IN-VITRO REGENERATION, GENETIC TRANSFORMATION AND EVALUATION OF MAIZE (*Zea mays* L.) FOR RESISTANCE TO MAIZE LETHAL NECROSIS DISEASE

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A Thesis Submitted to the Graduate School in Partial Fulfilment of the Requirements for the Doctor of Philosophy Degree in Plant Biotechnology of Egerton University

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DECLARATION AND RECOMMENDATION

Declaration

This thesis is my original work and has not been submitted or presented for examination in this or any other university for the award of degree.

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DEDICATION

This thesis is dedicated to my beloved parents for their contribution and provision of optimal environment that determined my social and moral destiny and my kids Beth-Annita and Milar-Aline, Didoo that are the source of my day-to-day motivation.

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ABSTRACT

Maize (*Zea Mays* L.) is one of the most important cereal crops in the world and over 300 million people in sub-Saharan Africa depend on it for food. The majority of Kenyan population, approximately 85%, directly depends on maize for food. However, since the emergence of maize lethal necrosis disease (MLN), Kenya has experienced losses of about US$ 140 million annually. Most of the existing MLN management approaches are ineffective. Gene silencing through transgene induced ribonucleic acid (RNA) is simple and rapid, where small RNA molecules pair with messenger RNA (mRNA) eliciting nuclease degradation or silencing. The method has been adopted for plant disease management. The objectives of this study were to: optimize a regeneration protocol for the International Maize and Wheat Improvement Center maize lines (CML) CML442 and CML444 inbred maize lines; transform maize with the P1, HC-Pro, or VPg transgenes of sugarcane mosaic virus (SCMV), identify transgenics and confirm transgene integration via conventional polymerase chain reaction (PCR) and restriction digestion, respectively and assay for MLN response. Plant regeneration from immature embryos was tested at 0, 2, 3 and 4 mg/L of 2, 4-D in Murashige and Skoog (MS) media in a completely randomized design. The best *in-vitro* regeneration performing maize inbred line was transformed with the P1, HC-Pro, or VPg transgenes. The presence of transgenes was confirmed by PCR and restriction digestion with restriction endonucleases; *AscI*, *BfmI* and *AsuII* for the VPg, P1 and HC-Pro, respectively. Resistance for MLN was conducted via detached leaf assay (DLA). Disease severity was scored on a CIMMYT scale of 1-5. Both CML444 and CML442 were not significantly different at ($p \leq 0.001$) during regeneration with CML442 recording a mean of 8.90 as compared to 6.85 in CML444. The lines were significantly different at ($p \leq 0.05$) during rooting with CML444 recording a mean of 35. 67 as compared to 28.67 in CML442 leading to selection of CML444 for transformation downstream. Digestion products of 303, 227; 535 bp, 169 bp; 1222, 163 bp for VPg, P1 and HC-Pro genes were detected, respectively. The DLA assay indicated that the VPg transgenics had a minimum disease severity score of 1.44, followed by 3.82 and 4.01 for the P1 transgenic and HC-Pro transformants, respectively. Based on our findings, the VPg transgenic indicated that gene silencing through transgenic induced RNA could be the solution for MLN management.

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LIST OF ABBREVIATIONS AND ACRONYMS

CaMV35S	Cauliflower mosaic virus promoter
CIMMYT	International Maize and Wheat Improvement Center
CML	CIMMYT-line
DNA	Deoxyribonucleic acid
FAOSTAT	Food and Agriculture Organization Corporate Statistical Database
GM	Genetically modified
HC-Pro	Helper Component Protein
HIGS	Host-induced gene silencing
IPM	integrated pest management
KALRO	Kenya Agricultural and Livestock Research Institute
MCMV	Maize Chlorotic Mottle Virus
MIGS	Micro-RNA induced gene silencing
MLN	Maize Lethal Necrosis Disease
MS	Murashige and Skoog Media
NAA	Naphthalene Acetic Acid
NOS	Nopaline synthase terminator
NPPOs	National Plant Protection Organizations
P1	Protease Gene 1
PCR	Polymerase Chain Reaction
QTL	Quantitative Trait Loci
RISC	RNA Inducing Silencing Complex
RNA	Ribonucleic acid
RNAi	Ribonucleic Acid Interference
RNase	Ribonuclease
SCMV	Sugarcane Mosaic Virus
SSA	Sub-Saharan Africa
USAID	United States Agency for International Development
USDA	United States Department of Agriculture
VIGS	Virus-induced gene silencing
VPg	Viral Genome Linked Protein

CHAPTER ONE

INTRODUCTION

1.1 Background information

Maize (*Zea mays* L.) is one of the most important crops worldwide. The USA leads in maize production globally followed by Argentina, Brazil and China in that order (Erenstein *et al*., 2022; USDA, 2023). Maize forms the basal diet for over 300 million people in the sub-Saharan region (FAO, 2023), where more than 85% of its production is used directly as food (FAO, 2022). Food security in Kenya is based on maize availability with 85% of the population relying on the crop for food. Maize production in Kenya is categorized into small and large-scale systems. However, a large proportion, about 85% of Kenya's maize production comes from smallholder farmers (Government of Kenya, 2023; Short *et al.*, 2012). Kenyan maize accounts for 3 % and 12% of the gross domestic product (GDP) and agricultural GDP, respectively. Furthermore, various studies estimate that maize contributes 20 % to agricultural production and 25 % to employment (CBK, 2023).

Despite the central role of maize globally, farmers realize average yields, relatively, lower than the potential estimates due to abiotic and biotic stresses (Rifai *et al.*, 2023; USDA, 2018). Bacteria, fungi, viruses, nematodes, and other pests are the main biotic constraints behind production deficits (He & Krainer, 2020). Maize lethal necrosis disease (MLN) is of major economic importance among these biotic constraints, significantly contributing to the current deficits as the disease is potentially capable of causing total yield losses. For instance, reports indicate that in 2014, Kenya accrued approximately US$291-339 million losses to MLN. Due to such constraints, Kenyan farmers average an annual maize production of about 100 metric tons. This has plunged the country into overreliance on maize importation to cover for the production deficits of about 700 metric tons (CBK, 2023; Johnmark *et al*., 2022).

Maize lethal necrosis can occur through maize chlorotic mottle virus (MCMV) single infection. The MCMV virus belongs to the family *Tombusviridae* and the genus *Machlomovirus* (Deng *et al*., 2014). The disease can also occur in MCMV co-infection with viruses of the *potyviridae* and potyvirus genus such as the sugarcane mosaic virus (SCMV), Maize dwarf mosaic virus (MDMV), and Wheat streak virus (WSMV). However, it is in co-infection that the severity of the disease is of economic concern (Regassa *et al*., 2022). Even though other viruses are potentially capable of causing MLN, MCMV-SCMV co-infection remains the common cause. Moreover, *Rioviridae*, Polerovirus, Nucleorhabdovirus and Totiviruses have been associated with MLN outbreaks (Bernardo *et al*., 2023; Chapwanya *et al*., 2021).

Maize lethal necrosis viruses gain entry into the host plant through mechanical injuries caused by either insect vectors or human activities. The disease can also be transmitted through infected seeds and soils. Transmission occurs in a non-persistent fashion (Kiruwa *et al*., 2016) through insect vectors such as thrips and chrysomelid beetles (Regassa *et al*., 2024), rootworms (King *et al*., 2011) and stem borers (Mekureyaw, 2017). MLN vector population can be influenced by environmental conditions such as humidity and temperature causing either an increase or a reduction in disease incidences (Canto *et al*., 2009). Besides, infected soils and seeds have also been identified as other transmission agents (Mekureyaw, 2017).

Sugarcane chlorotic mottle virus and MCMV viruses easily gain entry into the host because antiviral immune responses triggered via the resistance (R) genes are not typically classified as effector-triggered immune (ETI) responses during virus invasion. Moreover, viruses do not encode pathogen- or microbe-associated molecular patterns P/MAMPs or effectors. Thus, antiviral immune aspects are outside the plant innate immunity models (Schwessinger & Ronald, 2012). Host invasion is facilitated by virus suppressors of RNA silencing (VSRs) that act to antagonize plant anti-viral responses (Hussain *et al*., 2021).

Infected maize would show a range of symptoms from mild to severe chlorotic mottling, stunted growth, and death (Erenstein *et al*., 2022). Even though there is a close association of MLN symptoms with those of nitrogen and sulphur deficiencies, death of leaves can be witnessed in both young and old during MLN attack while only older leaves turn necrotic in nitrogen deficiencies. Crops in nitrogen deficiency soils would mature early while those infected with MLN do not reach maturity stage. On the other hand, plants with sulfur deficiency would display thin stems or light green leaves, red pigmentation on the abaxial surface and inward cupping of leaves as compared to yellowing and chlorotic mottling in MLN (Yu *et al*., 2021).

A number of methods including cultural, mechanical, biological, chemicals, crop rotation, and breeding for resistance have been put forth for MLN management however; the disease remains economically important (Johnmark *et al*., 2022). Literature indicates that MLN management remains a challenge, for instance, due to the non-persistent mode of its transmission Abera and Belei (2019), chemicals are rendered ineffective because of the short acquisition and inoculation periods of virus vectors before obtaining lethal insecticide doses (Garzo *et al.*, 2020). Besides, limited farmlands have constraint MLN management through field free seasons leading to frequent outbreaks caused by infected soils. Moreover, with the central role of maize in the sub-Saharan diet, actualization of crop rotation as a way of MLN management remains a major challenge as maize planting occurs year-round. Inadequate phytosanitary measures through the clean seed technology have also scuttled MLN management leading to frequent disease outbreaks (Chapwanya *et al.*, 2021). Improving susceptible varieties through natural crossing is far from reality as nearly all available commercial maize varieties are MLN susceptible (Johnmark *et al.*, 2022). Additionally, retrogration of MLN resistance is complex and this tool is limited by the genetic complexity of maize (Bocos-Asenjo *et al.*, 2022).

Therefore, development and transfer of resistance via biotechnological tools would be the most effective and sustainable way towards MLN management. Biotech tools such as transgenic RNA induced gene silencing are accurate and rapid (Kreuze & Valkonen, 2017). Imparting disease resistance through transgenic RNA induced gene silencing mimics the natural processes of gene regulation in eukaryotes. Such a tool would eliminate the current lengthy procedures involved before commercialization of genetically modified products thus, providing timely solutions to MLN management (Koch & Wassenegger, 2021).

Transgenic RNA induced gene silencing functions by complimentary base pairing of the transgenes with the intruding virus mRNA, creating an mRNA double strand RNA anomaly (Hung & Slotkin, 2021). The presence of this anomaly triggers the Dicer-like protein 1 (DCL1) and RISC reactions that lead to the elimination of the genomic parasite (Kong *et al.*, 2022). Because MLN is more severe in co-infection, introducing the SCMV P1, HC-Pro and VPg transgenes would base pair with the intruding SCMV P1, HC-Pro and VPg genes that are integral to SCMV and MCMV synergism, integration and movement. This would induce the RNA silencing complex to degrade the SCMV virus thus, lower MCMV titers and MLN expression, subsequently, conferring plant resistance or tolerance.

In-vitro adoption of transgenic induced RNA silencing has shown significant success in the management of plant viruses (Anikina *et al.*, 2023). For instance, reports of successful potyvirus management through transgenic RNA mediated disease resistance in maize; rice and wheat are evident (Kaur *et al.*, 2021). It is predicted that management of MLN through transgene RNA induced gene silencing would shorten breeding time and eliminate non-target trait transfer (Younis *et al*., 2014). This new technology of plant breeding would thus, reduce the frequency of pesticide applications during MLN invasion and render its management through field free seasons or crop rotation management redundant (Johnmark *et al*., 2022). Hence, our product would ascertain improved food security, economic and environmental sustainability.

1.2 Statement of the problem

Maize lethal necrosis disease (MLN) can occur during maize chlorotic mottle virus (MCMV) single infection or its co-infection with a potyvirus. The disease hinders the crop's photosynthetic activities thus, significantly lowering yields. The disease has ravaged maize fields across the world; from Americas to China and the Eastern Central Africa in the last decade. In Kenya, MLN was first reported in Bomet, and subsequently, spread to all of the country's 'maize basket regions'. Yield losses of 20-100% amounting to US$120 million annual losses have been reported. The rapid spread is mainly attributed to failure of the existing management measures; disease susceptibility in nearly all commercial maize varieties in Kenya, limited availability of resistance genes, difficulties associated with natural retrogration of resistance genes and poor understanding of the interaction of the two causal viruses. Without a proper solution, MLN would cause perennial food and nutrition insecurities in sub-Saharan Africa where maize is the main food crop. This calls for development of alternative approaches to address the problem. In this study, transgene silencing of the sugarcane mosaic virus in the control of MLN is addressed.

1.3 Objectives

1.3.1 General objective

To contribute to improved food, nutrition security and income generation through *in-vitro* regeneration, genetic transformation and evaluation of transgenic maize for resistance to maize lethal necrosis disease in Kenya

1.3.2 Specific objectives

i. To optimize regeneration protocol for the CML442 and CML444 maize inbred lines
ii. To transform and confirm transgene integration in putative transgenic maize inbred lines

iii. To determine the potential of P1, HC-Pro and VPg transgene expressions to silence sugarcane mosaic virus and evoke resistance against maize lethal necrosis disease

1.4 Hypotheses

i. There is no significant difference in optimized regeneration protocol for the CML442 and CML444 maize inbred lines
ii. There is no significant difference in transformation and transgene integration in putative transgenic maize
iii. There is no significant difference in P1, HC-Pro and VPg transgene expressions' to silence sugarcane mosaic virus and evoke resistance to maize lethal necrosis

1.5 Justification of the study

Maize being Kenya's primary source of food, a deficit in its production would lead to food, nutrition and income insecurity, hindering the achievement of sustainable development goals (SDGs); 2, 3 and 1, respectively (Cairns *et al*., 2021). Besides, food security is likely to contribute significantly to political stability (Subramaniam *et al*., 2023). Political stabilization is central towards the achievement of the Big Four Agenda and Vision 2030 in Kenya. For improved grain quality and yield, and increased resistance to pests and stresses, there is need to improve crop via genetic modification (Kavhiza *et al*., 2022).

There are existing reports that some genetically modified plants have altered biological soil properties such as the root associated microbiome indicating that the environment would influence genetically modified (GM) organisms adoption and integration. However, these cases are few as most GM plants do not require special soil conditions from the wildtype which is likely to lead to rapid integration of this technology (Ubalua, 2009). The technology would experience significant increase in profits due to reduced expenses on labour, agrochemicals and better yields. Decreased pesticide applications would also help in environmental conservation (Oloo *et al*., 2020). Nationally, the technology is likely to improve on food and employment security, increase national income and living standards and act as a cornerstone for environmental conservation and conservation of biodiversity (Azadi *et al*., 2022).

Introduction and stable integration of transgenes is dependent mainly on a reproducible *in-vitro* regeneration system as opposed to *in-planta* transformation procedures. Tissue culture, particularly through somatic embryogenesis is a critical step in genetic modification thus, the reason as to why an *in-vitro* regeneration protocol was optimized in this study (Mohammadi *et al*., 2023).

Assay for transgene integration is important in the identification of transgenics, for instance, this study required positive transgenic plants for assay of maize lethal necrosis disease (MLN) resistance downstream. Methods such as DNA sequencing and Southern Blotting have been used routinely for characterization of transgenes. However, these tools are labour intensive and time, consuming limiting their applications. Therefore, there is need to deploy much simpler, rapid and accurate techniques. Conventional PCR coupled with restriction digestion has been proved reliable, rapid and time saving during molecular characterization of transgenics. Moreover, these tools are user-friendly for researchers with limited bioinformatics background (Hermantara *et al*., 2024).

Because MLN is dependent on synergism, it was hypothesized that the introduction and expression of transgenic SCMV P1, HC-Pro or VPg genes would base pair the complementary intruding SCMV genes forming a double strand anomaly. This would act to induct the RNA Inducing Silencing Complex (RISC/DICER) against the intruding SCMV, subsequently, antagonizing MCMV replication leading to a reduction in MLN expression to confer disease tolerance or resistance (Ortolá & Daròs, 2024).

Transgenic RNA mediated gene silencing for improved resistance against potyviruses is evident. For instance, Jiang *et al.* (2022) demonstrated that plant resistance against potato virus X, potato virus Y, potato virus A, potato leafroll virus, and potato-spindle tuber viroid via transgenic RNA induced gene silencing was possible, which laid the foundation for this study.

Transgenic RNA induced gene silencing-based products are either early-stage or in late-stage development for open field applications leading to scanty literature in the quantification of their benefits. However, predictions show that crops improvement through biotechnological approaches such as gene silencing against biotic stresses would increase yield production by 20-80% (Munaweera *et al*., 2022). Management of MLN through our approach would require fewer pesticide applications reducing the cost of production downstream. Gene silencing through transgenic induced RNA silencing would also enhance environmental conservation through the elimination of frequent pesticide applications to unleash the potential of biotechnology to sustainable development (Kang, 2020). Even though the initial cost of production is higher in gene silencing technology, crop pest management becomes significantly cheaper downstream as compared to management of pests through breeding for resistance through conventional means. Furthermore, the current MLN management practices including crop rotation and crop free seasons that have significantly constraint maize production would be rendered redundant, ensuring food and income security.

1.6 Scope and limitation

Whole plant MLN resistance evaluation was not possible as the concerned authorities in the two organizations tasked with transgenic maize lethal necrosis disease (MLN) screening in Kenya namely, the Kenya Agricultural and Livestock Research Organization (KALRO) Headquarters and Kenyatta University (KU) Genetic Transformation Laboratory cited biosecurity concerns associated with accidental spillover. A project on transgenic MLN screening in KALRO had been completed and, the necessary screening infrastructures were not in place at the time this study was carried out. Thus, evaluation of MLN transgenic resistance was performed through the detached leaf assay (DLA). This study also focused only on the P1, VPg and HC-Pro genes of the sugarcane mosaic virus. The qRT-PCR was not used in this study because of the limitation of the research funds. Hence, assay of transgenes was used towards the characterization of transformed plants.

CHAPTER TWO

LITERATURE REVIEW

2.1 Origin and global distribution of maize lethal necrosis

Maize lethal necrosis disease (MLN) was first reported in Peru in 1973, with initial yield loss estimates of 10 to 15%. Later, an increase of up to 59% yield losses was reported according to Wangai *et al*. (2012). The disease spread to Kansas in the United States of America in 1977, where further increase in yield losses ranging from 50% to 90% was recorded. Later, MLN re-emerged in China, Kenya and Tanzania in 2011 and by 2014 the disease had colonized other Eastern and Central African countries such as Uganda, Rwanda, Democratic Republic of Congo, and Ethiopia (Mahuku *et al*., 2015a).

Kenya recorded its first MLN case in Bomet County in the South Rift in 2011. In a span of a year, the disease spread to other counties including Naivasha, Narok, Nakuru, Baringo, Trans-Nzoia, Uasin Gishu, Busia, Embu, Meru, Muranga, Kirinyaga, and Nyeri. Maize lethal necrosis diseasenhas had significantly affected Kenya's 'grain basket' regions including the Western, Eastern, Coast and Rift Valley regions (Wangai *et al*., 2012).

2.2 Factors influencing maize lethal necrosis outbreaks

Studies have demonstrated that plant disease outbreaks have been significantly escalated by the change in climatic conditions as such changes influence pathogen evolution and host-pathogen interactions changing pathogen mutation and plant susceptibility, respectively (Singh *et al*., 2023). Reports indicate that certain environmental conditions exacerbate MLN. For instance, highest MLN incidences were observed in the sub-humid areas, followed by semi-humid areas and semi-arid zones. In Kenya, Embu County recorded the highest disease incidences with seed recycling, irrigation and high amount of rainfall, extensive and continuous maize cultivation characterized by two planting seasons being cited as the major contributing factors. The short and long rains for instance, would provide a phase for MLN virus inoculum build-up and optimal breeding conditions for disease causing vectors leading to an increase in disease incidences (Eunice *et al*., 2021).

Higher disease incidences are also attributed to favorable climatic conditions such as elevated temperature that favour breeding of insect vectors (Yang *et al*., 2017). The list of counties with high MLN incidences in Kenya includes Nakuru, Elgeyo Marakwet, Pokot, Trans Nzoia, Baringo and Bungoma Kenya counties. Low disease incidences have been recorded in dry areas such as Makueni and Kajiado. This is attributed to seasonal maize planting and low water availability resulting in less to no MLN inoculum build up (Boddupalli *et al*., 2020; Eunice *et al.*, 2021). In addition, parasitic weeds such as striga also thrive in higher temperatures. Similar plant protein protection mechanism is expressed upon drought, striga and MLN constraints. The function of these proteins is compromised leading to a decreased effectiveness of RNA interference against MLN therefore, droughts exacerbate incidences of such diseases. Besides, the HSP70 proteins predicted to exacerbate MCMV infection has been found to be highly expressed during droughts (Biswal *et al*., 2022).

Literature also indicates that genotypic composition plays a significant role towards MLN resistance. For instance, the DK8031, a maize variety, recorded the highest mean incidence, while DUMA43 and DH04 had the least mean incidences. Similarly, reports show that different maize stages respond differently during MLN invasion. The growth stage (R5), which are physiologically mature plants, were found to have the highest disease incidences followed by the R3/milky stage and the vegetative stage. These findings form a clear illustration that the genetic composition and developmental stages are key determinants in plants' response to diseases (Eunice *et al*., 2021).

2.3 Maize lethal necrosis host plants

The two viruses host range mainly falls within the poaceous family with maize being the main natural host (Mbega *et al.*, 2016). With cases of the Kansas serotype *I* in *Zea mays* subsp. *Parviglumis* and *Zea luxurians* also being evident (Nault *et al*., 1983). However, a range of other plant species including *Bromus* spp., *Digitaria sanguinalis, Eragrostis trichodes, Hordeum* spp., *Panicum* spp., *Setaria* spp., *Sorghum* spp. and *Triticum aestivum* and *Zea mays* subsp. *Mays* and *mexicana* are prone to MLN (Biswal *et al*., 2022). This indicates that the disease has a wide host range, which might as well be the major limiting factor to its management.

Disease reservoirs play a central role in the spread of diseases. Molecular analyses have revealed the existence of sugarcane mosaic virus (SCMV) virus in a number of species including blackjack, Mexican sunflower, rice, onions, peas, peppers and cassava. This is because the common MLN vectors such as the corn thrips (*Frankliniella williamsi*) and aphid (*Rhopalosiphum maidis*) also feed on these plant species (Liu *et al*., 2017). Besides, MLN viruses have been detected in dead plant remains, contaminated soils and seeds that have further constraint the disease management measures (Boddupalli *et al*., 2020).

2.5 Maize lethal necrosis disease causal agents

Maize lethal necrosis occurs in either single infection of Maize Chlorotic Mottle Virus (MCMV) or in MCMV co-infection with viruses from the *potyviridae* family. The potyviruses include the Sugarcane Mosaic Virus (SCMV), Maize Dwarf Mosaic Virus (MDMV) or Wheat Streak Virus (WSMV) (Zhan *et al*., 2022). However, studies have shown that MCMV and SCMV co-infection is the most common cause of MLN. Disease severity in co-infection is greater with reports indicating that this increase in disease severity stems from the ability of SCMV to amplify MCMV titers. The causal agents are able to survive in plant remains, soils or seeds as virus particles a, scenario that has further scuttled MLN management through the proposed measures (Mbega *et al*., 2016).

Maize chlorotic mottle virus belongs to the family *Tombusvirideae* in the genus *Machlomovirus*. It is characterized by an icosahedral head of 30 nm in diameter composed of 25 kDa capsid protein subunit encasing a 4.4 kb positive sense single-stranded genomic RNA (Xie *et al.*, 2011). The MCMV genome encodes a coat protein (cp), two movement proteins (p7a and 7pb), two RNA-dependent RNA polymerases (p50 and p111), a protein aiding in systemic infection (p31) and a virulence protein (p32) (Scheets, 2000). Evidence also shows that the p31, p32, and p50 are involved in evading the host resistance mechanism via the suppression of the host of RNA silencing pathway however, these calls for further research (Scheets, 2016).

