavour. *Shangi* has a short cooking time and short tuber dormancy period (<1 month) and is highly preferred on the market with 42% of all traded varieties being Shangi (Janssens *et al*., 2013; CIP, 2018). However, it tends to easily fall apart when boiled, limiting its usefulness for certain cooking purposes. It also has the lowest storability compared to the Dutch Robjyn and Unica (Gikundi *et al.*, 2023). It is a drought and heat tolerant variety with relatively high productivity per unit area with yields of 30,000 – 40,000 kilograms (kg) per acre. It is an early-maturing variety, maturing in about 3 months. It does well in regions ≥1500 M.A.S.L, controlling more than 80% of the potato market share in the country's key potato producing regions. It does well in areas such as Nyandarua, Kiambu, Nyeri, Laikipia, Meru, Nakuru, Bomet, Narok, Kwale, Nandi, Kisii, Kericho, Elgeyo Marakwet, Uasin Gishu, Trans-Nzoia, West Pokot, Taita-Taveta, and Cherangani hills. *Shangi* seed is mainly produced by ADC-Molo and KALRO-Tigoni. *Shangi t*ubers are oval-shaped with white flesh and medium to deep eye

with pink pigmentation. The variety is also highly susceptible to bacterial wilt (Kaguora *et al.*, 2019).

Dutch Robjyn is mainly crisps variety with a long shelf life due to its long tuber dormancy period (3-4 months). This variety exhibits an average yield performance (≥ 14,000-16,000 kg/acre) while also enjoying widespread favor as a prominent selection due to its processing purposes. *Dutch Robjyn* does well in regions in high altitude of 1800-2600 M.A.S.L. It does well in areas such as Nakuru, Uasin Gishu, Kiambu, Trans Nzoia, Nyandarua, Nyeri, Meru; performing fairly well (and largely produced) in Bomet county (MoALF, 2017). Its tubers are round shaped with rough red skin and medium deep eyes.

Unica (formerly CIP392797.22), introduced in 2017, is a table and chips variety with a long tuber dormancy period (2.5-3.5 months) with a good flavor, texture, and appearance. It is virus resistant, drought and heat tolerant. *Unica* exhibits high performance (>45 tonnes/hectare). Its tubers are oblong-shaped, with creamy-coloured flesh and shallow eyes and red skin (Kaguora *et al.*, 2019). The variety is suited for all potato growing regions. *Unica* seed is mainly produced by ADC-Molo, GTIL, KALRO-Tigoni and Kisima Farm. It is moderately resistant to Late Blight and highly resistant to Potato Virus X (PVX) and resistant to Potato Leaf Roll Virus (PLRV). Despite its promising qualities, the popularization and marketing of new potato varieties such as *Unica* still receives relatively limited funding (Zhou & Kretschmer, 2023).

Different potato varieties exhibit unique characteristics consequently presenting variety specific management challenges (Muleta and Aga, 2019). A number of varieties grown in Kenya, such as *Shangi* and *Dutch Robjy*n were introduced over 20 years ago. Mugunyu (2020) reports that *Shangi* production in Nyandarua County is no longer producing expected high yields due to its high susceptibility to disease pointing to the need to promote the use of clean and certified seed and market new varieties with good promising performance. Andati *et al.* (2023) reported the adoption of seed management technologies (such as use of clean seed) enhanced potato yields for 350 farmers in Nyandarua County by up to 61%. The utilization of disease-free planting material is particularly important in

the context of potatoes. In this crop, the transmission of pathogens through the planting of tubers that harbor latent infections facilitates pathogen persistence, dissemination, and direct interaction with the host. van der Waals & Krüger (2020) highlighted the importance of seed inoculum due to the success demonstrated in significantly diminishing the occurrence of bacterial wilt in South Africa through the establishment of seed certification programs and the adoption of sound disease management strategies.

2.5 Rapid Multipication Techniques

Shortage of clean good-quality seed has been recognized as the single most important factor limiting potato production in Kenya. Fortunately, the potato has gained advantages from progress in both traditional and contemporary biotechnological methods. This has led to their application in addressing current issues associated with the cultivation and enhancement of potatoes in Kenya. The introduction, adoption and adaption of Rapid Multiplication Techniques (RMT's) in Kenya as a way of fast tracking the production of clean seed through strategies such as the '3G Seed'. '3G Seed' comprises rapid multiplication of seed through the production of mini-tubers from *In vitro* plantlets, which are then multiplied over two generations in the field increasing the supply of clean seed to farmers (Demo *et al*., 2015; Okello *et al.*, 2016). RMT's yield *In vitro* plantlets, micro-tubers, mini-tubers that are used as initial starter material (Abebe et al., 2014). Certified seed potato in Kenya is currently produced by over 30 registered seed merchants and growers who multiply seed on behalf of registered producers. However, the produced seed meets only 27-36% of potato farmers seed needs (International Potato Center, 2021).

The use of *In vitro* aseptic culture as an alternative to conventional propagation methods results in the production of true-to-type clones for the large scale multiplication of disease free plants (Hussain *et al.*, 2012). Under conventional propagation methods, the propagules (tubers) get infected by viruses and other pathogens during repeated cycles of cultivation for multiplication (Kaur *et al.*, 2017). *In vitro* culture is the technique employed for the cultivation of plant cells, tissues, and organs within an artificial medium,

either solid or liquid in an aseptic environment with stringent controls, facilitating the precise regulation of growth conditions.

2.5.1 Micro-propagation of Potato

Micro-propagation is defined as the rapid multiplication of stock plant material for the production of a large number of disease free plants using modern tissue culture methods (Namanda *et al.*, 2015). This process consists of several stages namely preparation and pretreatment of the plant, initiation of the explants, multiplication of the tissue, regeneration of whole plants and hardening for subsequent field planting. The associated benefits of micro-propagation include the rapid production of disease free plants over a short period of time in a small space with reduced labour (Shohael, 2008). Genetic integrity is maintained during the multiplication of clones via nodal cuttings/single node explants, micro-tubers or meristem tip cultures (Ebad *et al.*, 2015) making single node cultures a preferred method for agronomic bio-fortification experiments. Micro-propagation plays a crucial role in the propagation of highly heterozygous species like the potato, ensuring the generation of uniform plants (Hajare *et al.*, 2021).

The fundamental technique in potato biotechnology is shoot culture, serving as a rapid and effective means for establishing *In vitro* cultures by employing shoot tips as the starting material. Following establishment, shoots become a source of explants required for diverse techniques encompassing cell, tissue, and organ cultures. Given the pronounced apical dominance of potato shoots in *In vitro* culture, particularly owing to the robust nature of the potato plant, exogenous plant growth regulators (PGRs) are unnecessary for its growth (Vinterhalter *et al.*, 2008; Abeuova *et al.*, 2020).

Clonal multiplication of potato can be conducted via the initiation and multiplication of axillary shoots from *In vitro*-cultured nodal cuttings (Neumann, 2014; Harahagazwe *et al.*, 2018). Segmented stem nodes from mother plants are used to generate single node explants, containing an axillary bud. These segments are cultured for the generation of whole plants and have been found to develop at the fastest rates while also having the

highest genetic stability during propagation (Cimen & Ozge, 2012). For fast shoot multiplication, Vinterhalter *et al.* (2008) recommends cutting shoots of already established plants into single node explants and culturing them individually since single node explants liberated from apical dominance develop at the highest possible rate.

The efficacy of micro-propagation depends on factors encompassing the selection of explants and their inherent attributes, the processing and treatment of explants during their preparation for *In vitro* culture, the formulation of the culture media, the methodologies employed in the micro-propagation process, and the subsequent performance of the regenerated plantlets during acclimatization (Mohapatra & Batra, 2017; Abeuova *et al.*, 2020).

Plant regeneration through tissue culture can be accomplished using one of the three methods: meristem culture, somatic embryogenesis and organogenesis (Mweu, 2012).

Organogenesis: The formation of organs from the cultured explants (plant material such as stem nodes, roots, leaves and flowers). The organogenesis process is where the plant organs, either shoots or roots, are developed. It can either be direct or indirect. The production of direct buds or shoots from tissue with no intervening callus stage is called direct organogenesis. It is most used for clonal propagation since it ensures the production of uniform planting material without genetic variation (Gallego, 2020). Organogenesis is highly dependent on genotype, origin of explants, and the interaction between endogenous and exogenous hormones (Abdelaleem, 2015). This study focused on the agronomic bio-fortification with Calcium, of whole regenerated plantlets and therefore, no PGR's were utilized during shoot and root elongation and multiplication.

Plants derived from a regulated environment are favored over those cultivated in open fields due to their diminished susceptibility to fungal, bacterial, and viral contaminants. The initiation of explants exhibits greater efficacy when sourced from healthy and thriving plant material. In order to maintain an aseptic environment, it is important to sterilize all vessels employed in cultivation, media formulations, and instruments utilized during

tissue manipulation, including the explant itself when necessary (Mohapatra & Batra, 2017). All procedures should be conducted within a sterile cabinet equipped with laminar airflow.

2.5.2 Tissue Culture Media Formulation used for micro-propagation

The Murashige &Skoog (MS) nutrient medium is widely used as a conventional growth medium for the micro-propagation of a diverse group of plant species using nodal explants (Sevik & Guney, 2013; Shiji *et al.*, 2014; Cui *et al.*, 2019). The Murashige &Skoog (MS) nutrient medium is also the most widely used media in production of potato (Makau *et al.*, 2022). The amounts of various nutrient constituents in the medium vary for cultures of different species. However, MS medium was initially designed for tobacco calli cultures, not for shoot cultures, necessitating the need to optimize MS medium formulations to stimulate shoot growth for different species (Hunková *et al*., 2020). Several authors have reported issues with plants cultivated on conventional MS medium displaying physiological disorders including hyperhydricity (Machado *et al.*, 2014), chlorosis and necrosis (Ozgen *et al.*, 2011). The number of studies focused on culture media mineral composition is rising, paying particular interest to the effects of macro- and micro-nutrient medium constituents on plantlet performance and quality. Formulating a suitable growth medium tailored to a particular crop can be a complex task, given that the crop's reaction to the medium is frequently influenced by its genotype. Although the significance of mineral nutrients during *In vitro* plant propagation is well recognized, research on the medium constituents aimed at improving and accelerating growth of Kenyan potato varieties has been limited (Wada *et al.*, 2013; Lekamge *et al*., 2021)**.**

Regeneration of whole plants entails axillary shoot proliferation and root production. This requires use of plant growth regulators (PGRs). Hussey & Stacey (1981) demonstrated that PGR's are not necessary for the successful propagation of potato by shoot cultures. 4mm single node cuttings, sub-cultured onto fresh PGR-free MS medium produced healthy, upright positioned shoots with 6-12 nodes depending on the light and temperature regime. The technique advocated by Hussey & Stacey (1981) is highly recommended for

fast and uninterrupted *In vitro* propagation of potatoes ensuring high genetic stability through the employment of axillary buds as explants. Vinterhalter *et al.* (2008) found that potato (cv. '*Désirée*') shoot cultures cultured on a simple PGR-free MS medium supplemented with 2-3% sucrose supported shoot elongation and rooting but not axillary bud proliferation. Kozai *et al*. (1995) found that the volume and initial strength of MS medium affected the *In vitro* growth, photosynthesis, and ion uptake of potato plantlets.

Murashige & Skoog (1962) medium supplemented with different combinations of macronutrients, micronutrients, vitamins, organic acids have been used for tissue regeneration in potato. Munthali *et al.,* (2022) highlighted clear correlations between nutrient contents and uptakes of Nitrogen (N), Phosphorous (P), Potassium (K), Calcium (Ca) and Magnesium (Mg) and their effects on dry shoot biomass and morphological symptoms. The study authors found that at 100mM N, there was a significant decrease in shoot Ca and Mg uptakes while 10mM Ca resulted in increased uptake of shoot N, K and Mg. Niedz & Evens (2007) as well as Reed *et al*. (2013) identified secondary macronutrients ($CaCl_2.2H_2O$, KH_2PO_4, $MgSO_4$) present in the mineral composition of the Murashige and Skoog (MS) medium play a pivotal role in significantly enhancing the quality of shoots. Calcium used in tissue culture is mostly in the forms of calcium chloride and calcium nitrate in the concentration of 1-3Mm, which is the conventional Ca^{2+} content in tissue culture. Numerous studies have reported that increasing Ca content in MS medium is often beneficial to shoot development, with certain studies pointing to conventional MS medium formulations leading to physiological disorders during culture.

Hunková *et al*. (2020) established that 3x concentration of $CaCl_2.2H_2O$, KH_2PO_4, $MgSO_4$ in MS media compared to the 0.5x and 1x concentration resulted in a significant increase in the shoot number and shoot length for *Amelanchier alnifolia* var. *cusicki.* Habib *et al*. (2004) found that when the Ca^{2+} levels in the MS medium were increased from conventional 3mM Ca^{2+} to 15mM Ca^{2+}, shoot length of *Solanum kurtzianum* and *Solanum microdontum* shoot cultures were greatly improved. In MS medium containing 15mM Ca^{2+}, shoot fresh weights increased from 15 to 37% compared with 5mM Ca^{2+} (low Ca^{2+})

for *S. kurtzianum, S. microdontum, S. tuberosum* var. '*Bintje', S. tuberosum* var. *'Russet Bank'*. Ozgen *et al*. (2011) found that mean shoot length increased from 6.4cm to 15.0cm as Calcium concentration in culture medium increased from 1.4-3000µM during *S. tuberosum* L. cv. Dark Red Norland shoot culture. Vinterhalter *et al.*, (2008) concluded that MS medium in its standard formulation contains supra-optimal concentration of ammonium nitrate for growth of potato shoot cultures. Since Calcium is the study's main nutrient of interest, other nutrient and organic components were used in their standard concentrations.

Wada *et al.* (2013) reported the majority of genotypes of pear required substantially higher concentrations of $CaCl_2$ and KH_2PO_4 than contained in conventional MS medium. Makau *et al.* (2022) reported that 30g/L sucrose resulted in the fastest days it took *Shangi, Wanjiku* and *Unica* to initiate shoots compared to 40g/L sucrose in MS culture medium. Demo *et al.* (2008) used brown table sugar as the carbon source for the regeneration of three Kenyan varieties '*Asante*', '*Kenya Sifa*' and '*Tigoni*' achieving 100% plantlets survival. Kabira (2013) found that liquid media (non-solidified media) stimulated shoot growth while having no significant effect on root growth for 10 Kenyan potato varieties. Potato shoot cultures grow quite well on both liquid and solid medium. Veramendi *et al.* (1997) showed that solid medium, as a result of adding Gelrite, is advantageous for micro-propagation, promoting shoot elongation, rooting and micro-tuber production for *S. tuberosum 'Baraka'* when compared to agar. This calls for additional research to establish efficient protocols with specialized nutrient constituents of MS medium for Kenyan potato varieties, of significant economic importance, that hasten production of clean seed.

Machado *et al.* (2014) and Srikum *et al.* (2018) found that increased Ca levels in the culture medium decreased hyperhydricity and shoot necrosis occurrence. Shoot tip death causes the loss of apical dominance and promotes axillary shoot development (Ozgen *et al.*, 2005). Hyperhydric shoots result in poor survival rates during transfer to greenhouse/field conditions (Hazarika *et al.*, 2006).

Hyperhydricity: A physiological disorder that can occur during potato culture leading to the browning and subsequent death of the shoot tip, attributed to Ca deficiency during culture

Hyperhydricity is attributed to the limited transpiration that occurs during *In vitro* culture due to high humidity levels that limit the uptake and transportation of Ca, which is dependent on transpiration flow for delivery to the shoot (Nezami *et al*., 2015; Ibrahim *et al.*, 2016).

2.5.3 Micro-tuberization of Irish potato

Tubers are underground stems originating from stolons after the plane of cell division changes from elongation to promoting thickness after conditions are favourable for tuber initiation. Their primary function is for storage and vegetative propagation (Fernie & Willmitzer, 2001). Under *In vitro* conditions, tuberization is a developmentally complex process, is genotype dependent, regulated by plant growth regulators (PGR's) under the influence of environmental conditions such as temperature and day-length (Ghavidel *et al.*, 2012; Singh *et al.*, 2016). *In vitro* grown micro-tubers are genetically identical, high quality, and pathogen- free small-sized seed potatoes (about 0.5–1.5 cm diameter) which do not need acclimatization similar to the field-grown seed tubers. Similar to shoot cultures, micro-tuberization is often conducted on Murashige and Skoog (MS) media formulations, with varying concentrations of sucrose and PGR's. Rahman *et al.* (2015) found that micro-tuber weight of potato plantlets increased with the addition of 1.0 mg/L 6-Benzylaminopurine (BAP) to MS medium compared to micro-tubers with lower doses (0.5mg/L) of BAP.

Single nodal segments of *in vitro* generated plantlets are utilized for microtuberization on tuber-inducing liquid media as this media increases nutrition to the explant (Avila *et al.*, 1996). Short photoperiods with cool temperatures and low nitrogen concentrations have been found to also promote tuber initiation (Otroshy *et al.*, 2009; Al-Hussaini *et al.,* 2015). Additionally, elevated sucrose concentrations (5-8%) and cytokinins have been found to

be necessary for tuberization (Ali *et al.*, 2018; García-García *et al.*, 2019). Mamiya *et al.* (2020) found that MS medium containing 825 mg L^{-1} Ammonium nitrate +950 mg L^{-1} Potassium nitrate (half strength nitrogen) produced significantly higher total numbers of micro-tubers, although the differences within each size class were not significant.

Micro-tubers have tremendous advantages; their small size and weight accords them the benefits of being easily stored for a period of 3-4 months (due to dormancy) and easily transported as compared to bulky basic seed. Micro-tubers serve the function of germplasm conservation (Momena *et al.*, 2014). Despite these associated benefits, there have been no reports of commercial micro-tuber production in SSA and even in Kenya (Harahagazwe *et al.*, 2018). At the moment, germplasm conservation in Kenyan potato producing centers, both *In vivo* and *In vitro*, is insufficient to meet demand, with operations at KALRO-Tigoni having limited land and tissue culture facilities for both types of conservation (Muthoni *et al*., 2010). It has also been suggested that the storage and distribution of clean seed is important in availing seed to decentralized seed multipliers (Janssens *et al.*, 2013). Improving the efficiency of starter seed production through the development of regeneration protocols for micro-tuberization of the three varieties could be a cost-effective technique for rapid multiplication (Gildemacher *et al.*, 2009; Wandui *et al.*, 2013). Micro-tubers also offer other advantages such as being used as research tools under the areas of somatic hybridization, molecular farming and *In vitro* germplasm selection (Fufa & Diro, 2014). However, micro-tubers are delicate causing heavy losses during storage attributed to shrinkage and rotting (Naik & Karihaloo, 2007).

Arvin *et al*. (2005) studied the calcium concentration in media for micro-tuber production, and obtained highest yields with 10mM Ca (1470 mg L^{-1} calcium chloride dihydrate). Mamiya *et al.* (2020) established that higher calcium concentrations at 1760mgL^{-1} resulted in higher total yields, but the data showed no significant differences. The standard concentration of calcium chloride di-hydrate ($CaCl_2$) in full strength MS medium formulations is 880mg L^{-1} (8.8g/L $CaCl_2$ or 3mM Ca). Habib et *al.* (2004) reported that

increased calcium levels (15mM) in the medium caused significantly greater shoot fresh and dry weights.

2.6 Agronomic Calcium Bio-fortification and its associated benefits

Bio-fortification is a promising approach towards enriching plants with beneficial nutrients, for the plant and/or consumer benefits. Bio-fortification is an agronomical and biotechnological process in which targeted uptake and accumulation of certain nutrients occurs in food crops (Rouached & Rouached, 2013; Usmani *et al.*, 2019). Agronomic Calcium bio-fortification is conducted through the agronomic practice of increasing Ca content to plants by surplus Ca supply to cells and also increasing Ca storage within plant tissues (D'Imperio *et al.*, 2016) mainly through the application of Ca rich fertilizers in water-soluble forms. This calcium is found as free Ca (freely available and found in the cytosol) and bound Ca which has a strong affinity for macromolecules and is often found in crystalline forms e.g. calcium oxalate and sequestered/stored Ca in the cell wall where high levels of Ca are found (Kumar *et al.*, 2015). Agronomic bio-fortification generally relies on methods of fertilizer application and mineral element solubilization (Bhardwaj *et al.*, 2022).

Dussert *et al.* (1995) reported that calcium uptake occurs throughout the culture period. During *In vitro* culture, the root acquires calcium (Ca^{2+}) in its ionic form from the nutrient medium. The outer layers of the root come into contact with the nutrient medium containing calcium ions within the milli-molar range (mM). Calcium infiltrates the root epidermal cells via various calcium-permeable channels, traversing both apoplastic and symplastic pathways toward the cortex and subsequently the stele (Bairu *et al*., 2009; Hawkesford *et al*., 2012) Additionally, it has the potential to reach the xylem either apoplastically through extracellular spaces or symplastically by entering root cells and progressing from one cell to another through plasmodesmata *(*Sharma *et al*., 2017; Li, 2020)

Lekamge *et al.* (2021) reported MS media enhancement of macronutrients $CaCl_2$, K_2PO_4, and $MgSO_4$ by ≥2.0X (880-1100mg/L $CaCl_2$) while keeping other media components at conventional amounts significantly improved shoot elongation and leaf quality in *S. tuberosum 'Nishiyutaka', 'Okhotsk Chip'* and *'Sayaka'* compared to treatments grown on conventional half-strength MS media formulation (440mg/L $CaCl_2$). Calcium uptake also increased on 2.5x MS (1100mg/L $CaCl_2$) medium in *'Nishiyutaka'* by 1.5x*, 'cvs. 261',* and *'Okhotsk Chip'* by 2x compared to the conventional MS media formulation containing 440mg/L $CaCl_2$. MS media formulation containing 1320mg/L $CaCl_2$ resulted in plantlets with the highest total calcium content. Amalia *et al.* (2014) established that the addition of 1gL^{-1} calcium gluconate into MS medium containing 0.6mgL^{-1} BAP, at shoot induction stage, is recommended to prevent explant browning and plantlet shoot necrosis during raspberry micro-propagation.

Magnesium, zinc and iron are all crucial for plant cell proliferation and growth. Magnesium (Mg^{2+}) is an essential nutrient involved in chlorophyll synthesis, protein synthesis and enzyme activation during tissue culture (Ishfaq *et al.* 2022). Walker (1994) reported that biological antagonism between calcium and magnesium at the molecular level may have profound consequences for the optimization of biotechnological processes in cells. Mg^{2+} availability in cell culture can dramatically influence growth and metabolism of cells. Munthali *et al*. (2022) reported increased shoot N, K, and Mg associated with increased shoot Ca uptake showing a strong synergistic effect between Calcium and Magnesium during culture. Brewer *et al.* (1979) found calcium and zinc to have antagonist actions in a variety of systems. Zinc is involved in the folding, stability and/or activity of hundreds of proteins, being essential for several cellular functions (Klein *et al.*, 1962). Calmodulin-binding proteins are ubiquitous proteins playing essential roles in plant development and stress responses. Calmodulins are among the most dominant proteins in Ca^{2+} sensors (Mei *et al.*, 2021). However, Calmodulin protein function is known to be inhibited by low concentrations of Zinc (Brewer *et al.*, 1979).

Iron (Fe^{2+}) is also essential for chlorophyll synthesis, maintenance of chloroplast structure and function and photosynthesis (Rout & Sahoo, 2015). The micronutrient also plays a critical role in plant defense by triggering immune activation. According to a review of articles by Rietra *et al.* (2017) focusing on synergistic or antagonistic interactions among plant nutrients, there have been no previous studies focusing on interactions of Ca x Fe, especially during *In vitro* culture. However, Lekamge *et al.* (2021) found that increased uptake of 3x calcium and magnesium results in decreases in the uptake of iron during shoot propagation for *'Nishiyutaka'* cultivar. The study authors also established that increasing nutrient Ca content (together with potassium and magnesium) resulted in variety-specific increased iron uptake, improving shoot quality during tissue culture for *'Okhotsk Chip'* potato cultivar. Our study adds onto this growing body of research on Ca x Fe interaction during tissue culture.

Calcium also plays a role in plant disease resistance with Ca identified as a key structural element during cell wall reinforcement through deposition of Ca, forming complex polymers that confine invading pathogens (Agrios, 2005). Calcium promotes disease tolerance against pathogens such as *R. solanacearum,* through its effect on the composition of the cell wall. In addition, Calcium influences the production of defense related compounds e.g. enzymes implicated during the disease development e.g. peroxidases. Calcium is deposited on the cell wall reinforcing resistance to pathogen invasion (Yadeta & Thomma, 2013). Jiang *et al.* (2013) found that the highest Ca application in tomato (*Solanum lycopersicum* L. cv. Shanghai 906) plants resulted in reduced incidence of bacterial wilt due to strengthened cell walls. Calcium has also been found to have an effect on *R. solanacearum* and *Botrytis cinerea* pectinase activity, involved in the degradation of cell walls (Sasanuma & Suzuki, 2016; He *et al.,* 2017). Dolatabadian *et al.* (2013) established that in spring wheat (*Triticum aestivum* L. cv. Verinak) treated with foliar Ca, there was reduced hydro-peroxidation of membrane phospholipids and reduced antioxidant activity accompanied by increased proline activity, an amino acid implicated in defense responses against stresses.

2.7 Biotization using Arbuscular Mycorrhizal Fungi (AMF)

In nature, plants form associations with various soil microorganisms (beneficial or deleterious) that affect plant growth. Arbuscular Mycorrhizal Fungi (AMF) are considered natural bio-fertilizers that when absent or in very low levels, lead to a less efficient ecosystem functioning (Berruti *et al.*, 2016). During micro-propagation, bacterial or fungal colonies observed on plant tissue culture media are thrown out to control contaminations in other vessels. However, certain microbes called "endophytes" have been shown to be advantageous to plants, even during tissue culture (de Oliveira *et al.*, 2011; Kanani *et al*., 2020). Micro-propagated plantlets can exhibit high mortality rates upon their transfer to field or greenhouse conditions as a result of "transplantation shock" caused by abiotic and biotic stresses and weak root systems in the absence of beneficial microflora (Gosal *et al.*, 2010). The lower survival rate and poor establishment of *In vitro* plants in field conditions may also be due to the fact that the transferred plantlets do not find their natural micro-symbiont partner (Soumare *et al.*, 2021).

Inoculation of target growth substrate with AMF is a valid approach to minimizing mortality, improving the nutritional status and increasing stress resistance of tissue culture plantlets (Santos *et al*., 2010). Tissue culture plantlets exhibit hyper-juvenility, a state in which the plantlets exhibit poor resistance to both biotic and abiotic stresses increasing susceptibility to disease (Chandra et al., 2010). This presents the challenge of the plantlets having low disease tolerance to fungi, even weakly saprophytic fungi, that develops during the acclimatization period (Parvizi & Dashti, 2015). The fungi can become pathogenic to the plantlets causing diseases such as damping off leading to significant losses (Vettori *et al.*, 2010). These characteristics necessitate the gradual acclimatization of tissue culture plantlets to *ex vitro* conditions.

Biotization: A metabolic response of *In vitro*-grown plant material to a microbial inoculant(s), leading to the developmental and physiological changes enhancing biotic and abiotic stress resistance of the derived propagules (Nowak, 1998).

Bio-hardening/Biotization, an increasingly recognized biological strategy, is garnering attention as a potent method for alleviating acclimatization stress and facilitating accelerated growth in plantlets. Tissue culture plants exposed to the processes of biotization or bio-hardening undergo discernible physiological and developmental modifications, leading to heightened resilience against both biotic and abiotic stress factors upon inoculation with beneficial microorganisms (Divya *et al.*, 2022). Methods used to minimize mortality rates include the use of beneficial microbes such as Arbuscular Mycorrhizal Fungi and Rhizobacteria where these microbes improve the performance of tissue culture plantlets in stressful environment (Vettori *et al.*, 2010). In addition, these microbes can shorten the acclimatization period (Rai, 2001) and result in the production of higher value potato tissue culture plantlets since Kenyan farmers consider seed potato quality a major concern (Gildemacher *et al.*, 2009).

Sharif & Moawad (2006) reported that potatoes form a high frequency of AMF root infection with *Glomus fasciculatum* (>4000 spores kgG^{-1}) in fertile soils. TC plantlet inoculation with AMF during the beginning of the acclimatization phase has been shown to improve plantlet survival and performance by enhancing host plant growth and the formation of secondary metabolites such as phenolics (Chandra *et al.*, 2010). AMF inoculation with *Glomus clarum, Entrophospora colombiana* and *Acaulospora koskei* at 20, 416 and 23 spores/50mL soil under *ex vitro* conditions during the hardening stage of acclimatization, as compared to non-AMF inoculated plants, enhanced the survival rate and growth of micro-propagated ginger (*Zingiber officinale* Roscoe) when combined with phosphorous (Santos *et al*., 2010). Yao *et al.* (2002) reported an increase in tuber number, shoot fresh weight and root dry weight in micro-propagated potato plantlets after AMF-inoculation. Borriello *et al.* (2017) concluded that AMF inoculation of *Ranunculus asiaticus* ('*Juny*') plantlets using 10g of inoculum during the beginning of the acclimatization phase resulted in an increment in plantlet survival. However, AMF inoculation did not significantly influence plantlet survival rate. Mirjani *et al.* (2019) reported that inoculation of *Satureja khuzistanica* Jamzad micro-propagated plantlets with 300 spores of *Glomus fasciculatum* significantly improved plantlet survival, total

chlorophyll, a, b content and nutrient absorption of Zinc, Copper, Phosphorus, Calcium and Potassium.

Mycorrhizal inoculants acted as effective bio-fertilizers of potato -enhancing tuber yield and phosphorus nutrient of the shoot (Aguk, 2013). Plant protection by AMF results from a combination of mechanisms including improvement of plant nutrition, the compensation by the symbiosis of the damage caused by the pathogen and improving nutrient use efficiency that ultimately increase plant growth, health and yield (Soumare *et al.*, 2021). Hashem *et al.* (2019) reported that AMF inoculation and Ca application alleviated salt stress in *Bassia indica* Wight. These bio-inoculants have also acted as bio-control agents against *Ralstonia solanacearum* in potato (Aguk, 2013) and plant parasitic nematodes in banana (Elsen e*t al.*, 2008). Bio-hardening tissue culture plantlets with AMF induces metabolic and developmental changes that enhance tolerance to the various transplant stresses and also boosts nutrient availability and promotes soil stability (Young *et al.*, 2015).

2.8 Effects of Calcium application on Bacterial wilt

The *Ralstonia solanacearum* species complex (RSSC) is considered to be one of the most important phyto-pathogenic bacteria globally, because of the destruction it causes, its wide geographical distribution, and its ability to survive for extended periods in soil, water, on plant debris and within asymptomatic hosts. Bacterial wilt, caused by *Ralstonia solanacearum* species complex (RSSC), is the most destructive potato disease in Kenya. The RSSC also causes bacterial wilt in Solanaceous crops, such as tomato and pepper, but can also cause wilts in other important food crops such as fruit banana, plantain banana and cassava (Bragard *et al.*, 2019). The bacterium, *R. solanacearum* has an unusually wide and ever-expanding host range, infecting more than 450 plant species belonging to over 54 different botanical families (Wamani *et al.*, 2023). Throughout history, the predominant phylotypes in Africa have been identified as I and II, encompassing the notably virulent IIB1 strains (Nortj, 2015). In Kenya, RSSC phylotype II strains are distributed among all 14 counties associated with potato production in Kenya, with

phylotype II sequevar 1 strains, formerly known as race 3 biovar 2, primarily attacking potato in Kenya (Sharma *et al.*, 2022) (Figure 2). Latently infected tubers from visually asymptomatic plants and tubers are the primary source of pathogen dissemination throughout Kenya. The shortage of certified clean seed leads to the unrestricted movement of latently-infected potato tubers from informal seed systems resulting in disease dissemination within Kenyan counties (Demo *et al.*, 2015).

Kaguongo *et al.* (2014) reported that bacterial wilt affects 77% of potato farmers, with roughly 59% of potato field in the highlands and 86% of fields in areas below 2000 meters above sea level (M.A.S.L) infested with bacterial wilt due to the repeated vegetative propagation causing disease buildup in progenies (Muthoni *et al.*, 2014). Infected plants are often stunted and yellowed before they develop characteristic wilting symptoms, which usually lead to death of the entire plant. *R. solanacearum* gains entry into the roots through either wounds or natural openings, subsequently migrating towards developing vascular bundles and ultimately reaching the xylem vessels within susceptible potato roots (Caldwell *et al.*, 2017). Upon entry into the xylem network, *R. solanacearum* systematically disseminates throughout its host organism.

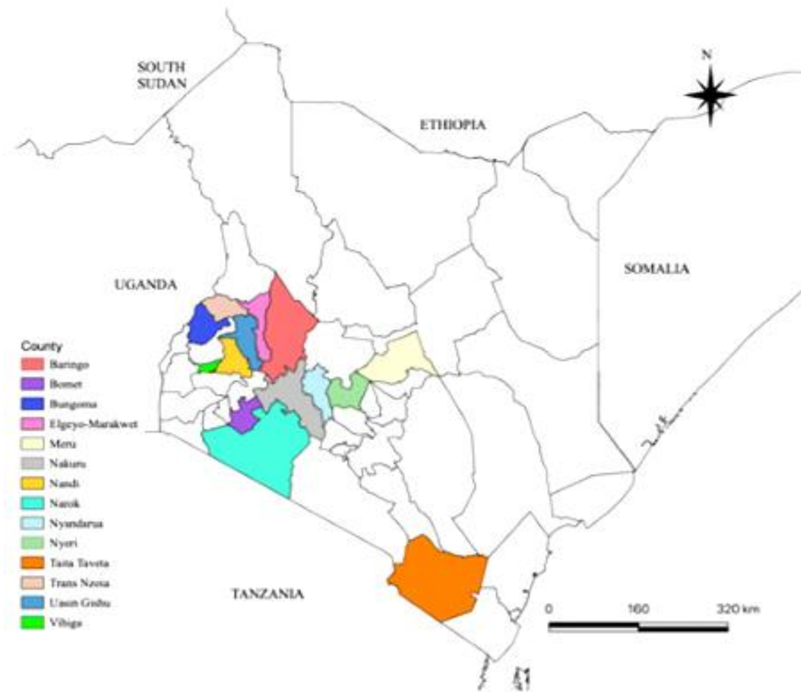


Figure 2.2: **Distribution of RSSC Phylotype II strains in potato producing counties** (Sharma *et al.*, 2022)

RSSC-infected plants exhibit distinctive phenotypic manifestations, characterized by wilting and necrosis of foliage, typically initiating in the lower leaves and progressing upwards (Genin & Denny, 2012). This pathological progression is accompanied by vascular discoloration and a decline in overall plant vigor. Additionally, affected plants may display a stunted growth phenotype, and in severe cases, a rapid and irreversible collapse of the entire plant system (Khairy *et al.*, 2021). These phenotypic alterations are indicative of the systemic and destructive nature of *R. solanacearum* infection on susceptible plant hosts. The precise cause of wilting in the context of RSSC infections remains elusive, although it is probable that multiple mechanisms collectively contribute to vascular dysfunction in plants (Lowe-Power *et al*., 2018).

Various techniques, including phyto-sanitation and cultural practices, are commonly employed for the management of bacterial wilt. The most popular method used by a majority of Kenyan potato farmers for control is negative selection that involves rouging

out diseased plants to minimize yield losses (Okello *et al.*, 2020). However, these methods are at times inefficient, expensive, tedious (Gildemacher e*t al.*, 2009). Tolerant cultivars of potatoes characterized with varying degrees of resistance, achieved through breeding, have been introduced for agricultural cultivation. Despite their availability, a significant challenge persists in the high frequency of latent tuber infections (Patil *et al.*, 2012).

Crop resistance is compromised by the emergence and resurgence of phyto-pathogens, necessitating the continuous pursuit of novel resistance through breeding efforts. This incurs costs for both farmers and seed companies. Farmers find themselves obliged to employ excessive quantities of chemical pesticides for the purpose of disease management and control, especially in the face of newly emerging pathogens (Mwangi *et al*., 2023). The employment of chemical management strategies involving bactericidal agents like streptomycin sulfate and fumigants such as 1,3-dichloropropene has proven efficacious in diminishing the prevalence of bacterial wilt (Han *et al.*, 2011; Mao *et al*., 2019). Chemical pesticides exert detrimental effects on the ecosystem, affecting both the soil and water bodies. Furthermore, their impact extends to non-target organisms, encompassing pollinators and beneficial microbial flora residing in the soil. The environmental limitations inherent in these approaches, coupled with the consequential emergence of elevated resistance levels, have deterred their widespread application (Yuliar *et al.*, 2015).

Biological, chemical, and cultural management strategies face challenges against *R. solanacearum,* a genetically diverse, soil-borne pathogen with a wide host range (Rostand *et al*., 2018). Nevertheless, the integration of biological control strategies, such as the application of mycorrhizal inoculants, along with cultural approaches like agronomic calcium bio-fortification, represents pivotal measures to control bacterial wilt, enhancing seed potato propagation and, consequently, boosting tuber yield production.

Calcium is known for strengthening the middle lamella and its involvement in hypersensitive responses to infection and also influencing soil pH (Hawkesford *et al.*, 2012). Calcium application and subsequent increased Ca concentrations in plants has been

shown to suppress pathogens such as *R. solanacearum*. Zhang *et al.* (2014) stated that transient and drastic changes in intracellular Ca^{2+} concentration in plant cells upon pathogen infection are known to be an essential early signaling event for plant defense responses. He *et al.* (2014) demonstrated that soils treated with $CaCO^3$ particles (0~1 mm) significantly inhibited the survival of *R. solanacearum*. The study authors also established that the growth of *R. solanacearum* was significantly inhibited by Ca^{2+} instead of Cl^- ions. Tuhwe (2015) showed that treating *Pectobacterium carotovora*-infected potatoes using $CaCl_2$ resulted in significantly lower disease severity scores compared to $CaSO_4$ and $Ca(NO_3)_2$ treatments. The study authors attributed this to calcium bridging of the plasma membrane components reducing electrolyte leakage and maceration by pectolytic enzymes since extracellular calcium is thought to help in maintaining the selective permeability of plasma membranes.

CHAPTER THREE

INVESTIGATING OPTIMAL *IN VITRO* CALCIUM BIO-FORTIFICATION, FOR REGENERATION AND MICRO-TUBERIZATION IN SELECTED POTATO VARIETIES

3.1 Abstract

The potato is the second most important food crop in Kenya, holding great economic importance for smallholder farmers. Despite increased demand for potato, production faces challenges including limited clean seed production and non-optimized nutrient protocols for potato TC plantlets. This necessitates the development of new strategies for enhancing production. Micro-propagation efficiency can be enhanced greatly through the modification of the basal culture media to include or enhance nutrients that promote plant regeneration and vigour while shortening the regeneration period. Laboratory experiments were set up to investigate the effect of *In vitro* calcium (Ca) bio-fortification on *Shangi*, *Unica* and *Dutch Robjyn* plantlet regeneration capacities and microtuberization. Single node cuttings of the three varieties were sub-cultured onto modified MS media with five levels of $CaCl_2$: 8.8g/L, 10.4g/L, 12g/L, 13.6g/L and 15.2g/L $CaCl_2$. In the micro-tuberization experiment, micro-tubers were induced on MS media modified with the five Ca levels, 60g/L brown sugar and 6mg/L 6-Benzylaminopurine. The experiments were set up in a completely randomized design and replicated five times. Nutrient analysis to determine total calcium content in plantlet whole root sections was conducted using atomic adsorption spectrophotometer. Performance determination was based on *In vitro* shoot-root regeneration, micro-tuber number, days to micro-tuber formation, micro-tuber fresh weight (FW) and root-zone mineral contents. Analyses of variance proved a significant and highly significant effect *($P \leq 0.01$)* on tested factors and their interactions on shoot and root development and micro-tuberization. There were no significant differences in shoot, leaf and node numbers of tested varieties. MS medium containing 10.4g-13.6g/L Ca levels significantly *($P<0.05$)* increased root numbers in tested varieties compared to conventional MS medium. There were significant differences *($P<0.05$)* in

the number of days it took *Shangi*, *Dutch Robjyn* and *Unica* to form a single micro-tuber. MS medium containing 10.4g/L Ca significantly reduced the days it took *Shangi* and *Unica* to induce a micro-tuber. Days in days to micro-tuber induction also resulted in reduced micro-tuber fresh weights. MS medium containing 13.6-15.2g/L Ca increased micro-tuber FW in *Shangi* and *Dutch Robjyn* compared to conventional MS medium. MS medium containing 12g/L Ca completely inhibited micro-tuberization in *Unica*. Ca content in the mid-stem + whole root zone sections of the tested varieties increased with increasing Ca content in culture media. Plantlets of the three varieties cultured on MS medium containing 13.6 g/L Ca had the highest Ca content in whole root zone sections. MS medium containing 12g/L Ca led to the highest magnesium and iron content in *Shangi* and *Dutch Robjyn* plantlets. MS medium containing 13.6 g/L led to the highest zinc content in *Unica*. Results confirm the optimization of MS nutrient protocol by Ca enhancement as a potential technology for scaling up the production of clean quality seed.

3.2 Introduction

Potato micro-propagation entails the rapid multiplication of stock plant material for the mass production of clean seed potato. Micro-propagation efficiency can be enhanced greatly through the modification of the basal culture media to include or enhance nutrients that promote plant regeneration and vigour while shortening the regeneration period. Macro-nutrients ($CaCl_2.2H_2O$, KH_2PO_4, $MgSO_4$) as a group of minerals in Murashige and Skoog (MS) medium, play an influential role during *in vitro* plant growth. Wada *et al.* (2013) found that enhancing $CaCl_2.2H_2O$, KH_2PO_4, $MgSO_4$ dramatically improved pear and blackberry micro-propagation respectively, by producing high quality shoots thereby contributing to a higher production of improved quality plantlets.

Calcium (Ca) is involved in a wide range of physiological processes such as promoting disease resistance to pathogens through the strengthening of cell wall structures and maintaining membrane stability (White & Broadley, 2003; Gilroy *et al.*, 2016). Calcium supplementation has been found to promote potato micro-tuberization and micro-propagation (Habib *et al.*, 2004) as well as potato tuber yield under filed conditions and

improve tolerance to diseases such as bacterial wilt (Jiang *et al.*, 2013). However, potato varietal response to agronomic calcium bio-fortification under *In vitro* conditions is not well documented. Agronomic calcium bio-fortification nutrient protocols for micro-tuberization of selected potato varieties have also not been properly investigated. This study therefore aimed at investigating the optimal calcium bio-fortification level for *In vitro* regeneration and micro-tuberization of three potato varieties in Kenya, for plantlet growth, micro-tuber production and tissue-culture plantlet vigour.

3.3 Materials and Methods

3.3.1 Study Site

The study was conducted under laboratory conditions at Institute of Biotechnology Research Laboratory (IBR) of Jomo Kenyatta University of Agriculture and Technology (JKUAT) in Juja sub-county, in 2021 and 2022.

3.3.2 Study Materials

Three Irish potato varieties (*S. tuberosum* L.) '*Shangi'*, '*Unica'* and '*Dutch Robjyn'* were evaluated for morphological and nutritional characteristics under five levels of calcium (8.8g/L, 10.4g/L, 12g/L, 13.6g/L, 15.2g/L $CaCl_2$) *In vitro*. Fifty (50) one-month old contamination-free Irish potato tissue culture plantlets of the three varieties were obtained from the KALRO Tigoni tissue culture laboratory. These varieties were selected on the basis of being the most preferred varieties among Kenyan farmers, consumers and seed producers (Kaguora *et al.*, 2019).