Potyviruses are 12-750 nm flexuous rod-shaped virions. The MLN causing potyviruses comprises of a single stranded, positive-sense genomic RNA. These viruses have a single large open reading frame (ORF) in their genome which translates into a single polyprotein. The polyprotein is auto-catalytically digested into the protein 1 (P1), helper component protein (HC-Pro), the third protein (P3), the first 6K protein (6K1), cylindrical inclusion protein (CI), the second 6K protein (6K2), viral genome-linked protein (VPg), nuclear inclusion protein a (NIa), nuclear inclusion protein b (NIb) and coat protein (CP) (Jiao *et al*., 2022) (Figure 2.1). Sugarcane Moasic Virus RNA in the host plant cells can be confirmed by the presence of the pinwheel or scroll-shaped inclusion bodies under the microscope. Analysis of plant species from different geographical regions has shown that the potyviruses genomes are highly varied (Mahuku *et al*., 2015).

Co-existence of viruses from other families with MCMV and Potyviruses in a single host is evident. For instance, there are reports of MCMV, SCMV and southern rice black-streaked dwarf virus of the family *Reoviridae* co-existence in maize plants. Similarly, co-existence of maize yellow mosaic virus MaYMV alongside SCMV and MCMV in maize plants is also evident. A study at the molecular level indicated that the Potyvirus Wheat Streak Mosaic virus (WSMV) in co-infection with MCMV+SCMV elevated MCMV titer contrary to MDMV, SCMV, and JGMV. The WSMV increased the MCMV severity on cellular organelles. However, there are no reports of WSMV in East Africa and Asia. In addition, most plants are tolerant to the virus. Research on the effect of MaYMV on MCMV or SCMV co-infected maize manifested no severe phytopathological effects. During MLND infection however, MaYMV suppressed SCMV increasing the MCMV titers a scenario that still caused MLND manifestation (Nel, 2021).

Figure 2.1: Genomic RNA organization of SCMV showing a single large polyprotein that is processed into ten mature proteins; the P1, HC-Pro, P3, 6K1, CI, 6K2, VPg, Nia, Nib and CP

Source: Jiao *et al*. (2022)

2.6 Maize lethal necrosis vectors

Transmission of MLN viruses mainly occur through piercing and biting insect vectors. However, in the case of high MCMV viral load transmission beyond biting insects is possible. Maize chlorotic virus is spread by *Sitophilus sp* and *Chaetocnema sp* beetles, *Busseola sp*, *Chilo sp*, and *Chilo sp* stem borers and *Diabrotica sp* rootworms (Nault *et al*., 1978). Sugarcane mosaic virus is spread through *Rhopalosiphum sp* and *Sitobion sp* aphids. However, when symptomatic plants harbour high concentrations of MCMV, disease transmission can occur beyond sucking insects such as *Frankliniella sp* thrips, which also form the main MCMV transmission agents. Transmission of MCMV occurs in a non-persistent manner, within seconds to a few hours of feeding on infected materials. Contrary, Cabanas *et al*. (2013) proposed a semi-persistent mode of MCMV transmission, within minutes to hours without latency. Nevertheless, both authors agreed that thrips are potentially capable of transmitting MCMV for up to six days. Longer feeding periods are associated with higher transmission efficiency, with a reduction in transmission over time.

2.7 Pathogen host entry and establishment in the host plant

Pre-existing conduits are necessary for any successful pathogen access into the host plant such channels can be injuries inflicted on plants mechanically by human or by feeding insects (Meisrimler *et al.*, 2021). However, most MLN transmissions occur through feeding insects. Insect vectors such as thrips have been found to transmit viral particles especially potyviruses in a non-persistent fashion which occurs when an insect spreads a layer of pre-digested materials containing viral particles at the feeding sites leading to virus access to the internal tissues of healthy hosts (Garzo *et al.*, 2020; Zhan *et al.*, 2022). Virus invasion requires the presence of a causative agent, a vector and a susceptible host (Singh *et al.*, 2023). Upon host entry, viruses spread translocate in short distances through the plasmodesmata channels to neighboring cells. Long-distance translocation takes place via the phloem leading to systemic invasions. Both short and long distance movements are aided by specialized virus proteins (Chaves-Bedoya *et al.*, 2011).

2.8 Maize lethal necrosis virus synergism and the role of specialized virus proteins in host plant infection

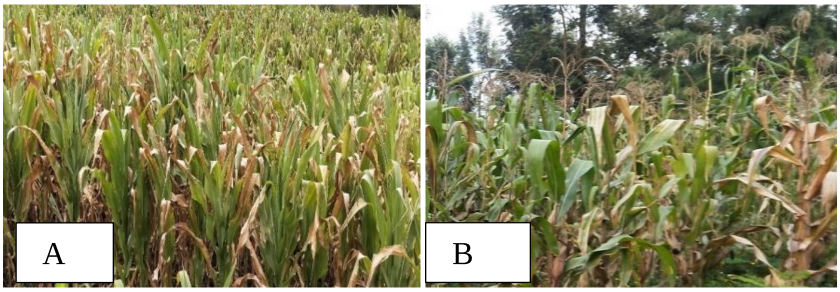
Two types of synergistic interactions including potyvirus-associated synergisms and non-potyvirus have been documented in plants (PooLPOL *et al.*, 1986). In potyvirus-associated synergism, one of the viruses belongs to the potyvirus family as opposed to non-potyvirus synergism which happens in the absence of potyviruses. Potyvirus-associated synergism has been reported as the most common type of association (Wangai *et al.*, 2012). For instance, the potyvirus plays a significant role during MLN infection. It increases the viral load of otherwise a less virulent MCMV virus (Zhang *et al.*, 2022). The potyvirus HC-Pro and NIb proteins function to suppress the RNA silencers of the host, increasing MCMV titers and potyvirus invasion (Shen *et al.*, 2020).

The HC-Pro protein constitutes the N-terminal constitutes, central domain and the C-terminus. The N-terminus is approximately 100 amino acids that functions to increase virulence and systemic movement. The central domain, which constitutes 200 amino acids, facilitates movement along the phloem and particles replication activities. The C-domain, a 150 amino acids segment harbours a cystein-type proteinase, which is involved in short distance movement, making the three protein domains integral for successful MLN invasion. The HC-Pro functions to counter plant defensive mechanism by suppressing post-transcriptional gene silencing (PTGS) an event that leads to successful virus invasion (Syller, 2006).

Another set of SCMV proteins, the NIa and VPg's synergistic interactions intensifies MCMV amplification (Atabekova *et al*., 2023). Moreover, the VPg protein acts as a primer for SCMV replication by binding the maize elongin C protein (*ZmElc*), which increase SCMV RNA accumulation. Other studies have demonstrated that the VPg- *ZmElc* complex leads to under expression and localization of *ZmElc* to specific plant parts, a scenario that antagonizes chlorophyll synthesis (Khan & Gross, 2019). The SCMV VPg polyproteins along with the P1 protein function to mediate synergism of the two viruses increasing disease pathogenicity (Urcuqui-Inchima *et al*., 2001). However, the mechanism of MCMV-potyvirus synergistic interaction remains poorly understood (Ohlson *et al*., 2022).

2.9 Maize lethal necrosis disease symptoms

Plant disease symptoms are the physical outcome caused by an infection. Symptoms in MLN vary in both co-infection and single infection with age and genotype of the plant and environmental conditions being cited as other influential factors (Deressa & Demissie, 2017). During early plant developmental stages, the symptoms are severe characterized by bright mosaic appearance, which causes tissue chlorosis, stunted growth and death or severe necrosis of terminal leaves (Niblett & Claflin, 1978) (Figures 2.1A). These symptoms compromise the normal physiological processes of infected plants leading to poor chlorophyll formation and reduced rates of photosynthesis (Redinbaugh & Stewart, 2018). Late infections may result into low severity (Figure 2.1B) or escalated symptoms such as small, partially filled and malformed to barren ears with other notable symptoms such as dwarfing, elongated yellow streaks parallel to leaf veins that may coalesce to create mild chlorotic mottle leading significant yield losses. Disease progression on young leaves is characterized by the onset of chlorosis from the margins progressing to the mid-rib, while chlorotic mottling normally starts at the base extending to the tips. Sometimes chlorotic mottling in the whorl before expansion also known as "dead heart" is common (Wangai *et al.*, 2012).



(Author's design)

Plate 2.1: (A) Early maize lethal necrosis onset associated with significant maize yield losses and (B) Late maize lethal necrosis onset associated with no significant losses

2.10 Economic impact of maize lethal nec disease

Maize lethal necrosis disease remains a major concern in the East African region due to continuous economic losses to farmers, seed companies and consumers (Boddupalli *et al.*, 2020). Small-scale farmers who form the greater component of maize farming are highly affected. In the East Africa for instance, Uganda incurred 58% yield losses while Ethiopia, Kenya, Rwanda, Tanzania, and Uganda had combined losses of USD 291-339 million in 2014. Since the first incident, Kenya has been losing approximately USD 140 annually (Mahuku *et al.*, 2015b; Zhan *et al.*, 2022). Maize lethal necrosis disease has had devastating economic effects on farmers, along the value chain and maize processors. Due to the decrease in demand for commercial maize seed varieties during the epidemic, maize seed companies registered heavy economic losses (Boddupalli *et al.*, 2020).

2.11 Molecular activities during maize lethal necrosis infection

Virus-host colonization requires a number of host protein factors for replication. Successful colonization may lead to two events, high disease severity due to suppression of the host defense system or elicitation of the host defense mechanism via gene silencing (Niehl & Heinlein, 2009). The expression of Class I β-1, 3-glucanase (GluI), and its synergistic interaction with plasmodesmata size exclusion proteins, has been found to expose plants to virus infections. In the case of MCMV, brassinosteroid (BR) pathway inductiony6 is fundamental for successful colonization. In addition, the expression of disulfide isomerases like protein ZmPDIL-1 and peroxiredoxin family protein ZmPrx5 are also fundamental. Sugarcane Mosaic Virus requires Maize Elongin C (ZmElc) protein for its colonization. Proteomic studies have revealed that overexpression of the ZmElc protein increases SCMV accumulation (Iglesias & Meins, 2000; Zhai *et al.*, 2015).

Even though the ZmElc protein is integral in SCMV replication, its role during MCMV-SCMV co-infection remains unclear (Biswal *et al.*, 2022). However, the ZmElc protein along with the maize initiation factor 4E (ZmeIF4E) have been hypothesized to increase SCMV accumulation as both proteins are central in viral proteins replication and translation, respectively upon their independent interaction with the VPg proteins. However, studies have shown that there is no direct interaction between ZmElc and ZmeIF4E and whether a downregulation of the former could decrease the latter's expression (Léonard *et al*., 2000). This also indicates that MLN viruses could be dependent on the host proteins for replication and post-translation activities. Therefore, a mutation of redundant host genes could as well provide a solution towards MLN virus management, as a number of redundant host genes are pivotal to their replication (Jiang & Laliberté, 2011; Zhan *et al*., 2022).

2.12 The role of host microRNAs during MLN invasion

MicroRNAs (miRNAs) contain highly conserved sequences ranging from 20-22 nucleotides. These sequences are integral to host plant disease resistance (Yang *et al*., 2021). To establish the role of miRNAs during MLN infection, maize inbred line B73 was challenged with MCMV and a combination of MCMV-SCMV. The expression profiles of three miRNAs; miR159, miR393, or miR394 that confer host-drought tolerance were assayed (Gupta *et al*., 2014). Results revealed a change of expression patterns of all the three miRNAs. Further, in-silico modelling demonstrated that these miRNAs would be involved in the synergistic interaction of the two viruses (Xia *et al*., 2019).

In a study in tobacco, the knockdown of miRNA159 led to the upregulation of plant resistance (PR) genes linked to defense and apoptosis. Contrary, silencing of the same miRNA in *Arabidopsis* did not confer similar results indicating that miR159 defense response varies across species. During droughts, miR159 is upregulated to silence MYB transcription factors that are involved in the positive regulation of ABA in *Arabidopsis* (Reyes & Chua, 2007). Additionally, miR159 imparts an ABA non-responsive trait allowing plants to thrive in low water stress. Drought-tolerant maize HKI-1532 was found to overexpress miR159. Similarly, the same miRNA was overexpressed in wheat during drought indicating that its downregulation during MLN infection would reduce plant tolerance to drought and expose them to other abiotic constraints (Aravind *et al*., 2017).

Downregulation of miR393 which functions to reduce stomata density and leaf chlorosis was found to improve on drought tolerance. The same miRNA was overexpressed in *Arabidopsis* during low and high-water stress, salt stress and upon ABA treatments. It was also revealed that the same miR393 was downregulated during SCMV/MCMV invasion, indicating that the MLN viruses would castigate detrimental effects during abiotic and biotic stresses (Sunkar & Zhu, 2004).

Overexpression of soybean miR394 improved drought tolerance in *Arabidopsis* and mung bean. However, its downregulation and that of miR159 during MCMV single infection or in SCMV/MCMV co-infection in maize leaves showed a reduced tolerance to drought in the infected plants (Xia *et al*., 2019). Further, studies revealed that the expression of miR167, which is integral in virus colonization and drought tolerance increased upon MCMV or SCMV infection or in co-invasion (Liu *et al*., 2022). However, contrasting findings showed that maize seedlings in low water stress had a low miR167 expression, probably because of the need to increase Phospholipase D mRNAs accumulation. The protein functions to induce a regulated ABA-induced stomatal activities and antioxidant defense for better survival (Wei *et al*., 2009).

2.13 Current maize lethal necrosis disease surveillance and detection approaches

Early disease surveillance is important for effective management. However, detection of MLN is complicated by the high recombination incidences in the causing viruses and the contagious nature of the disease. Management of MLN has also been baffled by the lack of resistance genes in all commercial germplasm leading to the adoption of integrated management approaches (CIMMYT, 2022). A regional response team was constituted in the East African region. The group deployed large-scale coordinated surveillance systems in MLN monitoring and disseminating of its information. The existing wheat stem rust (Ug99) surveillance system was adopted for MLN tracking. The outcome was promising as the system proved rapid and effective. However, the rates and relative importance of various MLN transmission mechanisms have not been fully exploited, a scenario that has limited its applications (Redinbaugh & Stewart, 2018).

Another project on MLN Diagnostics and Management was initiated in 2015 under CIMMYT in collaboration with National Plant Protection Organizations (NPPOs) and Aarhus University under the USAID funding leading to the establishment of an MLN surveillance system. Through this project, a standardized surveillance protocol was established in eight countries in the sub-Saharan region. This was followed by the establishment of a sampling approach and MCMV diagnostic protocols by CIMMYT and NPPOs later in the same year in Kenya, Uganda, Tanzania, Rwanda, Malawi, Zambia and Zimbabwe. A universal MCMV immunostrip diagnostic device was also adopted to update the public on MLN status (CIMMYT, 2022).

Harmonization of MLN monitoring systems regionally improved on its surveillance, diagnosis and awareness as information was updated in the CIMMYT website for public access. The coordinated surveillance majorly benefited the NPPOs in the Eastern and Southern Africa and farmers in other MLN-prevalent countries such as Ethiopia, Kenya, Rwanda, Tanzania, and Uganda. Besides, the tools were used for MLN monitoring in three major commercial maize producing but MLN-free countries namely Malawi, Zambia and Zimbabwe. Moreover, the monitoring devices including the open-data kit (ODK)-based smartphone survey app improved data collection on both MLN and other maize pest and diseases of economic importance. Collection of MLN data was based on visual inspection supported by standardized leaf sampling and validation by immunostrips testing (Redinbaugh & Stewart, 2018).

Information on annual sampling of seeds from agro-dealers and import sites was stored in the smartphone-based ODK kit. This was followed by MCMV-ELISA assay validation of the seed samples. Information obtained was stored in an online data management toolbox, which mainly constitutes field and seed surveys on MLN and major plant diseases in the SSA. The derived data at the national level were standardized and centralized at the continental level. Features of the MLN toolbox including; controlled access, secured storage, easy data editing, and data visualization through interactive maps and charts, and country-specific data export were considered. Data was verified by authorized country managers before public dissemination. Data on MLN status in the SSA region was collected from over 7000 field survey records in 8 countries and more than 400 seed survey records (Boddupalli *et al*., 2020).

During MLN outbreaks, rapid and sensitive diagnostic tools are important for efficient detection, easy and timely implementation of prevention measures. These tools are required because highly varied MLN symptoms have completed virus scouting through visual inspection. Therefore, the tools are integral in distinguishing viruses from non-virus infections and environmental stresses. Techniques such as serological tools and DNA-based detection techniques are capable of distinguishing virus infections from abiotic stressors (Mahuku *et al*., 2015).

Diagnostic kits made it possible to detect MLN at the farm level because the kits are easy to use, cost-effective, rapid and accurate. Additionally, field-based diagnosis and surveillance rendered the kits popular in remote areas (CIMMYT, 2022). However, constant challenges with serological-based detection paved way for RT-PCR, which could detect changes in gene expression and was rapid. However, an application of RT-PCR -based detection was found to be resource intensive and constraint by personnel capacity across the SSA region. Moreover, frequent mutations in MLN causing virus led to the deployment of the Next generation sequencing (NGS) technology. The NGS technology has proved affordable even though its implementation remains a challenge (Adams *et al.*, 2012). Despite the efforts, effective detection and management of MLN has been thwarted by the high cost of diagnostic tools, inadequate capacity and poor public awareness (Bacheller, 2017).

2.14 Current maize lethal necrosis management approaches

Management of plant diseases is critical for the attainment of potential yields. Plant disease management ranges from information dissemination and creation of awareness to the application of integrated pest management (IPM) tools (Richard & Fit, 2022). Integrated pest management includes cultural, mechanical, biological and chemical control measures. Cultural strategies entail the destruction of infected materials and the use of clean and resistant planting materials as described by (Rajashekhar *et al.*, 2024). Mechanical methods include incineration of infected materials to reduce farmland contamination and postharvest pathogens. Chemical method is the control of foliar pathogens and their vectors through soil fumigation. While biological methods revolve around the deployment of living agents for the elimination of plant pests (Mach, 2016).

Various organizations have advocated for a synergistic and integrated efforts in MLN management (Boddupalli *et al.*, 2020). For instance, the International Maize and Wheat Improvement Center (CIMMYT) partnered with the National Agriculture Research Organizations (NARs), National Plant Protection Organizations (NPPOs), commercial seed companies, several advanced research institutions in the USA and Europe, International Institute of Tropical Agriculture (IITA), non-government organizations, such as Alliance for Green Revolution in Africa (AGRA) and African Agricultural Technology Foundation (AATF) in quest for a long-lasting solution. These institutions advocated for a multi-disciplinary and multi-institutional coordination strategy for optimum management of MLN (CIMMYT, 2022).

To overcome MLN outbreaks, various management measures including agronomical, biological, entomological, and pathological control were adopted in the East Africa region (Marenya *et al.*, 2018). Planting MLN-free maize seeds, assaying for MLN-resistant maize varieties, and practicing maize crop rotations were also proposed to surmount MLN management challenges (Jafari Jozani *et al.*, 2020). Moreover, engagement with policy makers, awareness creation, and establishment of a phytosanitary community of practice were part of the proposal to enhance and accelerate MLN management (Prasanna, 2015).

2.14.1 Integrated pest management

This approach entails merging of different practices such as vector control, agronomic management and deployment of tolerant germplasm to counter crop diseases (Deguine *et al.*, 2021). Chemicals are important in shielding crops from disease vectors. Some of the common insecticides used include, Imidacloprid, Thiamethoxam, Deltamethrin, Abamectin, Permethrin, Endosalphan and Dimethoate. However, disease management through insecticides would be costly due to the need for frequent change of chemicals because of risks of development of resistance. Moreover, chemical-based insect vector control is unreliable in cases of semi-persistence transmission as the insects are able to transmit the disease before obtaining lethal doses. Chemicals are also non-selective and could lead to biodiversity erosion. Furthermore, chemical-based insect control is prone to environmental pollution and would increase the cost of planting materials (Mahuku *et al.*, 2015).

Improved plant nutrition is important in strengthening plant resistance against pests and diseases which is integral in plant disease management. Crop rotation and mono-cropping could also provide a sustainable solution towards MLN management. However, these methods are of no significance use particularly in the East Africa region where farmlands are small and maize is the central diet and therefore has to be planted year-round (Mahuku *et al.*, 2015). This has left the region with the need to explore MLN management alternative in biotech-based tools. Pest control through gene silencing is rapid and reliable. Moreover transgenes are economically and environmentally sustainable due to their ability to transfer disease resistance in a Mendelian fashion downstream (Vincelli, 2016).

2.14.2 Cultural practices

Cultural practices are tailored towards providing unfavorable conditions for pathogen multiplication and also to prevent contact with infectious agents (Harman *et al.*, 2019). Disease prevention through cultural practices includes the application of crop rotation, incineration of infected materials, improved growth environment and the use of tolerant germplasm. However, embracing these measures in MLN management remains a bottleneck (Wangai *et al.*, 2012). Practices such as crop rotation, season free cropping and food diversification would be difficult to apply in the sub-Saharan Africa because of the central role of maize in the region's diet. Hence, the year-round planting culture, which has caused high MLN viruses inoculum build-up and pathogen re-circulation (Eunice *et al*., 2021). The population density across the region has constrained farmlands limiting MLN management through season free cropping and crop rotation. Poor MLN awareness has further increased disease incidences. With nearly all available commercial germplasm being MLN susceptible, disease management through cultural measures has become inefficient (Marenya *et al*., 2018).

2.14.3 Quarantine and sanitation in maize lethal necrosis disease control

Pathogen quarantine is the prevention of disease establishment or entry in areas where it does not occur. Quarantine requires inspection of seeds before entry and exit in and within countries. The existing MLN quarantine measures were adopted from the Food and Agriculture Organization (FAO) framework (FAO, 2018). Removal and incineration of infected plants materials and reservoirs were also proposed for MLN management (Biswal *et al*., 2022). In addition, vigilance at entry and exit border points and deployment of accurate and highly sensitive MLN detection devices were also considered (Boddupalli *et al*., 2020). However, some regions have poor phytosanitary measures, porous entry and exit border points, and sub-standard regulatory framework leading to escalation of MLN cases. Furthermore, poor MLN detection devices, low sensitization and the existence of cultural practices involving seed sharing have baffled management through quarantine and the application of the clean seed technology approach (Bacheller, 2017).

2.14.4 The clean seed strategy

The clean seed technology focuses on the importance of pest and seed-borne diseases testing to ensure dissemination of health seed materials to the farmers (Dell'Olmo *et al*., 2023). Based on the fact that maize seeds are potentially capable of causing 0.4 to 3.9% MLN transmission rates and that this transmission percentage is genotype dependent, the African Agricultural Technology Foundation (AATF), Alliance for Green Revolution in Africa (AGRA) International Maize and Wheat Improvement Centre (CIMMYT) and USAID have been championing for MCMV free commercial seeds in the region. A scenario that saw both CIMMYT and USAID initiate a seed inspection project to foster the use of MLN free certified seeds in the East Africa region (Boddupalli *et al*., 2020; CIMMYT, 2022). Through this project, CIMMYT, AATF, AGRA, researchers, seed companies and other extension agents developed MLN free seed production checklist and Standard Operational Procedures (SOPs), which have become feasible in Kenya, Tanzania and Ethiopia.

Currently, more than 30 seed stakeholders in Kenya, Uganda, Rwanda, Tanzania and Ethiopia have implemented the MLN management checklist. The checklist is predicted to enhance MLN free seed production. The stakeholders constitute both the public and private sector institutions and extension agencies that are advocating for efficient MLN management through the checklist. The checklist is updated in the MLN portal for seamless information sharing to encourage the production and exchange of disease free seeds. Basing on the fact that seed companies are the major sources of MLN outbreaks, the initiative would reduce disease incidences significantly (CIMMYT, 2022). However, implementation of the clean seed strategy is still at the grassroots level and its application is constraint by the substandard phytosanitary measures, porous entry and exit points and sub-standard regulatory framework across MLN prone countries in Africa. Moreover, traditional practices such as seed sharing, at the smallholder farm level across the MLN prone countries have constraint the effectiveness of the clean seed strategy (Bacheller, 2017; CIMMYT, 2022).