3.3.3 General Materials, Reagents and Media preparation

All chemicals were of analytical grade and were obtained from Loba Chemie Pvt Ltd, Unichem Chemicals, Panreac Quimica SLU and Duchefa Biochemie. For the shoot and root regeneration experiment, five different $CaCl_2.H_2O$ levels T_0=8.8g/L $CaCl_2$ (Conventional Murashige and Skoog (MS) media/Control), T_1= 10.4g/L $CaCl_2$, T_2= 12g/L

$CaCl_2$, T_3= 13.6g/L $CaCl_2$, T_4= 15.2g/L $CaCl_2$, were amended into basal MS solution and subsequently supplemented with 3% (w/v) sucrose and 0.28% (w/v) Gelrite. All other MS medium constituents, apart from $CaCl_2$, were kept at conventional levels for all five different MS treatment levels. The pH value for the media was adjusted to 5.8 using 0.1M HCl or 0.1M NaOH. For the microtuberization experiment, 6% (w/v) sucrose and 6.0mg/L 6-Benzylaminopurine were added into MS media containing the five different $CaCl_2.H_2O$ levels. All other MS medium constituents, apart from $CaCl_2$, were kept at conventional levels for all 5 $CaCl_2$ treatment levels. The culture media, forceps and surgical blade holders were sterilized in an autoclave at 121˚C for 15 minutes at 15 pounds per square inch (psi). Sterilized media was then kept in the culture room for three days before use to avoid inoculation of contaminated plantlets. All initiation procedures were conducted within a sterile cabinet equipped with laminar airflow.

3.3.4 Culture conditions of regeneration and microtuberization experiments

Single node cuttings (12-15mm with one axillary bud) of 1-month-old tissue culture plantlets of the three Irish potato varieties were evaluated for shoot, node and leaf and root regeneration response under five levels of Ca (control 8.8g/L (T_0), 10.4g/L (T_1), 12g/L (T_2), 13.6g/L(T_3), 15.2g/L (T_4) $CaCl_2$) in 25ml of media dispensed into 300mL culture jars. The cultures were maintained in a culture room at 21 ± 2°C, under cool white fluorescent tubes at 36µmol s^{-1} m^{-2} and examined on a weekly basis for a period of 4 weeks where every change in growth was carefully observed and recorded. For the microtuberization experiment, 1-month-old single node cuttings obtained from the regeneration experiment were cultured onto 20mL solid medium in 300mL glass jars. Cultures were maintained in a growth room at 21± 2°C in complete darkness until tuber formation occurred. Assessments were done on a weekly basis and micro-tubers harvested after formation. Every change in growth was carefully observed and recorded up to 90 days after culture initiation. Culture physical conditions were the same for all the treatments during regeneration and microtuberization experiment.

3.3.5 Experimental Design and Data Collection

Experiments on shoot formation and multiplication, root formation and multiplication, and microtuberization under five different levels of calcium (control 8.8g/L (T_0), 10.4g/L (T_1), 12g/L (T_2), 13.6g/L(T_3), 15.2g/L (T_4) $CaCl_2$) were laid out in a completely randomized design following a 3x5 factorial set-up with 5 replications. Data on regeneration capacity (shoot initiation and multiplication, leaf number, node number and root formation) were collected after every 7 days then analyzed 28 days after culture. Data on number of micro-tubers per explant cutting, days to micro-tuberization and micro-tuber fresh weight of harvested micro-tubers per cutting were recorded. Days required for *In vitro* tuberization was recorded at visible swelling of micro-tubers.

3.3.6 Nutrient Analysis

After completion of the regeneration experiment, five 45-day-old tissue culture plantlets were randomly selected from each of the three varieties (*Shangi, Dutch Robjyn* and *Unica*) and each of the five different $CaCl_2$ treatments. The plantlets were removed from their TC jars and rinsed under running water until all traces of culture media were removed. The tissue culture plantlets were patted dry and left to air dry at ambient temperature. Tissue culture plantlets fresh weights (FW) was recorded, then the mid-stem and root section was excised from each of the tissue culture plantlets and weighed. The plant materials were then oven dried at 70°C in an oven for 24 hours. Oven-dried mid-stem and root zone samples were ground in a pestle and mortar, digested and analyzed for total Calcium (Ca), Magnesium (Mg), Iron (Fe) and Zinc (Zn) according to Welz & Sperling (2008) using atomic adsorption spectrophotometer (Shimadzu AA-7000 series, Shimadzu Corporation, Japan). Plantlets with highest total calcium content of the five different MS media formulations were regarded as best fortified plantlets.

3.4 Statistical Analysis

Data obtained from the *In vitro* fortification, regeneration and microtuberization experiment were subjected to two-way analysis of variance (ANOVA) using GLM procedure in SAS University Edition (SAS®, Version 9.4, SAS Institute Inc., Cary, NC, USA) (SAS Institute, 2020). Treatment means were separated by Tukey's HSD (Honestly Significant Difference) test at $P \leq 0.05$.

3.5 Results and Discussion

3.5.1 Effect of MS media Ca Bio-fortification on *In-vitro* growth of *Shangi*, *Dutch Robjyn*, and *Unica* at 28 Days after culture (DAC)

3.5.2 Shoot Number

Analyses of variance proved a significant and highly significant effect ($p < 0.01$) on tested factors (Variety, Calcium amount in MS medium) and their interactions (Variety x Calcium amount in MS medium) on the shoot number of all the three varieties (Table 3.1). Shoot numbers in *Shangi* and *Dutch Robjyn* exhibited no significant differences within the five treatment levels over the 28 days of culture. MS medium containing 10-12g/L $CaCl_2$ (T1-T2) resulted in the highest shoot number for *Shangi* (4.14±0.86) representing 52% increase, compared to the conventional MS (8.8g/L $CaCl_2$) medium (2.71±0.47) (Table 3.1). Shoot numbers doubled 21-28 days after culture onset for *Shangi* across all 5 treatments compared to the conventional MS media (2.71±0.47) formulation after 28 days of culture.

MS medium containing 15.2g/L $CaCl_2$ (T4) delayed shoot initiation in *Shangi* 7 days after culture compared to other treatments. There were no significant differences in shoot numbers between treatments for *Shangi* over 28 days of culture. MS medium containing 10.4-15.2g/L $CaCl_2$ resulted in the formation of at most 2 shoots for *Dutch Robjyn*. MS medium containing 13.6g/L $CaCl_2$ delayed shoot formation in *Dutch Robjyn* 7 days after culture onset but also promoted a 9% increase in shooting after 14 days of culture

(1.57±1.57) compared to the conventional MS medium formulation containing 8.8g/L $CaCl_2$ (1.43±1.43). MS medium containing 13.6g/L $CaCl_2$ also resulted in the highest number of shoots (2.29±0.36) for *Dutch Robjyn* after 28 days of culture. In *Unica*, Calcium level significantly influenced ($p<0.0001$) shoot number within the five treatments. MS media with 8.8g/L $CaCl_2$ (T0) resulted in the highest number of shoots (5.57±1.21) compared to all other treatments in *Unica* (Table 3.1) after 21 days of culture. MS medium with 10.4g/L-15.2g/L$CaCl_2$ resulted in the regeneration of one dominant apical shoot in *Unica* over the 28 days of culture. Conventional MS medium (8.8g/L $CaCl_2$) resulted in the formation of chlorotic axillary shoots in *Unica* (Plate 3.1) associated with hyperhydricity. There were no significant differences in shoot numbers between treatments in Unica, 7-21 days after culture onset.

Hunková *et al.*, (2020) reported significant effects of Cultivar and Cultivar x Macronutrient concentration in MS medium and their interactions on the shoot number and shoot length in *Amelanchier alnifolia* var. *cusickii*, *Rubus fruticosus* and *Vaccinium corymbosum* cultivars which is consistent with the results of our study. Previous studies that showed that supplemental Ca (added to conventional MS medium formulation) promoted dominant apical shoot growth (Ozgen *et al*., 2005; Machado *et al.*, 2014). One reported constraint associated with the tissue culture of Irish potato is shoot tip necrosis that results in the death of apical shoots, loss of apical dominance and the formation of axillary shoots (Ibrahim *et al.*, 2016). Conventional MS media formulation containing 8.8g/L $CaCl_2$ resulted in the formation of axillary shoots in *Unica* 21 days after culture onset while MS medium containing 10.4-15.2g $CaCl_2$ promoted the growth of one dominant apical shoot. Srikum *et al.* (2018) found that 880mg/L $CaCl_2$ level in the culture medium, combined with 0.5mg/L BAP, significantly decreased shoot necrosis occurrence in *Elsholtzia stachyodes* and resulted in 100% shoot induction. This difference could be attributed to genotype differences and their response to the treatments. Ozgen *et al*. (2011) noted statistically significant increases in the number of axillary shoots observed after 30 days of culture on MS medium formulations containing 1.4-680mM calcium but no axillary shoots after 13 days of culture in *S. tuberosum* '*Dark Red Noland'*. MS medium

formulations with calcium concentrations ranging between 1500-3000mM significantly reduced axillary shoot formation after 30 days of culture.

Table 3.1: Effect of MS media Calcium enhancement on shoot regeneration of *Shangi, Dutch Robjyn*, and *Unica* potato varieties over 28 days of culture (DoC)

		7 DoC	14 DoC	21 DoC	28 DoC
Variety	Treatment	No. of shoots	No. of shoots	No. of shoots	No. of shoots
Shangi	Control (T0)	1.00 ±0.00[ab]	1.43 ±0.20[a]	2.00 ±0.22[a]	2.71 ±0.47[a]
	T1	1.00 ±0.00[ab]	1.14 ±0.14[a]	2.29 ±0.29[a]	4.14 ±0.86[a]
	T2	1.00 ±0.00[ab]	1.29 ±0.18[a]	2.14 ±0.40[a]	4.14 ±0.63[a]
	T3	1.14 ±0.14[a]	1.57 ±0.30[a]	2.14 ±0.26[a]	2.43 ±0.30[a]
	T4	0.57 ±0.20[b]	1.86 ±0.34[a]	2.29 ±0.18[a]	2.86 ±0.55[a]
	p value	0.012	0.3069	0.946	0.131
Dutch Robjyn	T0	0.86 ±0.14[a]	1.43 ±1.43[a]	1.71 ±0.18[a]	2.14 ±0.34[a]
	T1	1.00 ±0.00[a]	1.14 ±1.14[a]	1.43 ±0.20[a]	1.71 ±0.18[a]
	T2	1.00 ±0.00[a]	1.29 ±1.29[a]	1.43 ±0.30[a]	1.86 ±0.34[a]
	T3	0.71 ±0.18[a]	1.57 ±1.57[a]	2.00 ±0.38[a]	2.29 ±0.36[a]
	T4	1.00 ±0.00[a]	1.86 ±1.86[a]	1.14 ±0.14[a]	1.43 ±0.30[a]
	p value	0.227	0.3069	0.194	0.33
Unica	T0	1.29 ±0.29[a]	1.29 ±0.18[a]	1.43 ±0.30[a]	5.57 ±1.21[a]
	T1	0.86 ±0.14[a]	1.14 ±0.14[a]	1.14 ±0.14[a]	1.43 ±0.30[b]
	T2	1.00 ±0.00[a]	1.00 ±0.00[a]	1.00 ±0.00[a]	1.14 ±0.14[b]
	T3	1.00 ±0.00[a]	1.29 ±0.29[a]	1.29 ±0.29[a]	1.29 ±0.18[b]
	T4	1.00 ±0.00[a]	1.00 ±0.00[a]	1.14 ±0.14[a]	1.14 ±0.14[b]
	p value	0.331	0.5658	0.646	<.0001
Anova(p value)	Trt	0.3956	0.6582	0.5642	0.0014
	Vty	0.3004	0.0052	<.0001	<.0001
	Trt*Vty	0.0166	0.2730	0.5953	<.0001

not significantly different for each treatment (Tukey's HSD test, $P \leq 0.05$).

Analys ... ve ... facto
Calcium amou ... MS medium) a ... eir interactions ... ty x Calcium
MS medium o ... eaf number ove ... ays of culture fo ... hree varieties (Table 3.2).

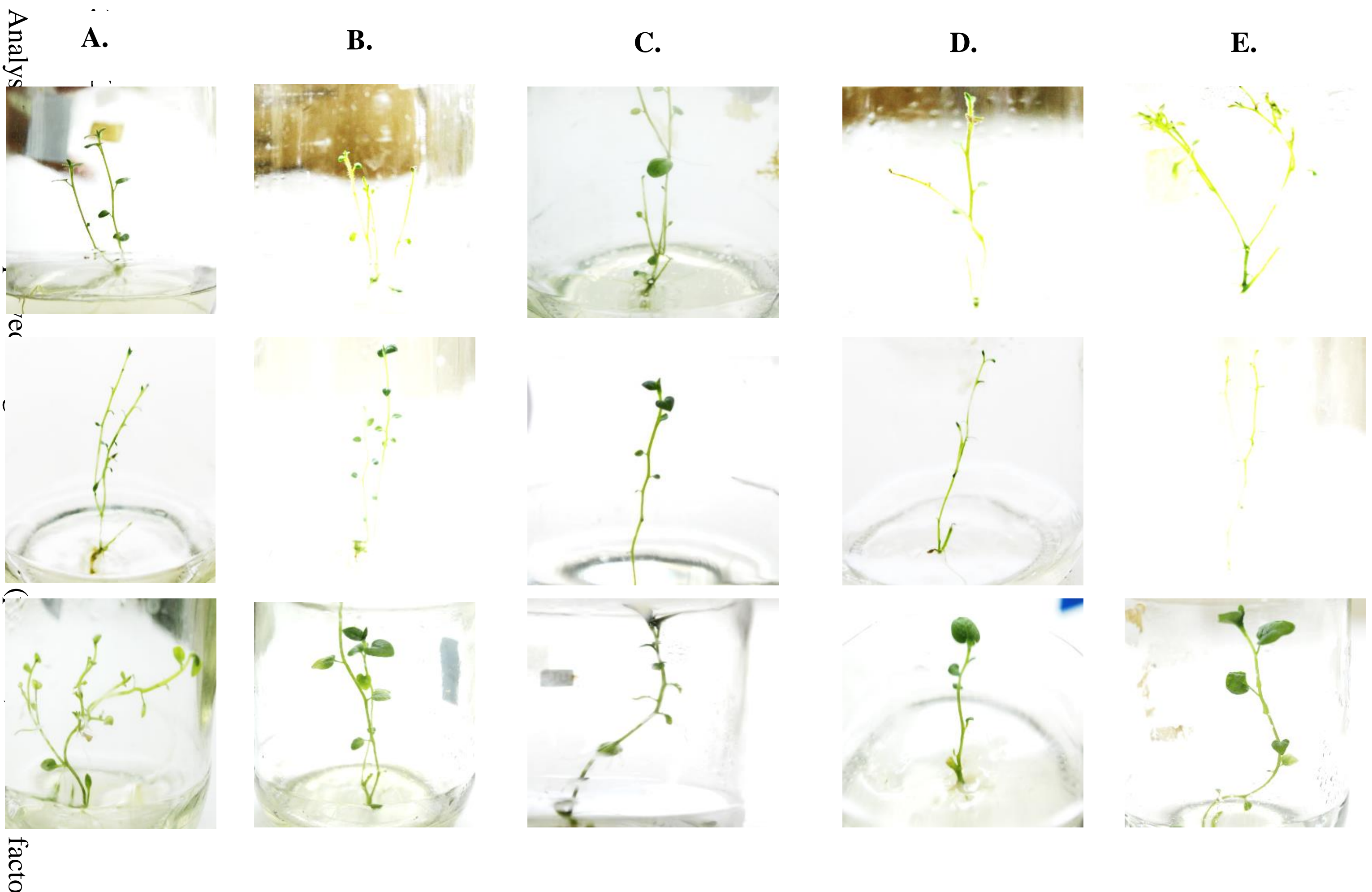


Plate 3.1: TC plantlets of *Shangi* (i.), *Dutch Robjyn* (ii.) and *Unica* (iii.) showing shoot development after 28 days of culture in MS medium containing 8.8g/L Ca (A.), 10.4g/L Ca (B.), 12g/L Ca (C.), 13.6g/L Ca (D.), 15.2g/L Ca (E.). MS medium containing 8.8g/L Ca resulted in the formation of multiple axillary shoots in *Unica*. MS medium containing 12-15.2g/L Ca typically resulted in the development of one dominant apical shoot in *Dutch Robjyn* and *Unica*.

There were no significant differences in leaf numbers between the five treatments in *Shangi*. MS medium containing 10.4g/L $CaCl_2$ (T1) resulted in the highest leaf number in *Shangi* (10.14± 1.98) after 28 days of culture. MS media containing 12g-15.2g/L $CaCl_2$ promoted twice the number of leaves produced by *Shangi* plantlets (2-2.14 leaves) after 7 days of culture set-up, compared to the control (0.71±0.18). There were significant differences (p=0.016) in *Dutch Robjyn* leaf numbers between the five treatments observable from 21 days of culture. Treatment T1 (MS media containing 10.4g $CaCl_2$) resulted in the highest number of leaves in *Dutch Robjyn* plantlets (12.71±0.52), 1.1 times higher compared to the control (11.29±0.61) after 28 days of culture. Treatment T4 resulted in the lowest number of leaves in *Dutch Robjyn* (8.43±0.84) after 28 days of culture.

There were significant differences (p=0.0071) in *Unica* leaf numbers between the five treatments observable from culture onset (Table 3.2). Conventional MS medium resulted in the highest $CaCl_2$ number of leaves in *Unica* (10.43±1.13). MS medium containing 15.2g/L $CaCl_2$ (T4) resulted in a 27% increase in *Unica* leaf number (4.00±0.65) after 7 days of culture compared to the control (3.14±0.51). MS medium containing 12-15.2g/L $CaCl_2$ resulted in an average of 6-8 leaves per plantlet after 28 days of culture (Table 3.2). Al-Mayahi (2019) reports that Ca addition to MS media (combined with boron) improved shoot multiplication rate of date palm (*Phoenix dactylifera* L.), resulting in 46% increase in shoot number. *Unica* plantlets grown in conventional MS medium had a higher number of leaves due to axillary branch development (Plate 3.1). Calcium is an integral component of the cell wall and plays an important role in maintaining membrane integrity. Ozgen *et al.* (2011) reported increased calcium in the culture medium (340-3000μM) remedied the loss of apical dominance caused by the death of apical shoots in potato which is similar to our results. Amalia *et al.* (2014) demonstrated that incorporating calcium gluconate, an organic calcium source, into Murashige and Skoog (MS) medium with 0.6 mg/L benzyladenine (BA) during the shoot induction stage led to the most significant enhancement in shoot development (increased number of shoots and leaves) among three raspberry cultivars.

Table 3.2: Effect of MS media Calcium enhancement on leaf regeneration of *Shangi*, *Dutch Robjyn*, and *Unica* potato varieties over 28 days of culture (DoC)

		7 DoC	14 DoC	21 DoC	28 DoC
Variety	Treatment	No. of leaves	No. of leaves	No. of leaves	No. of leaves
Shangi	Control (T0)	0.71 ±0.18^{a}	3.57 ±0.75^{a}	6.14 ±0.63^{a}	7.43 ±0.72^{a}
	T1	1.43 ±0.30^{a}	4.57 ±0.37^{a}	6.43 ±0.65^{a}	10.14 ±1.98^{a}
	T2	2.00 ±0.44^{a}	4.00 ±0.31^{a}	7.43 ±0.65^{a}	10.00 ±0.69^{a}
	T3	2.43 ±0.48^{a}	4.71 ±0.36^{a}	6.57 ±0.37^{a}	7.86 ±0.51^{a}
	T4	2.14 ±0.77^{a}	4.14 ±0.74^{a}	7.43 ±0.65^{a}	9.71 ±0.64a
	p value	0.1141	0.5881	0.4336	0.2352
Dutch Robjyn	T0	2.29 ±0.47^{a}	5.00 ±0.38^{a}	7.86 ±0.40^{ab}	11.29 ±0.61^{ab}
	T1	1.71 ±0.29^{a}	6.43 ±0.57^{a}	9.86 ±0.40^{a}	12.71 ±0.52^{a}
	T2	1.00 ±0.69^{a}	5.00 ±0.49^{a}	7.71 ±0.61^{ab}	9.86 ±0.83^{ab}
	T3	1.14 ±0.40^{a}	5.57 ±1.13^{a}	8.00 ±0.79^{ab}	10.29 ±1.23^{ab}
	T4	2.29 ±0.57^{a}	4.43 ±0.48^{a}	7.14 ±0.74^{b}	8.43 ±0.84^{b}
	p value	0.2367	0.2967	0.0414	0.016
Unica	T0	3.14 ±0.51^{ab}	6.43 ±0.20^{a}	9.00 ±0.82^{a}	10.43 ±1.13^{a}
	T1	1.71 ±0.36^{b}	3.43 ±0.57^{b}	5.71 ±0.42^{b}	6.86 ±0.70^{b}
	T2	2.86 ±0.59^{ab}	4.86 ±0.34^{ab}	7.00 ±0.38^{ab}	8.43 ±0.37^{ab}
	T3	1.86 ±0.51^{ab}	4.57 ±0.75^{ab}	6.00 ±0.62^{b}	7.29 ±0.47^{b}
	T4	4.00 ±0.65^{a}	5.71 ±0.42^{a}	7.57 ±0.30^{ab}	8.86 ±0.40^{ab}
	p value	0.0285	0.0023	0.0012	0.0071
Anova(p value)	Trt	0.0527	0.9300	0.5593	0.2757
	Vty	0.0022	0.0101	0.0014	0.0007
	Trt*Vty	0.0368	0.0041	0.0002	0.0018

letters are not significantly different for each treatment (Tukey's HSD test, $P \leq 0.05$).

3.5.4 Node Number

Analyses of variance proved a significant and highly significant effect ($p < 0.01$) on tested factors (Variety, Calcium amount in MS medium) and their interactions (Variety x Calcium amount in MS medium) on the node number after 28 days of culture for the three varieties (Table 3.3). There were no significant differences in node numbers between treatments for *Shangi* right from culture onset. MS medium containing 10.4-12g/L $CaCl_2$ resulted in <10 nodes per plantlet compared to conventional MS medium (7.00±0.72). T1-T2 (MS medium containing 10.4g-12/L Ca) resulted in the highest number of nodes in *Shangi* (<10 nodes) after 28 days of culture.

There were significant differences (p=0.0158) in node numbers between treatments for *Dutch Robjyn* right after 28 days of culture. Plantlets cultured in T1 (MS medium containing 10.4g/L Ca) had the highest number of nodes in *Dutch Robjyn* (12.71±0.52) compared to plantlets in conventional MS medium (10.86±0.67) after 28 days of culture (Table 3.3). However, *Dutch Robjyn* plantlets cultured in T0 and T4 had at most 2 nodes after 7 days of culture compared to plantlets grown on MS medium containing 10.4g/L Ca (T1).

There were significant differences (p=0.0085) in *Unica* node numbers between treatments after 28 days of culture. MS medium containing 15.2g/L Ca resulted in 130% increase in the number of nodes in *Unica* (3.29±0.52) compared to the control (1.43±0.30) after 7 days of culture. *Unica p*lantlets grown on MS medium containing 8.8g/L Ca had at most 10 nodes. A higher number of nodes in *Shangi* and *Dutch Robjyn* points to increased plant regeneration vigour that may lead to larger leaves and increased shoot size (Sachs, 1999). Lekamge *et al.* (2021) reported that MS medium enhancement using 880-1320mg/L Ca resulted in significant improvements to shoot elongation. Morphologically, shoot growth consists of formation of leaf and node initials and elongation of internodes (Junttila, 1991). Calcium plays an important role in stimulating cell elongation by rigidifying the cell wall (Burstrom, 1968).

Table 3.3: Effect of MS media Calcium enhancement on node regeneration of Shangi, *Dutch Robjyn*, and *Unica* potato varieties over 28 days of culture (DoC)

		7 DoC		14 DoC		21 DoC		28 DoC	
Variety	Treatment	No. of nodes		No. of nodes		No. of nodes		No. of nodes	
Shangi	Control (T0)	0.57	±0.20[a]	3.14	±0.46[a]	5.71	±0.61[a]	7.00	±0.72[a]
	T1	1.00	±0.22[a]	3.71	±0.29[a]	5.86	±0.63[a]	10.29	±1.89[a]
	T2	1.43	±0.20[a]	4.14	±0.40[a]	7.00	±0.38[a]	9.71	±0.64[a]
	T3	1.57	±0.30[a]	4.43	±0.20[a]	6.57	±0.48[a]	8.00	±0.58[a]
	T4	2.00	±0.72[a]	4.00	±0.69[a]	7.43	±0.65[a]	9.29	±0.52[a]
	p value	0.1165		0.3202		0.1727		0.1686	
Dutch Robjyn	Control (T0)	2.29	±0.47[a]	4.86	±0.34[a]	7.71	±0.42[ab]	10.86	±0.67[ab]
	T1	1.57	±0.20[a]	6.29	±0.52[a]	9.71	±0.42[a]	12.71	±0.52[a]
	T2	1.00	±0.00[a]	4.29	±0.64[a]	7.00	±0.62[b]	9.71	±0.75[ab]
	T3	1.57	±0.43[a]	5.57	±1.13[a]	7.86	±0.70[ab]	10.14	±1.20[ab]
	T4	2.29	±0.57[a]	4.57	±0.48[a]	7.14	±0.74[b]	8.43	±0.84[b]
	p value	0.1276		0.2512		0.0231		0.0158	
Unica	T0	1.43	±0.30[b]	4.71	±0.29[ab]	8.00	±0.76[a]	10.14	±1.06[a]
	T1	1.43	±0.37[b]	3.14	±0.46[b]	4.57	±0.43[c]	6.71	±0.71[b]
	T2	1.57	±0.37[ab]	3.14	±0.34[b]	6.14	±0.51[abc]	8.14	±0.46[ab]
	T3	1.57	±0.53[ab]	3.29	±0.61[b]	5.57	±0.43[bc]	7.29	±0.42[b]
	T4	3.29	±0.52[a]	5.00	±0.31[a]	7.57	±0.30[ab]	8.86	±0.40[ab]
	p value	0.0179		0.0038		0.0002		0.0085	
Anova(p value)	Trt	0.0016		0.5695		0.4123		0.3178	
	Vty	0.0848		0.0002		<.0001		0.0004	
	Trt*Vty	0.1744		0.0086		<.0001		0.0010	

T0=8.8g/L $CaCl_2$; T1=10.4g/L $CaCl_2$; T2=12g/L $CaCl_2$; T3=13.6g/L $CaCl_2$; T4=15.2g/L $CaCl_2$. Trt= Treatment. Vty= Variety. Values indicate means±SE. Mean values within the same variety grouping followed by the same letters are not significantly different for each treatment (Tukey's HSD test, $P \leq 0.05$).

3.5.5 Root Number

Analyses of variance proved a highly significant effect ($p < 0.01$) on tested factors (Variety, Calcium amount in MS medium) and their interactions (Variety x Calcium amount in MS medium) on the root number of the three varieties after 28 days of culture (Table 3.4). There were significant differences ($p=0.0003$) in *Shangi* root number between the five treatments. MS medium containing 12g/L Ca (T2) promoted 2x the number of roots (9.86±0.70) compared to the control (T0) (4.86±0.83). Treatment T4 (MS media containing 15.2g/L Ca) delayed root formation after 7 days of culture initiation for *Shangi* compared to T0-T3, but after 14 days of culture, promoted root regeneration 1.7 times (5.28±0.80) compared to the conventional MS medium (3.00±0.38). MS medium containing 10.4g-13.6g/L Ca significantly promoted root growth (<9 roots) in *Shangi* compared to plantlets grown in conventional MS medium (4.86±0.83) after 28 days of culture (Plate 3.2).

There were highly significant differences ($p<0.0001$) in *Dutch Robjyn* root numbers between the treatments. In *Dutch Robjyn,* root regeneration was significantly ($p<0.0001$) influenced by Ca level especially after 14 days of culture. MS medium containing 10.4g/L Ca promoted 1.3 times the number of roots in *Dutch Robjyn* (13.29±0.89) compared to the control (10.43±0.81) after 28 days of culture. MS medium containing 8.8g-10.4g/L Ca resulted in the highest number of roots (<13 roots) in *Dutch Robjyn*. Treatment T2 (7.00±0.72) also resulted in 57% increase in root growth for *Dutch Robjyn* 14 days after culture establishment, compared to the control (3.00±0.58). There were highly significant differences ($p<0.0001$) in *Unica* root numbers between the treatments. Calcium levels in culture medium significantly influenced root regeneration from culture onset for *Unica* (Table3. 4). MS medium containing 15.2g/L Ca resulted in 105% increase in root number (5.00±0.62) after 7 days of culture, compared to the conventional MS medium (2.43±0.37). This trend remained constant over the 28 days of culture since *Unica* plantlets grown in T4 had the highest number of roots after 28 days of culture

(15.00±0.79). *Unica* plantlets (15.00±0.79) under T4 significantly outperformed *Shangi* (9.86±0.70) and *Dutch Robjyn* (13.28±0.89) in root regeneration capacity (Table 3.4).

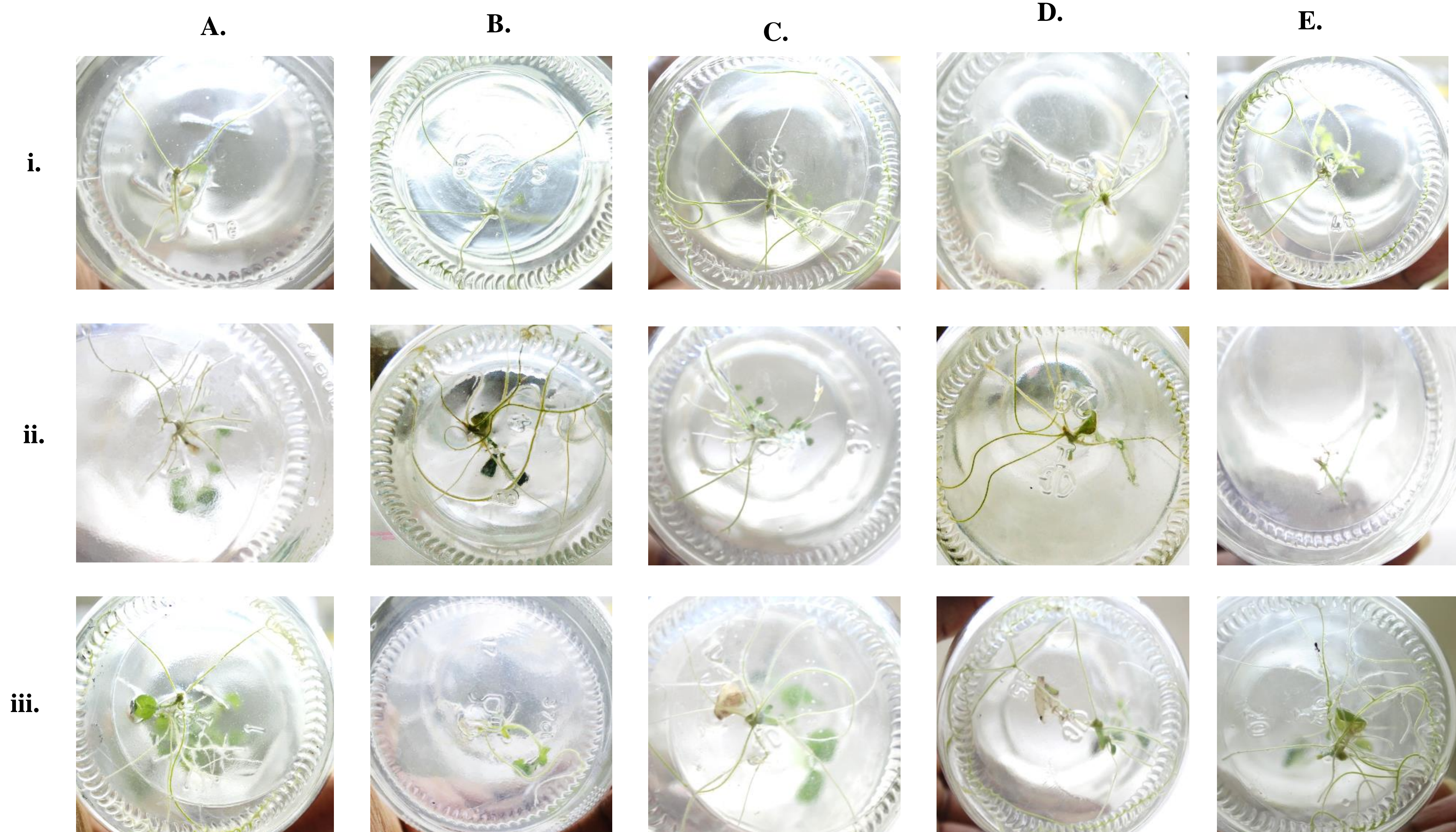
Calcium plays a role in promoting primary root development and maintaining the root hair growth machinery by influencing the direction of microtubule growth (Bibikova and Gilroy, 2009; Leitão *et al.*, 2019). Calcium impacts primary root development through modulating auxin accumulation, transport and signaling (Zhang *et al.*, 2020). Lekamge *et al.* (2021) found that supplemental Ca caused extensive *in vitro* root growth for cultivar *Okhotsk Chip*. Munthali *et al.* (2022) reported increased and improved root growth with increased shoot Ca uptake caused by Ca enhance in MS medium.

In conclusion, MS medium containing 10.4g-13.6g/L $CaCl_2$ promoted earlier and increased *In vitro* shoot development in *Shangi*, *Dutch Robjyn* and *Unica*. MS medium containing 10.4g-15.2g/L Ca reduced axillary shoot development in Unica while promoting strong apical shoot dominance in *Shangi* and *Dutch Robjyn*. MS medium containing 12-13.6g/L Ca significantly promoted root regeneration in Shangi, while 8.8-10.4g/L Ca promoted root development. T4 stimulated rapid and earlier root regeneration in *Unica* after 7 days of culture onset.

Table 3.4: Effect of MS media Calcium enhancement on root regeneration of *Shangi, Dutch Robjyn*, and *Unica* potato varieties over 28 days of culture (DoC)

		7 DoC	14 DoC	21 DoC	28 DoC
Variety	Treatment	No. of roots	No. of roots	No. of roots	No. of roots
Shangi	Control (T0)	1.86 $\pm0.40^{a}$	3.00 $\pm0.38^{a}$	4.00 $\pm0.53^{a}$	4.86 $\pm0.83^{b}$
	T1	1.29 $\pm0.52^{a}$	3.43 $\pm0.95^{a}$	4.86 $\pm0.77^{a}$	6.14 $\pm0.67^{b}$
	T2	1.43 $\pm0.65^{a}$	4.43 $\pm0.78^{a}$	6.14 $\pm0.80^{a}$	9.86 $\pm0.70^{a}$
	T3	1.14 $\pm0.40^{a}$	3.57 $\pm0.20^{a}$	5.00 $\pm0.38^{a}$	7.00 $\pm0.58^{b}$
	T4	0.71 $\pm0.57^{a}$	5.29 $\pm0.81^{a}$	6.14 $\pm0.67^{a}$	6.86 $\pm0.59^{b}$
	p value	0.6332	0.1602	0.1202	0.0003
Dutch Robjyn	Control (T0)	1.86 $\pm0.70^{a}$	3.00 $\pm0.58^{b}$	6.00 $\pm0.44^{b}$	10.43 $\pm0.81^{ab}$
	T1	2.00 $\pm0.69^{a}$	7.00 $\pm0.72^{a}$	10.14 $\pm1.06^{a}$	13.29 $\pm0.89^{a}$
	T2	1.57 $\pm0.84^{a}$	2.29 $\pm0.64^{b}$	4.71 $\pm0.75^{bc}$	7.86 $\pm0.94^{bc}$
	T3	1.29 $\pm0.52^{a}$	3.86 $\pm0.88^{b}$	4.43 $\pm0.65^{bc}$	5.14 $\pm0.80^{cd}$
	T4	1.00 $\pm0.44^{a}$	1.71 $\pm0.52^{b}$	2.71 $\pm0.68^{c}$	3.43 $\pm0.65^{d}$
	p value	0.8138	<.0001	<.0001	<.0001
Unica	T0	2.43 $\pm0.37^{b}$	3.57 $\pm0.43^{b}$	7.86 $\pm0.83^{b}$	11.14 $\pm1.49^{b}$
	T1	3.57 $\pm0.48^{ab}$	4.00 $\pm0.49^{b}$	4.57 $\pm0.43^{c}$	5.14 $\pm0.63^{c}$
	T2	2.29 $\pm0.71^{b}$	5.71 $\pm1.04^{ab}$	8.57 $\pm0.53^{ab}$	12.57 $\pm0.53^{ab}$
	T3	1.86 $\pm0.46^{b}$	3.43 $\pm0.43^{b}$	3.86 $\pm0.40^{c}$	6.00 $\pm0.31^{c}$
	T4	5.00 $\pm0.62^{a}$	6.86 $\pm0.51^{a}$	11.00 $\pm0.69^{a}$	15.00 $\pm0.79^{a}$
	p value	0.0019	0.0014	<.0001	<.0001
Anova(p value)	Trt	0.3320	0.0190	0.0004	<.0001
	Vty	<.0001	0.0248	<.0001	<.0001
	Trt*Vty	0.0224	<.0001	<.0001	<.0001

T0=8.8g/L $CaCl_2$; T1=10.4g/L $CaCl_2$; T2=12g/L $CaCl_2$; T3=13.6g/L $CaCl_2$; T4=15.2g/L $CaCl_2$. Trt= Treatment. Vty= Variety. Values indicate means±SE. Mean values within the same variety grouping followed by the same letters are not significantly different for each treatment (Tukey's HSD test, $P \leq 0.05$).



late 3.2: TC plantlets of *Shangi* (i.), *Dutch Robjyn* (ii.) and *Unica* (iii.) showing root development after 28 days of culture

n MS medium containing 8.8g/L Ca (A.), 10.4g/L Ca (B.), 12g/L Ca (C.), 13.6g/L Ca (D.), 15.2g/L Ca (E.)

3.6 Effect of MS media Ca enhancement on *In vitro* micro-tuberization of *Shangi, Dutch Robjyn*, and *Unica*

3.6.1 Days to Micro-tuber formation

Single nodal cuttings (SNC) of *Shangi*, *Dutch Robjyn*, and *Unica* sub-cultured onto MS media modified with $CaCl_2$ at different levels, exhibited significant differences ($p<0.05$) in tuber weight and days to tuberization depending on the variety, whereas tuber number did not differ significantly. Micro-tuberization is influenced by, but not specifically dependent on mineral nutrition as Tibebu (2016) found significant varietal differences ($p\leq0.05$) on tuber number in four Irish potato varieties under three tuber sizes. Results from the current study are consistent with previous findings on the factors affecting potato tuber weight and days to tuberization depending on variety. Analyses of variance proved a significant ($p < 0.05$) and highly significant effect ($p < 0.01$) on tested factors (Variety, Calcium amount in MS medium) and their interactions (Variety x Calcium amount in MS medium) on the days it took single node cuttings of the three varieties to form a micro-tuber (Table 5).

MS media containing 10.4g-15.2g/L Ca (T2, T3 and T4) had significantly variable effects on the number of days to tuber formation (Table 5). The tuberization process begun with the formation of a single stolon for all three varieties under T0 (8.8g/L Ca), T2 (12g/L Ca), T3 (13.6g/L Ca), T4 (15.2g/L Ca), subtending over the surface of the nutrient media as aerial stolons (Plate 3.3). MS media containing 10.4g/L Ca (T1) led to a 54%, 59% and 43% formation of sessile micro-tubers at the axillary buds of explants for *Shangi, Dutch Robjyn* and *Unica* (Plate 3.3). Calcium enhancements into MS medium significantly *($p<0.0001$)* influenced the days it took *Shangi* explants to form a single micro-tuber. There were significant differences in *Shangi* days to micro-tuber formation between the treatments. In *Shangi*, T1 and T2 promoted earlier tuberization (21.7-22.6 days) compared to the control (43.50±3.50). MS media containing 13.6g/L Ca (T3) resulted in *Shangi* forming a micro-tuber 21.7-43.5 days after culture initiation, the shortest duration to a

single tuber being formed (50% decrease in days to tuber formation) compared to the control (T0).

Calcium enhancements into MS medium significantly *($p<0.0001$)* influenced the days it took *Dutch Robjyn* explants to form a single micro-tuber (Table 5). There were significant differences in the days it took *Dutch Robjyn* to form a micro-tuber between the treatments. Micro-tuber initiation was significantly delayed (29 more days to form a micro-tuber) compared to the control in *Dutch Robjyn* when the culture media was Ca fortified at 10.4g/L Ca (55.34±2.47). Treatment T0, T2, and T3 also promoted earlier micro-tuberization in *Dutch Robjyn* (26.1-32.0 days) compared to MS media containing 10.4g/L Ca (T1) (55.43±2.47) and T4 (47.13±3.36). Calcium enhancements into MS medium significantly *($p<0.0001$)* influenced the days it took *Unica* explants to form a single micro-tuber and there were significant differences in the number of days it took *Unica* to form a single micro-tuber. Treatment T1 (10.4g/L Ca) reduced the number of days to tuber initiation by 16.83 days in *Unica* (21.67±0.33) compared to the control (38.50±2.25) (Table 5). MS medium containing 13.6g/L $CaCl_2$ delayed tuberization in *Unica* (94.00±3.00) by up to 100%.

Upadhyaya *et al.* (2016) observed that addition of Ca^{2+} (from 3mM to 9mM) in tuber induction medium significantly improved the period of tuberization induction reducing days to tuber induction to 4-5 days from 8-10 days in the control. This is consistent with our results for *Shangi* and *Unica* where 10.4-12g/L Ca in the micro-tuberization medium reduced the days it took explants of both varieties to induce a single micro-tuber. Reduction in the days it took to induce a micro-tuber was found to be associated with increased expression of the Ca^{2+} dependent genes and *LOX* mRNA transcript. Cheng *et al.* (2020) reported increased exogenous BAP concentration in MS medium (6-13mg/L) increased the days it took *S. tuberosum* L. cv. *Atlantic* single node cuttings to induce a micro-tuber. We used 6mg/L BAP to stimulate tuberization during the tuberization experiment.

Table 3.5: Effect of different Calcium levels on number of days to micro-tuberization, tuber number and tuber weight in *Shangi, Dutch Robjyn* and *Unica* varieties

Variety	Treatment	Days to micro-tuberization		Micro-tuber fresh weight (mg)	
Shangi	T0	43.5	±3.50^{a}	38	±0.00^{c}
	T1	22.6	±1.47^{b}	26.2	±0.00^{d}
	T2	21.75	±0.48^{b}	29.5	±0.00^{d}
	T3	42	±1.00^{a}	43.5	±0.00^{b}
	T4	49.67	±1.67^{a}	57.33	±0.00^{a}
	p value	<.0001		<.0001	
Dutch Robjyn	T0	26.13	±1.23^{b}	14.5	±0.00^{b}
	T1	55.43	±2.47^{a}	16.71	±0.00^{b}
	T2	32	±1.33^{b}	34	±0.00^{a}
	T3	25.89	±2.37^{b}	17.33	±0.00^{b}
	T4	47.13	±3.36^{a}	18.13	±0.00^{b}
	p value	<.0001		<.0001	
Unica	T0	38.5	±2.25^{c}	29.83	±0.01^{a}
	T1	21.67	±0.33^{d}	23.67	±0.00^{a}
	T2	*			*
	T3	94	±3.00^{a}	21	±0.00^{a}
	T4	57.33	±1.86^{b}	13.33	±0.00^{a}
	p value	<.0001		0.224	
	ANOVA				
	(p-value)	Trt	<.0001		0.0363
		Vty	<.0001		<.0001
		Trt*Vty	<.0001		<.0001

$T0=8.8g/L\ CaCl_2$; $T1=10.4g/L\ CaCl_2$; $T2=12g/L\ CaCl_2$; $T3=13.6g/L\ CaCl_2$; $T4=15.2g/L\ CaCl_2$. Trt= Treatment. Vty= Variety. Values indicate means±SE. Mean values within the same variety grouping followed by the same letters are not significantly different for each treatment (Tukey's HSD test, $P \leq 0.05$).

3.6.2 Micro-tuber Number

MS medium containing 10.4-12g/L $CaCl_2$ promoted micro-tuberization response by 150% and 100% respectively compared to the conventional MS medium. Treatment T1 (10.4g/L Ca) promoted the highest overall number of micro-tubers for *Shangi*. MS medium containing 12g-13.6/L Ca caused an overall increase in number of micro-tubers for *Dutch Robjyn*, with T2 (MS medium containing 12g/L $CaCl_2$) increasing tuberization response by 25% compared to the conventional MS medium.