The USAID MLN Diagnostics and Management was another project established for creation of MLN awareness and acceleration of its detection for better management across MLN-prevalent countries in Africa. This initiative targets seed companies, seed trade associations, contract growers, National Agricultural Research Systems (NARS) institutions and regulatory and extension agencies in Kenya, Uganda, Ethiopia, Tanzania and Rwanda. The preference and selection of the targeted institutions was based on the potentiality to disseminate SOPs and the MLN checklist. A total of 574 participants were drawn from National Plant Protection Organizations (NPPOs) and NARS institutions, 544 from commercial seed companies, and 2313 small-scale contract seed growers in the Eastern Africa for training in the period 2016–2019. The primary goals of this training were to better farm scouting, leaf and seed sampling, and immunostrips testing (CIMMYT, 2022).

2.15 The mechanism of natural plant disease resistance and contemporary crop resistance improvement tools

Plant disease resistance is an evolutionary process arising from diversified selection, recombination and gene conversion or retro-element insertion (Meyers *et al.*, 2005). Plant disease resistance is classified into quantitative and qualitative resistance. Qualitative resistance which is also referred to as major gene resistance is influenced by a single major-effect resistance (R). Qualitative resistance is a high-level and race specific characterized by necrosis around the spot of pathogen entry. Quantitative resistance involves the action of many genes with additive or partially dominant effects. Quantitative resistance is non-race specific characterized by intermediate to high-level resistance that is influenced by epistasis and gene by environment interaction. Breeding for resistance in maize is mainly achieved through quantitative genes because of its genetic diversity and the ability to out-cross (Pilet-Nayel *et al*., 2017).

Plant virus management is of significant economic importance, and, currently, there are no direct remedies for crop virus management necessitating the need for advanced research on host plant resistance. With the contemporary molecular tools and techniques including novel gene mapping tools and emerging technologies such as transgenic mediated gene silencing, trait improvement through transgenesis is inevitable (Medina-Lozano & Diaz, 2022).

2.15.1 Transgenic RNA dependent gene silencing

Gene silencing via RNA occurs naturally in eukaryotes. The process helps in regulating growth and development. Additionally, gene silencing is required by plants for protection against genomic parasites, pathogens and abiotic stresses (Kwon *et al*., 2020). The process works through effecting gene mutations or tampering with the processes of replication and translation helping plants evade natural enemies. Adoption of RNA-based gene silencing has demonstrated tremendous results against plant pest management in addition to microbial and viral infections that constraint production (Gebremichael *et al*., 2021). Previously, a number of viruses have been managed through RNA interference successful cloning and transformation of maize with the SCMV CP gene demonstrated success in the management of SCMV. Sequence complementarity plays a key role in identifying target sequences for degradation in RNA induced gene silencing. Small RNAs of about 20-22 sequences base pair with target complementary sequences forming a duplex capable of triggering RISC/DICER destruction (Widyaningrum *et al*., 2021).

Approaches that entail transgenic dependent RNA interference include virus-induced gene silencing (VIGS), host-induced gene silencing (HIGS) and micro-RNA induced gene silencing (MIGS). Virus-induced gene silencing is a rapid approach deploying either viral DNA or RNA where target genes are cloned into a binary vector with multiple clonal sites, and delivered into the host (Tzean *et al*., 2019). The VIGS technique is dependent on the availability of double strand sequence of RNA. Upon transgene entry and integration, amplification of the target copies follows via RNA-dependent RNA polymerase (RdRp). The transgenic RNA transcripts base pairs with the target pathogen mRNA leading to a double stranded (dsRNA) anomaly. The dsRNAs are digested by DICER (DCLs) into short dsRNAs and loaded into the RISC as guide strands for downstream destruction of homologous sequences (de Felippes *et al*., 2012). The siRNAs occur in many forms such as *trans*-acting siRNA (tasiRNA), natural antisense transcript-derived siRNAs (natsiRNA), heterochromatic siRNA (hcsiRNA) and repeat-associated siRNA (rasiRNA) (Zheng *et al*., 2018).

Virus-induced gene silencing is classified into the first generation and the second generation. The first generation VIGS entails deletion of pathogenic genes and addition of inverted repeats causing hairpins formation (Senthil-Kumar & Mysore, 2011). Its vector systems constitute of more than 35 DNA/RNA viruses such as the Tobacco Mosaic Virus (TMV), Tomato Golden Mosaic Virus (TGMV), and Potato Virus X (PVX) (Ruiz & Baulcombe, 1998). However, these systems are associated with a number of limitations such as having few susceptible hosts. Therefore, the first generation VIGS are weak suppressors or lack gene silencing ability. The second generation VIGS have a wide host-range and occur in a systemic fashion for instance, the Bamboo Mosaic Virus (BaMV) binary vectors. The second generation VIGS are strong gene silencers with a wide host-range (Liou *et al*., 2014).

In Host Induced Gene Silencing (HIGS), inverted repeat sequences are administered into the host via *Agrobacterium* or VIGS binary vectors leading to the production of double stranded RNAs (Harvey *et al*., 2011). The transgenic RNA transcripts bind its target via homologous base-pairing triggering destruction of the dsRNA anomaly via the RISC/DICER pathway. The HIGS then advances to VIGS, which works by suppressing specific viral genes via the expression of RNAi constructs (Zheng *et al*., 2018).

MicroRNA (miRNA)-induced gene silencing (MIGS) is a method that exploits a 22-nucleotide miRNA of *Arabidopsis thaliana* also referred to as miR173. The miR173 is capable of triggering the production of another class of small or short interference RNAs called trans-acting small interfering RNAs (tasiRNAs). The tasiRNAs function to trigger gene silencing of the corresponding endogenous gene (de Felippes *et al*., 2012). The first use of miR173-mediated MIGS was in tested in *Arabidopsis* and tobacco for the induction of double gene silencing. This study was followed by silencing of the phosphoglycerate dehydrogenase I (PGDH1) gene in *Arabidopsis*, the C-terminal protein 1 (CEP1) gene in *M. truncatula* and those of chalcone synthase (CHS) and phytoene desaturase (PDS) genes in

Petunia. Silencing of genes through sRNAs in MIGS is preferred because it is more potent and convenient as compared to the hpRNA and amiRNA in VIGS. This feature has widened the applications of MIGS across a range of plant species such as *Arabidopsis*, tobacco, soybean, and petunia. Moreover high success rates have been recorded in the silencing of wheat streak mosaic virus (WSMV) (Zheng *et al*., 2018).

Adoption of MIGS comes with a number of benefits. The amiRNA designing in MIGS is dependent on many PCR reactions that guarantee proper orientation of the mature amiRNA in the precursor backbone while the hpRNAi in VIGS can only be inserted in an antisense orientation. The guide miR173_ts capability to prime different targets enables multiple target genes by a single MIGS vector. The guide miR173 in MIGS is potentially capable of fusing similar MIGS vectors resulting in a co-expression as opposed to VIGS that only works with specific virus vectors (de Felippes *et al*., 2012; Zheng *et al*., 2018). Moreover, construction of MIGS vectors is easy due to the use sequences ranging between 200-500 nucleotides which require a one-step PCR reaction. Similarly, designing of VIGS amiRNA is also simple as it requires a single adaptor of between the 22 nt, a miR173_ts target site and a segment of the gene to be silenced. However, reports of transformation via MIGS in monocot plants are unavailable (Zheng *et al*., 2018). Furthermore, the application of gene silencing through RNA is restricted by off target effects, limited transgene delivery vectors, and low transgene uptake. Moreover, public acceptance of RNAi derived products remains low (Kang *et al*., 2022).

Spray induced gene silencing (SIGS) is another advanced RNA silencing approach applied in both monocots and dicots. It involves administration of a double stranded RNA, targeting particular sequence/s into a plant of interest via syringe infiltration, irrigation methods, foliar spray, or adjuvant (Hoang *et al*., 2022). The SIGS method is environmentally friendly and appropriate for pre- and post-harvesting applications. In addition, SIGS is prone to fewer off-target effects. Success with SIGS crop improvement has been reported in the management of Alfalfa Mosaic Virus (AMV), Pepper Mild Mottle Virus (PMMoV), and Tobacco Etches Virus (TEV). The use of syringes to deliver transgenes further simplifies the application of SIGS compared to HIGS and VIGS. The method offers the best platform for the transformation of recalcitrant genotypes. However, SIGS has reported low transgene accumulation and poor elicitation of RNAi (Hoang *et al*., 2022). Moreover, the transfer of transgene/s through gentle rubbing or soft brushes in SIGS remains inapplicable in field and greenhouse. This has left the use of spray-based transgene delivery as the only alternative (Akbar *et al.,* 2022). Mechanical inoculations through rubbing cause increased disease resistance due to thigmo-morphogenesis which renders plants weak in priming defense responses for subsequent challenges. Reversing such kind of plants to their original status has also been found to be time consuming due to chronic alterations of gene expression patterns (Brenya *et al*., 2020).

The major setback of transgenic induced gene silencing is the knockdown of unintended genes, which demands for precision during siRNA designing. Precise fabrication is pivotal for the elimination of non-target base pairing sequences (Hannus *et al*., 2014). However, this siRNA designing remains a bottleneck due to poor prediction of miRNA seed matches. In cases of such mismatches, accidental knockdowns have been reported. Therefore, it is predicted that whole transcriptome sequencing would be important before siRNA molecules fabrication. Sequencing of both target and siRNA sequences would reduce off-target effects and improve on base-pairing precision. For instance, chemical modification of position 2 of the 5' end by the addition of a 2'-0-methylation chemically linked to the 4'-carbon of the ribose in the siRNA guide-strand seed matching regions would ensure a reduction in mismatches and off-target effects. This modification functions to weaken the interaction between the guide and target strand. Since the seed matches in the guide strands are short, small disruptions would result into destabilization and poor interactions with target sequences as compared to siRNA segments which are fully bound to their targets (Jackson *et al.,* 2006; Seok *et al*., 2018).

Besides, administration of less siRNA would be another alternative to mismatch elimination. One disadvantage with this approach is that it has low on-target activities, thus, calling for siRNA pooling, which directs multiple positions to individual siRNAs. The siRNA contains a salient off-target signature coupled with same on-target signature within a pool. This mediates for a synergistic mode of action on a specific RNA. In complex pools, it requires low individual siRNA concentrations to counter miRNA off-target binding. Pooling can be done in three ways, through SmartPools which involves a cocktail of four individual siRNA. These pools are not complicated and the dilution effects are not certain. The second pooling is based on the endoribonuclease-produced siRNAs (esiRNAs) where long dsRNA molecules are digested using RNAase III. However, the pools are complex comprising multiple siRNAs. The third pool constitutes a highly complex but well-defined siPOOLs with 30 siRNAs. Each siRNA concentration is adjusted to a picomolar level to dilute and reduce off-target effects. The siRNA pooling has been predicted as the solution to off-target effects and is commonly used in the knockdown studies and genome-wide RNA silencing (Neumeier & Meister, 2021; Seok *et al.*, 2018).

2.15.2 Gene silencing through base editing

Crop improvement through conventional breeding tools has been a challenge due to diverse gene pools. Traditional genetic engineering was deemed as the solution; however, the technology has been associated with a number of challenges such as the random transgene insertion and off-target silencing effects (Nerkar *et al.*, 2022). Moreover, studying of essential genes via conventional genetic engineering could pose deleterious risks to the organism/s (Zhang *et al.*, 2021). These challenges led to the birth an advanced tool in genome editing. Gene silencing through base editing has enabled precision deletion or insertion of single nucleotides and large DNA fragments providing a solution to the contemporary breeders' well-established challenges. The technology mimics natural processes of DNA repair eliminating the integration of foreign DNA materials which has been the central point of ethical concerns (Das *et al.*, 2023). Furthermore, coupling CRISPR with high-throughput sequencing techniques has efficiently advanced functional genomic studies leading to exponential rise in its applications. Gene editing has shown success in crop improvement for better quality and yield and improved resilience against abiotic and biotic constraints. Research on CRISPR edited MLN resistance maize is in progress in Kenya and if successful, it is predicted to curb MLN yield losses to negligible levels (Zhang *et al.*, 2021).

2.16 In-vitro regeneration systems

In-vitro regeneration is the process of growing cells, tissues or organs in an artificial and sterile environment to obtain plants for the purpose of multiplication or genetic transformation. The technique is dependent on the ability of cells to differentiate and de-differentiate; in this case, a single cell possesses the capacity to produce multiple cells capable of forming a full plant (Morinaka *et al.*, 2023). Moreover, successful regeneration is dependent on a number of parameters such as physicochemical conditions. These factors are critical due to their ability to spur physiological and biochemical changes in the cultures leading to the formation of fully developed plants. *In-vitro* regeneration is crucial for breeders for mass production, clean material generation and integration of desirable traits. The technology is also time saving, less laborious and season flexible (Martini *et al.*, 2022).

In-vitro regeneration falls in two pathways; somatic embryogenesis which is achieved through culture initiation of explants and the production of bipolar structures from somatic cells. Somatic embryogenesis can further be classified into direct somatic embryogenesis, where somatic embryos form directly from the explant and indirect somatic embryogenesis which involves callus formation and subsequent somatic embryo development for plant regeneration (Oseni *et al.*, 2018).

De novo organogenesis another form of *in-vitro* regeneration is a pathway that involves direct organs formation from adventitious meristems. *De novo* organogenesis can further be divided into direct organogenesis, which involves direct organ formation from meristematic tissues and indirect organogenesis where regeneration goes through the callus phase. Regeneration via organogenesis is unsuitable for genetic manipulation purposes as it entails significant de-differentiation cycles that are likely to cause transgene mutations (Ahmad & Mukhtar, 2017).

2.17 In-vitro plant growth regulators

In-vitro regeneration requires certain physicochemical parameters such as plant growth regulators (PGRs) that are pivotal for the growth and development of the explant. Exogenous PGRs are normally required in small quantities and in varied concentrations throughout different culture phases. They work to trigger explant proliferation or to induce the secretion of endogenous percussors that play a similar role (Bidabadi & Jain, 2020). Some of the important PGRs used for plant regeneration include auxins, cytokinins, gibberellins, ethylene and Abscisic acid.

Auxins determine the fate of somatic embryo formation in most plant species. Exogenous auxins initiate callus formation via eliciting the production of endogenous precursors such as 1-aminocyclopropane-1-carboxylic acid of ethylene synthesis (Sidek *et al.*, 2022). However, the constant presence of exogenous auxins beyond the initial stages of callus induction is not required during *in-vitro* regeneration as they only function to induce the initial secretion of endogenous hormones. In addition, their constant presence could cause other detrimental effects such as poor explant regeneration downstream showing that plant regeneration is less dependent on the PGR (Vennapusa *et al.*, 2015). Despite the existence of reports that root formation is possible in auxin free media in some crop species, contradiction still remains as others indicate that media incorporation of the PGR is integral during the rooting phase. Different species require different auxin concentrations during callus induction due to genotypic differences. However, most studies have shown that callus induction in most species is optimal at 2 mg/L concentrations (Miri, 2020).

Cytokinin is a class of PGRs that include Benzyl Amino Purine (BAP), Isopentenyl-adenine (2-ip), Furfurylamino Purine (kinetin), Thidiazuron (TDZ) and zeatin. These hormones are central to cell divisions (Hönig *et al.*, 2018). Cytokinins transform the cell fate, eliciting cell competency and formation of cell masses. These PGRs are widely used for the induction of callus, somatic embryos as well adventitious rooting and shoot formation (Asghar *et al,* 2021). In equal proportions, auxins and cytokinin are potentially capable of inducing callus. However, higher cytokinin-auxin ratio has been reported to induce shoot formation and explant sprouting while the reverse is integral to the formation of roots (Zhang, 2023).

2.18 Types of callus and their influence on transformation

Two types of callus are likely to be observed during *in-vitro* regeneration namely Type I callus and Type II callus. Type I Callus is more organized and compact. It constitutes a mixture of late embryogenic, late organogenic and meristematic domes. This type of calli is covered by an epidermal layer which renders this type of calli less transformable. Type I Callus is also complex with differentiated tissue whose individual cells are dependent on the surrounding tissues for growth. This lack of cell autonomy has further complicated transformation of this type of calli because if transformation of such cell occurs they are not likely to develop further during selection due to dependent totipotency. The second type of callus is Type II Callus which consists of early embryogenic tissues comprising of rapidly growing friable and embryogenic cell aggregates (Armstrong & Green, 1985). This type of callus is easy to transform and have been reported to confer stable transgene integration because they do not undergo significant dedifferentiation. Rather a simple redirection of growth is enough to form a fully developed plant. Significant de-differentiation would cause genome stress leading to transgene mutations (Kausch *et al.*, 2021).

2.19 Genes related to callus formation and regeneration

Assaying for genes responsible for callus induction and regeneration has become possible courtesy of molecular assay tools. These tools are capable of detecting DNA sequences or their change in expressions which is significant to cell differentiation and dedifferentiation (Ikeuchi *et al.*, 2013). A number of genes responsible for callus formation have been identified. For instance, quantitative trait nucleotides (QTNs) technique was used for the identification of 40 genes responsible for callus regeneration, cell fate, seed germination and embryo development. These genes include GRMZM2G108933, GRMZM2G130442, GRMZM2G315375, GRMZM2G163761, GRMZM2G412611, GRMZM2G066749, and GRMZM2G371033. The GRMZM2G108933 gene annotated as *WOX2* is an embryonic transcription factor associated with callus browning and callus differentiation (Ma *et al.*, 2018). To test one of these genes involvements in callus formation, the WOX2 gene which is homologous to GRMZM2G108933 was introduced into maize and the results showed that callus browning was inhibited in the transgenics (Nardmann *et al.*, 2007). Callus browning polymerizes enzymes that are involved in metabolic activities leading to inhibition of anabolic and catabolic activities which causes culture deaths.

The Potri.004G118700, Potri.018G014800, Potri.015G078200, Potri.004G118700, Potri.009G066100 and Potri.015G078200 genes were shown to be integral to callus formation in contrast to the Potri.018G014800 gene whose expression was antagonistic to the same process. Both the Potri.006G222700 and Potri.012G083800 expressions are bound to a co-expression network involving 77 genes that includes the KNUCKLES (KNU). The KNU gene is involved in a network that mediates the repression of WUSCHEL (*WUS*) genes. This gene is responsible for floral meristem-induction and embryogenic transition. The KNU gene is homologous to the *AT5g14010* and *AT2g35390* genes that are also involved in cellular biosynthesis and microtubule organization, respectively (Tuskan *et al.*, 2018).

Experiments have shown that the PLETHORA (PLT) genes are involved the regulation of callus regeneration in *Arabidopsis*. The PLT3, PLT5, and PLT7 function by activating the expression of root stem cell regulators PLT1 and PLT2, which are responsible for the establishment of pluripotency and shoot progenitors formation. The PLT3, PLT5, and PLT7 gene expressions on the other hand act to complete the process of shoot regeneration by increasing the expression of shoot-promoting factor Cup-shaped cotyledon1 (CUC1) and Cup-shaped cotyledon2 (CUC2). Further research indicated that inhibition of the ICK gene, a cyclin-dependent kinase (CDK) inhibitor also improved the regeneration of embryonic callus (Cheng *et al.*, 2015).

Similar to callus formation, shoot regeneration is also genotypic dependent and involves interplay of genetic factors. For instance, the expression of the GRMZM2G130442 gene was found to accelerate callus greening (Ingram *et al.*, 2000). The APETALA2/ETHYLENE RESPONSIVE FACTOR (AP2/ERF) and the wound induced dedifferentiation1 (WIND1) were identified to influence *in-vitro* shooting. Iwase *et al.* (2015) also indicated that upregulation of the enhancer of shoot regeneration1 (ESR1) gene would trigger transcription of the AP2/ERF transcription factor, which is responsible for shoot formation. Through QTL analysis, two QTLs on chromosomes 1 and 9 that are responsible for green shoot re-differentiation in rice were as reported by Wu *et al.* (2022). The GRMZM2G315375 gene known br2 was also found to encode the P-glycoproteins which are central to embryo differentiation and auxin transport. Equally, embryo development, a process similar to callus re-differentiation, was found to be dependent on the br2 and br3 genes, an indication that the GRMZM2G130442 and GRMZM2G315375 are key players of callus regeneration (Pasternak *et al.*, 2002; Yang *et al.*., 2012).

Two hundred and sixty three SNPs that determine the root architectures at the seedling stage in maize were also identified in Lowe *et al.* (2016) studies. The GRMZM2G153722 gene located on chromosome 4 in maize was found to contain nine significant SNPs, confirming that SNPs expression can occur in both roots and shoots. Moreover, the identification of other genes that modulate maize leaf architecture was identified through the Nested Association Mapping (NAM) technique (Pace *et al.*, 2015; Tian *et al.*, 2011). The GRMZM2G315375 was found to increase the rate of rooting in callus. However, a member of the HD-Zip (homeo domain-leucine zipper) family, the GRMZM2G130442, annotated as *Zea mays* outer cell layer (*ZmOCL*) family was found to define the formation of different regions of the epidermis for root initiation (Ingram *et al.*, 2000).

2.20 Organogenesis and precotious germinations

The appearance of bud-like structures on the embryogenic sites of immature embryos is referred to as precotious germination or precotious germinating embroids. These structures are developing somatic embryos prior to maturation. Their occurrence varies across different genotypes. Precotious germinations are deleterious such that their presence hinders callus formation. They also deter callus from producing secondary embryogenic tissues such as somatic embryos. Therefore, their excision is important for optimal development of callus. However, other authors argue that precotious germinations promote embryoid bodies’ and their excision should be avoided (Lange *et al.*, 1998).

All callus cells have root meristem characteristics irrespective of the explant source. Meristematic cells are highly totipotent due to the xylem-pole pericycle like cells that are the origin of lateral roots. Moreover, a greater number of genes responsible for callus formation are also important in lateral root formation with evidence that adventitious root formation does not require exogenous hormones (Zhang *et al*., 2022). However, initiation of adventitious root premodia requires polar auxin and local auxin transport in the pro-cambium cells, with further evidence indicating that genotypes without callus forming ability lack adventitious root formation ability (Olatunji *et al*., 2017). Auxin hormones are important in the transitioning of the cell fate to adventitious root formation via the initiation of the WUSCHEL RELATED HOMEOBOX11 (*WOX11*) genes in the procambium. Similarly, the initial stages of cell fate transitioning to callus and adventitious roots are dependent on the expression of the *WOX11* and its homolog *WOX12* confirming that the expression of the WOX gene in rice is the baseline for the development of crown roots which are a form of adventitious roots (Liu *et al*., 2014).

2.21 Somatic embryo maturation

Somatic embryos are finger-like projections on callus surfaces that originate from single cells in events that are similar to both direct and indirect somatic embryogenesis. Formation of these structures is stimulated by culture stresses such as heat, nutrient depletion, solute-based water stress or elevation of ABA levels (Valencia-Lozano *et al*., 2021). Somatic embryo maturation is a critical stage during *in-vitro* regeneration as this is the phase that determines the success in callus-plant transitioning. Evidence has shown that incomplete callus maturation causes low to no plantlet formation. Plant growth regulators hinder somatic embryo formation and maturation therefore, their omission is necessary (Puspasree & Rath, 2012).

2.22 Molecular activities behind maturation of somatic embryos

Besides the culture environment, genetic factors play a significant role towards the development and maturation of somatic embryos. Somatic embryo genes function by enhancing cell proliferation, differentiation and morphogenesis of the explant (Salaün & Dubreucq, 2021). The genes including the *Histidine Kinase Homologs1* (*AHK1*), cytokinin signaling genes (*AHK3, AHP4, ARR1*) and auxin signaling *ARF5* related coding gene have been found to influence SAM and RAM formation in *Arabidopsis* (Nguyen *et al*., 2016; Zhao *et al*., 2010). These genes interact with the *WUSCHEL* and *WOX5* homeo domains, the *BBM, LEC1, FUS3* and *AGL15* morphogenic genes that are involved in meristem maintenance and embryogenesis (Uddenberg *et al*., 2011). During high osmotic stress, the *histidine kinase 1* (*AHK1*), *histidine-containing phosphotransfer* (*AHP4*) and *auxin response factor 1* (*ARR1*) are upregulated whereas the *histidine kinase 3* (*AHK3*) is down regulated leading to the formation of somatic embryos. Similarly, the *WUSCHEL, WOX5* (a *WUSCHEL* related homeobox) and *auxin response factor 5* (*ARF5*) level surges in high osmotic conditions promoting the formation of somatic embryos (Zhao *et al.*, 2014).