MS medium containing 12g-15.2g/L Ca greatly impacted tuber production in *Unica*. Treatment T2 (12g/L Ca) completely inhibited microtuberization and instead, promoted the growth of etiolated shoots and apical hooks in *Unica* (Plate 3.3). MS medium formulation containing 10.4g/L $CaCl_2$ (T1), which is an 18.18% increase in Ca compared to the control, in micro-tuberization media influenced the formation of sessile micro-tubers for the three varieties, which might indicate that T1 enhanced a strong tuberization stimulus in the explants of the three varieties (Seabrook *et al.*, 1993). Potato micro-tubers originating from individual-node cuttings represents a more effective approach for handling and preservation when compared to those derived from sprouts (Mohamed & Girgis, 2023). However, using single node cuttings resulted in at most one micro-tuber being formed per single node explant for all three varieties. Seifu & Deneke (2017) reported significant interaction effects between potato varieties and calcium nutrients for average tuber yield. Application of 5-15g/pot of $CaNO_3$ alone and a combination of $CaCl_2$ and $CaNO_3$ significantly increased potato tuber yield both in *Shenkola* and in *Gera* potato varieties similar to our results for *Dutch Robjyn*. The findings of our study corroborate Hossain *et al.* (2019) who found plantlets of varieties *Asterix*, *Granola*, and *Diamant* produced 1 micro-tuber even with the use of BAP (known to promote tuberization) and

gibberellin scrubbers. Cheng *et al.* (2020) established BAP concentrations above 3mg/L in the MS medium inhibited *In vitro* micro-tuber formation. Cytokinins are known as important participants in cell proliferation and differentiation, morphogenetic processes occurring during potato tuberization. For our study, we used 6mg/L 6-Benzylaminopurine to trigger tuberization during culture.

Upadhyaya *et al.* (2016) reported increased tuber growth and yield with Ca^{2+} supplementation could be attributed to the increased phosphorylation of tuberization-specific enzyme by enhanced expression of the Ca^{2+} dependent genes, the *CaM1* and *StCDPK*. Increased calcium in the microtuberization medium promoted tuber yield in *Shangi* and *Dutch Robjyn.* Gargantini *et al.* (2009) reported higher expression of *StCDPK1* in swelling stolons which plays a key role in Gibberellic Acid (GA) signaling during tuberization. Upadhyaya *et al.* (2016) reports that a higher expression of *StCDPK1* potentially increased tuber growth and yield. However, GA production during culture is known to inhibit tuberization and plays a role in promoting the growth of etiolated shoots in darkness (Ozgen & Palta, 2005; Kusnetsov *et al.*, 2020) but further studies are required on the important roles Ca^{2+} and Ca^{2+} dependent proteins play in signal transduction pathway triggering tuber formation.

Arvin *et al.* (2005) established that increased Ca concentration multiplied micro-tuber number for cultivars *Bintje* and *Russet Bank.* Increasing calcium concentration also increased micro-tuber numbers for *Sassy*, *Cynthia*, *Alowa* and *Jagakids* purple potato varieties but there were no significant differences in the tuber numbers *(Mamiya et al.*, 2020). Habib *et al.* (2004) reported an increase in the number of micro-tuberized single node cuttings in treatments with high Ca^{2+} concentrations in the growth medium compared to the low Ca^{2+} growth medium.

3.6.3 Micro-tuber Fresh Weight

Analyses of variance proved a highly significant effect ($p < 0.01$) on tested factors (Variety) and their interactions (Variety x Calcium amount in MS medium) on the micro-

tuber fresh weight of all the three varieties. However, Ca treatments did not significantly influence fresh tuber weight of the varieties. MS media containing 10.4g-13.6g/L Ca (T1-T2) had the most significant effect (p <0.01) on the total number of micro-tubers imitated for all three varieties. Additionally, 10.4g-15.2g/L Ca significantly (*p<.0001*) affected micro-tuber fresh tuber weight of *Shangi* and *Dutch Robjyn*, while enhanced calcium had no significant effect on *Unica* micro-tuber fresh weight.

MS media containing 12g/L Ca (T_2) caused a significantly variable effect (p<.0001) on the tuber weights of the three varieties (Table 5). Treatment T2 increased tuber weight in *Dutch Robjyn* 2.3 times compared to the control. Enhanced calcium (10.4g-15.2g/L $CaCl_2$) resulted in a 15-134% increase in the micro-tuber fresh weight of *Dutch Robjyn* compared to the control. MS media containing 10.4g/L Ca (T1) and 12g/L Ca (T2) caused a 31% and 22% reduction in tuber weight for *Shangi* respectively, while MS media containing 13.6g/L Ca (T3) and 15.2g/L (T4) caused an increase in tuber fresh weight by 14% and 50% respectively, compared to the control (Table 5).

Rykaczewska (2016) and Salem & Hassanein (2017) reported that micro-tubers usually have a fresh weight ranging between 24 to 273 mg. *Shangi* micro-tuber weights ranged between 26-57mg, while *Dutch Robjyn* micro-tuber weights ranged between 14-34mg and *Unica* micro-tuber fresh weights ranged between 13-29mg. Micro-tubers were graded into two weight classes of 0.3–0.5 g, 0.5–1 g according to Mamiya *et al.* (2020). The study authors also noted that micro-tubers smaller than 0.3g easily shrank during cold storage, which was also observed during our experiments. Cheng *et al.* (2020) found that using 6mg/L BAP to induce micro-tubers on MS medium decreased micro-tuber fresh weight which would explain the low micro-tuber weights recorded in our experiments. The authors attributed this to BAP concentrations above 3mg/L in the microtuberization medium downregulating patatins storage proteins, known as primary storage proteins in potato tubers, that accompany the developmental transition of stolons into potato tubers. Hossain *et al.* (2019) also reported lower micro-tuber weights for potato varieties '*Asterix'*, '*Granola'* and '*Diamant'* compared to their control which was attributed to the

use of BAP used in this experiment to trigger tuberization and chlorocholine chloride. Arvin *et al.* (2005) reported an increase in micro-tuber fresh weight in '*Bintje*' with increased calcium concentration in the tuberizing medium, and a 17% reduction in fresh tuber weight in variety '*Russet Burbank*'. Habib *et al.* (2004) reported that 15mM Ca^{2+} improved micro-tuber weight by an average of 25% for all tested potato varieties compared to potatoes grown in nutrient media containing 5mM Ca^{2+}.

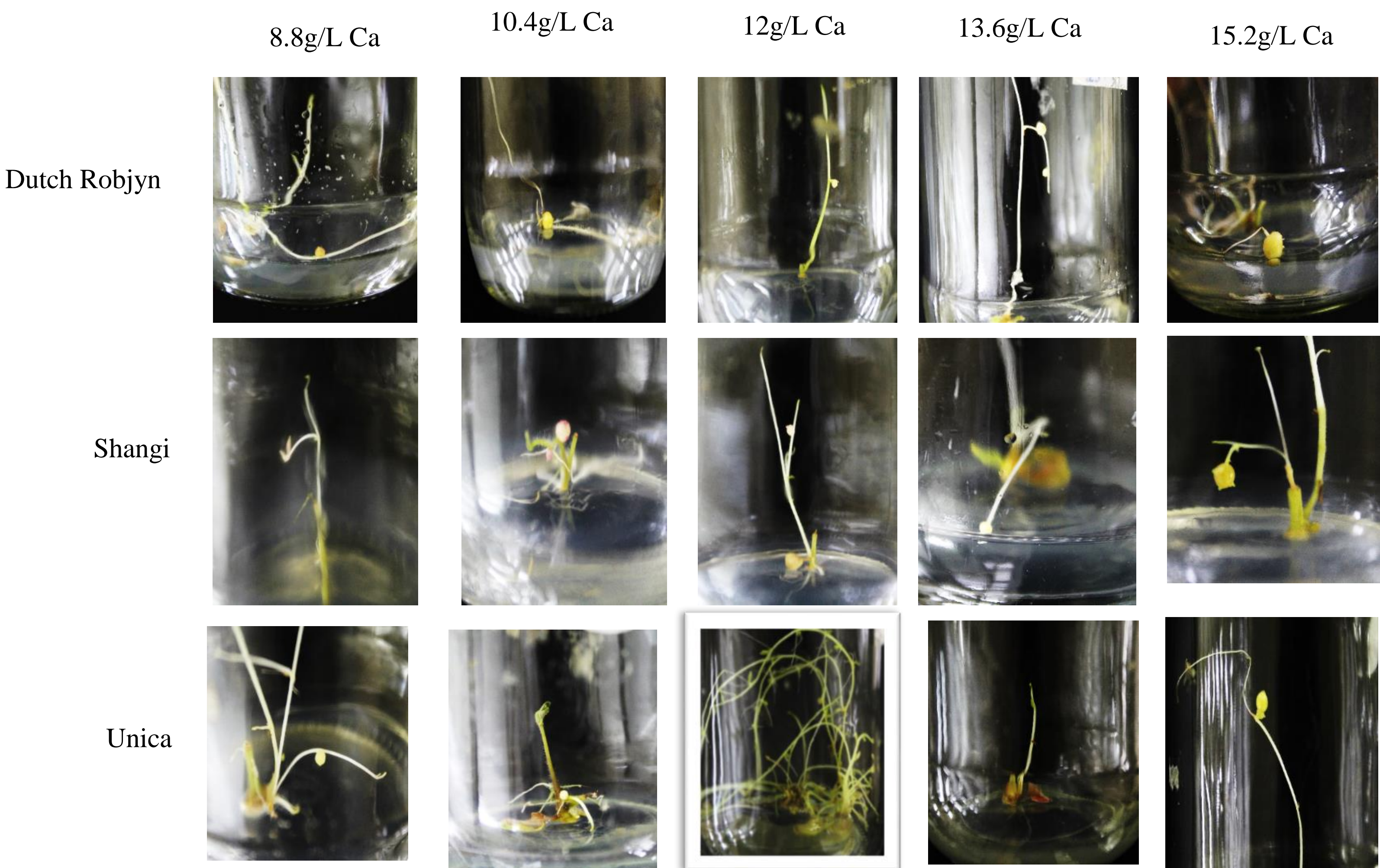


Plate 3.3: Micro-tuber formation in *Dutch Robjyn, Shangi* and *Unica* at 45 days of culture. Sessile micro-tubers were formed in the three varieties on MS medium containing 10.4g/L Ca. MS medium containing 12g/L Ca resulted in the formation of etiolated shoots with apical hooks in *Unica*.

Table 3.6: Total Calcium (Ca^{2+}) content in mid-stem + whole root section of calcium fortified *Shangi*, *Dutch Robjyn* and *Unica* under five Ca levels

	Calcium (Ca^{2+}) content (mg/g DW) in whole root + mid-stem section				
Variety	8.8g/L Ca (T0)	10.4g/L Ca (T1)	12g/L Ca (T2)	13.6g/L Ca (T3)	15.2g/L Ca (T4)
Shangi	6.60	5.74	6.41	9.59	8.15
Dutch Robjyn	2.94	6.70	6.99	7.79	5.27
Unica	2.09	3.23	6.00	6.31	4.00

Mean weights (mg/g) of 45-day-old plantlets composited after oven drying for 24hrs. milligrams/gram Dry Weight (mg/g DW).

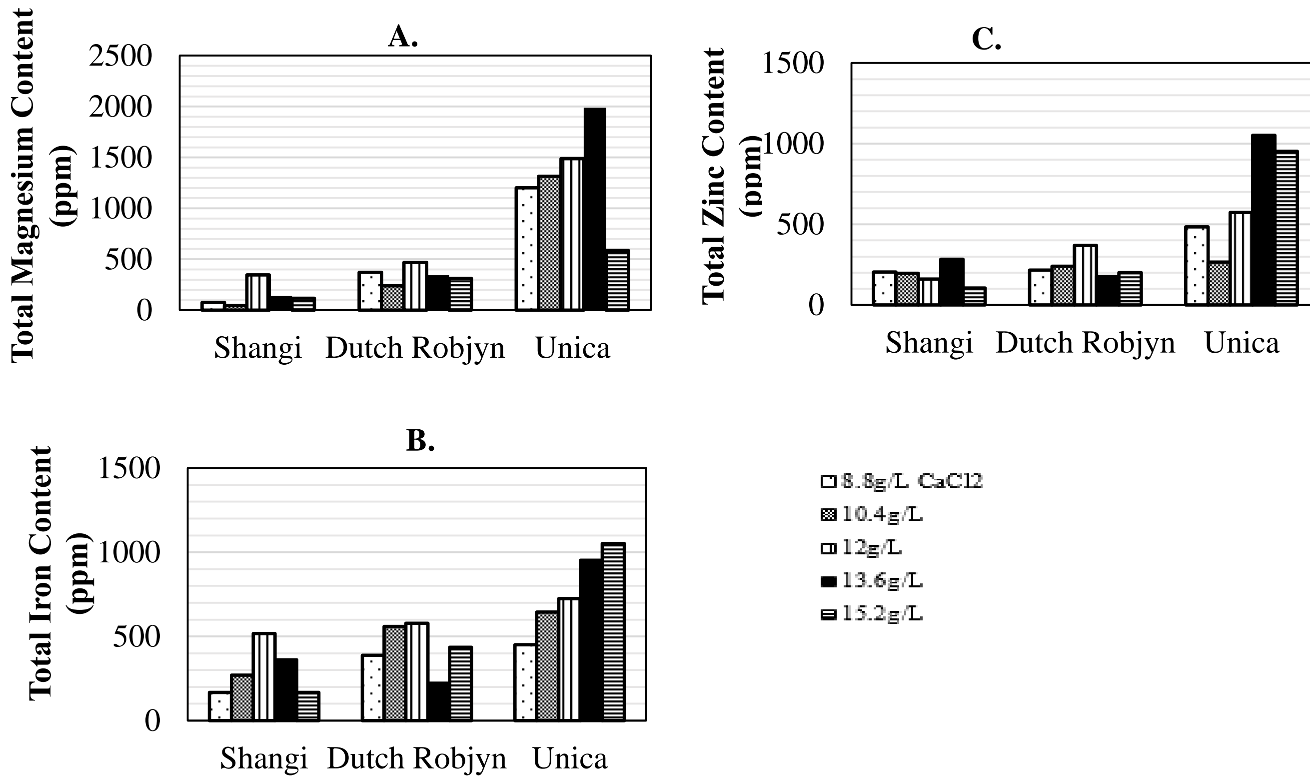
3.7 Effect of MS media Ca enhancement on tissue concentrations of Ca^{2+}, Mg^{2+}, Fe^{2+}, Zn^{2+} of *Shangi*, *Dutch Robjyn* and *Unica* potato plantlets

3.7.1 Total Tissue Ca^{2+}, Mg^{2+}, Zn^{2+} and Fe^{2+} in mid-stem + whole root sections of *Shangi*, *Dutch Robjyn* and *Unica* potato plantlets

Results showed that Ca content in the mid-stem and root zone sections of the three potato varieties increased with increasing Ca content in the regeneration media, with the highest Ca^{2+} tissue content observed in MS media containing 13.6g/L $CaCl_2$ (T3). Clear inter-variety differences were apparent when comparisons in Ca^{2+} content in the whole root and mid-stem section were done.

Percent increases in tissue Ca^{2+} were recorded in *Unica* by 202%, *Shangi* by 45%, and *Dutch Robjyn* by 165% compared to the control (T0) plantlets (Table 6). Increased total Ca^{2+} levels were observed in plantlets from the three varieties with the highest (9.59mg/g DW) and lowest (2.09mg/g DW) Ca levels observed in *Shang*i and *Unica* respectively. In *Shangi*, MS media containing 13.6g-15.2g/L Ca (T3-T4) increased tissue Ca^{2+} in mid-stem + whole root section by an average of 34% compared to the control. *Shangi* plantlets

grown on MS medium containing 13.6g/L Ca had the highest tissue Ca content (9.59mg/g DW) in their mid-stem + whole root



Variety

Figure 3.1: Total Magnesium (**A**.) Iron (**B**.) and Zinc content (**C**.) in mid-stem + whole root sections of *Shangi, Dutch Robjyn* and *Unica*. Parts per million (ppm).

MS media containing 10.4g-15.2g/L Ca (T_1-T_4) increased tissue Ca^{2+} by an average of 127% and 133% for *Dutch Robjyn* and *Unica* varieties respectively. *Dutch Robjyn* and *Unica* plantlets grown on MS medium containing 13.6g/L Ca also recorded the highest Ca^{2+} content in their mid-stem and whole root sections (7.79mg/g DW, 6.31mg/DW respectively) compared to plantlets grown on conventional MS medium (Figure 3.1). Treatment T4 (MS medium containing 15.2g/L Ca) caused a decrease in tissue Ca content for the three varieties. Plantlets of the three varieties grown on conventional MS medium had the lowest total Ca^{2+} content in their mid-stem + whole root sections.

Increasing calcium content in the culture medium resulted in increased tissue Mg^{2+} in the mid-stem + whole stem sections of the three varieties. Plantlets of *Shangi* and *Dutch Robjyn* grown on MS medium containing 12g/L Ca had the highest Mg content of all treatments (0.35mg/g DW, 0.47mg/DW respectively). *Shangi* and *Dutch Robjyn* plantlets cultured in T2 (MS medium 12g/L Ca) had 4x and 1.2x magnesium content when compared to their plantlets grown on the conventional MS medium (Figure 3.1). Magnesium uptake remarkably decreased in *Shangi*, *Dutch Robjyn* and *Unica* on MS medium containing 13.6-15.2g/L Ca (T3-T4). *Unica* plantlets grown in Treatment T3 had the highest Mg^{2+} content (1.99mg/g DW) of all *Unica* plantlets. MS medium containing 10.4g-13.6g/L Ca (T1-T3) resulted in an average increase of 45% in Mg^{2+} content in *Unica* mid-stem + whole root section, compared to the control (Figure 3.1). MS medium containing 15.2g/L Ca resulted in the lowest Mg^{2+} content in *Unica*.

Increasing calcium content in the culture medium resulted in variable Zn^{2+} content in the mid-stem + whole root sections of *Shangi*, *Dutch Robjyn*, and *Unica*. Treatment T1-T3 (MS media containing 10.4g/L-13.6g/L $CaCl_2$ resulted in a 40%, 68%, and 119% increase in total Zinc mineral content in the root-zone sections of *Shangi, Dutch Robjyn* and *Unica* compared to the control. *Shangi* plantlets grown on MS medium containing 13.6g/L Ca had the highest Zn^{2+} content (0.28mg/g DW) in the mid-stem + whole root section compared to the plantlets grown on conventional MS medium (0.20mg/g DW). Treatment T4 resulted in the lowest Zn^{2+} content (0.10mg/g DW) in the mid-stem + whole root

sections of *Shangi* plantlets. Treatment T2 resulted in the highest Zn^{2+} content (0.37mg/g DW) in the mid-stem + whole root sections of *Dutch Robjyn* plantlets. Treatment T1 also resulted in 9% increase in Zn^{2+} content in *Dutch Robjyn* mid-stem + whole root tissue sections of *Dutch Robjyn* plantlets. MS medium containing 13.6-15.2g/L Ca (T3-T4) resulted in the lowest Zn^{2+} (0.18-0.20mg/g DW) content in mid-stem + whole root tissues of *Dutch Robjyn* compared to plantlets grown in conventional MS medium (0.22mg/g DW). In *Shangi* and *Unica*, MS medium containing 13.6g/L Ca (T_3) resulted in a 40% and 118.75% increase in total Zn^{2+} respectively compared to conventional MS medium. *Dutch Robjyn* plantlets grown in treatment T2 (MS medium containing 10.4g/L Ca) had a 68% increase of tissue Zn^{2+} compared to plantlets grown on conventional MS medium. *Unica* had the highest zinc concentration (0.67 mg/g DW on average) in the root and mid-stem section while *Shangi* had the lowest zinc concentration (0.19 mg/g DW on average) (Figure 3.1)

Increasing calcium content in the culture medium resulted in increased Fe^{2+} content in the mid-stem + whole root sections of *Shangi*, *Dutch Robjyn*, and *Unica*. *Shangi* plantlets had the lowest (0.17-52mg/g DW) iron content compared to *Dutch Robjyn* (0.22-58mg/g DW) and *Unica* (0.45-1.05mg/g DW) plantlets. *Shangi* plantlets grown on MS medium T1, T2 and T3 had an average increase of 125% in Iron content. *Shangi* plantlets grown on MS medium containing 12g/L Ca had the highest iron content (0.52mg/g DW). Treatment T4 and T1 resulted in the lowest Fe^{2+} content (0.17mg/g DW) in mid-stem + whole sections of *Shangi* plantlets. *Dutch Robjyn* plantlets grown on MS medium containing 12g/L Ca had the highest (0.58mg/g DW) iron content of all treatments. The treatment resulted in a 48.7% increase in iron in *Dutch Robjyn*, compared to the control. Treatments T1, T2 and T4 resulted in an average 34% increase in total iron content in mid-stem + whole root sections of *Dutch Robjyn*. MS medium containing 15.2g/L Ca resulted in the highest iron content (1.05mg/g DW) in *Unica* plantlets. There was a progressive increment in iron content in *Unica* plantlets with increasing calcium in MS medium. Treatment T1-T4 led to a progressive increase in total Iron for *Unica*, with an average increase of 87%.

Our study results are consistent with previous studies that found increased media Ca contributed to increased TC plantlet Ca content and promoted TC plantlet quality (Sarkar *et al.*, 2005). Habib *et al*. (2004) established a clear inter-cultivar differences in calcium uptake and content betw Cv. Alpha, Bintje, Russet Burbank, Kennebec, Green Mountain, and Shepody grown in low and high medium Ca^{2+}. However, most studies tend to focus on shoot Ca content, whole plantlet Ca content or tuber Ca content, not root Ca calcium for bio-fortification purposes, aimed at improving callus and *In vitro* plantlet growth, micro-tuberization and addressing phenotypic abnormalities (Sarkar *et al.*, 2005; Kim *et al.*, 2006; Amalia *et al.*, 2014; Nezami *et a*l., 2015; Al-Mayahi, 2019). Our study focuses on increasing Ca content within the whole root and mid-stem section since these regions are primarily attacked by the *R. solanacearum* pathogen. The study also proves that *In vitro* nutritional status carries over during greenhouse and/or field establishment. Munthali *et al.* (2022) reported that Ca supplies of above 10Mm (up to 30Mm Ca) had no effect on *In vitro* potato plant growth even when compared to 10mM Ca (resulted in optimum shoot biomass) suggesting excessive Ca supply. The study authors also attributed a steady increase in shoot Mg^{2+} content associated with increasing Ca^{2+} in tissue culture medium which is similar to our study findings where supplemental Ca^{2+} (10.4-13.6g/L Ca) in the culture medium lead to increased tissue Mg content in the whole root sections of the three varieties. The *Shangi* variety could be a potential "Calcium packer" due to its ability to take up larger amounts of Ca even under normal/control MS media Calcium levels. This could be attributed to an enhanced molecular ability in *Shangi* that enables it to engineer a higher expression of Ca^{2+} transporters and Ca^{2+} binding proteins (Kim *et al.*, 2006). However, further studies need to be conducted to validate this finding.