Through gene expression assays, it was revealed that the upregulation of *ARR7-ARR15* and *histidine-kinase 3* (*AHK3*) in a non-osmotic environment inhibited SAM and RAM development which hindered somatic maturation (Su *et al.*, 2015). These genes function by silencing the *AHK1, AHP4* and *ARR1* genes that are involved in drought tolerance and seed maturation Singh *et al*. (2015) an event that causes the subsequent blocking of the switching on of *WUS-WOX5* and *LEC1-FUS3-BBM-AGL15* stem cell maintenance genes the that are involved in embryogenesis. To test this hypothesis, the re-introduction of osmotic stressors caused the expression of the *AHK1, AHP4* and *ARR1* genes an event that resulted into the activation of *BBM, LEC1, MP,* and *FUS3* somatic embryo genes which are responsible for the initiation of embryogenic competence and regeneration (Dussert *et al.*, 2018).

Furthermore, the *BABY BOOM* (*BBM*) genes *LEC1, LEC2, FUS3* and *AGL15* have been found to play an integral part in the process of somatic embryogenesis (Horstman *et al.*, 2017). Upregulation of the *BMM* genes was reported to cause a surge in cytokinin response factors (CRFs) via the cytokinin signaling Valencia-Lozano *et al*. (2021), the *LEC1* (a nuclear transcription factor Y Subunit B-9 responsible for embryo maturation and cellular differentiation) Pelletier *et al.* (2017) and the *FUS3* gene, a regulator of the late embryogenesis (Bäumlein *et al.*, 1994). Studies on *Arabidopsis* showed that *lec1, lec2, fus3* mutants repressed somatic embryogenesis because they conferred poor adaptation to desiccation. In a different study, Gaj (2004) indicated that somatic embryo maturation and stem cell maintenance, cytokinin signaling and embryogenesis were connected. During somatic maturation and stem cell maintenance, *AHK1* is upregulated. The *AHK1* works by transferring the phosphates to the *AHP4* gene products causing a downregulation of the *AHP1, AHP2* and *AHP3* genes. The *AHK1* is an osmoregulatory that controls desiccation processes during seed maturation and low water stress in a process similar to somatic embryo maturation (Singh *et al.*, 2015). Moreover, the expression of the *AGL15* gene of the MADS-box domain family has also shown to be critical in somatic embryogenesis and regeneration processes (Valencia-Lozan *et al.*, 2021).

2.23 Challenges associated with immature embryo as an explant for genetic transformation

A number of factors have crippled maize immature embryo transformation. Some of the challenges that have been reported include a few available transformation protocols, low transformation efficiency and genotypic recalcitrance (Yan *et al.*, 2023). In addition, extraction of immature embryos is laborious and time-consuming, is available within a limited window period and explant availability is also season dependent (Kausch *et al.*, 2021). However, the expression of certain explant genes has been found to enhance the development of particular receptors in immature embryos that encode *Agrobacterium* docking receptors (vitronectin) that are required for a successful transformation event. Vitronectin mutants are transformation recalcitrance; genotypes that are limited to genetic transformation (Tzfira & Citovsky, 2002).

2.24 *Agrobacterium* mode of transgene delivery and the significance of Acetosyringone

Agrobacterium remains the main transgene delivery method due to its ability to confer stable transgene integration. The transfer of T-DNA in *Agrobacterium* is facilitated by a cascade of events. The VirA genes are phosphorylated which inducts phosphorylation of the VirG. These two events lead to priming of the vir boxes with their respective promoters and the activation of the transcription of the vir gene operon. Transcription of Vir operon leads to the expression of the virulence proteins that are essential in the delivery and integration of the T-DNA region into the target cell (Toh *et al.*, 2020).

Delivery of transgenes through *Agrobacterium* is dependent on a number of factors. *Agrobacterium* activity is determined by the explant type and the culture conditions. *Agrobacterium* has been found to thrive at pH 5.2 and at temperatures of 20˚C. Explant treatment with enhancers such as Acetosyringone also influence on the induction of T-DNA transfer hence, transgene transfer into the explant Manfroi *et al.* (2015) because monocots do not produce this phenolic compound. Acetosyringone function to chemotactically attract *Agrobacterium* into the explant, an event that triggers T-DNA transfer and integration into the explant. Moreover, antioxidants media incorporation has been found to improve on culture survival rates thus, increasing the transformation frequency (Xi *et al.*, 2018).

The type of the expression vector deployed and *Agrobacterium* strain and cell density used are also important. For instance, transgene expression in *Agrobacterium* through the pMD35 vector has shown significant results. Bacterial densities affect the stable and transient transformation frequency. Densities of $OD_{500\text{-}600nm} = 0.4$ have shown highest GUS expression which is a reflection of better transgene integration. Optimal densities also buffer bacterial overgrowth which prevents culture deaths thus, increasing transformation efficiency (Du *et al.*, 2019; Su *et al.*, 2023).

The use of more than three ears per transformation event should also be considered as it reduces the frequently encountered ear-to-ear variations during genetic transformation. Immature embryos intended for genetic transformation should be within 1.5–2.0 mm, at this size, the explant is highly totipotent an event that increases the rate of transformation frequency (Kang *et al.*, 2022). Explants obtained from low water stress environment are not recommended as they possess an additional layer of wax to the cell wall that inhibits *Agrobacterium* activities (Du *et al.*, 2019).

2.25 Significance of the co-cultivation phase

The use of enhancers during genetic engineering is integral to transgene transfer to the explant. One of the enhancers in Acetosyringone acts to induct the *vir* genes of *Agrobacterium*, which subsequently causes the transfer of the T-DNA containing transgene/s to the target explant (Yong *et al.*, 2006). Acetosyringone concentration 100 µM was reported to trigger optimum Agrobacterium activities on maize explant (Du *et al.*, 2019). However, Bhatt *et al.* (2021) reported effective *Agrobacterium* activities in sorghum with as low as 50 µM Acetosyringone concentration. Contrary, other studies have shown that the concentration of Acetosyringone is not a critical factor rather its presence (Matheka *et al.*, 2019). Moreover, media incorporation of antioxidants such as proline and L-Cysteine during this phase is also important. Antioxidants function to reduce or completely stop phenol production and lower the sensitivity of the scutellum to *Agrobacterium* which reduce culture deaths. Such a reduction in callus necrosis is projected to improve on transformation efficiency. The co-cultivation media pH of 5.2 has been found to favour optimum *Agrobacterium* activities (Wang *et al.*, 2023). Heat treatment of the explant could improve explant transformation competence. For instance, exposure of maize and rice explants to 43°C temperatures was found to improve on transformation efficiency (Gurel *et al.*, 2009). Therefore, enhancement of transgene transfer requires interplay and optimization of a range of factors.

2.26 Significance of the resting phase

The resting phase is the period during genetic transformation that follows the co-cultivation phase. This phase occurs in a slightly lower pH at 5.8 and requires exclusion of

Agrobacterium selection agent to allow for better transgene integration. The resting phase grants explants time for transgene integration, and recovery from *Agrobacterium* shock and damages, which ensures improved transformation efficiency. It also provides sufficient time for the initiation of explant differentiation and sprouting genes which is pivotal to culture regenerability. It is also during this period that the explants adapt and acclimatization itself to downstream culture pressures (Matheka *et al.*, 2019).

2.27 Research gaps in this study

Abiotic and biotic constraints are the major causes of sub optimal crop yields leading to food insecurity. Development of both biotic and abiotic constraints resilient crops is key towards sufficient food production. However, crop improvement through traditional ways remains a challenge calling for the application of the new breeding technologies such as gene silencing. Biotech-based crop improvement tools are highly precise hence, deemed to provide rapid solutions to the current food crisis. However, one main requirement of crop improvement through bioengineering is existence of an existing in-vitro regeneration platform. However, in-vitro regeneration remains a bottleneck due to genotypic recalcitrance.

The origin of maize chlorotic mottle virus (MCMV) in East Africa remains unclear. A number of hypotheses have been put fourth including the use of highly susceptible planting materials. Moreover, the change of climatic conditions leading to the exponential increase in population of thrips and the central role of maize in the East African diet could have also led to the escalation of the disease. Questions still linger on MLN re-emergence, how the disease spread, the contributing factors to its vast spread, what might have caused its vast spread within a short time and if the spread was caused by a non-indigenous pathogen or a lag in the host immune system. Therefore, understanding the MCMV epidemiology, preventive measures, and transmission mechanisms through seed and soil would be important. The existing risk index-based epidemiology and hot spots mapping remains underexploited. Reports also show that researcher-farmer communication on MLN remains low, a scenario deemed to be the contributing factor to the frequent outbreaks.

Information on MLN resistance gene (s) and their mechanism of action is scanty. Similarly, the information on the interaction between MCMV-potyviruses and other putative MLN associated viruses such as poliovirus and totivirus is limited. Moreover, information, particularly on the beetle and thrip species, vector population dynamics, the causes of long and short distance movement and the diversity of MCMV transmitting vectors is inadequate.

Not excluding clarity on the role of social and economic practices towards MLN management and agronomic practices that might have exacerbated disease incidences. Therefore, providing answers to these questions would be a step forward towards effective MLN management particularly, in the sub-Saharan region where food security is based on maize availability.

2.28 Ongoing research on genetic engineering for MLN resistance

Kenya is a frontier of biotech in the East Africa region. In partnership with Corteva Agriscience (USA), Kenya Agricultural and Livestock Research Organization (KALRO) and CIMMYT on MLN resistance CRISPR-Cas9 edited maize is ongoing in Kenya. Four CIMMYT parent maize inbred lines and two MLN susceptible commercial hybrids were selected for genetic improvement through CRISPR/Cas 9. In CRISPR-edited MLN-resistant maize, the host genes were targeted for silencing while in the present study; genes of SCMV, one of the MLN causing viruses were targeted. Views of the public were also considered during the development of the MLN resistant CRISPR edited maize. This was important as lack or inadequate public engagement has caused rejection or low adoption of key biotechnological inventions globally. The MLN resistant CRISPR edited maize project is anticipated to produce a low-cost product for accelerated adoption, particularly, in MLN prevalent countries. However, implementation of this project remains a challenge due to its cost and ethical concerns. Subject to compliance to regulatory framework, commercial seeds of CRISPR-edited MLN-resistant maize would be available by 2025. The seed materials are predicted to be of economic significance particularly to smallholder farmers who are mostly affected (Boddupalli *et al*., 2020; CIMMYT, 2022). Both transgenic RNA induced gene silencing and base editing through CRISPR-Cas9 approaches utilize gene silencing. However, the former functions by reducing gene expression while the later completely and permanently silence the target gene/s.

CHAPTER THREE

OPTIMIZATION OF IN-VITRO REGENERATION SYSTEM FROM IMMATURE EMBRYOS OF MAIZE INBRED LINES

Abstract

Maize (*Zea mays* L.) is a popular crop and one of the most important cereals worldwide. International Maize and Wheat Improvement Center maize lines (CML), CML444 and CML442 are commonly deployed in maize breeding due to high general and specific combining abilities (GCA/SCA). However, the two inbred lines are highly susceptible to maize lethal necrosis disease (MLN). To provide a platform for bioengineering for MLN resistance, this study optimized a regeneration system for the two inbred lines. Successful maize regeneration is evident despite the existence of genotypic recalcitrance. The objective of this experiment was to test the *in-vitro* regeneration ability of CML444 and CML442. Plant growth regulators (PGRs) are critical during *in-vitro* regeneration, thus the influence of different PGRs were tested in different regeneration phases. Callus were induced at 0, 2, 3 and 4 gm/L concentrations of 2, 4-dichlorophenoxyacetic acid (2, 4-D) in Murashige and Skoog (MS) media; shoot regeneration was tested at 0 mg/L 6-benzyl aminopurine (BAP)/ 0 mg/L *α-napthaleneacetic acid* (NAA); 0.5g/L BAP/ 1 mg/L NAA; 1mg/L BAP/1.2 mg/L NAA and 1.5 g/L BAP/1.4 g/L NAA; and root formation tested at 0, 0.2, 0.3 mg/L indole-3-butyric acid (IBA) levels. The experiment was laid in a completely randomized design (CRD) with three replications. Callus induction was tested in MS medium supplemented with 0, 2, 3 and 4 gm/L of 2, 4-D. This was followed by callus maintenance in MS supplemented with 2 mg/L of 2, 4-D. Somatic embryo maturation was achieved in MS medium supplemented with 60 g/L of sucrose devoid of PGRs. The 2, 4-D rates had significantly different effects at ($p \leq 0.001$) for callus induction. The highest number of callus was obtained at 2 mg/L followed by 3, 4 and 0 mg/L at 96.50%; 74.17%; 26.67% and 2.50%, respectively. The interactive effect of line × rate demonstrated that 2 mg/L of 2, 4-D had the highest callus induction. However, CML444 had the highest callus induction frequency of 51.42% as compared to CML442, which recorded a frequency of 48.50%. During shooting, responses of the lines were significant at ($p \leq 0.001$). The inbred line CML442 recorded a mean of 8.90 as compared to 6.85 of CML444. The PGR rates and the interaction of lines × PGR was not significant in both maize lines. The lines, rates and the interaction IBA rates × lines were significant at ($p \leq 0.05$) during root induction. This protocol was effective for plant regeneration, therefore, can be considered for future genetic transformation applications.

3.1 Introduction

Establishment of a suitable regeneration system is a prerequisite for recombinant DNA technology-based crop improvement (Kalaitzandonakes *et al*., 2023). Genetic transformation can be performed via *in planta* or *in-vitro* regeneration. Of the two, transformation through *in-vitro* regeneration is the most preferred as it ensures stable transgene integration. However, genotypic recalcitrance poses a major challenge during *in-vitro* regeneration, particularly, in maize, rendering most genotypes inaccessible to recombinant DNA (rDNA)-based upgrading (Muppala *et al*., 2020). Furthermore, *in-vitro* regeneration has been constraint by the type of explant, culture media and culture conditions used as reported by Mostafa *et al*. (2020).

In-vitro regeneration can be achieved either through organogenesis or somatic embryogenesis. However, regeneration through somatic embryogenesis has a couple of scientific benefits. Somatic embryogenesis constitutes of rapidly growing transformable embryogenic cells. The method enables for the production of bipolar structures that are potentially capable of germinating directly into a full plant evading several de-differentiation cycles in organogenesis that would cause transgene mutation (Kessel-Domini *et al*., 2022). Conservation of genetic integrity is also guaranteed in somatic embryogenesis because the shoots and roots of zygotic embryos are well connected having originated from a single or mass of closely related cells. Furthermore, somatic embryogenesis could allow for automation and mass productions, particularly, with the deployment of bioreactors (Fraga *et al*., 2023). Pawar *et al.* (2015) also reported that somatic embryogenesis provides a platform for production and encapsulation of somatic embryos into artificial seeds. This culture system can also accelerate propagation through the elimination of apical dominance which guarantees efficient and reliable multiplication of seeds.

Because *in-vitro* regeneration is performed in precisely controlled environmental conditions, evasion of the effect of tropism or external growth stimuli is eminent. Tropism influences cells to take multiple stimuli converting them into auxin transport that drives tissue level growth and remodelling, causing change in plant shape and position with respect to stimuli, a scenario that cause deformation in the culture products (Moulton *et al*, 2020). Moreover, *in-vitro* regeneration allows for the use of diverse explants, such as mature embryo, coleoptile nodes nodal regions, leaf tissues, anther, tasselled, ear meristems, protoplast culture and shoot meristem (Ye *et al*., 2022).

Despite the availability of a wide range of explants, immature embryos remain the most preferred *in-vitro* regeneration explant. Immature embryos are highly competent and therefore easy to induct callus. However, it requires that this type of explant be used within a specified window which has significantly constraint their applications in *in-vitro* regeneration (Abebe *et al*., 2008).

Various studies have demonstrated success in *in-vitro* regeneration of maize through immature embryos. However, the main challenge in maize regeneration lies on genotypic composition of the explant and the ability to respond to plant growth regulators (PGRs) presenting a significant challenge in the improvement of elite varieties through genetic transformation (Mostafa *et al*., 2020). *In-vitro* PGRs play a critical role during *in-vitro* regeneration. For instance, 2, 4-dichlorophenoxyacetic acid (2, 4-D), is the most utilized PGR during callus induction and maintenance. Moreover, experiments in wheat have confirmed that 2, 4-D synthesis is at its prime during callus induction, an indication that auxins are the cornerstone for callus formation. Maize regeneration can occur in the absence of plant growth regulators (Oduor *et al.* 2006) however, both auxins and cytokinins have also been utilized for callus induction, with literature indicating that auxins alone are less influential on callus induction in wheat as compared to the combination of the two (Malik *et al.*, 2004). Although root formation can also occur in the absence of PGRs, some genotypes have a poor root induction potential in the absence of PGRs. In such cases, a supplement of auxins such as indole-3-butyric acid (IBA) is important to trigger root formation. In addition, research has proved that IBA is potentially capable of enhancing rooting and/or reducing rooting durations (San José *et al*., 2012). Therefore, testing for different PGR ability to induct callus, shoots and rooting as a parameter of *in-vitro* regeneration in CML44 and CML442 was pivotal.

3.2 Materials and Methods

3.2.1 Experimental site and study materials

The experiment was conducted at Field 3 and the Kenya-China Joint Laboratory for Crop Molecular Biology at Egerton University for the period June 2020 to March 2021. Maize inbred lines CML444 and CML442 were acquired from CIMMYT-Kiboko, Kenya. Selection of the lines was based on high general and specific combining abilities and MLN susceptibility.

3.2.2 Growing of immature embryo donor maize

Donor plants were grown in the greenhouse according to Abebe *et al*. (2008) and Masters (2020) protocols with modifications. Black polythene bags measuring 30×20×20 cm filled with 18 kg of normal garden soil were used for sowing (Figure 3.1) and four seeds sown per bag. One hundred grams of fertilizers, N-P-K in the ratio15-9-12, was introduced into each bag 2 weeks of planting because stored food in the sowed seeds was depleted. This would foster shoot and root development for improved nutrient acquisition in the rapidly developing seedlings. Essential agronomic practices were maintained and watering was done daily in the evening. Seedlings were thinned to 2 per pot at 14 days of planting. Planting was staggered at 14 days' interval to ensure a continuous supply of immature embryos. Upon tassel emergence, both ears and tassels were covered with brown bags to curb cross pollination. Three days post tasselling, covered tassels were bent and tapped for pollen collection. Silk ear bags were removed and with the aid of a sterile blade, silks were cut and selfing performed out. Screening for embryo size was performed from day nine of pollination and harvesting done from day 21-28 post pollination.

3.2.3 Harvesting of immature embryos and sterilization

Immature embryos were selected and harvested between of 21-28 days post pollination. Explant sterilization was carried out based on Abebe *et al*. (2008) and Masters (2020) protocols with modifications. The cobs were dehusked and soaked in 50 % NaOCl and 0.01% TWEEN20® solution for 10 minutes. Rinsing was done three times in sterile distilled water with 3 min of each rinse. The materials were transferred to 70% ethanol for 3 min and rinsed again in cold autoclaved distilled water three times with 3 min of each rinse. Ears were cut open at the top and immature embryos picked out.

3.2.4 Media preparation and explant inoculation

The experiment was laid in a completely randomized design with three replicates. Explant inoculation, callus induction and maturation were carried based on Bohorova *et al*. (1999) and Holderbaum *et al*. (2019) with modifications. Media was prepared by amending Murashige and Skoog (MS) media with 100 mg L^{-1} casein hydrolysate, 2 g/L L-Proline, 10 mg/L silver nitrate, 30 g/L sucrose and four levels of 2,4-D (0, 2, 3 or 4 mg/L) reconstituted. Media pH was adjusted to 5.8 followed by the addition of 3 g/L Gelrite and autoclaving at 121˚C for 15 min. Exactly 30 ml of MS media was dispensed into 100 mm × 15 mm diameter petri plates. Ten embryos were placed in each petri plate with embryo axis in contact with callus induction media. A total of 320 embryos were inducted for each line. Plates were kept in the dark at 26 ± 2˚C. Excision of precocious germinations followed two days post-inoculation. Callus induction was carried out for 2-weeks with every 2-day observation interval and the induction rate recorded. Callus induction was followed by callus maintenance for 4 weeks in MS media amended with 30 g/L sucrose and 2 mg/L of 2,4-D following the same media preparation procedure as callus induction media. Cullus were sub cultured at 2-week interval and necrotic calli were discarded in every sub-culture. Callus induction rate was observed after every 3-day interval for 2-week induction period. Callus were also observed after every 5-day during callus maintenance.

Calli were transferred to maturation media on the sixth week of callus induction. MS media was prepared by adding 100 mg/L casein hydrolysate, 2 g/L of L-proline, 10 mg/L silver nitrate and 60 g/L sucrose to MS media. Plant growth regulators were excluded during this phase. Media pH was adjusted to 5.8 followed by the addition of 3 g L^{-1} Gelrite and autoclaving at 121˚C for 15 min. Exactly 30 ml of media was dispensed into 100 mm × 15 mm diameter Petri plates. Ten calli of about 10 mm were placed in each petri plate. A total of 280 calli for CML444 and 210 calli for CML442 inbred lines were subjected to maturation for 4 weeks with fresh media change at 2-week interval. Calli were observed after every 3-day interval for the 4-week culture period.

3.2.5 Plant regeneration

Shoot, induction was based on the protocol by Pathi *et al.* (2013). The experiment was laid in a completely randomized design with three replicates. Murashige and Skoog media amended with 30 g/L sucrose and four treatments of BAP and NAA (0 mg/LBAP and 0 mg/L NAA, 0.5g/L BAP and 0 mg/L NAA, 1mg/L BAP and 1.2 mg/L NAA, 1.5 g/L BAP/1.4 mg/L NAA). Media pH adjustment and autoclaving were performed as in the callus induction section. Exactly 40 ml of the media was dispensed into each culture bottle of 100 × 50 mm. Three calli of approximately 10 mm were inoculated in each culture bottle and placed at 26 ± 2˚C under 16 h light/8 h dark photoperiod. A total of 250 calli for CML444 and 190 calli for CML442 were tested for shooting. White fluorescent lamps emitting approximately 130 $\mu mol/m^2/s$ of photosynthetically active radiation (PAR) at the surface of the bottles were used for lighting. Plant formation was observed after every 5-day interval for the 4-week culture period and data on the number of shoots formed recorded.

Root induction was based on Pathi *et al.* (2013) protocol. The experiment was laid in a completely randomized design with three replications. MS media with varied IBA treatments of 0 mg/L, 0.2 mg/L and 0.3 mg/L was reconstituted. Media pH adjustment, autoclaving and dispensing was performed as in the shoot induction section. Five CML442 and 3 CML444 shoots of about 3 cm were excised and transferred into rooting media. Plant incubation was performed as indicated in the shoot induction section. Root formation was observed after every 5-day interval for the 4-week culture period and the number of plants that were able to form roots recorded.

3.2.6 Acclimatization and greenhouse transfer

The plantlets were washed with tap water to remove culture media and transplanted into 3 × 3 × 3-inch pots containing autoclaved pre-wetted peat moss. The pots were covered with a plastic humidity dome, with two 1mm holes and placed on a 30 cm x 50 cm tray. The pots were then placed on the laboratory bench at 25˚C Day/20˚C night for three days in natural light. Domes were removed after 5 days and plants transferred to the greenhouse with an average temperature of 32˚C day and 20˚C night at Egerton University (35˚56' 5.04" E; 0˚22' 28.08" S). Watering was done moderately to avoid drying and waterlogging to encourage proper root development. For complete recovery from post-transplant stress, plants were left undisturbed in peat moss for two weeks before transfer to a 1:1 ratio of loam-sand soil mixture in larger pots of 6 × 6 × 6-inch. Frequent watering was maintained for proper plant development. Plant formation was observed after every 5-day interval for the 4-week culture period.

3.3 Data collection and statistical analyses

Data was collected on the rate of callus formed in the four 2, 4-D regimes, plants regenerated per line and roots formation. Data on frequence of callus induction, shoot and root formation was transformed using square root transformation and was subjected to general linear model (GLM) for analysis of variance (ANOVA) using SAS software version 8.2. Mean separation was conducted using Fisher's Least Significant Difference test at $P \leq 0.05$ level of significance.

Analysis of the effect of 2, 4-D on callus induction was based on the model below.

$$Y_{ijkl} = R_i + L_i + T_k + LT_{ik} + \varepsilon_{ijkl} \ldots\ldots\ldots\ldots\ldots\ldots \text{Equation 1}$$

Where, Y_{ijkl}=observation of the experimental units, R_i=effects due to replicates, L_i=effects due to lines, T_k=effects due to treatments (2, 4-D hormones), LT_{ik}=interaction effects due to lines and treatments, ε_{ijkl}=residual

Analysis for the effect of BAP and NAA on shoot formation was based on the model below

$Y_{ijkl} = R_i + L_i + H_k + LH_{ik} + \varepsilon_{ijkl}$……………………………………………..Equation 2

Where, Y_{ijkl}=observation of the experimental units, R_i=effects due to replicates, L_i=effects due to lines, H_k=effects due to BAP and NAA hormones, LH_{ik}=interaction effects due to lines and hormones, ε_{ijkl}=residual

Analysis for the effect of IBA on root formation was based on the model below

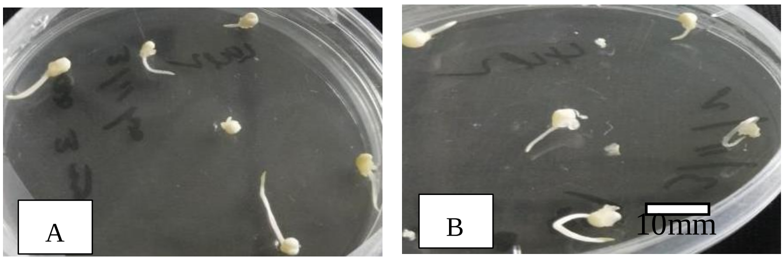
$Y_{ijkl} = R_i + L_i + I_k + LI_{ik} + \varepsilon_{ijkl}$…………………………...…………………..Equation 3

Where, Y_{ijkl}=observation of the experimental units, R_i=effects due to replicates, L_i=effects due to lines, I_k=effects due to IBA hormone, LI_{ik}=interaction effects due to lines and IBA, ε_{ijkl}=residual.

3.4 Results and discussion

3.4.1 Callus induction

One day after inoculation, immature embryos had increased in size due to imbibition. Callus emergence was signified by the opening of the scutellum and the appearance of a white to pale yellow amorphous growths from day three and day five of callus induction for CML444 and CML442, respectively. The onset of callus was also characterized by the appearance of precocious germinations (Plate 3.1).



CML444 CML442

Plate 3.1: Precocious germinations at day 3 of callus induction in CML444 and CML442.

Effects due to maize lines were not significant ($p \leq 0.05$) for callus induction. However, the effects of 2, 4-D rates were significant at ($p \leq 0.001$) on callus induction while the interaction effects due to maize lines x 2, 4-D rates were not significant at ($p \leq 0.05$) on callus induction (Table 3.1).

Table 3.1: Mean squares of the analysis of variance for callus induction for maize lines CML442 and CML444 at different rates of 2, 4-D hormone in MS media

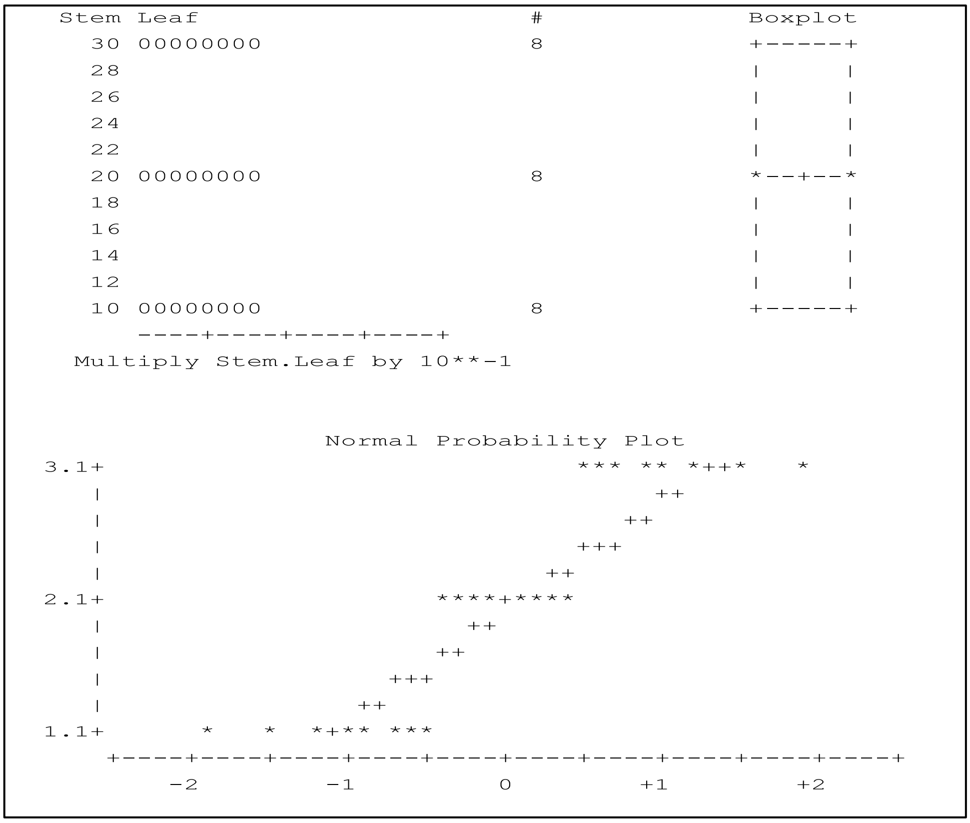
		Callus induction			
Source of variation	**df**	**Sum of squares**	**Mean squares**	**F-value**	**P-value**
Replicates	2	8.33	45.167	1.30	0.3030
Maize Lines	1	51.042	51.042	1.47	0.2452
2, 4-D Rates	3	33281.79	11093.931***	319.80	<.0001
Lines × Rates	3	28.13	9.375	0.27	0.8458
Error	14	6799.34	485.667		
R^2			0.986		
CV (%)			11.590		

*** Significant at $p \leq 0.001$, rates= 2, 4-D, CV=coefficient of variation

The normality test from Shapiro wilk test (**W**=0.797272) showed that the data was normally distributed (Table 3.2). Furthermore, the normal probability plot test showed that the data was symmetrically distributed (Figure 3.1).

Table 3.2: Shapiro wilk test for shooting induction

Tests for Normality				
Test	**Statistic**		**p Value**	
Shapiro-Wilk	W	0.797272	Pr < W	0.0003
Kolmogorov-Smirnov	D	0.218061	Pr > D	<0.0100
Cramer-von Mises	W-Sq	0.264485	Pr > W-Sq	<0.0050
Anderson-Darling	A-Sq	1.87881	Pr > A-Sq	<0.0050



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    Stem Leaf                     #             Boxplot
      30 00000000                 8             +-----+
      28                                        |     |
      26                                        |     |
      24                                        |     |
      22                                        |     |
      20 00000000                 8             *--+--*
      18                                        |     |
      16                                        |     |
      14                                        |     |
      12                                        |     |
      10 00000000                 8             +-----+
        ----+----+----+----+
     Multiply Stem.Leaf by 10**-1