Kozai *et al.* (1995) reported increasing Ca alone negatively influences magnesium uptake limiting growth in *Solanum tuberosum* L. Our study found that increasing Calcium in the culture medium (10.4-12g/L $CaCl_2$) promoted magnesium uptake in *Shangi*, *Dutch Robjyn*, and *Unica.* Magnesium contributes to increased leaf development by increasing chlorophyll content and consequently increasing photosynthetic efficiency (Rodrigues *et al.*, 2021), however, there was a reduction in leaf number in Unica in treatments with

10.4g-15.2g/L $CaCl_2$. Lekamge *et al.* (2021) reported that increase in $CaCl_2$ (880-1100mg/L) (combined with $MgSO_4$, and KH_2PO_4) improved Iron uptake in *'Nishiyutaka'* and *'Okhotsk Chip'* which is similar to our results since increased medium Ca resulted in increased root iron uptake and content in whole roots sections of *Shangi, Dutch Robjyn* and *Unica*. Iron concentrations in standard MS with high $CaCl_2$, $MgSO_4$, and KH_2PO_4 contributed to shoots without physiological disorders since iron is an essential micronutrient with significant effects on plant morphology and metabolism. Iron is involved in the synthesis of chlorophyll, and is essential for the maintenance, structure and function of the chloroplast. *Shangi* had the lowest iron content of the three varieties, which is consistent with Gikundi *et al.* (2021) who reported *Shangi* tubers significantly having the lowest iron content compared to *Unica* and *Dutch Robjyn* tubers. Conventional MS medium resulted in *Shangi* plantlets with the lowest iron content in the mid-stem + whole root section. Increasing calcium in the culture medium (10.4-13.6g/L $CaCl_2$) improved iron uptake for *Shangi*.

Our study revealed that *Unica* plantlets had the highest zinc content in the mid-stem + whole root section. These results are consistent with Gikundi *et al.* (2021) who reported that *Unica* tubers had the highest zinc content. The study authors also reported that *Dutch Robjyn* had the lowest zinc content. Our study established that *Shangi* instead had the lowest zinc content compared to *Dutch Robjyn* and *Unica* plantlets. Zinc, a divalent cation (Zn^{2+}), plays a functional and structural role in enzymatic reactions (Hawkesford *et al.*, 2012). This could be attributed to environmental and cultural differences under which the sampled Irish potato varieties were grown.

3.8 Conclusion

The results of our study demonstrate the cumulative effect of Calcium increment in the culture medium in achieving an optimum state of shoot and root growth, micro-tuberization while improving macronutrient and micronutrient concentrations in plantlets of *Shangi*, *Dutch Robjyn* and *Unica.* MS medium containing 10.4-13.6g/L resulted in plantlets of the three varieties having the best shoot, leaf and node and root regeneration

compared to conventional MS medium. However, there significant differences in for shoot, leaf and node numbers after 28 days of culture on MS medium containing 8.8-15.2g/L $CaCl_2$. Calcium supplementation using T4 and T1 significantly improved *Unica* and *Dutch Robjyn* root regeneration respectively. Increasing calcium content in the culture medium (10.4-15.2g/L $CaCl_2$) affected the days it took *Shangi*, *Dutch Robjyn* and *Unica* varieties to induce micro-tubers. MS medium containing 10.4g/L Ca significantly reduced the days it took *Shangi* and *Unica* to form micro-tubers. However, reducing the days taken to induce micro-tubers resulted in lower micro-tuber fresh weights for both varieties. Our results also demonstrated the effect of increased concentrations of Calcium in culture medium influenced the mineral uptake rates of rates of iron, magnesium and zinc. With increasing calcium concentrations (10.4-13.6g/L $CaCl^2$), compared to conventional MS medium concentration, increased uptake of iron, zinc and magnesium also occurred for all varieties tested, showing clear synergistic effects between Ca and Mg, Fe and Zn on shoot and root regeneration, microtuberization and agronomic calcium bio-fortification. MS medium containing 13.6g/L Ca resulted in plantlets of three varieties having the highest Ca content in the mid-stem + whole root sections, achieving *In vitro* agronomic calcium bio-fortification of the three varieties.

CHAPTER FOUR

EVALUATING THE EFFECT OF I*N VITRO* CALCIUM BIO-FORTIFICATION AND AMF BIOTIZATION ON PLANTLET PERFORMANCE AND MANAGEMENT OF BACTERIAL WILT IN *SHANGI*, *DUTCH ROBJYN* AND *UNICA*

4.1 Abstract

Potato is the second most important staple crop after maize and plays a major role in national food and nutritional security. However, the crop is susceptible to many diseases and insect pests. In most potato growing areas in Kenya, bacterial wilt is considered an important disease contributing to poor yields, high post-harvest losses, and poor quality of farm-saved seed. Despite the associated benefits of enhancing cellular calcium nutrient levels and AMF Biotization in managing bacterial wilt, there have been little to no reports on the combined use of these strategies on local potato varieties at improving plant performance and combating bacterial wilt disease development *in vivo*. A greenhouse experiment was set up to investigate the effect of calcium (Ca) fortification and AMF mycorrhizal inoculation on plantlet performance and mycorrhizal colonization of *Shangi*, *Unica* and *Dutch Robjyn*. Non-Ca fortified and Ca-fortified plantlets (plantlets with highest mineral Ca content in root-zone section) from the first experiment plantlets were inoculated with a commercially available mycorrhizal inoculant in a completely randomized design with six replications. The plantlets were then infected with *R. solanacearum* after one month of establishment. Performance determination was based on plantlet height and leaf number, total chlorophyll content, percent mycorrhizal colonization and bacterial wilt disease severity. Analyses of variance proved a significant and highly significant effect *(P≤0.01)* on tested factors and their interactions on plantlet height, plantlet leaf number, mycorrhizal colonization and disease severity in *Shangi, Dutch Robjyn* and *Unica*. There were significant differences (*P*< 0.05) in plantlet height of *Shangi* and *Unica*. Agronomic *In vitro* calcium bio-fortification combined with mycorrhizal inoculation resulted in 9% increase and 40% decrease in *Shangi* and *Unica*

plantlet height respectively. Calcium bio-fortification resulted in the tallest *Dutch Robjyn* plantlets (244.02mm). Biotization increased the number of leaves in all varieties. Mycorrhizal inoculation resulted in a 12.7% increase in total chlorophyll content in *Shangi*, 16.12% increase in *Dutch Robjyn* and a 22% increase in *Unica* compared to non-mycorrhizal plantlets. Agronomic calcium bio-fortification strongly and significantly *P<.0001*) influenced the number of propagules in 1cm root fragments of *Shangi*, *Dutch Robjyn* and *Unica*. Agronomic *In vitro* calcium bio-fortification combined with mycorrhizal inoculation resulted in the highest colonization frequency in *Dutch Robjyn* plantlets. *Unica* plantlets recorded the lowest colonization percentage and frequency compared to *Shangi* and *Dutch Robjyn*. All plantlets in all treatments showed disease symptoms highlighting the pathogen's aggressiveness. Bacterial wilt infection was more pronounced in *Shangi* (71.71%) and *Unica* (74.92%) compared to *Dutch Robjyn* (69.87%). Non-fortification combined with non-biotization resulted in *Shangi* and *Dutch Robjyn* plantlets with the highest bacterial wilt severity score. Calcium bio-fortification combined with biotization resulted in *Unica* plantlets with the highest disease severity score of all the treatment combinations.

4.2 Introduction

Arbuscular mycorrhizal fungi (AMF) is a symbiotic associations between roots of ~80% of terrestrial plants, playing a major role in processes associated with nutrient acquisition, soil aggregation and ecosystem function (Xu *et al.*, 2018; Muiruri *et al.*, 2023). AMF improves plant stress resistance and is involved in the enhancement of plant defense mechanisms of host plants, fulfilling the role of plant bio-protectors when used for plant pathogen control (Deja-Sikora *et al.*, 2023). As bio-trophs, AMF depend on carbohydrates and lipids provided by plants for the completion of their life cycle. AMF hyphae are generally non-septate and may grow either extra or intraradically. Intra-radical mycelium produces characteristically branched structures inside the cortical cells called arbuscules. The spores of AMF are asexual, multinucleated and produced directly by the mycelium, both inside and outside the root. Potatoes are reported to establish symbiosis with different

AMF species that are an inherent part of the agricultural soil biome e.g. *Funneliformis mosseae*, *Glomus mosseae, Rhizophagus intraradices (*formerly *Glomus intraradices), Glomus etunicatum* etc (Silvana *et al*., 2020). AMF colonize root tissues through mature spores or substrate containing mycorrhizal fragments.

Calcium, as an essential plant nutrient, plays a key role as a structural component of cell walls by cross-linking negatively charged carboxyl groups of de-esterified pectin in the middle lamella. Calcium also stabilizes the plasma membrane by bridging phosphate and carboxylate groups of phospholipids and proteins. However, in its most important role as a secondary messenger in signaling networks, distinct $[Ca^{2+}]_{cyt}$ perturbations encode cellular responses to specific biotic and abiotic stimuli (White & Broadley, 2003). Atim *et al.* (2013) reported that increased nutrient concentrations of Ca (with Nitrogen and Potassium) during tissue culture and greenhouse development showed less disease development. However, despite the associated benefits of enhancing cellular calcium nutrient levels and AMF biotization, there have been little to no reports on the combined use of these strategies on *Shangi, Dutch Robjyn* and *Unica* at improving *In vivo* plantlet performance and combating bacterial wilt disease severity.

4.3 Materials and Methods

4.3.1 Study Site

The study was conducted under greenhouse conditions at Jomo Kenyatta University of Agriculture and Technology (JKUAT) in Juja sub-county, in 2022 and 2023.

4.3.2 Study Materials

One-month old fortified (plantlets grown on T3 culture medium) and non-fortified (T0 grown plantlets) of *Shangi, Unica* and *Dutch Robjyn* obtained from the regeneration experiments were evaluated for plant performance and Bacterial Wilt disease development. Plantlets of the three varieties grown on T_3 culture medium contained the highest tissue Ca^{2+} content in their mid-stem + whole root sections. Though no significant

differences existed within shoot, leaf, node numbers of the three varieties, MS medium containing 13.6g/L $CaCl_2$ resulted in the higher root numbers for *Shangi* compared to other conventional MS medium, higher shoot numbers in *Dutch Robjyn* compared to the plantlets grown in conventional MS medium and healthy shoot development in *Unica* plantlets compared to the plantlets grown in conventional MS medium formulation. Plantlets of *Shangi*, *Dutch Robjyn*, and *Unica* grown on MS medium containing 13.6g/L Ca also had higher magnesium, iron and zinc tissue concentrations compared to plantlets grown on conventional MS medium.

4.3.3 Biotization and Acclimatization of Calcium fortified and non-fortified plantlets

Pure river sand was washed and sieved to remove debris. The sand was then sterilized by autoclaving thrice at 120°C for 15minutes at 15 PSI to kill all organisms. Sterilized sand was then distributed into 7x7x6.5cm sterile plastic propagation plugs and covered with autoclaved foil to prevent contamination at experimental onset. All procedures were conducted within a sterile cabinet equipped with laminar airflow (Plate 4.1). Fortified and non-fortified plantlets were removed from their culture jars and the adhering gel washed off under running water for about 2 minutes taking special care not to damage the fine roots formed. The plantlets were then transferred into the plastic plugs with fortified and non-fortified plants inoculated with 2g commercial mycorrhizal product Rhizatech™ per plug and left for four weeks in a greenhouse for mycorrhizal colonization. RHIZATECH ® TM contains spores, colonized root fragments, and other propagules of Arbuscular Mycorrhizal Fungi (AMF) in a granular carrier (50 propagules/cm^3). The plugs soil surface was covered using sterilized aluminium foil to prevent cross-contamination.

4.3.4 Experimental Design

Treatments included: Fortified (T3) *Shangi* + Rhizatech™ (AMF), Control (T0) *Shangi* + Rhizatech™, Fortified (T3) *Unica* + Rhizatech™, Control (T0) *Unica*+ Rhizatech™, Fortified (T3) *Dutch Robjyn* + Rhizatech™, Control (T0) *Dutch Robjyn* + Rhizatech™.

Each treatment had 6 replicate plants arranged in a completely randomized design. The plantlets were maintained under greenhouse conditions. Each plantlet was regularly watered with 30ml of modified Hoagland's nutrient solution, and the sand culture routinely weeded and tilled to minimize the growth of mold.

Plate 4.1: Packing sterilized sand into sterilized containers under sterilized cabinet equipped with laminar airflow to prevent cross-contamination

4.3.5 Chlorophyll content analysis

Chlorophyll is an important photosynthetic pigment to a plant, largely determining photosynthetic capacity and hence plant growth. Extraction of photosynthetic pigment was done one month after plantlet establishment following procedures by Mohd Amin *et al*. (2023), 1g of dark green leaves from the experimental set-up were placed in a mortar and macerated using 50ml of 100% acetone until the residue became colourless. The mixture was centrifuged at 3500*x g* for 10min. The absorbance values of the extracted chlorophyll content in the supernatant were collected at 663nm and 645nm for chlorophyll a and b respectively using electronic absorption spectra recorded on a Shimadzu (Shimadzu Japan) UV/VIS 1800 spectrophotometer. Acetone (100 percent) was used as the reference in the UV-Vis spectrophotometer (Shimadzu, Japan). Chlorophylls a and b and total chlorophyll (mg/g fresh weight) were then calculated according to the method by Pérez-Patricio *et al.* (2018).

Chlorophyll a = (12.7*A663) – (2.59*A645)

(mg/g) (1)

Chlorophyll b = (22.9*A645) – (4.7*A663) (mg/g/) (2)

Total chlorophylls = (8.2* A663) + (20.2 A645)

(mg/g) (3)

Where A663 and A645 were the absorbance measured.

4.3.6 Bacterial Wilt isolation, culture and inoculum preparation

Infected tomato (*Solanum lycopersicum*) plants from JKUAT HOSA farms were evaluated for *R. solanacearum* infection by bacterial streaming test where stems of infected, symptomatic tomato plants were suspended in 90ml of distilled water (Plate 4.2). The suspension was then filtered and 20 µL of the bacterial suspension was spread on plates containing Casamino Acid-Peptone-Glucose (CPG) media amended with 0.05% Tetrazolium chloride to obtain a virulent *R. solanacearum* inoculum (Plate 4.2). Cultures were incubated at 28°C±2°C overnight for 48hrs. Virulent colonies of *R. solanacearum* were identified by their colour, size, elevation and fluidal nature. Inoculum was harvested from the CPG plates by flooding Petri dishes with distilled water. The suspension was adjusted to an optical density of 0.06 at 660nm on a Shimadzu (Shimadzu Japan) UV/VIS 1800 spectrophotometer corresponding to 10^8 colony forming units per milliliter (CFU/ml) and diluted 1 time using double-distilled water.

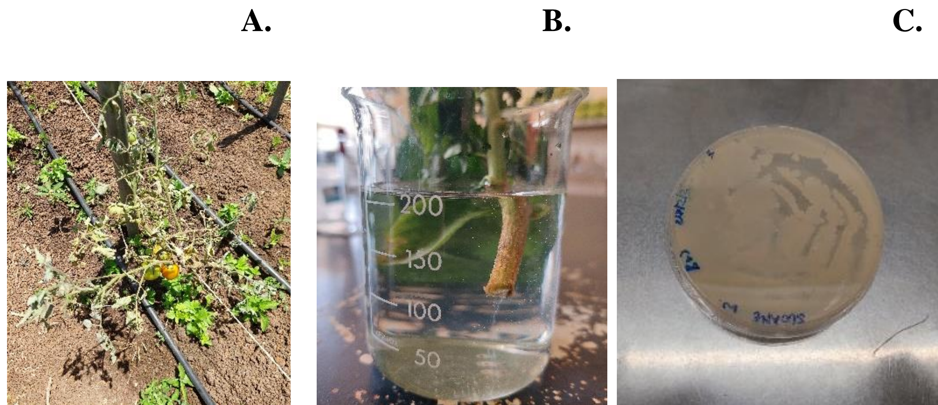


Plate 4.2: A: Source of *R. solanacearum* inoculum from infected tomato plant showing symptoms of bacterial wilt B: Bacterial streaming from cut tomato stem; a sign of *R. solanacearum* infection C: Virulent *R. solanacearum* colonies on CPG medium with distinct fluidal, cream-coloured, opaque colonies after 48 hours of culture

4.3.7 Greenhouse Assay and Assessment of growth parameters

Roots of one-month old biotized and non-biotized plantlets raised under greenhouse conditions were wounded using a sterile razor and inoculated by soil drenching 20ml of the 10^8 CFU/ml bacterial suspension per pot at the plant base. Disease development was evaluated daily to observe for symptoms of Bacterial Wilt using a disease score based on 4 plants per treatment. The evaluation begun when the first symptoms were observed on the leaves and continued until symptoms became stable. The following scoring criteria was used: 0 = no leaves wilted, 1 = one leaf wilted, 2 = two leaves wilted, 3 =three leaves wilted, 4 = four leaves wilted excluding the tip and 5 = wilting of the whole plant (death) (Kiirika *et al.*, 2013).

Disease severity index was calculated as follows (Jiang *et al.*, 2016):

$$\textbf{Severity index} = \sum \left[\frac{\text{(Number of diseased plants in the index * Disease index)}}{\text{(Total number of plants investigated * Highest disease index)}} \right] * 100$$

Plant height and leaf number were evaluated 4 weeks after removal from culture jars. Leaf number was evaluated by physically counting the leaves from the base of the stem to the shoot.

4.3.8 Root colonization assessment after one month of establishment

Rhizospheric roots from the three varieties were assessed for mycorrhizal colonization intensity and frequency according to procedures described by Muiruri *et al*. (2023). Five centimeter (5cm) root fragments were harvested from plants through destructive sampling and washed free of adhering substrate under running tap water. The samples were then treated using 2.5% KOH solution (25g KOH in 1000ml water) for 60 min. in an oven at 70°C, in order to empty the cell of their cytoplasmic contents which facilitates their coloring. The 5cm root samples were then placed in alkaline hydrogen peroxide (60ml of 28- 30% NH_4OH, 90 ml of 30% H_2O_2 and 840 ml distilled water) at 70°C for 20 minutes to remove phenolic contents. The roots were washed 3 times with sterilized distilled water and acidified with 1% hydrochloric acid (HCL) for 30min. Without rinsing the samples with water, the roots were placed in acidic glycerol (500 ml glycerol, 450 ml water, 50 ml of 1% HCl) solution containing 0.05% trypan blue in lacto-phenol solution, and taken back to the oven for 1hr at 70°C according to the method described by. The segments were then decanted, and a de-staining solution of acid glycerol was added. The 5cm root samples were then chopped into 1cm root segments and 25 samples of 1cm root randomly picked and mounted on microscope slides and examined under bright field- light under x40 magnification to obtain colonization percentage of AMF by assessing the intensity and frequency of colonization by methods developed by Kormanik *et al.* (1982).

$$\text{Colonization percentage \%} = \frac{\text{No. of colonized root segments}}{\text{Total no. of segments examined}} \times 100$$

The frequency and intensity of colonization by the AMF were calculated and recorded according to Mumo (2012):

$$\text{Frequency (\%)} = \frac{\text{No. of root fragments infected with AMF}}{\text{(Total no. of root fragments observed)}}$$

4.4 Data Analysis

Data obtained from biotization experiment were arcsine-transformed and means reported back after back transforming. Means were subjected to three-way analysis of variance (ANOVA) using R (R Core Team, 2018) and treatment means separated using Tukey's HSD (Honestly Significant Difference) test at $P \leq 0.05$.

4.5 Results and Discussion

4.5.1 Effect of *In vitro* Agronomic Ca bio-fortification and AMF Biotization on plant growth parameters of *Shangi, Dutch Robjyn* and *Unica* potato plantlets

Agronomic calcium bio-fortification and biotization showed variable effects on plant height and number of leaves of the varieties tested (Plate 4.3). Analyses of variance proved a significant ($p < 0.05$) and highly significant effect ($p < 0.01$) on tested factors (Variety, AMF Biotization) and their interactions (Variety x Calcium, Variety x AMF Biotization) on the plantlet height of all the three varieties (Table 4.1). *Dutch Robjyn* had the tallest plantlets (202.98mm), *Shangi* plantlets had an average height of 128.96mm and *Unica* plantlets had an average plantlet height of 194.13mm. Calcium bio-fortified plantlets (T3) of the three varieties were taller (180.94mm) than non-bio-fortified plantlets (169.77mm). Non-biotized plantlets of the three varieties were on average taller (191.11mm) than biotized plantlets (159.60mm). Analyses of variance proved a significant and highly significant effect ($p < 0.01$) on tested factors (Variety, Calcium, AMF Biotization) and their interactions (Variety x Calcium, Variety x AMF Biotization, Calcium x AMF Biotization) on the plantlet leaf number of the three varieties (Table 4.1).

Plantlets of the three varieties under T0 treatment, regardless of biotization status, had a higher number of leaves compared to calcium bio-fortified plantlets. Biotization using 2g/pot Rhizatech™ also resulted in *Shangi*, *Dutch Robjyn* and *Unica* plantlets with a higher leaf number than non-biotized plantlets.