                     Normal Probability Plot
   3.1+                              *** ** *++*   *
      |                                   ++
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   2.1+                     ****+****
      |                       ++
      |                     ++
      |                  +++
      |                ++
   1.1+      *   *  *+** ***
       +----+----+----+----+----+----+----+----+----+----+
           -2        -1         0        +1        +2
```

Figure 3.1: Probability plots showing normality for rooting induction

Callus induction means of the lines were not significant at (*$p \geq 0.05$*) (Table. 3.3). However, callus induction means for the 2, 4-D rates were significant at (*$p \leq 0.05$*) for callus induction. The hormone 2, 4-D at 2 mg/L had the highest induction frequency followed by 3 mg/L, 4 mg/L and 0 mg/L, respectively (Table 3.4).

Table 3.3: Means for callus induction for the two genotypes (CML442 and CML444) in MS media.

Maize lines	Callus induction (%)
CML444	51.417a
CML442	48.500a
LSD $_{0.05}$	5.1572

Means followed by same letter are not significantly different at $p \leq 0.05$

Table 3.4: Means for callus induction at different rates of 2, 4-D for maize lines CML444 and CML442**.**

2, 4-D rates	**Callus induction (%)**
0	2.50d
2	96.50a
3	74.17b
4	26.67c
LSD $_{0.05}$	7.30

Means followed by same letter are not significantly different at $p \leq 0.05$

The interaction between the lines and rates showed that CML444 had the highest induction rate of 99 at 2 mg/L as compared to 94±0.372 in CML442. Among the four rates 2 mg/L had the highest callus induction mean followed by 3 mg/L, 4 mg/L and 0 mg/L, respectively in both CML442 and CML444 (Table 3.5). There was low callus necrosis at 2 mg/L as compared to 4 mg/L.

Table 3.5: Means for callus induction in CML lines 442 and 444 at different 2, 4-D concentrations.

Lines	**2, 4-D rates**	**Callus Induction (%)**	**±**	**se**
CML442	0	1.667	±	2.236
CML442	2	94.000	±	0.372
CML442	3	71.667	±	0.902
CML442	4	26.667	±	0.559
CML444	0	3.333	±	3.162
CML444	2	99.000	±	0
CML444	3	76.667	±	1.319
CML444	4	26.667	±	1.118

CML= CIMMYT Line, Se=standard error

The use of 1.6-2 mm immature embryos and orientation of the embryo axis in contact with the media in this study was in line with. Between 1.5-2 mm immature embryos are highly totipotent leading to the rapid formation of amorphous growths referred to as callus. Placing the thin-walled scutellum region in contact with media also allowed for efficient entry of nutrients as reported by Wu *et al*. (2022). Similar to other plants, the scutellum region of maize has been found to possess a dense pack of highly totipotent parenchyma cells to allow for imbibition of nutrients required for embryo development. These cells multiply rapidly upon nutrient acquisition to form callus during *in-vitro* culture (López-Ruiz *et al*., 2019).

Appearance of callus in day 4 and 5 of callus induction in both CML444 and CML442 inbred lines was in agreement with findings of Abebe *et al*. (2008). This could be due to optimal growth conditions leading to the influx of substances into the scutellum region and an increase in embryo volume. Plant growth regulators (PGRs) such as 2, 4-D auxin induce callus formation in many plant species. As an illustration, the docking of exogenously supplied 2, 4-D in both maize lines caused a decrease in the cell wall pressure rendering plant cells water permeable. This caused an influx in intracellular solutes as reported by Saputro *et al*. (2017) leading to volume doubling and callus formation. Noor *et al*. (2022) illustrated that 2, 4-D and other physical/environmental parameters could have stimulated CML444 and CML442 embryos to expand and form amorphous cell growth, also known as callus. In addition, studies by Pradipunt *et al*. (2022) demonstrated that 2, 4-D can be used singly or in combination with a cytokinin for callus initiation. For instance, 2, 4-D has been used in combination with 6-Benzylamunopurine (BAP), or Kinetin for callus induction. Auxins or auxin in combination with other PGRs function to trigger processes of cell division and elongation leading to callus formation as reported by Wijerathna-Yapa and Hiti-Bandaralage (2023).

Both Pawar *et al*. (2015) and Ward *et al*. (2001) reported that precotious germinations also referred to as vivipary or pre mature germination can occur while seeds are still attached to the parent plant during *ex-vitro*. The process occurs when the embryo completes its normal development leading to the appearance of appendages that are also referred to as weak seedlings. The presence of precotious germinations as observed in this study is common in immature/mature embryo-initiated cultures. Germinations of these outgrowths has been found to be caused by the loss of embryo development inhibitors due to the removal of the seed coat. This event highlights separation these inhibitors from the seed coat an event that eliminates seed dormancy. However, precotious germinations have been reported to inhibit the morphogen potential of the region and that of somatic embryos multiplication. Therefore, their excision is important to encourage callus development despite the existence of contradictory findings.

Similar to this study, variation in callus induction rates have been witnessed across different 2, 4-D regimes, however, according to Çabuk and Özgen (2016), optimum callus induction for most *poaceae spp* occurs at 2 mg/L. These variations could be influenced by physicochemical and/or genotypic factors. For instance, Ramsay *et al*. (2003) reported that basal meadia, PGRs and dark and light treatments could influence callus formation. Therefore, the choice of callus induction media has a significant influence on callus induction. For instance, MS media has been found to confer better callus induction ability.

Light and dark conditions could also have influenced callus formation in this study based on Zenser *et al*. (2001) findings. For instance, phenolic oxidation normally occurs under light conditions leading to browning and death of cultures. Therefore, carrying out callus initiation and maintenance in dark conditions as in the case of the present study was important for optimal development. Darkness functions by degrades auxins hence, regulating auxin-cytokinin ratios leading to optimum callus formation. Moreover, Dai and Castill (2007) reported incubation in dark conditions reduces cell wall formation through restricting cell wall deposits, an event that leads to intracellular influx of substances leading to optimal formation of callus. Dark conditions can also function to induce genetic factors in favour of optimal callus formation based on Yu *et al*. (2019) findings. The upregulation and expression of somatic embryogenesis marker genes, such as *WUS*, *BBM*, and leafy cotyledon 2 (*LEC2*) is highly dependent on the availability of dark conditions. These genes have been found to positively influence callus formation.

Media incorporation of casein hydrolysate and L-Proline in the case of the present study was fundamental in the induction of callus as Khaleda and Al-Forkan (2006) findings indicated that the two supplements were integral to callus formation. The two are important sources of amino acids that function as protein building blocks. Casein hydrolysate enhances nitrogen availability, improving on its processing and transportation from organic sources. A process that functions to accelerate callus development and the improvement of morphogenetic activities of the explant leading to better callus formation, particularly, embryogenic callus. Embryogenic callus are associated with high induction of somatic embryo that have a higher plantlet conversion rate. Moreover, somatic embryos offer a potential platform for genetic transformation due to their tolerance to *Agrobacterium* and fewer de-diferentiation cycles that are likely to present fewer chance of transgene mutation (Pasternak & Steinmacher, 2024). On a study conducted on wheat Hassan and Islam (2021) reported that the inclusion of silver nitrate as in the case of the present study enhances the formation of embryogenic callus, and reduce hyperhydricity, thus, improving on tissue regeneration.

Moreover, Pawar *et al*. (2015) demonstrated that the use of amino acids as in the present study is important in the acceleration of embryo growth, callus formation and regeneration. For instance, casein hydrolysate contains about 18 amino acids making this media constituency integral for *in-vitro* regeneration as compared to proline and glutamine. However, incorporation of glutamine and/or proline into callus induction media has been found to intensify callus formation in rice. This is because both amino acids are important sources of organic nitrogen. Quality calli have a significant influence on the overall *in-vitro* regeneration and transformation. Studies have demonstrated that there was a higher callus activities in media incorporated with both amino acids. Similarly, the same media was reported to have a higher regeneration potential. However, both callus induction and regeneration are highly varied in monocots leading to significant differences in media constituents. Moreover, casein hydrolysate can be substituted by proline, serine or glutamine during somatic embryo/ callus maturation.

Pawar *et al*. (2015) also indicated that the level of sucrose and agar could cause variations in callus induction. Sucrose is an important energy source during *in-vitro* culture besides its use in osmotic potential maintenance. Immature embryos require sucrose concentrations of 8% to 30% because younger embryos require higher osmotic potentials that mimic that of the embryo sac. Moreover, higher osmotic potentials were found to prevent precotious germinations by keeping cells in mitotic state rather than elongation state. Agar should be maintained at certain concentrations, for instance, 0.5% to 1.8% is optimal for embryo culture. Higher concentrations of agar could have negatively influenced culture the development of CML444 and CML 442 by reducing water availability.

The variations in callus formation in CML444 and CML442 could have also been influenced by genetic factors. Tomes and Smith (1985) reported that the presence and expression of primary response genes or the expression of genes in chromosomes 1, 2, and 9 could cause variations in callus induction in maize. Furthermore, the expression of QTLs in chromosomes 1, 3 and 8 has been found to cause variations in callus formation in maize (McFarland *et al*., 2023). Fan *et al*. (2012) demonstrated that the ectopic expression of primary response genes such as lateral boundary domain (LBD) transcription factors would also negatively influence callus formation. Thus, incorporation of 2, 4-D in the culture media was important for the inhibition of LBD expression based on Long *et al*. (2022) findings.

Auxin-dependent upregulation or downregulation of callus-induction transcription factors such as MYB15 in CML444 and CML442 could have triggered callus formation as reported by (Gao *et al.* 2019). Gene MYB15 has the potential to modulate the auxin-inducible genes through its interaction with Auxin Response Factors 43 (ARFs43) (Shin *et al.*, 2007). The surge in the expression of MYB15, IAA18 and IAA29 cause CDKB2-2 and CYCD3-1 upregulation which promote auxin-cytokinin signalling pathways during callus development (Abel *et al.*, 1995). Similarly, Panda *et al.* (2018) demonstrated that the upregulation of GH3.1 and A-ARR5 genes transcription factors are important for callus appearance which could have been the case with CML444 and CML442. These transcription factors are important in the regulation of other pathways such as DNA replication, zeatin biosynthesis, glutathione metabolism and photosynthesis processes that are pivotal to callus development as reported by Roudier *et al.* (2003) and Stals *et al.* (2001). For instance, zeatin biosynthesis, DNA replication and glutathione metabolism are central to the initiation of cell division and callus formation processes enhance antioxidant defence, nutrient acquisition, and regulation of cellular events during callus initiation and proliferation (Gao *et al.*, 2019).

The expression of GRMZM2G315375 (br2) which is responsible for encoding the P-glycoproteins in maize could have also influenced callus formation and its variations in CML444 and CML442 as reported by Nakashima *et al.* (2023). These proteins are central to embryo differentiation and auxin translocation. The br2 gene products cause DNA hyper methylation, an event that maintains high mitotic activities in the cell which leads to callus formation. Genetic reprogramming of parenchyma cells in the CML444 and CML442 inbred lines might have also acted to influence the formation of callus. Cellular reprogramming however is dependent on a cascade of reactions including cytokine-auxin-based and wound-induced routes that lead to the formation of meristematic cells that in turn accelerate cell division and callus formation (Fehér, 2019).

Pawar *et al.* (2015) reported that the use of immature embryo or embryo culture contains a number of scientific benefits. For instance, embryo culture could easily overcome seed dormancy thus, shortening breeding cycles. Dormancy in seeds is influenced by physicochemical parameters such as temperatures, light, plant hormones and embryo immaturity. Some of these factors are localized in the testa and extraction of the embryos leads to its separation from the influence of these factors leading to rapid germination. Besides, embryo culture is fundamental in basic research particularly in nutrition and metabolism studies of the embryo. These studies are integral to the provision of insights on sites of germination enhancers and inhibitors which are important for embryogenesis and cryopreservation. Generation of haploids during embryo culture has also been used in the fusion of important traits particularly in the fusion of maternal traits where paternal genes have been deactivated. In this case, fertilization takes place, however, the male paternal chromosomes are eliminated through embryo culture and a homozygote is created through chromosome doubling. Embryo extraction also causes 'cooling' during germination significantly reducing the generation time. Embryo cultures can be used to test for regeneration potential and for vegetative propagation. Embryo cultures are important in the study of precotious germinations for understanding activities during embryo development including the physicochemical factors behind embryo development. Embryo culture can also be used in the evaluation of seed viability and for rapid testing of seed viability after the elimination of seed dormancy barriers. Immature embryos are highly meristematic, juvenile tissues such as immature embryos are reliable for the induction of organogenesis and somatic embryogenesis. Despite the significant benefits of using immature embryos during *in-vitro* regeneration, the use of mature seeds during in-vitro activities has been found to possess a couple of benefits as they are available throughout the year with no limited window. Mature seeds are less likely to be exposed to chemicals and various agents during extraction and sterilization which would cause mutation as compared to immature embryos.

3.4.2 Callus maintenance

Adventitious rooting was observed on the callus surface from week 2 of callus maintenance (Plate 3.2 A: C: D). Two distinct types of calli were present; type I compact and type II friable callus (Plate 3.3 A, B). CML444 had the highest frequency of Type II callus of 80% at 2 mg L^{-1} 2, 4-D and a minimum frequency of 20 % at 4 mg/L. CML442 had a maximum of 70% embryogenic (Type II) callus at 2 mg/L 2, 4-D with a minimum frequency of 20 % at 4 mg/L (Table 3.6). However, both lines had a lower frequency of Type I Callus averaging at 10%. The onset of callus greening was also observed at the fifth week of callus induction.

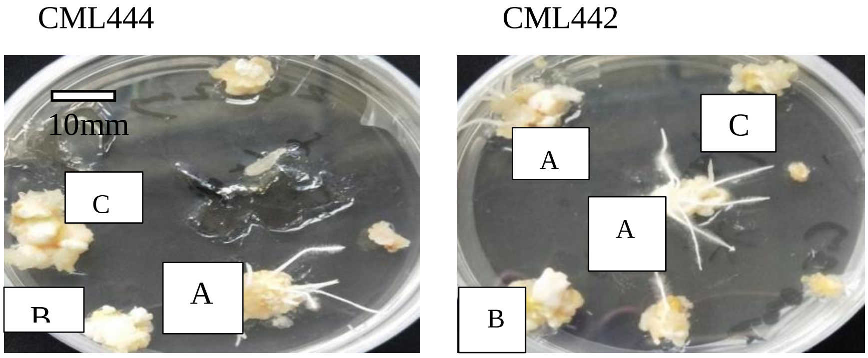


Plate 3.2: Maize line CML444 and CML442 at day 7 of callus maintenance showing (A) Adventitious roots during callus maintenance callus with cream tentacle like structure (B) Type I Callus (C) Type II Callus.

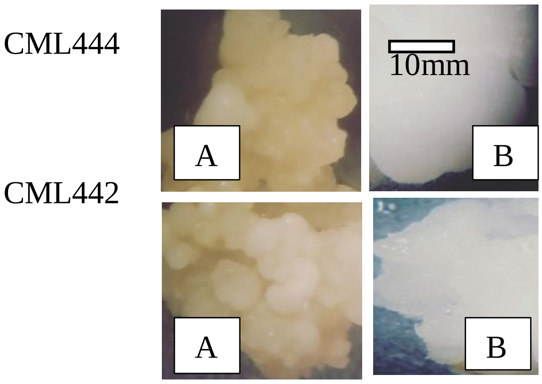


Plate 3.3: Types of callus at day 14 of callus maintenance showing A) Type II Callus B) Type I Callus in CML444 and CML442 under the Leica ZOOM® Stereo Microscope, USA at X35 magnification.

Table 3.6: Mean embryogenic and organogenic callus frequency for CML444 and CML442 maize lines under 2 mg/L 2, 4-D rates.

		Induction (%)	
2,4-D rates	**Callus Type**	**CML444**	**CML442**
2 mg/L	Embryogenic calli	80.00	70.00
2 mg/L	Non embryogenic calli	20.00	30.00

Literature indicates two types of callus are likely to be observed during *in-vitro* regeneration. Formation of these types of calli is dependent on genotypic and physicochemical factors. Similar to He *et al*. (2006) studies, two types of callus were observed in this study namely the Type I and Type II callus where the former had a lower frequency as compared to the latter. Ge *et al*. (2016) discovered that the ability to form both Type II Callus and Type I Callus is influenced by the ability of the explant to express and respond to endogenous hormones and exogenous PGRs. Moreover, the appearance of either type of calli can be caused by the different gene expression profiles. For instance, McFarland *et al*. (2023) indicated that the expression of *Wox2a* and *MYB15* genes was integral to the formation of embryogenic callus in maize. Krakowsky *et al*. (2006) reported that the presence and interaction of allele H99 at both loci resulted into a higher percentage of Type I Callus while the presence of the Mo17 allele at one locus and the H99 allele at the other caused a significant reduction in Type I Callus formation. This demonstrated that formation of Type I Calli could be dependent on quantitative loci (QTL) and epistasis interactions.

Tomes and Smith (1985) also reported that the appearance of both Type I and Type II Calli can be caused by genetic factors including additive gene effects or heterosis. For instance, a block of genes such as the WOX genes were discovered to play a significant role towards the induction of embryogenic callus. Moreover, *ex-vitro* studies have indicated that the maternal effect plays a key role in the formation of somatic embryos. For instance, embryogenesis emanates from reproductive tissues such as the nucellus and synergid cells, and somatic cells in ovules which originate from the female gametophyte. Similarly, Willman *et al*. (1989) affirmed that somatic embryogenesis and gene expression patterns in maize are controlled by a single gene or a block of genes. Binott *et al*. (2008) also reported that the formation of Type I and II embryogenic callus is also dependant on the expression of auxin or auxin analog receptor gene families such as *Aux/IAA* (auxin/indole-3-acetic acid), *GH3* (growth hormone 3) and *SAUR* (small auxin-upregulated RNA). For instance, Spartz *et al*. (2012) stated that the *ZmSAUR15* genes are responsible for the mediation of embryogenic callus formation in maize.

The appearance of adventitious rooting observed in this study could have been caused by the presence of 2, 4-D. Cortleven *et al*. (2019) reported that 2, 4-D plant growth regulator is capable of inducing adventitious roots in cultures, similar to the presence of endogenous cytokinin. Cytokinin, functions by transforming the cell fate leading to the formation of adventitious roots.

3.4.3 Callus maturation

The tentacle-like projections (somatic embryos) observed during culture induction and maintenance were intense during the callus maturation phase (Plate 3.4A). Most of the somatic embryos were pale yellow to cream while others had a greenish appearance (Plate 3.4B). Callus browning was also observed during this phase (Plate 5.5).

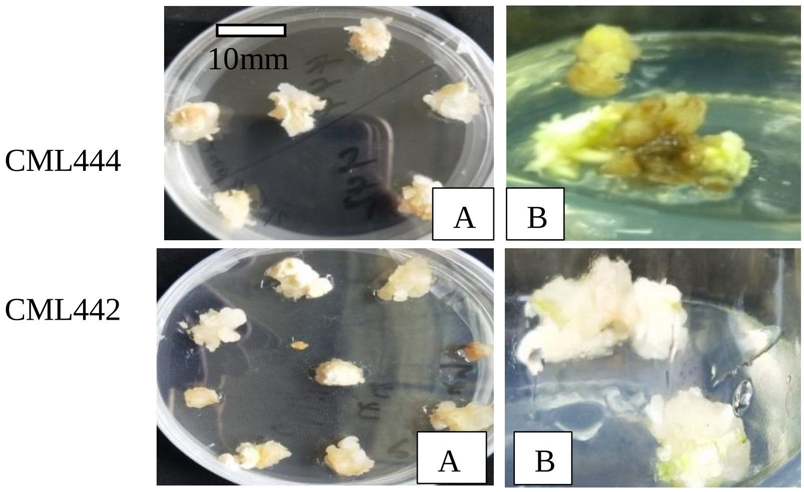


Plate 3.4: Maturation of callus showing (A) pale yellow and greenish somatic embryos at day 14 of callus maintenance (B) inbred lines at day 14 of maturation period in CML444 and CML442.

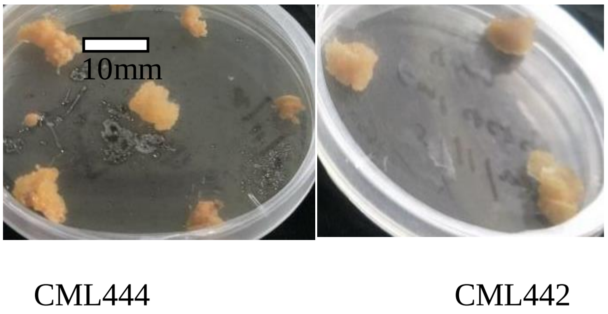


Plate 3.5: Browning of callus at day 7 of maturation period during in CML444 and CML442 inbred line.

Maturation of embryos is media specific. There is a wide spectrum of basal culture medium including Murashige and Skoog (MS), N6, Woody Plant Medium (WPM), and B5 that can be used for callus maturation. Saeed and Shahzad (2015) reported that MS as as used in this study improves maturation and guarantee higher rates of somatic embryo conversion. Sundararajan *et al*. (2017) also reported that MS media is a strong maturation enhancer which could have been the reason for maturation of CML444 and CML442 embryos. In comparison, N6 media is associated with better regeneration activities while intensification of somatic embryos development is associated with WPM (Saeed & Shahzad, 2015).

Carbon sources are also pivotal to somatic embryo maturation. Glucose, sucrose, and maltose are the commonly used energy sources during somatic embryo maturation based on Long *et al*. (2020) findings. However, despite the existence of a wide spectrum of carbon sources, sucrose is a preferred alternative since it can be used to induce maturation in diverse plant species as reported by Lipavská *et al*. (2012) which could have been the reason as to why callus maturation was achieved in CML44 and CML442 cultures.

Osmotic stress induction during callus maturation is a critical step in plant regeneration. For instance, Reyes-Díaz *et al.* (2018) indicated that higher concentrations of sucrose encourage somatic embryo formation however; beyond 80 g/L concentrations sucrose is likely to have inhibitory effects. Elevated sugar concentrations lead to a stressful osmotic environment that acts to enhance somatic embryo maturation while at low concentrations, sucrose has been found to promote somatic embryo formation (Nowakowska *et al.*, 2022). Ombori *et al.* (2008) indicated that osmotic stress functions to promote protein proliferation and in the downregulation of the expression of cell nuclear antigens, nucleases, proteases and glucosidases. These events act to reduce cell division and increase cell growth thus, accelerated development and formation of somatic bodies. Chen *et al.* (2022) discovered that callus greening as observed in this study, could be caused by overexpression of chlorophyll a and b coding proteins. Greening of callus is important as it improves nourishment during culture development as opposed to callus browning, which causes culture deaths. Ko *et al.* (2014) also reported that the maturation phase is significant towards successful regeneration as most of the genes responsible for root and shoot developments are also upregulated during this phase. For instance, upregulation of ABCG14 gene responsible for cytokinin translocation during shoot formation was observed during this phase.

Callus browning observed in this study was also reported by Singh (2018), a study that indicated that phenolic compounds produced from the injuries inflicted during explant extraction could be one of the causes. According to Eray *et al.* (2020), production of phenolic compounds and callus browning could also be triggered by abiotic and biotic stress. Therefore, the choice of carbon source is a critical factor towards the reduction of culture browning. Malik *et al.* (2017) discovered that the use of maltose reduced culture browning and recorded higher conversion rates of somatic embryos as compared to glucose and sucrose. However, this study preferred maturation under glucose because of its compatibility with diverse plant species as reported by Lipaviska *et al.* (2012). Callus browning in this study could also be caused by the denaturation of chlorophyllase or klorofilin enzyme due to abiotic stresses in the culture environment. Hu *et al.* (2021) reported that chlorophyllase enzyme or klorofilin enzyme is responsible for chlorophyll synthesis and its degradation would cause lack of greening in callus.

The time of explant collection could have influenced browning in this study. Das *et al.* (2016) illustrated that explant collection before the peak of phenols production particularly, before the onset of biotic and abiotic stresses should be considered. Stress exposure causes phenolic compounds accumulation in the explant which is responsible for culture browning. Furthermore, Gemechu and Amante (2021) indicated that media incorporation of antioxidants is important. Antioxidants such as nitric oxide, citric acid, ascorbic acid, activated charcoal and polyvinyl pyrrolidine and of 2-aminoindane 2-phosphonic acid function to reduce phenol production in the cultures. Moreover, the use of liquid culture or micrografting in the course of culture processes has been found to reduce phenol production. Liquid culture or micro grafting antagonize phenylalanine-ammonia-lyase activities, an enzyme responsible for polyphenol production (Jones & Saxena, 2013).

The ability of the explant to express certain protein could be the cause of callus browning in CML444 and CML442 cultures. For instance, the expression of polyphenol oxidases (PPOs) and peroxidase (POD) has been cited to be the cause of browning in cultures. Zhao *et al.* (2021) reported that the conversion of phenolic compounds to quinines by PPOs and POD enzymes contribute significantly to callus browning. The accumulation of phenolic compounds intensifies as the concentration and hyperactivity of PPOs increases. Browning of cultures occurs because phenolic compound production polymerizes cell proteins leading to a reduction in cell metabolism causing culture deaths.

Phenolic compounds production in CML444 and CML442 cultures could also have been triggered by overexpression of differential genes (DEGs) such as the Q03808_J11, GQ03806_D05, GQ03307_E08, GQ036606_J07, GQ03815_M16, WS00740_J05) and GQ01301_K10 as reported by Zhang *et al.* (2019). However, the expression of GRMZM2G108933 annotated as *WOX2* could reduce callus browning which might be the case with the regenerated CML444 and CML442 callus.

3.4.4 Shoot induction

Shoots were observed a day post shoot induction (Plate 3.6A) with multiple shoots observed a week later (Plate 3.6B). From the total 250 CML444 calli and 190 CML442 calli tested for root formation, eleven plants were formed in CML444 compared to 6 plants in CML442. Root formation was also observed during this phase despite the absence of the rooting plant growth regulators (Plate 3.6 B; C). It was noted that not all calli were able to form plantlets. Other callus in both CML444 and CML442 failed to shoot; generating roots instead (Plate 3.6C).

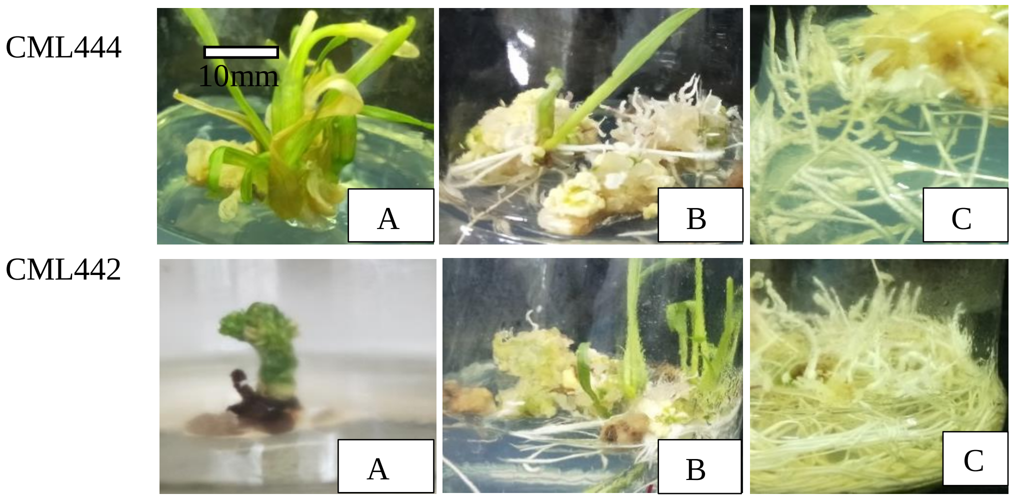


Plate 3.6: CML444 and CML442 showing (A) shooting onset at day 7 of shoot initiation and (B) developed shoots (C) adventitious rooting under shooting conditions at day 14 of shoot