There were significant differences ($p<0.05$) in *Shangi* plantlet height and *Shangi* leaf number between treatments (Table 4.1). Treatment T3/AMF (biotized and bio-fortified) resulted in the tallest *Shangi* plantlets (148.65±9.13) compared to the non-fortified and non-biotized plantlets (135.36±4.25). Biotization using 2g/pot Rhizatech™ (Treatment T0/AMF) resulted in 21% decrease in *Shangi* plantlet height (106.33±2.84) compared to plantlets in the control treatment (135.00±4.25).

Table 4.1: Effect of *In vitro* Calcium bio-fortification and Biotization on plantlet height and leaf number of *Shangi, Dutch Robjyn* and *Unica* varieties

Variety	Treatment		Plant height (mm)		Leaf number	
Shangi	T0	AMF0	135.36	±4.25^{ab}	12	±0.45^{b}
		AMF	106.33	±2.84^{c}	18	±1.10^{a}
	T3	AMF0	125.51	±2.51^{bc}	17	±1.67^{a}
		AMF	148.65	±9.13^{a}	17	±1.24^{a}
Dutch Robjyn	T0	AMF0	188.45	±2.66^{b}	10	±0.52^{b}
		AMF	170.17	±17.85^{b}	15	±1.41^{b}
	T3	AMF0	244.02	±18.05^{a}	16	±1.86^{b}
		AMF	209.27	±10.54^{ab}	21	±0.75^{a}
Unica	T0	AMF0	236.51	±12.90^{a}	26	±2.00^{a}
		AMF	181.78	±5.58^{b}	27	±0.58^{a}
	T3	AMF0	216.84	±9.48^{ab}	13	±1.06^{b}
		AMF	141.38	±6.99^{c}	9	±0.88^{c}
	ANOVA *(p-value)*	Variety (V)	<0.001		0.0002	
		Calcium (Ca)	0.0590		0.0006	
		AMF	<0.001		0.0245	
		V*Ca	<0.001		<.0001	
		Ca*AMF	0.6692		0.0008	
		V*AMF	0.0002		0.0425	
		V*Ca*AMF	0.0208		0.0054	

T0= 8.8g/L Ca; T3= 13.6g/L Ca. AMF= 2g/per plant Rhizatech™; AMF0=No AMF added. Values indicate means±SE**.** Mean values within the variety grouping followed by the same letters are not significantly different for each variety (Tukey's HSD test, $p \leq 0.05$).

Treatment T3/AMF0 (calcium bio-fortification) resulted in a 7% decrease in *Shangi* plantlet height (125.51±2.51) compared to the control plantlet height. Calcium bio-fortification and biotization using 2g/pot Rhizatech™ promoted leaf numbers in *Shangi* (Table 4.1). Treatment T0/AMF spurred 1.5 times the number of leaves (18 leaves) compared to plantlets in the control treatment (12 leaves), while also promoting the highest leaf number for *Shangi* compared to the other treatments (Table 4.1). This represents a 50% increase in the number of leaves compared to plantlets in the control treatment. T3/AMF and T3/AMF0 facilitated 1.4 times the number of leaves (17 leaves), which is 41% increase in leaf number, compared to the control (T0/AMF0).

A. **B.**

Plate 4.3: A. Non-biotized plantlets B: Biotized plantlets of calcium bio-fortified and non-calcium bio-fortified *Shangi*, *Dutch Robjyn* and *Unica* after one month of establishment.

There were significant differences ($p<0.05$) in *Dutch Robjyn* plantlet height and plantlet leaf numbers between treatments. Agronomic *In vitro* calcium bio-fortification (Treatment T3/AMF0) resulted in the tallest *Dutch Robjyn* plantlets (244.02±18.05). This represented 29% increase in *Dutch Robjyn* plantlet height compared to plantlets under the control treatment (188.45±2.66). Biotization using 2g/pot Rhizatech™ (T0/AMF) caused 9.7% decrease in *Dutch Robjyn* plantlet height (170.17±17.85) compared to the control plantlets. Agronomic *In vitro* calcium bio-fortification combined with biotization using

2g/pot Rhizatech™ (T3/AMF) caused a 11.1% average increase in plantlet height (209.27±10.54) compared to the T0/AMF0 plantlets (188.45±2.66). Treatment T3/AMF resulted in the highest *Dutch Robjyn* plantlet leaf number (21±0.75) compared to other treatments. Agronomic *In vitro* calcium bio-fortification combined with biotization using 2g/pot Rhizatech™ (Treatment T3/AMF) resulted in 110% increase in *Dutch Robjyn* plantlet leaf number (21±0.75) compared to the control treatment (10±0.52). Treatment T3/AMF0 resulted in 60% increase in *Dutch Robjyn* plantlet leaf number (16±1.86). Biotization using 2g/pot Rhizatech™ (T0/AMF0) resulted in 50% increase in *Dutch Robjyn* plantlet leaf number (15±1.41).

There were significant differences ($p<0.05$) in *Unica* plantlet height and plantlet leaf number between treatments. *Unica* plantlets in treatment T0/AMF0 were the tallest plantlets (236.51±12.90) compared to other treatments. Agronomic *In vitro* calcium bio-fortification combined with biotization using 2g/pot Rhizatech™ (Treatment T3/AMF) resulted in the shortest *Unica* plantlets (141.38±6.99) of all treatments, which represents 40.2% decrease in plantlet height compared to the control plantlets (236.51±12.90). Treatment T0/AMF resulted in 23% decrease in *Unica* plantlet height (181.78±5.58) compared to *Unica* plantlets under the control treatment. Agronomic *In vitro* calcium bio-fortification (T3/AMF0) resulted in 8% decrease in *Unica* plantlet height (216.84±9.48) compared to the control. Biotization using 2g/pot Rhizatech™ (T0/AMF) resulted in the highest leaf number (27±0.58) for *Unica* plantlets, representing a 3% increase in *Unica* leaf number. Agronomic *In vitro* calcium bio-fortification combined with biotization using 2g/pot Rhizatech™ (T3/AMF) resulted in 65% decrease in *Unica* leaf number (9±0.88). Agronomic *In vitro* calcium bio-fortification (T3/AMF0) resulted in 50% decrease in *Unica* leaf number (13±1.06) compared to the control treatment (26±2.00).

Divya *et al.* (2022) reported that leaf numbers and fresh weights of micro-propagated black pepper roots inoculated with *Piriformospora indica* were significantly higher than in non-inoculated *P. indica* treated plants. Susiana *et al.* (2019) showed that mycorrhizal application using 25mg/L *Trichoderma* spp. significantly increased potato plant height

and the number of leaves. Our study results agree with Susiana *et al.* (2019) and Divya *et al.* (2022) since biotization influenced plantlet heights and leaf numbers of *Shangi, Dutch Robjyn* and *Unica*. Seifu & Deneke (2017) established that pre-harvest application of combined 5-15g $CaNO_3$ with $CaCl_2$ significantly ($P<0.05$) affected plant height of *Gera* and *Shenkola* potato varieties. This is similar to our results since agronomic calcium bio-fortification influenced *Dutch Robjyn* plantlet height. Due to significant differences in *Shangi* plantlet height and leaf number, plantlets that were fortified with calcium combined with AMF biotization did have notably higher leaf numbers that non-fortified and non-biotized *Shangi* plantlets. Kibiro (2014) reported that potato plants inoculated with *Glomus mosseae* and *Glomus intraradices* had the highest number of leaves. Rhizatech™ is partly composed of *Glomus mosseae* and *Glomus intraradices* spores and mycelial fragments. Ci *et al*. (2021) found that AMF combined with Ca^{2+} was better than AMF or Ca^{2+} alone at improving peanut growth in saline alkali soil. This suggests a synergistic effect between Calcium and AMF on influencing leaf development in *Shangi*. Tahat (2012) reported that plantlets infected with *Glomus mosseae* (an constituent ingredient of Rhizatech™) were taller than those infected with other mycorrhizal fungi which is consistent with our results for *Dutch Robjyn*. Contrary to expectation, biotization using 2g/pot Rhizatech™ (T0/AMF) resulted in shorter *Shangi* and *Unica* plantlets with more leaves than their non-biotized plantlets. Wang *et al.* (2023) reported that *Artemisia ordosica* biomass was reduced by AMF inoculation with 15g/pot *Funneliformis mosseae*, but only under low water and nutrient conditions. The study authors attributed this to the AMF suppressing the direct pathway of water and nutrient absorption by the plant roots themselves. Calcium bio-fortification resulted in shorter *Shangi* and *Unica* plantlets. Roosta *et al.* (2023) established that the lowest internode length in *Mentha pulegium* L. was observed in field cultivation systems with calcium carbonate foliar spraying.

4.5.2 Chlorophyll a and b content and Total chlorophyll content

Biotized treatments (T0/AMF and T3/AMF) resulted in increased total chlorophyll content for the three varieties (Table 4.2).

Table 4.2: Effect of Calcium Bio-fortification and Biotization on Chlorophyll a, Chlorophyll b and Total chlorophyll content of *Shangi, Dutch Robjyn* and *Unica*

Variety	Calcium	AMF	Chlorophyll a (mg/g)	Chlorophyll b (mg/g)	Total chlorophyll (mg/g)
Shangi	T0	AMF0	24.743	21.473	58.132
		AMF	24.743	21.473	65.510
	T3	AMF0	31.753	31.693	63.809
		AMF	21.261	18.484	55.776
Dutch Robjyn	T0	AMF0	30.298	21.743	52.415
		AMF	31.045	29.454	60.858
	T3	AMF0	29.640	20.034	50.043
		AMF	31.477	29.075	60.919
Unica	T0	AMF0	30.477	24.464	55.308
		AMF	32.395	34.725	67.483
	T3	AMF0	29.787	22.284	52.435
		AMF	30.055	18.191	48.627

T0=8.8g/L Ca; T3=13.6g/L; AMF=2g/pot Rhizatech™; AMF0= No AMF added. Fragments of leaves (1g) randomly selected and composited for chlorophyll content analysis. mg/g FW denotes milligrams per gram, fresh weight.

Treatment T0/AMF resulted in a 12.7% increase in total chlorophyll content in *Shangi*, 16.12% increase in *Dutch Robjyn* and a 22% increase in *Unica* compared to their controls.

Treatment T3/AMF0 resulted in the highest chlorophyll a (31.75mg/g) and chlorophyll b (31.69mg/g) in *Shangi* plantlets. Treatment T0/AMF resulted in the highest total chlorophyll content in *Shangi* plantlets (65.50mg/g). Treatment T3/AMF resulted in a 14% reduction in chlorophyll a (21.26mg/g) and a 13% decrease in chlorophyll b (18.48mg/g) in *Shangi* plantlets compared to the control (T_0/AMF0) (24.74mg/g, 21.47mg/g respectively). Treatment T3/AMF0 resulted in a 28% increase and a 47% increase in chlorophyll a and chlorophyll b respectively for *Shangi*. T_3/AMF0 also resulted in an increase of 9.7% in total chlorophyll content for *Shangi* compared to the control (Table 4.2).

In *Dutch Robjyn* leaves, T3/AMF resulted in the highest total chlorophyll content (60.91mg/g) and chlorophyll a (31.47mg/g). Treatment T0/AMF resulted in the highest chlorophyll b content (29.45mg/g) in *Dutch Robjyn* plantlets. T0/AMF (60.85mg/g) and T3/AMF (60.92mg/g) led to an increase in total chlorophyll content by 16% compared to the control T_0/AMF0 (52.41mg/g) in *Dutch Robjyn* plantlets. Consequently, T_0/AMF and T_3/AMF resulted in an increase in chlorophyll a and chlorophyll b content by 2% and 35% respectively in *Dutch Robjyn*. However, T_3/AMF0 led to a 4% reduction in total chlorophyll content in *Dutch Robjyn*, with chlorophyll a (29.64mg/g) and chlorophyll b (20.034mg/g) also reduced by 2% and 7% compared to the control (30.30mg/g; 21.743mg/g. Treatment T0/AMF0 and T3/AMF caused a 16.22% increase in total chlorophyll content for *Dutch Robjyn*, but led a to reduction in total chlorophyll content for *Shangi* and *Unica* by 4% and 12% in that same order.

Treatment T0/AMF resulted in the highest total chlorophyll content (67.48mg/g), chlorophyll a (32.39mg/g), and chlorophyll b (34.72mg/g) content in *Unica* plantlets. T_0/AMF (67.48mg/g) increased total chlorophyll content 1.2 times compared to T_0/AMF0 (55.31mg/g) in *Unica* plantlets. This was also reflected in a percentage increase in chlorophyll a and chlorophyll b content by 6% and 41% respectively in *Unica*. T3/AMF resulted in a 1.3% and 25% decrease in chlorophyll a and chlorophyll b for *Unica* compared to T0/AMF0. T3/AMF0 also resulted in a 5% reduction in total chlorophyll

content compared to T0/AMF0, with chlorophyll a and chlorophyll b also decreasing by 2% and 8% respectively (Table 4.2).

Agronomic calcium bio-fortification combined with 2g/pot Rhizatech™ promoted chlorophyll content production in *Dutch Robjyn* plantlets. Cui *et al.* (2019) established that AMF and AMF + Calcium (20 mmol/L $Ca(NO_3)_2$) increased chlorophyll content in peanut (*Arachis hypogaea* L.) seedlings due to the shared GA and flavonoid-mediated pathway. Hashem *et al.* (2019) also established that exogenous calcium application (50mM $CaCl_2$;10mL/plant) and AMF inoculation (100 spores/g trap soil) with *Glomus mosseae, Glomus intraradices* and *Glomus etunicatum* enhanced chlorophyll content under cadmium stress in *Bassia indica* Wight. Barogh *et al.* (2023) reported AMF inoculation with *Funneliformis mosseae* and *Rhizophagus intraradices* (co-inoculated with *Pseudonomas putida*) of potato mini-tubers resulted in increased chlorophyll content by up to 57%. Xu *et al.* (2018) established that maize plants inoculated with *Glomus tortuosum* (AM plants) had higher chlorophyll content than non-AM maize plants.

Agronomic calcium bio-fortification promoted total chlorophyll content in *Shangi* plantlets. This could be due to an associated overexpression of calcium binding proteins and Ca^{2+} sensors that enhance photosynthetic efficiency and thus promotes plant growth. Raina *et al.* (2021) established that transgenic tobacco leaves overexpressing *StCaM2* (a calcium-binding messenger protein associated with multiple stress in potato) showed delayed chlorophyll senescence under abiotic stress conditions. Cheval *et al*. (2013) reported that an increase in free Ca^{2+} levels (in our case due to fortification) in a cell compartment acts as a signal carrying specific information to be translated into appropriate responses, such as the upregulation of calcium-binding proteins for transportation within the cell/plant. Treatment T3/AMF0 (agronomic calcium bio-fortification) caused an earlier increment in total Magnesium content in the whole root + mid-stem section of *Unica* by up to 65%, which was expected to influence total chlorophyll content as reported by Sheng *et al.* (2008) since magnesium forms an important part of the chlorophyll pigment.

4.5.3 Assessment of AMF colonization in *Shangi*, *Dutch Robjyn* and *Unica* plantlets

There was successful root colonization in all plants in the biotized treatments, namely T0/AMF and T3/AMF due to the observation of arbuscules (Plate 4.4) in the infected roots; no colonization occurred in the control experiments (T0/AMF0) and non-biotized treatments (T0/AMF0; T3/AMF0). Analyses of variance proved a highly significant effect ($p < 0.01$) on tested factors (Variety, Calcium bio-fortification) and their interactions (Variety x Calcium) on mycorrhizal colonization of all the three varieties (Table 4.3).

There were highly significant differences ($p < 0.01$) within mycorrhizal propagule numbers of the three varieties. Treatment T0/AMF had no significant effect on the number of propagules in a 1cm root fragment ($p=0.3483$) in the three varieties, and there were no significant differences between the treatments (Table 4.3). Agronomic calcium bio-fortification (T3/AMF) strongly and significantly influenced ($p<.0001$) the number of propagules in 1cm root fragments of *Shangi*, *Dutch Robjyn* and *Unica* (Table 4.3). *Unica* T0/AMF, *Dutch Robjyn* T0/AMF and Dutch Robjyn T_3/AMF showed 100% colonization percentage (Table 4.3).

There were highly significant differences ($p < 0.01$) in mycorrhizal propagule numbers for *Shangi* plantlets between treatments (Table 4.3). Treatment T0/AMF resulted in a higher number of propagules (191.75 ±10.33) in *Shangi* compared to T3/AMF0 (107.61±10.58). There was a marked reduction in colonization percentage in *Shangi* T3/AMF plantlets compared to *Shangi* T0/AMF plantlets by 9% (Table 4.3). There were highly significant differences ($p < 0.01$) in mycorrhizal propagule numbers for *Dutch Robjyn* plantlets between treatments (Table 4.3). *Dutch Robjyn* recorded the highest colonization frequency, regardless of biotization and/or fortification status (Table 4.3). *Dutch Robjyn* plantlets under T3/AMF had the higher number of AMF propagules (222.72±3.80) compared to plantlets in the T0/AMF. T3/AMF resulted in 21% increase in the number of propagules in 1cm root fragments of *Dutch Robjyn*, compared to T0/AMF treatment. *Unica* plantlets recorded the lowest colonization percentage and frequency compared to *Shangi* and *Dutch Robjyn*, largely due to T3/AMF. There were

highly significant differences ($p < 0.01$) in mycorrhizal propagule numbers between treatments in *Unica* plantlets (Table 4.3). T_3/AMF resulted in a 90% decrease in mycorrhizal propagules in 1cm root fragments of *Unica* compared to *Unica* T0/AMF

Table 4.3: Effect of Calcium Bio-fortification and Biotization on colonization percentage, frequency and number of propagules in 1cm-root fragments of *Shangi*, *Dutch Robjyn* and *Unica*

Variety	Treatment	Frequency	Colonization Percentage (%)	Number of propagules in 1cm fragment
Shangi	T0/AMF	0.88	88	191.75±10.33[a]
	T3/AMF	0.80	80	107.61±10.58[b]
	p value			<.0001
Unica	T0/AMF	1.00	100	205.2±12.83[a]
	T3/AMF	0.76	76	20.1667±1.72[b]
	p value			<.0001
Dutch Robjyn	T0/AMF	1.00	100	183.58±8.56[b]
	T3/AMF	1.00	100	222.72±3.80[a]
	p value			.<0001
ANOVA *(p-value)*	Variety (V)			<.0001
	Calcium (Ca)			<.0001
	V*Ca			<.0001

T_0/AMF= 8.8g/L Ca + 2g/pot Rhizatech™; T_3/AMF= 13.6g/L Ca + 2g/pot Rhizatech™. Values indicate means±SE. Mean values in the same variety grouping followed by the same letters are not significantly different for each variety (Tukey's HSD test, $p \leq 0.05$).

AMF colonization is largely promoted when plants are exposed to stressful conditions (abiotic or biotic) (Begum *et al.*, 2019; Moukarzel, 2020) however that was not the case for Unica T3/AMF. The explanation for this different result is not clear to us but it has been established that the physiological status of the plant is important in determining

successful AMF and plant associations (Song *et al.*, 2019) since plants influence microbial community structures in the rhizosphere. As we previously reported, supplemental calcium (10.4g-15.2g $CaCl_2$) resulted in lower chlorophyll content compared to other treatments, fewer micro-tubers, lower total chlorophyll content and shorter plants with fewer leaves, which might point to a deeper physiological deficiency in *Unica* plantlets grown under excess calcium conditions.

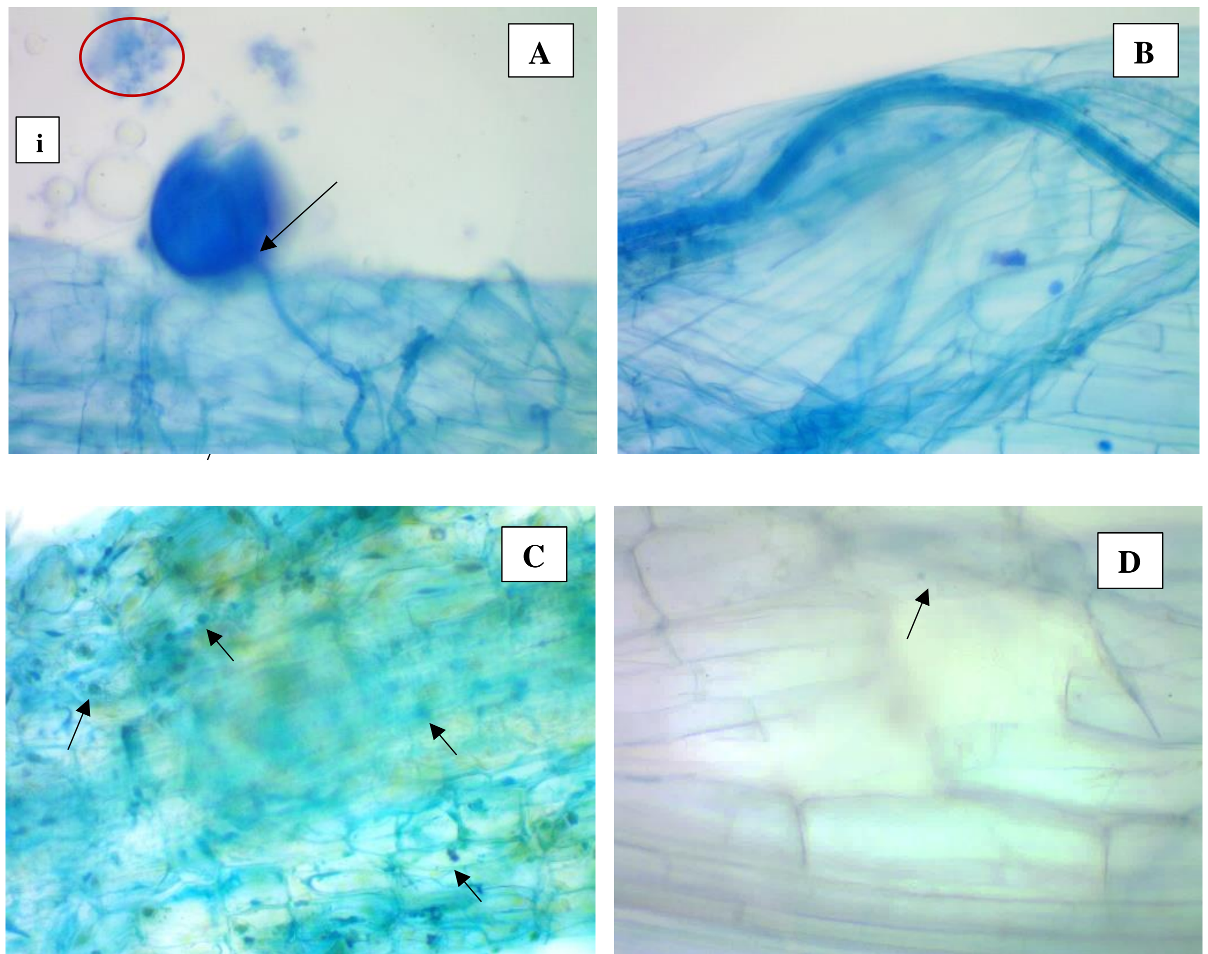


Plate 4.4: Randomly selected 1cm root fragments from biotized treatments showing mycorrhizal propagules. Arbuscular mycorrhizal structures in A. Arbuscules (i) and a Spore in *Shangi* T3/AMF B. Fungal hyphae in *Dutch Robjyn* T0/AMF root fragment C. Vesicles in *Unica* T0/AMF D. Vesicles in Unica T3/AMF root fragment which was poorly infected by mycorrhizal propagules

Calcium bio-fortification significantly influenced the number of propagules in 1cm root fragments of *Shang*i, *Dutch Robjyn* and *Unica*. Calcium bio-fortification significantly improved mycorrhizal inoculation in *Dutch Robjyn*. Cui *et al.* (2019) reported that peanut roots inoculated by *F. mosseae (*syn. *Glomus mosseae)* under Ca^{2+} -sufficient conditions had a significantly higher number of fungal colonizers compared to Ca^{2+}-deficient conditions. The study authors attributed this to Ca^{2+} playing a vital role in the formation of AM symbiosis by up-regulating and specifically inducing transcripts of AMF-specific marker genes in biotized plants. However, calcium bio-fortification also reduced mycorrhizal colonization in *Shangi* and *Unica*. This could be linked to increased cell wall stiffness caused by increased Ca^{2+} content within the roots. Kemat & Krens (2019) reported that linearly demethylation of galacturonic acid (constituent of pectin that makes up the cell wall) residues allows strands of pectin to be linked more efficiently via Ca^{2+} bonds, leading to enhanced cell wall stiffness. Further studies need to be done to determine the exact cause of reduced mycorrhizal propagules in calcium bio-fortified *Shangi* and *Unica* plantlets.