Effects due to maize lines were significant ($p \leq 0.001$) for shooting induction. However, both the hormone effects and lines x rates interaction did not have significant effects on shooting induction (Table 3.7).

Table 3.7: Mean squares for shooting induction for maize lines CML444 and CML442 at different rates of BAP and NAA.

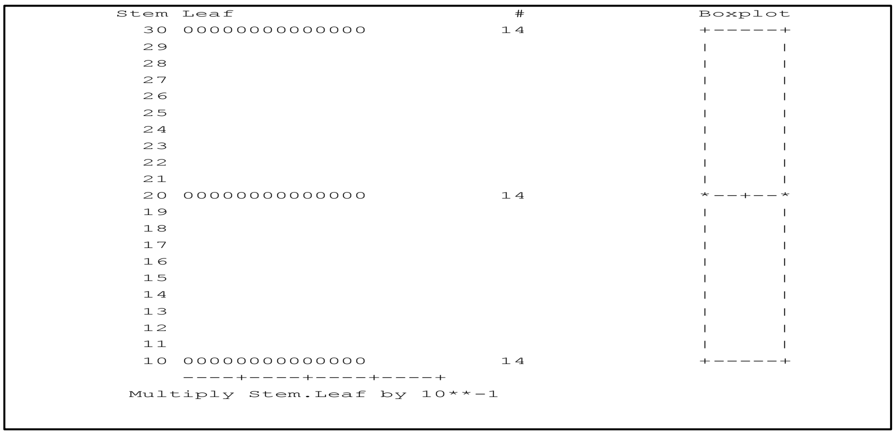
		Shooting induction			
Source of variation	df	Sum of squares	Mean square	F-value	P-value
Replicates	2	48.04	24.024	13.98	<0.00003
Maize Lines	1	42.88	42.875***	24.94	<0.00002
BAP/NAA	1	0.446	0.446	0.26	<0.61323
Lines × Rates	1	0.02	0.018	0.01	<0.92090
Error	36	61.88	1.719		
R^2			0.599		
CV (%)			16.634		

*** Significantly different at $p \leq 0.001$, CV=Coefficient of variation.

The normality test from Shapiro wilk test (**W**=0.781752) and normal probability plot showed that the data was normally distributed (Table 3.9; Figure 3.2).

Table 3.8: Shapiro wilk test for shooting induction

Tests for Normality				
Test	**Statistic**		**p Value**	
Shapiro-Wilk	W	0.793978	Pr < W	<0.0001
Kolmogorov-Smirnov	D	0.220209	Pr > D	<0.0100
Cramer-von Mises	W-Sq	0.469158	Pr > W-Sq	<0.0050
Anderson-Darling	A-Sq	3.350298	Pr > A-Sq	<0.0050



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  Multiply Stem.Leaf by 10**-1
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Figure 3. 2: Probability plots showing normality for shooting induction

However, the shooting induction means of the lines and that of PGRs were not significant at (*p*≥0.05). CML442 had a higher shooting induction mean than CML444. In comparison to NAA, BAP resulted to a higher shooting induction (Table 3.9; 3.10).

Table 3.9: Maize lines and different rates of BAP and NAA means during shooting induction.

Maize lines	**Shooting induction**
CML442	8.905a
CML444	6.857b
LSD $_{0.05}$	0.821

Means followed by same letter are not statistically different at *p*≤0.05.

Table 3.10: The different rates of BAP/NAA means during shooting induction.

PGRs	**Shooting induction**
BAP	8.00a
NAA	7.79a
LSD $_{0.05}$	0.82

Means followed by same letter are not statistically different at $p \leq 0.05$.

The interaction between the lines and PGRs showed that CML442 had a higher mean for shoot induction in both BAP and NAA as compared to CML444. BAP had a higher mean of 9 ± 0.408 for shooting induction compared to 7 ± 0.866 in NAA in both inbred lines (Table 3.11).

Table 3.11: Means for shoot induction in CML442 and CML444 in BAP and NAA**.**

		Shooting induction		
Lines	**Hormones**	**Mean**	±	**se**
CML442	BAP	9.000	±	0.408
CML442	NAA	8.833	±	0.347
CML444	BAP	7.000	±	0.866
CML444	NAA	6.750	±	0.772

Se=standard error

Shoot regeneration is a critical stage during *in-vitro* regeneration as reported by Zenser *et al*. (2001) reported that shooting can be affected by a number of factors such as plant growth regulators (PGRs), genetic factors, basal media used, and light and dark treatment. For instance, Gaspar *et al*. (2003) reported that auxins negatively influence shoot formation therefore, their complete omission from regeneration media would have no significant effect. However, Fatima *et al*. (2011) demonstrated successful regeneration in the presences of both auxin-cytokinins is possible. Further, their study illustrated that a higher cytokinin to auxin ratios has shown better results in most plant species. Moreover, Ahmad *et al*. (2017) also discovered that regeneration in maize was possible even in as low as 0.2 mg/L auxin concentrations. Plant BAP could have influenced shoot formation in CML444 and CML442 because cytokininis have a significant control over the cell cycle. According to Sosnowski *et al*. (2023), cytokinin is responsible for the stimulation of cell division, release of lateral dormancy and growth of lateral buds and in the induction of adventitious bud formation. Based on Majda and Robert (2018) findings, auxins could have also influenced the regeneration of CML444 and CML442 through inducting activities that include cell wall formation and meristematic restructuring thus, giving rise to shoot and root formation. Therefore, a combination of both auxin and cytokinin acted to trigger a certain organogenic program that influenced a certain developmental pattern leading to plant formation as reported by Su *et al*. (2011).

Ombori *et al.* (2008) also indicated that maize regeneration in PGR free medium is possible. The ability of maize to regenerate in hormone free environment or in very low supplements of PGRs could be due to the presence and the ability to respond to endogenous hormones. The effect of endogenous auxin supplement at the initial regeneration stages could function to trigger subsequent production of endogenous auxins leading to regeneration in PGR free media as reported by Oduor *et al.* (2006). Anami *et al.* (2010) demonstrated that exclusion or introduction of auxins at lower levels could initiate shoot formation, suggesting that shoot induction is hormone independent. Lack of a statistical difference during regeneration in this study shows that the presence or absence of BAP and NAA hormones could be attributed to the production of endogenous hormones and the ability of the explant to respond to the same hormones (Azizan & Zakaria, 2017).

Pawar *et al.* (2015) demonstrated that PGRs are not so significant during embryo culture particularly, in maize species which was in line with the present study. Addition of is only important during the callus induction period. Cytokinins are also less significant during *in-vitro* regeneration as compared to intensive growth and differentiation in cultures that has been reported during the deployment of both auxin and cytokinin combinations. Despite their important role during *in-vitro* regeneration, addition of PGRs has been found to cause soma clonal variations.

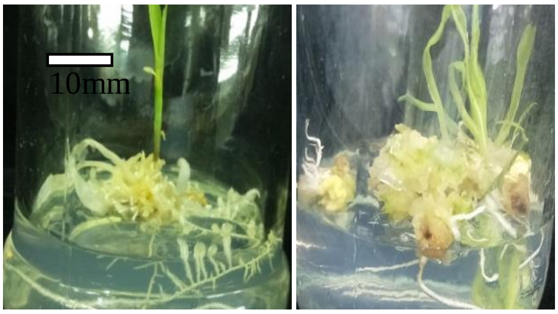
A few CML444 and CML442 calli regenerated into plantlets, according to Abebe *et al.* (2008), failure to form shoots can be caused by poor expression of embryogenesis genes. However, Che *et al.* (2006b) demonstrated that the upregulation of certain growth factors such as the ethylene responsive (ERF/AP2) transcription factors could enhance shoot formation as well. In addition, lack of the expression of stress related transporter encoding chloroplast component and photosynthetic encoding proteins could lead to failure of shoot formation in maize (Ombori *et al.*, 2008). The varied number of plantlets formed in CML444 and CML442 could be attributed to the differences in the explant genetic makeup as reported by Johnson *et al.* (2023). Appearance of such shoots could be influenced by the expression of cytokinin genes leading to the upregulation of endogenous cytokinins. Cytokinin hormones are responsible for explant regeneration in a genotypic dependent fashion (Nowakowska *et al.*, 2022).

The abiotic environment can also influence *in-vitro* regeneration. For instance, Murashige and Skoog (MS) media has recorded optimum regeneration results as reported by Saeed and

Shahzad (2015) which could be the reason behind the successful regeneration of CML444 and CML442. Li *et al.* (2002) also reported that photoperiods are integral to successful shoot formation however, higher shoot formation is observed under light conditions. Farhadi *et al.* (2017) indicated that the auxin-cytokinin ratios are influenced by light which function to accelerate apical meristem differentiation and shoot and root formation. Moreover, Miler *et al.* (2019) and Ramírez-Mosqueda *et al.* (2017) demonstrated that the use of photosynthetically active radiation (PAR) lamps enables precise light regulation as opposed to traditional cool-white fluorescent lamps. Photosynthetically active radiation lamps are optimal in the production of photomorphogenic radiation which is pivotal to plant morphogenesis. Photomorphogenic radiation is associated with precise regulation of cellular redox reactions and antioxidative metabolic activities. These processes are integral to culture regenerability (Gupta & Karmakar, 2017).

3.4.5 Root induction

Most of the rooting was observed during the shoot induction phase (Plate 3.7) however, other plants only formed roots upon introduction root induction media. Interestingly, adventitious roots were also observed during callus maintenance and maturation phases.



CML444 CML442

Plate 3.7: Fully developed plantlet with well-established shooting and rooting system at day 21 of shoot induction in CML444 and CML442.

Rooting induction was significantly ($p \leq 0.001$) different due to effects of maize lines and hormone rates. On the other hand, interaction effects of maize lines x rates were significant ($p \geq 0.05$) for rooting induction (Table 3.12).

Table 3.12: Mean squares for rooting induction for maize lines CML442 and CML444 at different rates of IBA

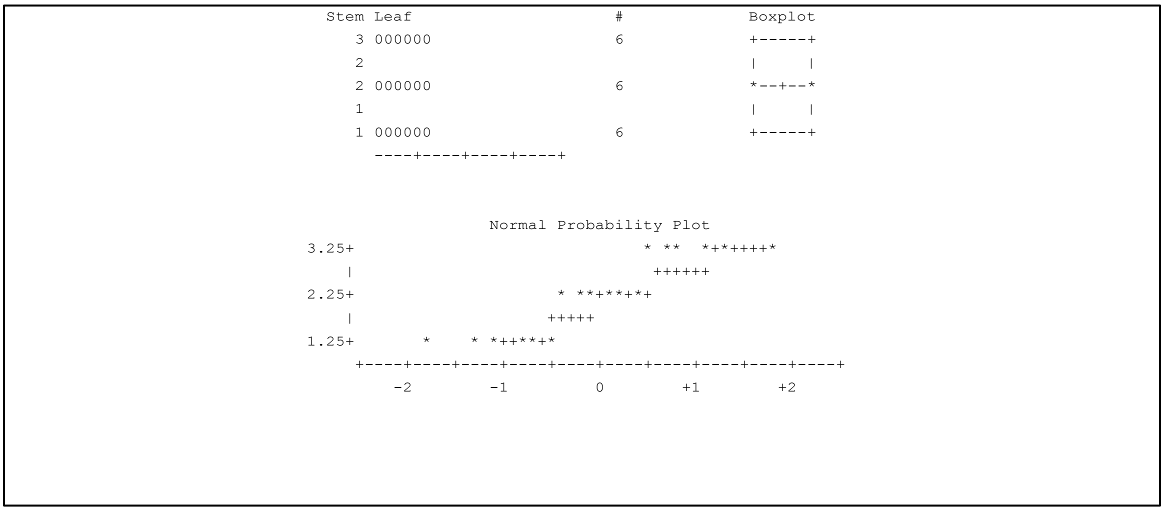
		Rooting induction			
Source of variation	**df**	**Sum of squares**	**Mean square**	**F-value**	**P-value**
Replicates	2	46.33	23.167	1.88	<0.39270
Maize Lines	1	220.50	220.500***	17.93	<0.00083
IBA Rates	2	496.33	248.167***	20.18	<0.00008
Line × Rates	2	134.33	67.167*	5.46	0.04226
Error	14	172.20	12.300		
R^2			0.879		
CV (%)			10.903		

* Significant at *p*≤0.05, ** Significant at *p*≤0.01, *** Significant at *p*≤0.001, Rates=IBA, CV=co-efficient of variation.

The normality test from Shapiro wilk test (**W**=0.800992) and normal probability plot showed that the data was normally distributed (Table 13; Figure 3.3).

Table 3.13: Shapiro wilk test for rooting induction

Tests for Normality				
Test	**Statistic**		**p Value**	
Shapiro-Wilk	W	0.800992	Pr < W	0.0016
Kolmogorov-Smirnov	D	0.216357	Pr > D	0.0242
Cramer-von Mises	W-Sq	0.196296	Pr > W-Sq	<0.0050
Anderson-Darling	A-Sq	1.388965	Pr > A-Sq	<0.0050



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  Stem Leaf                     #             Boxplot
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     2                                        |     |
     2 000000                   6             *--+--*
     1                                        |     |
     1 000000                   6             +-----+
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                   Normal Probability Plot
3.25+                             * **  *+*+++++*
    |                              ++++++
2.25+                     * **+**+*+
    |                    +++++
1.25+       *    * *++***+*
     +----+----+----+----+----+----+----+----+----+----+
         -2        -1         0        +1        +2
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Figure 3. 3: Probability plots showing normality for rooting induction

Genotype means were significant during root induction, CML444 had a higher root induction mean than CML442. IBA rates were also significant at ($p \leq 0.05$) during root induction, with rate 0 mg/L having the highest mean of 38.333 followed by rates 0.3 mg/L and 0.2 mg/L at 32.667 and 25.500, respectively (Table 3.14; 3.15).

Table 3.14: Means for root induction at the interaction of maize lines × IBA.

Maize lines	**Rooting induction (%)**
CML444	35.667a
CML442	28.667b
LSD $_{0.05}$	3.684

Means followed by same letter are not significantly different at $p \leq 0.05$

Table 3.15: Means for root induction at different levels of IBA.

IBA rates	**Rooting induction (%)**
0	38.333a
0.2	25.500c
0.3	32.667b
LSD $_{0.05}$	4.512

Means followed by same letter are not statistically different at $p \leq 0.05$

In the interaction between the lines and rates, CML442 had a higher mean of 32.667 at 0.3 mg/L while CML444 recorded a higher mean of 45.000 at 0 mg/L. However, IBA rate 0.2 mg/L had a lower mean in both lines (Table 3.16).

Table 3.16: Means for root induction in CML442 and 444 maize lines under different IBA rates

		Rooting induction		
Lines	**IBA rates**	**Mean (%)**	**±**	**se**
CML442	0	31.667	±	0.513
CML442	0.2	21.667	±	0.620
CML442	0.3	32.667	±	1.125
CML444	0	45.000	±	0.650
CML444	0.2	29.333	±	0.213
CML444	0.3	32.667	±	0.440

CML=CIMMYT Line, Se standard error

Rooting is a highly interesting developmental pathway. It is one of the main routes of plant regeneration, alongside callus induction, somatic embryogenesis and shoots formation. During root formation, the cells undergo an apparent reversal of differentiation turning meristematic, an event that is genotypic dependent and highly varied (Lardon & Geelen, 2020). Auxin application, particularly, indole-3-butyric acid (IBA), as used in our study has proved effective for root induction during *in-vitro* activities as reported by Han *et al.* (2009). Moreover, Hunt *et al.* (2011) also discovered that auxin inclusion could induce lateral rooting in difficult to root regenerants which was in line with the present study. Our observation of significant root formation in PGR free media disagreed with Akter *et al.* (2016) findings, which demonstrated poor rooting in IBA free media. The findings from Hunt *et al.* (2011) that the presence of exogenous auxin and their concentration is critical during root induction also differed with our findings where roots were regenerated in PGR free media. The appearance of roots in IBA free media in CML444 and CML442 might have been caused by the effect of endogenous 2, 4-D at the initial stages of regeneration. Panda *et al.* (2018) reported that endogenous auxin concentrations in immature embryo surge 30 days post callus induction, which determines the fate of downstream root formation. Bariya and Pandya (2014) also indicated that for the explant to root, it must contain the required genes for the production endogenous auxins and its receptors and the ability to response to both exogenous and exogenous auxins.

Different auxins have been used for *in-vitro* root initiation. Oduor *et al.* (2006) reported that NAA at 0.1 mg/L concentration was optimal for root induct in maize. Ombori *et al.* (2008) observed a promotive effect of IBA on rooting and demonstrated a variation in root formation in staggered IBA levels which was in agreement with the present study. Even though Bariya and Pandya (2014) and Ombori *et al.* (2008) agreed that rooting activities decreased with an increase in IBA, the former had demonstrated less rooting activities beyond 1 mg/L while the later reported decreased rooting activities above 3 mg/L because at concentrations greater than 3 mg/L, IBA start producing ethylene root growth inhibitor which antagonizes root initiation.

A variation in root formation at different IBA treatments as witnessed in this study could also be caused by genotypic differences in CML444 and CML442 or the culture conditions. The ability of the explant to express specific pericycle cells leads to IBA docking which converts them into founder cells and subsequent lateral root development as demonstrated in Casimiro *et al*. (2003). Certain spatial patterns witnessed during lateral root formation are genotypic dependent (Dubrowsky *et al*., 2009). For roots to form, expression of key regulators such as the *ARF6* (*AUXIN RESPONSE FACTOR 6*) and *ARF8* (*AUXIN RESPONSE FACTOR 8 WOX11/12* (*WUSCHEL-RELATED HOMEOBOX* 11 and 12) and their interaction with auxins is required according to Fambrini *et al*. (2022) studies. Upon the formation of auxin-*ARF6* or auxin-*ARF8* complex, root induction is initiated in the root founder region. These response factors function in promoting the ACS4 gene encoding 1-aminocyclopropane-1-carboxylate (ACC) synthase expression, which induce ethylene biosynthesis. Subsequently, the expression of ethylene-responsive transcription factor occurs which promote root formation (Růžička *et al*., 2007). Ethylene also modulates the expression of the Weak Ethylene Insensitive 2 and 7 (*WEI2* and *WEI7*) genes that encode tryptophan biosynthesis rate-limiting enzyme anthranilate synthase, which determines the level of auxin concentrations downstream (Stepanova *et al*., 2005). Upon a surge in auxin levels, the expression of *ARF18* and *ERF RAP2*-12 gene regulators is upregulated while that of *IAA18* and *IAA29* genes is downregulated. The two key processes are responsible for the induction of root. These genetic activities are also central to the moderation of other downstream pathways such as cutin, suberin, wax, and phenylpropanoid metabolism, which are important in the formation of cellular activities such as the specialized cell wall during plant regeneration (Li *et al*., 2015).

3.4.6 Acclimatization and greenhouse transfer

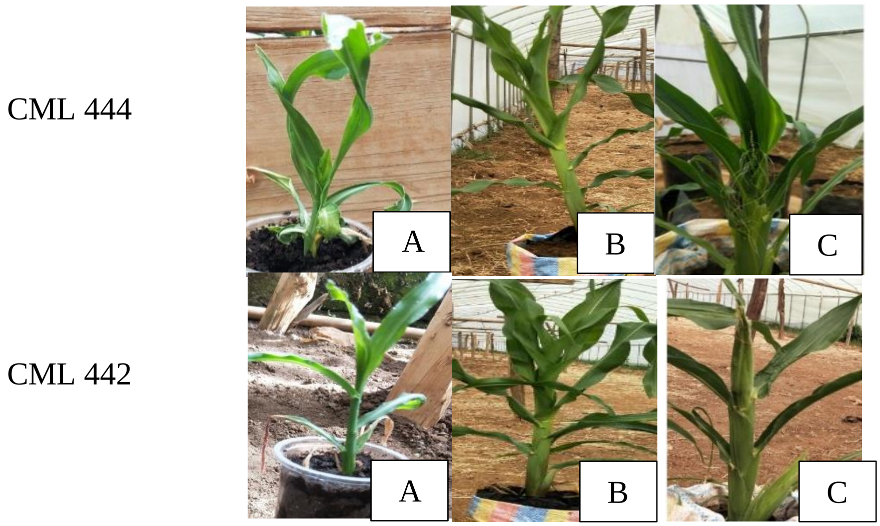


Plate 3.8: CML444 and CML442 inbred lines (A) hardening in peat moss at day 7 of the hardening phase B) fully developed plantlets in the garden soil bags in the greenhouse

at day 21 post peat moss hardening C) tussel skeletonization at day 60 post peat moss hardening.

Due to the lowly established rooting and shooting systems, gradual introduction of *in-vitro* regenerated plants into the *ex-vitro* conditions is fundamental for improved survival rates. Acclimatization in slightly lower temperatures and elevated humid conditions as in our study is required for optimal light and nutrient absorption to encourage root and shoot systems development in quest for enhanced survival of the culture products (Klanrit *et al*., 2023). One key observation during acclimatization was tassel skeletonization or tassel blasting, an event that was also observed by Ye *et al*. (2022). Tassel blasting as shown in (Figures 3.7 B and C) is a condition that results into underdeveloped tassels that are not able to produce pollen due to epigenetic mutations caused by the tissue culture environmental stress. Epigenetic changes such as DNA methylation and histone modifications are a form of physiological responses to the *in-vitro* environment stresses. The rate of these changes is highly influenced by *in-vitro* culture conditions, species type and the genotypic composition among same species. For instance, tassel blasting in this study could be caused by the effect of culture components such as IBA in our case as reported by Pawełkowicz *et al*. (2021). Such variations can be observed at morphological, cytological, cytochemical, biochemical, or molecular levels (Leva *et al*., 2012).

Epigenetic mutation is common as compared to genetic mutations. This is because modulation of DNA methylation patterns at the chromatin state, where development programs resetting epigenetic patterns in plants occur, remains common during plant development and organ or tissue differentiation. However, Ferreira *et al*. (2023) shows that it is the lack of precision during this resetting that causes epigenetic variations. Soma clonal variation occurs when somatic differentiation processes such as endopolyploidy, polyteny and amplification or diminution of DNA sequences, which causes gross changes of the genome, occurs. These changes caused by minor or major genes result into cellular dedifferentiation and/or re-differentiation leading to deletion or duplication of DNA sequence(s). The changes in DNA sequence(s) can be attributed to the *in-vitro* culture environment, genotypic composition, the source of the explant and the age of the explant. Such chromosomal changes take place in the initial stages of tissue culture. Activities such as variation in chrome structures and numbers can be caused by chromosomal breaks, acentric and centric fragments, ring chromosomes, deletions and inversions that are commonly observed in regenerants. In other cases, events including switching on of transposable elements, putative silencing of genes and varied methylation patterns in single-copy sequences can also be witnessed. These activities can cause loss of genes, gene functions, switching on of initially inactive genes and the expression of recessive genes. These changes can sometimes be silent or amplified to affect critical cellular processes such as photosynthesis. Sometimes these irregularities can cause positive changes in the host plant such as the production of normal regenerants from initial abnormal parents. Various deoxyribonucleic (DNA) markers have been designed to check on fidelity at different levels including morphological, physiological and molecular in various crops to curb for economic losses that might arise from such events (Leva *et al*., 2012).

Other causes of soma clonal variations as reported by Pawar *et al*. (2015) include regeneration systems such as micro-propagation of structures including shoot tips or nodal explants, adventitiously derived shoots, somatic embryogenesis and organogenesis. During *in-vitro* regeneration, cellular control which is the cornerstone for plant growth is lost leading callus formation. The loss of cellular control is also likely to cause soma clonal variation in cultures. Chances of soma clonal variation can also be increased during somatic embryogenesis system as compared to organogenesis system due to the introduction of callus phases and the number of sub culture involved. However, cases of soma clonal variations have reported in organogenesis systems due to the occasional loss of meristem stability.

The explant sources are a key factor in determining the occurrence of soma clonal variation events. Genetic precision is largely dependent on the deployment of meristematic tissues as young explants significantly reduce chances of soma clonal variation. Differentiated tissues have been found to possess varied soma clonal variation potential. Moreover, the use of explants from a single donor plant could also increase chances of soma clonal variation events. Soma clonal variations can be caused by both the adverse effects of the culture media and the already existing explant mutations. Tissues with pre-existing variations would give rise to extensive variations in the first soma clonal variation as compared to subsequent events where the variation can be eliminated or stabilized. This shows the importance of the source of the explant with respect to its inherent genetic composition and genome uniformity in any of its components.

Media constituents could be significant to the occurrence of soma clonal events. For instance, literature indicates that the type and concentration level of plant growth regulators (PGRs) form the basis of soma clonal variations. Unbalanced cytokinin-auxin ratios were reported to cause polyploidy whereas low or absent PGRs show normal ploidy. Addition of auxins to culture media for callus induction has also been reported to cause soma clonal variations through increasing the rate of DNA methylation. The presence of 2, 4-D can increased replication processes of the nuclear DNA in the absence of mitosis intensifying polyploidy and DNA synthesis activities that are central to soma clonal variations. Similarly, most PGRs have been reported to increase multiplication of genetically aberrant cells. For instance, benzyladenine (BA) was found to increase the number of chromosomes. However, higher concentrations of cytokinins have not been found to influence soma clonal variation directly. The duration and the culture cycles are also integral to occurrences of soma clonal variation. An increase in the number of sub cultures and their durations would lead to higher cases of soma clonal events. Moreover, each genome responds differently to *in-vitro* conditions or stresses indicating that genotypic composition has a significant influence on soma clonal variation. The varied genetic stability is based on the genetic composition as different genetic components respond differently to *in-vitro* culture pressures. For instance, the repetitive genome sequences, which would cause soma clonal events, in different plant species differ in quantity and quality.

3.5 Conclusion and recommendation

Both inbred lines were regenerated leading to the rejection of our null hypothesis that there is no *in-vitro* regeneration protocol for both CMLL444 and CML442. The regeneration protocol herein was effective and therefore, could be considered for future genetic transformation activities.

CHAPTER FOUR

AGROBACTERIUM MEDIATED TRANSFORMATION OF CML444 MAIZE INBRED LINE WITH SCMV PI, HC-Pro AND VPg GENES

Abstract

One of the major concerns in maize production in Kenya is maize lethal necrosis disease (MLN), which can potentially cause significant yield losses. Maize lethal necrosis is caused by the Maize Chlorotic Mottle Virus (MCMV) and the Sugarcane Mosaic Virus (SCMV) of the Tombusviridae and *Potyvirdae* families, respectively. The viral protein genome linked (VPg), helper component protein (HC-Pro) and the proteinase 1 (P1) genes of SCMV are required for MCMV integration, replication and translocation. The P1 protein of the potyviruses functions as a primer and enhances genome amplification. The HC-Pro possesses a number of functions including elevating viral pathogenicity through blocking the endonuclease activities of antiviral defense proteins of the host plants to enhance systemic translocation. Moreover, the HC-Pro in synergistic with the VPg proteins aids in the production of virus suppressors RNA (VSR) for the suppression of the host RNA silencing inducing complex to accelerate systemic invasion of MLN causing viruses. Blocking or lowering the expression of these genes is hypothesized to curb MLN expression. The objective of this study was to: transform inbred line CML444 with P1, HC-Pro or VPg of sugarcane mosaic virus (SCMV). Immature embryos were transformed with *P1, HC-Pro* and *VPg* genes and subjected to *Hygromicin* selection. Transgenics were regenerated *in-vitro*, the number of plants recorded, and transformation efficiency determined. DNA was isolation from the T_0 putative transgenics following the Total Volume of Reagents Kit® (TPS) protocol with target DNA isolated from *pMDC35* and non-transformed maize materials being used as checks. Specific SCMV gene sequences were retrieved through SerialCloner2-6 and specific primers designed using the ApE-A Plasmid Editor v2.0.53c. Conventional PCR was carried out for the three transgenes and bands visualized under the UV transilluminator Boier™. Further, the VPg, P1 and HC-Pro transgenes were digested with *AscI*, *BfmI* and *AsuII* enzymes to confirm the presence of transgenes. The putative transgenics exhibited expected band sizes of 556 bp, 705 bp and 1489 bp in that order for the VPg, P1 and HC-Pro transgenics. Expected digestion products of 303 bp and 227 bp; 535 bp and 169 bp, and 163 bp and 1222 bp from the VPg, P1 and HC-Pro transgenic DNA, respectively were obtained confirming the presence of transgenes in T_0 plants. The VPg transgenic had a higher transformation frequency of 2.5% while both P1 and HC-Pro events recorded a transformation frequency of 1.67%.

4.1 Introduction

Despite the central role of maize globally, its production has been constraint by a number of factors; one of them being maize lethal necrosis diseases (MLN). A number of solutions have been proposed towards its management however, these tools have fell short of expectations (Giroux *et al.*, 2023). Genetic transformation which is central in plant breeding and crop improvement is deemed as the ideal solution to MLN. Genetic transformation offers benefits such as high precision, which avoids transfer of non-target traits. The method is also rapid, eco-friendly and economically viable. Moreover, reports have indicated that tools such as transgenic-induced RNA silencing are highly successful in crop virus management (Chen *et al.*, 2023).