Table 4.4: Effect of *In vitro* Calcium Bio-fortification and Biotization on bacterial wilt severity in *Shangi, Dutch Robjyn and Unica*

Variety	Treatment		Bacterial Wilt Severity Index (%)	
Shangi	T0	AMF0	83.33	±6.66[a]
		AMF	77.33	±0.93[a]
	T3	AMF0	73.00	±0.97[a]
		AMF	68.00	±0.68[a]
Dutch Robjyn	T0	AMF0	79.00	±0.44[a]
		AMF	63.00	±2.59[b]
	T3	AMF0	71.00	±6.71[ab]
		AMF	66.00	±0.92[b]
Unica	T0	AMF0	65.00	±3.65[b]
		AMF	57.00	±1.74[b]
	T3	AMF0	83.00	±4.11[a]
		AMF	93.00	±2.50[a]
	Anova *(p-value)*	Variety (V)	0.1948	
		Calcium (Ca)	0.0597	
		AMF	0.5198	
		V*Ca	<.0001	
		Ca*AMF	0.9284	
		V*AMF	0.7932	
		V*Ca*AMF	0.0379	

T0/AMF0=8.8g/L Ca; T0/AMF= 8.8g/L Ca + 2g/pot Rhizatech™; T3/AMF0=13.6g/L Ca; T3/AMF= 13.6g/L Ca + 2g/pot Rhizatech™. Values indicate means±SE. Mean values in the same variety grouping followed by the same letters are not significantly different for each variety (Tukey's HSD test, $p \leq 0.05$).

4.5.4 Effect of AMF colonization and *In vitro* Calcium Bio-fortification on Bacterial Wilt severity in *Shangi*, *Dutch Robjyn* and *Unica* plantlets

Agronomic calcium bio-fortification combined with biotization showed variable effects on disease severity in the varieties tested. Analyses of variance proved a significant and

highly significant effect ($p < 0.01$) on tested factors (Calcium) and their interactions (Variety x Calcium, Variety x Calcium x AMF) on disease severity of all the three varieties (Table 4.4).

Bacterial wilt infection was more pronounced in *Shangi* (71.71%) and *Unica* (74.91%) compared to *Dutch Robjyn* (69.88%). Non-biotized (AMF0) plantlets of *Shangi*, *Dutch Robjyn* and *Unica* displayed higher disease severity scores (73.31%) than biotized plantlets of the same varieties (71.03%). Fortified plantlets (T3) of the three varieties had a higher disease severity score (75.75%) than non-fortified (T0) plantlets (68.58%). There were no significant differences ($p < 0.05$) in disease severity scores between treatments for *Shangi* plantlets. Non-fortification combined with non-biotization resulted in *Shangi* plantlets with the highest bacterial wilt severity score (83.33±6.66). T0/AMF, T3/AMF0 and T3/AMF treatments resulted in lower bacterial wilt infection by 7%, 12% and 18% respectively compared to control T0/AMF0 plantlets in *Shangi* (Table 4.4).

There were significant differences ($p < 0.05$) in disease severity scores between treatments for *Dutch Robjyn* plantlets. Non-fortification combined with non-biotization resulted in *Dutch Robjyn* plantlets with the highest bacterial wilt severity score (79.00±0.44). *Dutch Robjyn* plantlets under treatments T0/AMF and T3/AMF resulted in lower disease severity scores (63.00±2.59; 66.00±0.92 respectively) compared to T0/AMF0 (79.00±0.44) and T3/AMF0 (71.00±6.71) treatments, reducing Bacterial Wilt severity by 20% and 16% compared to the control. T3/AMF0 resulted in a 10% decrease in Bacterial Wilt severity in Dutch Robjyn compared to the control (Table 4.4).

There were significant differences ($p < 0.05$) in disease severity scores between treatments for *Unica* plantlets. Calcium bio-fortification combined with biotization (T3/AMF) resulted in *Unica* plantlets with the highest disease severity score (93.00±2.50) compared to T0/AMF0 treatment (65.00±3.65) (Table 4.4). T3/AMF0 resulted in 27% increase in disease expression in *Unica* plantlets (83.00±4.11), compared to the control. *In vitro* calcium enhancement detrimentally affected *Unica* plantlets response to *R. solanacearum*. T0/AMF resulted in a 12.3% decrease in Bacterial Wilt severity in *Unic*a (57.00±1.74), compared to the control.

All plantlets in all treatments showed disease symptoms indicating that the pathogenic strain was highly aggressive (Plate 4.5). *R. solanacearum* forms biofilm-like aggregates that fill vessels obstructing water flow resulting in characteristic wilting symptoms which usually lead to death of the entire plant (Lowe-Power *et al.*, 2018). Since the experimental set-up was conducted on a pure sand culture in a green house, the medium was constantly wetted to prevent drying out, which possibly affected the aggressiveness of *R. solanacearum* (Jiang *et al*., 2021). The pathogen is mainly affected by temperature and moisture, high temperature and high moisture which promote infectivity of the pathogen, promoting disease development *(*Muthoni *et al*., 2014). As previously discussed, calcium bio-fortification most likely affected *Unica* plant vigour and health, leading to increased plant host susceptibility to *Ralstonia solanacearum*, the causal agent of Bacterial Wilt (Hosack & Miller, 2017). Biotization led to a reduction in disease severity for *Unica, Shangi* and *Dutch Robjyn*. Wang *et al.* (2018) reported that AMF-inoculated plants exhibited enhanced plant resistance against soil-borne pathogenic fungi by elevating superoxidase dismutase and peroxidase, enzymes implicated in plant defense. However, there were no strongly significant and positive improvements to all the varieties reaction to infection with *R. solanacearum* within the treatments. Despite AMF mitigating wilt severity to a very small degree, Baradar *et al.* (2021) confirms that biological control bio-agents are more efficient when used in combination with other bio-control agents such as Plant Growth Promoting Bacteria than using them individually. Response to *In vitro* calcium fortification and biotization was highly variety specific, with the treatments

influencing plant response in different ways. Fritz *et al*. (2022) reported that plant genotype influenced AMF symbiosis suggesting different degrees of functional compatibility between specific AMF strains and plant species.

Felix *et al.* (2010) and Muthoni *et al.* (2014) recorded that Dutch Robjyn variety is rated as being highly susceptible to bacterial wilt. Calcium fortification, AMF biotization with Rhizatech™ combined with calcium bio-fortification resulted in a lower bacterial wilt disease severity score, even when compared to *Shangi* and *Unica*. Jiang *et al.* (2013) documented the highest Ca application in tomato (*Solanum lycopersicum* L. cv. Shanghai 906) plants resulted in the reduced incidence of bacterial wilt due to an increased production of hydrogen peroxide (H_2O_2) implicated in the strengthening of the cell wall due to the role Ca^{2+} plays in the triggering changes in H_2O_2 concentration (Agrios, 2005; Petrov & Van Breusegem, 2012). Ngadze (2018) documented that boosting the calcium levels in plants has demonstrated improved capacity to fortify resistance against bacterial phyto-pathogens that induce the breakdown of plant tissues. Calcium enhances the resilience of potato stems and tubers against maceration induced by pectolytic enzymes, such as pectate lyase and polygalacturonase produced by pathogens. The importance of cellulose degradation lies in its pivotal role in enhancing the virulence of *R. solanacearum* whereas the contribution to virulence stemming from pectin degradation is comparatively minor (Lowe-Power *et al.*, 2018). He *et al.* (2014) reported that increasing Ca^{2+} (10-40Mm) significantly reduced *R. solanacearum* pectinase activity. The study authors stated that with reduced pectinase activity, the tobacco cell wall was difficult to degrade by bacteria in Ca^{2+} solutions and thus the entrance of bacteria into the host plant was delayed.

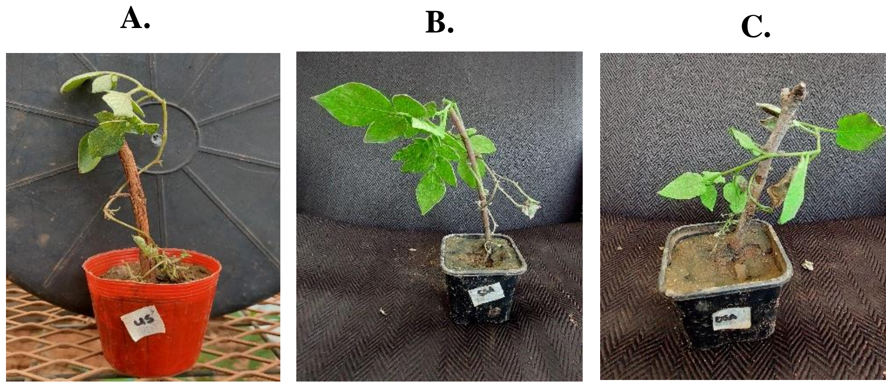


Plate 4.5: Plantlets in treatment A. *Unica* T3/AMF0 B. *Shangi* T3/AMF0 and C. *Dutch Robjyn* T3/AMF 14 Days after introduction of *R. solanacearum* inoculum showing symptoms of bacterial wilt disease

Kibiro (2014) demonstrated that inoculation of potato plants with *Glomus mosseae* and a mixture of *Glomus mosseae* and *Glomus intraradices* conferred a degree of reduction in severity of potato blight. The study author also noted that though there were no significant differences between then control (non-AMF inoculated plants) and the treatments, the controls had a higher number of infected leaves than all the treatments which is similar to our results. The authors also established that minimum root colonization occurred 30 days after initial inoculation for all the treatments, and colonization reached maximum 16 weeks after the inoculation. High bacterial wilt severity scores could also be attributed to the short duration in between biotization and infection with *R. solanacearum*. Yao *et al.* (2002) reported that inoculation of micro-propagated potato plantlets *Goldrush* with *G. etunicatum* led to a significant reduction in *Rhizoctonia solani* disease severity.

Jiang *et al.* (2013) reported that increasing Ca nutrition to near the level of physiological sufficiency significantly reduced the severity of tomato wilt caused by *R. solanacearum*. The authors also reported that hydrogen peroxide levels in high Ca treatment rose faster and reached a higher peak with 10.86 µM gFW−1(31.32 %) which was greater than in medium Ca plants. The study authors attributed this to the reduction in bacterial wilt severity due to the increased H_2O_2 concentration stimulated by Ca. Our study observed

lower severity scores in *Shangi* and *Dutch Robjyn* plantlets that were bio-fortified using calcium compared to non-fortified plantlets.

4.6 Conclusion

Agronomic calcium bio-fortification and biotization showed variable effects on plantlet performance and bacterial wilt severity. Treatment T3/AMF resulted in the tallest *Shangi* plantlets compared to the non-fortified and non-biotized *Shangi* plantlets. Treatment T0/AMF resulted in a 21% decrease in *Shangi* plantlet height while T0/AMF resulted in the highest total chlorophyll content in *Shangi* plantlets. T3/AMF resulted in a 9% decrease in mycorrhizal propagules in *Shangi* while also resulting in the lowest bacterial wilt severity score. There were significant differences ($p < 0.05$) in *Dutch Robjyn* and *Unica* plantlet performance between treatments. T3/AMF0 resulted in the tallest *Dutch Robjyn* plantlets while T3/AMF resulted in the highest plantlet leaf number and highest total chlorophyll content in *Dutch Robjyn* plantlets. *Dutch Robjyn* recorded the highest colonization frequency, regardless of biotization and/or fortification status. T3/AMF resulted in 21% increase in the number of propagules in 1cm root fragments of *Dutch Robjyn* and the lowest bacterial wilt severity score. T0/AMF0 resulted in the tallest *Unica* plantlets with the highest number of leaves. T3/AMF resulted in the shortest *Unica* plantlets with the lowest number of leaves. T0/AMF resulted in the highest total chlorophyll content in *Unica* and the lowest disease severity score. Agronomic calcium bio-fortification combined with proved to be somewhat beneficial in managing bacterial wilt, but only to a small degree since severity scores were high in all treatments. Biotization using 2g/pot Rhizatech™ proved to be beneficial in improving plantlet performance for the three varieties.

CHAPTER FIVE

SUMMARY, CONCLUSION AND RECOMMENDATIONS

5.1 Summary

Seed potatoes (tissue culture plantlets) can benefit from *In vitro* calcium bio-fortification and the application of commercial mycorrhizal formulations to improve regeneration capacity, microtuberization, nutrition and health during *In vitro* culture and greenhouse establishment, respectively. Analyses of variance proved a significant and highly significant effect ($p < 0.01$) on tested factors and their interactions during *In vitro* plantlet development of all the three varieties. MS medium containing 10.4-15.2g/L Ca promoted apical dominance in *Dutch Robjyn* and *Unica* while the conventional MS media formulation resulted in the formation of axillary shoots in *Unica*. MS medium containing 10.4-15.2g/L Ca improved root regeneration in all *Shangi*, *Dutch Robjyn* and *Unica*. MS medium containing 10.4-15.2g/L Ca delayed micro-tuberization in *Shangi* and *Unica* while promoting tuber formation in *Dutch Robjyn.* The results from this study reveal the positive effects of *In vitro* calcium addition using 10.4-13.6g/L Ca on impacting regeneration capacity and micro-tuberization are highly variety dependent. This study also establishes that calcium bio-fortification, compared to conventional MS media, has a significant impact on the days taken to a regenerate a micro-tuber, either by delaying tuberization (as in *Dutch Robjyn*) or hastening the micro-tuberization process (as was observed in *Unica*). The knowledge generated on the effects of calcium bio-fortification on *Shangi*, *Dutch Robjyn* and *Unica In vitro* development (regeneration and micro-tuberization) is important in devising suitable macronutrient (in this case Calcium) concentrations for the purpose of *In vitro* agronomic calcium fortification.

The study also illustrates the compounded benefits of combined *In vitro* calcium bio-fortification and biotization (inoculation with mycorrhizal compounds) on *Shangi* and *Dutch Robjyn* plantlet growth and performance *In vivo* during greenhouse establishment. Calcium bio-fortification combined with biotization using 2g/pot Rhizatech™ boosted

total chlorophyll content in *Dutch Robjyn*, while biotization promoted chlorophyll content in *Shangi* and *Unica*. However, it was observed that *In vitro* calcium bio-fortification caused a marked reduction in chlorophyll content in *Dutch Robjyn* and *Unica*. AMF colonization (number of propagules, frequency and percentage) was also significantly ($p<0.05$) impacted by *In vitro* calcium bio-fortification in *Shangi*, *Dutch Robjyn* and *Unica*. The study results highlight calcium bio-fortification and biotization combined effect on lowering bacterial wilt disease development in *Shangi* and in *Dutch Robjyn*, both reported to be highly susceptible to *Ralstonia solanacearum.* The study also reveals supplemental calcium shows a particularly significant interaction that affects *Unica* growth not only during *In vitro* culture but also during greenhouse establishment and development. Little to no information is reported on the continued effect of *In vitro* calcium bio-fortification during *In vivo* establishment on seed potato, specifically tissue culture plantlets, instead multiple studies focus and highlight the effects of exogenous calcium application, most especially on tuber yield. There is little information on the effects of *In vitro* calcium bio-fortification and mycorrhizal inoculation, and the effect of their interaction on the performance of tissue culture seed potato plantlets, in greenhouse and field conditions. The interaction between *In vitro* calcium bio-fortification and AMF mycorrhizal inoculants should be further investigated and considered in the commercial production of seed potato plantlets.

5.2 General Conclusion

i. MS medium containing **10.4g-13.6g/L $CaCl_2$** promoted earlier regeneration and increased *In vitro* shoot and root numbers in *Shangi*, *Dutch Robjyn* and *Unica*

ii. MS medium containing **10.4g/L $CaCl_2$** promoted earlier micro-tuberization in *Shangi* and *Unica*

iii. MS medium containing **13.6g/L $CaCl_2$** optimized calcium bio-fortification in *Shangi*, *Dutch Robjyn* and *Unica* root sections during *In vitro* culture

iv. MS medium containing **12g-13.6g/L $CaCl_2$** promoted Magnesium, Iron and Zinc uptake improving the root nutritional status of *Shangi*, *Dutch Robjyn* and *Unica*

v. **Bio-fortification with 13.6 g/L Ca** combined with **Biotization with 2g/pot Rhizatech™** promoted *Shangi* and *Dutch Robjyn* plantlet height and leaf number while **Biotization with 2g/pot Rhizatech™** promoted *Unica* plantlet height and leaf number during greenhouse establishment, improving plantlet functional traits

vi. **Biotization with 2g/pot Rhizatech™** increased total chlorophyll content during greenhouse establishment improving plantlet photosynthetic traits of *Shangi*, *Unica* and *Dutch Robjyn*

vii. ***In vitro* calcium bio-fortification alone**, and/or **calcium bio-fortification combined with biotization with 2g/pot Rhizatech™** reduced bacterial wilt disease severity in *Shangi* and *Dutch Robjyn* by an average of 12% and 15%, respectively.

5.3 Recommendations

The study demonstrates how calcium bio-fortification protocols for *In vitro* regeneration can be developed by enhancing calcium macronutrient levels in culture medium and recommends calcium bio-fortification and biotization as potential applications in promoting plantlet performance.

i. This study recommends Ca fortification for optimized *In vitro* plantlet regeneration of *Shangi, Dutch Robjyn* and *Unica*

ii. The study recommends Calcium bio-fortification to promote earlier micro-tuberization in *Unica* and *Shang*i while promoting micro-tuber yield in *Dutch Robjyn*

iii. The study recommends the adoption of mycorrhizal inoculation during acclimatization to boost plant performance and plant defense against bacterial wilt

iv. The study recommends further investigation should be conducted on Ca bio-fortification effects on *Unica* regeneration and micro-tuberization and the interaction between Ca bio-fortification and mycorrhizal inoculation in potato

v. This study also recommends field trials to evaluate performance of AMF-inoculated and fortified plantlets

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APPENDICES

Appendix I: Murashige and Skoog constituent chemicals and reagents

Components	Source	Molecular Weight (g/mol)	Concentration in stock solution (mg/L)
NH_4NO_3	LOBA Chemie	80.0	33
KNO_3	LOBA Chemie	101.10	38
$CaCl_2.2H2O$	LOBA Chemie	147.02	8.8
$MgSO_4.7H2O$	LOBA Chemie	246.47	7.4
KH_2PO_4	LOBA Chemie	136.09	3.4
H_3BO_3	Panreac Quimica SLU		124mmg
$MnSO_4.4H2O$	LOBA Chemie		446mg
$ZnSO_4.7H2O$	Duchefa Biochemie		214mg
Micronutrients			
$Na_2MoO_4.2H_2O$	LOBA Chemie		5mg
$CuSO_4.5H_2O$	Unichem Chemicals	249.71	0.5mg
$CoCl_2.5H_2O$	LOBA Chemie		0.5mg
KI	Panreac Quimica SLU	166.01	16.6mg
Iron (fe)			
Na_2EDTA	RANKEM™		745mg
$FeSO_4.7H_2O$	RANKEM™		557mg
Organic constituents			
Glycine	LOBA Chemie		40mg
Meso-inositol 99%	LOBA Chemie	180.16	200mg
Nicotinic acid	LOBA Chemie		10mg
Pyridoxine HCl (B_6) 99.5%	LOBA Chemie	205.64	10mg
Thiamine HCl (B_1) 99%	LOBA Chemie	337.27	10mg
Plant Growth Regulators Gibberellic acid (GA)	LOBA Chemie		
Gelrite	Duchefa Biochemie		2.8g
Table sugar			30

Appendix II: Hoagland's Nutrient Solution

Components	Concentration in stock (g/L)
Stock 1	
KNO_3	182
KH_2PO_4	13.6
$MgSO_4$ (hydrated)	49.30
Stock 2	
$Ca(NO_3)_2$	147.61
Stock 3	
H_3BO_3	0.806
$MnCl_2$	0.5
$Zn(SO_4)$	0.0555
$CuSO_4$	0.0222
$MoO3.H_2O$	0.025
Stock 4	
$FeSO_4$	0.6944
Na_2EDTA	0.9167