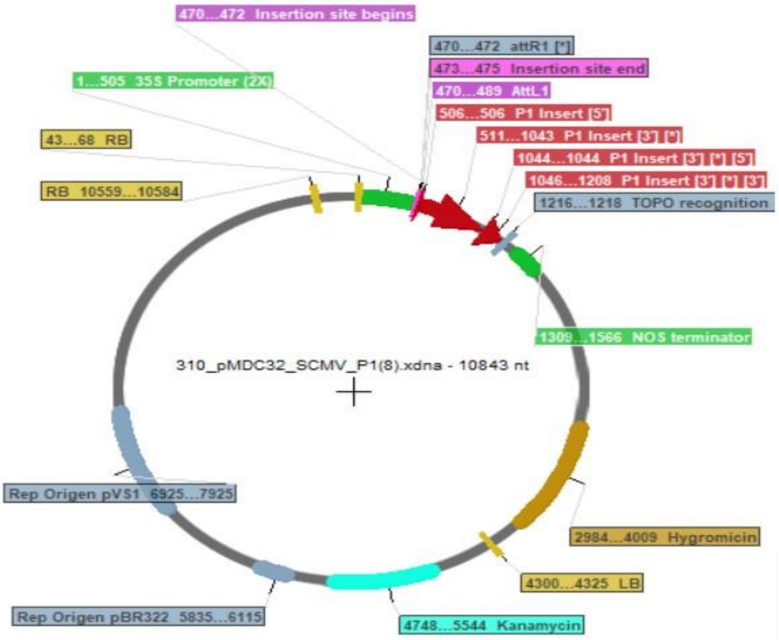
However, the pre-requisite for any successful genetic transformation relies on the type of DNA vectors used, the mode of gene delivery and the genetic composition of the explant (Girijashankar & Swathisree, 2009). For instance, *Agrobacterium* 3101 strain was discovered to enhance transgene delivery (Gelvin, 2003). Moreover, deploying *Agrobacterium* 3101 alongside the *pMDC32* vector demonstrated higher explant transformability. The *pMDC32* vector contains a highly active *35s* promoter, and a *NOS* terminator both of which drive the transgene expression. The vector also has a *Hygromicin* selection marker that allows for transgenic selection. The *att* region offers a one-step avenue for transgene transfer and also ensures that transgenes are integrated in the right orientation in all the three reading frames. Moreover, the 8bp restriction sites for *AscI/PacI*, flanking the *att* guarantees easy identification of the new recombinant clones that have substituted the *att* recombination site (Curtis & Grossniklaus, 2003).

A complete cycle of genetic transformation and transgenic identification entails transgene cloning, transgene delivery, transgenic DNA integration, *in-vitro* regeneration of the transgenics, and confirmation of transgene presence (Feng *et al.*, 2023). Transgene delivery can be performed through protoplast transfection, *Agrobacterium*, and gene gun. However, it's the *Agrobacterium*-based gene delivery that has dominated the current genetic transformation activities. This approach allows for easy selection of single and unfragmented transgene insertions leading to high level transformation frequency (Cordeiro *et al*., 2023). Furthermore, it allows for the use of high expression plasmids such as the *pMDC32*. The *pMDC32* plasmids contain a highly active promoter and a reliable selection marker, which has significantly improved transgene expressions and transgenic selection processes (Toprak *et al*., 2014) hence, our choice of this vector.

4.2 Materials and Methods

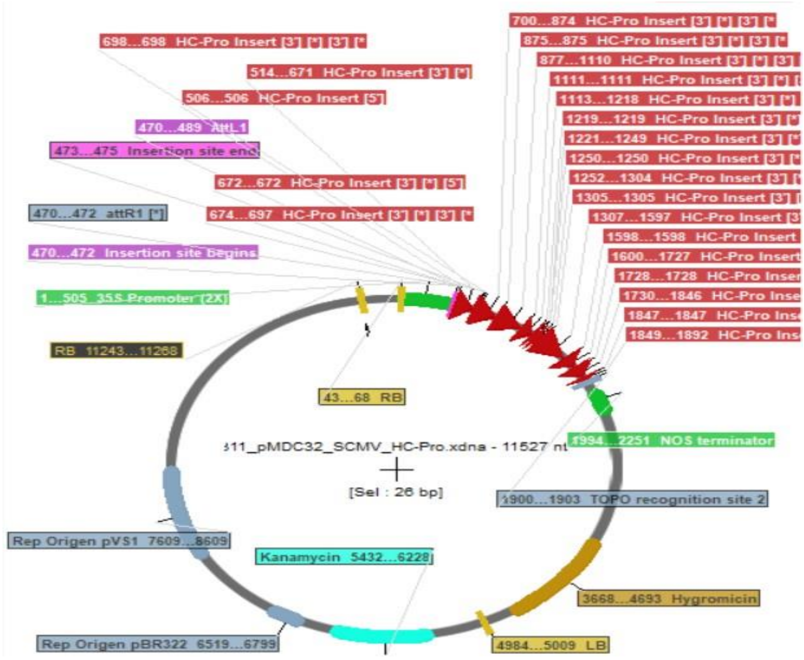
4.2.1 Experimental materials

The maize inbred line CML444 was selected for transformation for improved MLN resistance because it has superior *in-vitro* regeneration qualities as demonstrated in chapter three. *Agrobacterium* immobilized on filter paper disks was obtained from the University of Nebraska-Lincoln Center for Virology. All cistrons as shown in figures 4.1; 4.2 and 4.3 were contained in the *pMDC32* vector.



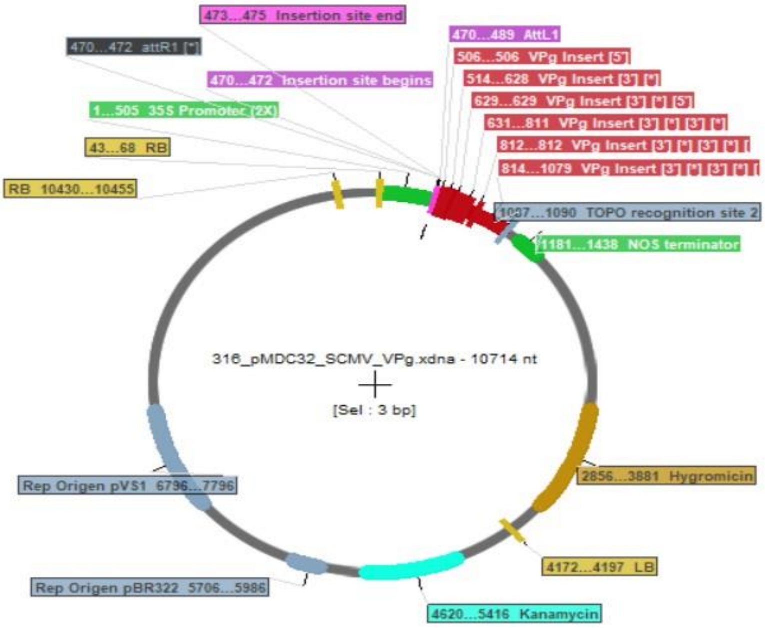
Key: Transgene: colour coded red

Figure 4.1: pMDC32 (310 code) containing SCMV P1 insert (colour coded red) under 35S promoter and NOS terminator with a Hygromicin selection gene.



Key: Transgene: colour coded red

Figure 4.2: pMDC32 (311 code) containing SCMV HC-Pro insert (coded red) under 35S promoter and NOS terminator with a Hygromicin selection gene**.**



Key: Transgene: colour coded red

Figure 4.3: pMDC32 (316 code) containing SCMV VPg insert (colour coded red) under 35S promoter and NOS terminator with a Hygromicin selection gene.

Source: SerialCloner2-6.

The transgenes were separately inserted between the *35S* promoter and *NOS* terminator of the *pMDC32* vector with a *Hygromicin* selection marker. *Agrobacterium* on filter paper disks containing each transgene were broken into 6 pieces. A single piece was processed leaving the other five for later uses. The 1/6 piece was placed in a 1.5 mL tube and covered with liquid broth (LB) containing 50μg/mL Kanamycin for the first selection. The inoculum was incubated overnight at 4˚C for multiplication. Using a sterile pipette, 1 mL of the inoculum was taken and serially diluted into 10-fold, 100-fold, and 1000-fold and incubated overnight at 4˚C. A 250 mL inoculum was transferred to 100 mm × 15mm diameter petri plates with solid LB containing 50μg/mL Kanamycin and 35μg/mL Rifampicin for the second selection at 28˚C for 48 hr. A single colony was picked from the culture and transferred to 5 mL LB containing 50μg/mL *Kanamycin* and 35μg/mL *Rifampicin* and incubated at 28˚C overnight for later use.

4.2.2 Generation of immature embryos

This was done as eluded in section 3.2.3

4.2.3 Media preparation

Preparation of transformation media was based on Bedada *et al.* (2016) and Ishida *et al.* (2007) protocols. Infection media was prepared by amending 4.4 g/L MS media with Casein hydrolysate 1 g/L, MES 0.5 g/L, 2, 4-D 1.5 mg/L, Proline 0.7 g/L, Glucose 18 g/L and Sucrose 34.5 g/L. Media pH was adjusted to 5.2 with KOH/HCl followed by the addition of 3 g/L Gelrite. Autoclaving was done at 121˚C for 15 min followed by the addition of 100 µM/L Acetosyringone to the cooling media. Exactly 30 ml of the media was dispensed into petri plates in the laminar flow chamber for explant inoculation.

Co-cultivation media was prepared through supplementing 4.4 g/L MS media with MES 0.5 g/L, 2, 4-D 1.5 mg/L, Proline 0.7 g/L, 2 mg/L 2, 4-D, 18 g/L Glucose and 34.5 g/L sucrose. Media pH was adjusted to 5.8 with KOH/HCl followed by the addition of 3 g/L Gelrite. Media autoclaving was done at 121˚C for 15 min followed by the addition of 100 µM/L Acetosyringone to the cooling media. Exactly 30 ml of the media was dispensed into petri plates in the laminar flow chamber for explant inoculation

Resting media was constituted from 4.4 g/L MS media supplemented with MES 0.44 g/L, 2, 4-D 1.5 mg/L, Proline 0.72 g/L, 2 mg/L 2, 4-D and Sucrose 30 g/L. Media pH was adjusted to 5.8 with KOH/HCl followed by the addition of 3 g/L Gelrite. Media autoclaving was done at 121˚C for 15 min followed by the addition of 7.5 mg/L *Kanamycin* and 7.5 mg/L *Cefotaxime* to the cooling media at 37˚C. Exactly 30 ml of the media was dispensed into petri plates in the laminar flow chamber for explant inoculation

First selection and callus induction were synchronized and media was prepared by amending 4.4 g/L MS media with 100 mg/L casein hydrolysate, 0.7 mg/L L-Proline, 30 g/L sucrose and 2 mg/L 2, 4-D. Media pH was adjusted to 5.8 with KOH/HCl followed by the addition of 3 g/L Gelrite. Media autoclaving was done at 121˚C for 15 min followed by the addition of *Hygromycin* 50 mg/L and 7.5mg/L concentrations of both *Kanamycin* and *Cefotaxime* to the cooling media at 37˚C. Exactly 30 ml of the media was dispensed into petri plates in the laminar flow chamber for explant inoculation

Second selection and callus maintenance were synchronized and media was prepared by amending 4.4 g/L MS media amended with 2 mg/L 2, 4-D and 30 g/L sucrose. Media pH was adjusted to 5.8 with KOH/HCl followed by the addition of 3 g/L Gelrite. Media autoclaving was done at 121˚C for 15 min followed by the addition of *Hygromicin* 150 mg/L and 7.5mg/L concentrations of both *Kanamycin* and *Cefotaxime* to the cooling media. Exactly 30 ml of the media was dispensed into petri plates in the laminar flow chamber for explant inoculation

Maturation was also synchronized with second selection. Media was prepared by amending 4.4 g/L MS media with 0.7 g/L L-Proline, 2 mg/L 2, 4-D and 60 g/L sucrose. Media pH was adjusted to 5.8 with KOH/HCl followed by the addition of 3 g/L Gelrite. Autoclaving was done at 121˚C for 15 min followed by the addition of 7.5 mg/L *Kanamycin* and *Cefotaxime* and 150 mg/L *Hygromicin* to the cooling media at 37˚C. Exactly 40 ml of the media was dispensed into culture bottles in the laminar flow chamber for explant inoculation.

Plant regeneration media involved amending 4.4 g/L MS media with 0.7 g/L L-Proline and 30 g/L sucrose. Media pH was adjusted to 5.8 with KOH/HCl followed by the addition of 3 g/L Gelrite. Media autoclaving was done at 121˚C for 15 min followed by the addition of 150 mg/L *Hygromicin*, and 7.5 mg/L of both *Kanamycin* and *Cefotaxime* to the cooling media at 37˚C.

4.2.4 Explant infection, co-cultivation, selection and regeneration

Explant infection was based on Bedada *et al*. (2016) with modifications. One loop of about 3 mm of *Agrobacterium* was scraped from an overnight culture and suspended in 25 mL of MS infection liquid media amended with 100 µM/L Acetosyringone in 50 mL Falcon tube. The tube was wrapped in aluminium foil and horizontally fixed on a bench-top shaker. Shaking was done at low speed, 250 revolutions per min (rpm) for 2 h 30 min, at 28˚C. 5 mL infection media was diluted to OD550 0.3–0.4 using a UV-VIS spectrophotometer, Shanzhai®. Using a sterile pipette, 1 mL of dilute infection media was taken and added to 250 immature embryos that had been pre-washed in 5 mL bacteria-free MS infection media for 2 h. this was repeated for the three transgenic events. The content in the petri plate for the three transformation events were swirled 5 times and incubated at room temperature in the dark for 5 min. The final wash of infection media was sucked with a sterile pipette. Excess *Agrobacterium* suspension medium in petri plates was further removed with the aid of an 85mm dry sterile Whatman filter paper.

The embryos for each transformation event were co-cultured on co-cultivation media in 100 mm×15 mm diameter petri plates. Each plate was inoculated with 20 embryos of about 3 mm with the embryo axis in contact with the medium. Plates were wrapped in a parafilm and incubation in the dark for 3 days at 19˚C.

A hundred and twenty immature embryos for each transformation event were transferred from the co-cultivation media to the resting media. Twenty embryos were placed in each petri plate and incubated at 28˚C in the dark for 7 days. Callus induction was carried out synchronously with transgenic selection. Co-cultivation cultures were transferred to induction media amended with 50 mg/L *Hygromicin* for first selection and incubated at 28ºC. The growing radicle and plumule were excised and cullus maintenance performed as described in section 3.4.2.

Fifty, forty and twenty-nine *Hygromicin*-resistant calli for the P1, HC-Pro and VPg transgenes, respectively were transferred to maturation media amended with 150 mg/L *Hygromicin*. Callus maturation was performed as described in section 3.4.3. Data on the number of plants regenerated after selection was collected and transformation frequency calculated following Jayashree *et al.* (1993).

$$TF = \frac{No.of\ transgenic\ plants}{total\ number\ of\ explants} \times 100\%$$ ……………………………………………Equation 1

4.2.5 Plant acclimatization and hardening

Acclimatization and hardening were performed as described in section 3.4.6. Transgenics (T_0) were selfed to give T_1 to multiply the seeds for downstream MLN assay.

4.2.6 Molecular characterization and confirmation of P1, HC-Pro and VPg putative genes in T0 transgenics

Fresh leaves were taken from T_1 plants of the three transgenic events. Isolation of DNA was performed based on the TPS protocol. Plasmid isolation from *Agrobacterium* GV3101 strain and non-transformed maize were used as checks. Plasmid isolation was based on the (Generay Biotechnology, Shanghai, China). Specific SCMV gene sequences were retrieved using SerialCloner2-6. Primer sequences were designed through ApE-A Plasmid Editor v2.0.53c. Forward and reverse primer sequences targeting the three genes (Table 4.1) were sent to Synbio Technologies, Shanghai, China for primer synthesis.

Table 4.1: Forward and reverse primer sequences for the P1, HC-Pro and VPg genes

Gene	Forward primer	Reverse primer
P1	F-ATGGCGGGAACGTGGACCT	R-TTGGATATTGAGCACTACTAGTG
HC-Pro	F-CAGATCCCCAGGCTAATGA	R-CGCGAATACATAGTAGGATAGTGA
VPg	F-ATGGGGAAAAACAAACGCA	R- GGGTTGCACACGAGTAGTGA

Source: Authors' design

Transgenic plant DNA was subjected to conventional PCR with non-transformed plant and *Agrobacterium GV3101* DNA being used as checks. The PCR mix and PCR program were based on Bedada *et al*. (2016) with modifications. A total of 25 µL PCR reaction was reconstituted by mixing 0.5 µL F and R Primers (10 pmol. $µ1^{-1}$ each), 2.5 µL of PCR buffer (×10), 0.5 µL dNTPs (10mM), 1.2 µL $MgCl_2$ (25mM), 0.5 µL *Taq* Polymerase (5 U/ µL and 1 µ template (20 ng). Exactly 18.5 µL of nuclease free sterile water was used to adjust the total volume. The PCR program was set for 10 min at 98˚C for initial denaturation followed by 3 sec at 94˚C for 35 cycles of final denaturation, primer annealing at 60˚C for 30 sec, initial extension for 2 min at 72˚C and final extension at 72˚C for 15 sec. DNA was quantified on a nano-drop, spectrophotometer 2000 (Thermo Scientific). Gel loading was carried out in 0.8 % (w/v) agarose and 1×TAE buffer followed by electrophoresis at 130V for 30 min. Bands were compared to the corresponding ladder (NEB) under the UV transilluinator (Boier^{TM}).

DNA purification was performed as per the Generay Biotechnology, Shanghai, China reclamation kit where gel blocks of about 4 mm×2 mm ×2 mm were excised using a sterile scalpel and transferred into 1.5 mL centrifuge tubes. Exactly 400 µL of DEA-A solution was pipetted into the content in the tube. The tubes were placed on a 70˚C hot plate for 10 min with every minute of inversion. Tubes were taken out of the hot plate and 200 µL of DE-B was added and the mixture allowed standing for 2 min. A total of 600 µL volume of the content was transferred to a 2 mL separation column tube and centrifuged at 12000 g for 1 min. Exactly 500 µL of Wash I solution (W1) was added to the content in the separation column and centrifuged at 12000 g for 30 sec. This was followed by the addition of 200 µL W2 to make a 700 µL total and centrifuged at 12000 g for 1 min. The content in the separation column along with the column were transferred to another 1.5 mL tube. Three drops of PCR elution warm water was added to the content in the separation column followed by centrifugation at 12000 g for 1 min. The eluent, putative genes of interest, were quantified on a Nano-drop spectrophotometer 2000 (Thermo Scientific) and subjected to restriction digestion.

Digestion of DNA followed the Generay Biotechnology, Shanghai, China DNA digestion Kit: Exactly 2 µL digestion buffer was dispensed into a 1.5 mL tube using a sterile pipette, followed by the addition of equal amount of the target DNA and restriction enzyme. The VPg, P1 and HC-Pro genes were separately restricted with *AscI*, *BfmI* and *AsuII* enzymes, respectively (Appendix iv; v, vi). Vortexing was carried out for 2 min followed by incubation on the hot plate for 4 h at 37˚C. PCR was carried out and bands visualized under the UV trans illuminator Boier^{TM}. Data on band sizes was compared to the NEB ladder.

4.3 Data collection and analysis

Data on the number of infected embryos inducted, callus obtained from first and second selection and transgenic plants generated was recorded. Data was transformed using square root transformation and subjected to analysis of variance on SAS version 9.4. DNA banding was visualized under agarose gel electrophoresis.

4.4 Results and discussion

4.4.1 Explant infection and regeneration

One hundred and twenty callus from the three transgenics were subjected to *Hygromicin* selection. The P1, HC-Pro and VPg trensgenes had survival rate means of 13.17, 10 and 7.5, respectively in the first selection. The cultures had a survival rate of 8.33, 6.67 and 4.83

means for the P1, HC-Pro and VPg trensgenes, respectively (Table 4.2; Appendix vii). Non-transformed callus turned brown-black and necrotic upon selection (Figures 4.1 A; B) while transformed callus flourished and developed into well-established plantlets (Figure 4.1 B; C) that were considered as putative transgenics of P1, HC-Pro and VPg genes.

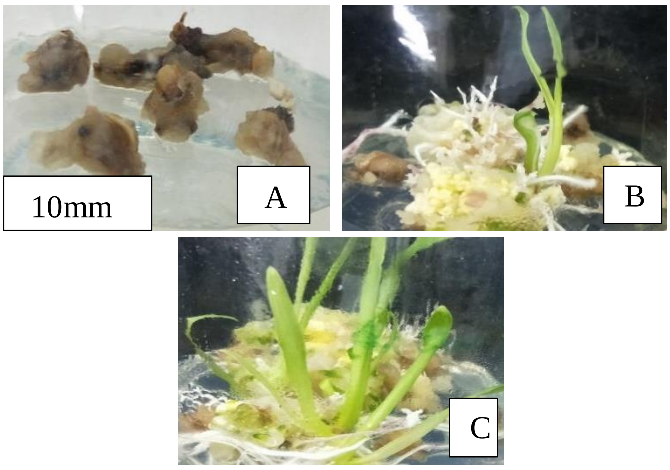


Plate 4.1: (A) Necrotic maize callus under 50 mg/L Hygromicin at the day 14 of selection I, (B) Necrotic callus and green shooting callus at day 7 of transgenics shoot induction, and (C) fully developed transgenic plantlet at 21 days of shoot induction.

Similar to this study, McCaw *et al.* (2021) reported successful *Agrobacterium* mediated transgene delivery on immature embryos of FFMM and FFMM-AT maize inbred line. *Agrobacterium* gene delivery has gained popularity in genetic transformation due to its efficiency in transgene delivery, which subsequently increases transformation efficiency. *Agrobacterium* guarantees stable transgene expression because of its ability to transfer one or more copies of the full-length T-DNA. This method of transgene delivery is also less resource intensive and it is compatible with a wide range of expression vectors and diverse tissue culture techniques. Moreover, the method is less invasive, causing low attrition rates or deleterious effects on the growth of the explant as indicated in Kharb *et al.* (2022). However, the success of *Agrobacterium* is dependent on the strain type and expression plasmids deployed. In addition, parameters such as infection and screening conditions, culture environment, co-cultivation duration, and physiological status of the recipient tissue are important as well. Sometimes *Agrobacterium* requires wounding which might damage the explant. Besides, the use of chemicals such as *Acetosyringone* may significantly increase the research cost (Li *et al.*, 2022).

Similar to this study, Li *et al.* (2022) incorporated 100 µM/L Acetosyringone in the infection and co-cultivation media. Unlike dicots that are capable of secreting this phenolic compound, maize requires exogenous supplements of Acentosyringone. The chemical acts to induct the *Agrobacterium vir* genes leading to the transfer of the T-DNA region into the cultures. A three-day co-cultivation in this study was also in agreement with McCaw *et al.* (2021), study that demonstrated that a sufficient co-cultivation duration guarantees complete transgene transfer. Other studies have confirmed better GUS activities from day 3 of co-cultivation period, indicating superior transgene integration from day three of explant infection. Staining through GUS is a confirmatory test used to assay for transgene transfer and integration (Feng *et al*., 2018).

Moreover, Segatto *et al.* (2022) studies revealed that *Gus* staining was stronger after day 5 of the resting stage, an indication that there was improved transgene integration. Frame *et al.* (2002) indicated that the resting phase is important as it provides sufficient time for callus formation in small and slow responding embryos. The resting period allows for optimal transgene integration, and recovery of the explant from *Agrobacterium* injuries sustained during the infection phase. This phase also allows for sufficient time for acclimatization of the explant to culture pressures downstream and the switching on of regeneration genes as reported by Merrick and Fei (2015). It has also been reported that resting phase gives the opportunity to small and slow responding embryos to form callus (Frame *et al*., 2002).

The transfer of the T-DNA containing the three transgenes from *pMDC35* into the immature embryo might have been influenced by the explant genetic composition. For instance, Didoné *et al.* (2018) indicated that the expression of vitronectin protein in the explant is important for effective transgene DNA uptake. This receptor protein is genotypic specific and necessary for *Agrobacterium* docking. Furthermore, the *chvB*, *pscA*, and *att* mutant have shown to have a reduced *Agrobacterium* binding ability as these genes code for *Agrobacterium* docking receptors. These genes are responsible in triggering bacterial movement toward and attachment to the wounded plant cell (Hwang *et al*., 2017). In a different study, Maren *et al.* (2022) indicated that the explant ability to accept transgene DNA can also be attributed to the ability to express the *LEC1*, *LEC2*, LEC1- LIKE (L1L), B3, *WUS2*, *BBM* and *ZmKn1* genes. Expression of these genes has been found to increase explant transformation efficiency. The ability of explants to accept transgenes is dependent on their ability to express certain genes.

For instance, the genes downstream or upstream the *BMM* genes have been found to enhance transformation efficiency. Downstream the *BMM* are the *LEC1* and *LEC2*, the *LEC1* initiate the expression of *AGL15*, which improves transformation efficiency. Expression of the *SAUR15*, an auxin-inducible negative regulator, is known to enhance callus formation in maize. However, its mutant *zmsaur15* increases maize transformation efficiency. Expression of these genes in CML444 would have been fundamental towards the acquisition of the target P1, HC-Pro and VPg transgenes according to Chen *et al*. (2022) and Jia *et al*. (2013).

Reports also indicate that the expression profile of certain genes in non-transformable genotypes would trigger transgene DNA uptake. For instance, CML444 inbred line might have overexpressed the *WUS2* and *BBM* genes that are known to enhance transformation efficiency, somatic embryos induction and regeneration as reported by Hoerster *et al*. (2020). Similarly, overexpression of maize *STM* homolog *Knotted1* (*ZmKn1*) might have increased chances of CML444 explant transformation (Hu *et al*., 2016). *STM* genes act to induce *IPT7* genes which function to increase cytokinin biosynthesis, accelerating transformability of the explant. Literature also indicates that higher expression of the *GRF5* or its homolog boosts transformation efficiency in recalcitrant maize genotypes. The *GRF5* functions to bind the *PpnCKX1*, a complex that buffers cytokinin breakdown, hence improving meristematic induction. Maize transformation efficiency can also be enhanced by overexpression of the *STM* dependent or independent *CUC1* and *CUC2* genes that function in stem cell renewal (Maren *et al*., 2022).

4.4.2 Plant acclimatization

Non-transformed callus turned necrotic upon *Hygromycin* exposure. Fewer callus were obtained as the selection pressure was increased. The VPg transformation event had the highest acclimatization mean of 0.5 as compared to 0.33 in each of the P1 and HC-Pro (Table 4.2; Appendix vii).

Table 4.2: Mean transformation events for P1, HC-Pro and Vpg genes

Transformation events (%)	P1	HC-Pro	Vpg
Callus induction	20	20	20
First selection	13.17	10	7.5
Second selection	8.33	6.67	4.83
Callus Maturation	8.33	6.67	4.83

Regeneration	0.33	0.33	0.5
Acclimatization	0.33	0.33	0.5

The VPg had the highest number of plants which translated to highest transformation frequency of 8.62%. The HC-Pro was second with 4.17% transformation frequency while P1 had the least transformation frequency of 3.33% (Table 4.3; Appendix vii).

Table 4.3: Transformation frequency for the P1, HC-Pro and VPg transgenic events

Genes	Number of explant	Survival under selection	Plants obtained	Transformation frequency (%)
P1	120	41.67	4.00	3.33
HC-Pro	120	33.33	5.00	4.17
VPg	120	24.17	10.34	8.62

Li *et al*. (2013) reported that upon the increase of *Hygromicin* concentration, culture necrosis is imminent in the case of non-transformed events. The low number of transgenics could be attributed to the higher concentration of *Hygromicin* which is likely to cause retardation of cultures and inhibition of shoot regeneration or even culture deaths particularly in non-transformed callus. Retardation of cultures occurs in the transformed tissues because initially, they are not able to compete with the non-transformed tissues before their elimination. Moreover, the fewer number of transgenics or low transformation frequency in this study could be attributed to maize sensitivity to *Hygromicin* as compared to other more tolerant species.

However, Abou-Alaiwi *et al*. (2012) reports there are existing reports that improved transformation frequency can be achieved through two approaches; step-wise selection or gradual increase of the selection agent. The two techniques are strategies to allow for sufficient time for recovery of the transformed cultures from *Hygromicin* stress. Milojević *et al*. (2012) also proposed that delayed selection or delaying the transfer of the culture products to the selection media after co-cultivation can be used to reduce the detrimental effects of *Hygromicin*. In contrast to the present study, Li *et al.* (2013) utilized a first selection with a higher concentration of *Hygromicin* as compared to the second selection in quest to accelerate the elimination of non-transformed cultures before initiating nutrient competition with their transformed counterparts.

Similar to the present study where *Hygromicin* concentrations of 50 mg/L and 150 mg/L for the first and second selection, respectively were used, a broad range of concentrations has been deployed during transgenic selection. For instance, Bibi *et al*. (2013) indicated that a concentration range of 25 mg/L to 150 mg/L can be considered during transgenic selection. The ability of the explant to survive the selection agent is attributed to the integration of transgenes DNA within the T-DNA region (Figure 4.7B) which enables callus proliferation in *Hygromicin* stress. A reliable selection agent is required during genetic transformation. In most transgenic selection experiments, *Hygromicin* and *Kanamicin* are commonly used. However, literature indicates that *Hygromicin* is more effective during selection of some species. The antibiotic at higher doses functions to inhibit polypeptide elongation during protein synthesis, causing cell death (Li *et al*., 2013).

Transgenic identification is important in allowing for the continued production of only organisms carrying the required trait for downstream applications. In the present study, *Hygro* marker gene was used as a selection marker gene. Miki and McHugh (2004) demonstrated that marker genes are DNA sequences used for the identification of transgenics. These genes can be harboured within the same DNA fragment with gene/s of interest in a vector as in the case of *Hygro* resistant gene in the *pMDC*32 vector as in the case with present study or sometimes outside the selectable markers. Marker genes are used to flag a particular gene of interest for its selection from a pool of 'unwanted' genes. Moreover, Breyer *et al*. (2014) indicates that besides antibiotic marker genes, herbicide and reporter genes are also in existence. Antibiotic as in the case of *Hygromicin* resistant gene confer resistance, while reporter genes are detected visually.

4.4.3 Molecular characterization and confirmation of P1, HC-Pro and VPg T1 transgenes

Expected band sizes of 556bp for the VPg, 705 bp for the P1 and 1489 bp for the HC-Pro were observed, respectively upon the gel analysis of the DNA samples (Figures 4.8; 4.9; 4.10: S3/S4/S5; S3/S4; S3/S4). The results were in agreement with the gel results obtained from non-transformed plant and *Agrobacterium GV3101* checks (Plates 4.2; 4.3; 4.4: S1/S2; S1/S2; S1/S2) confirming that the VPg, P1, and HC-Pro transgenes were detected in the tested materials of the putative transgenic plantlets.

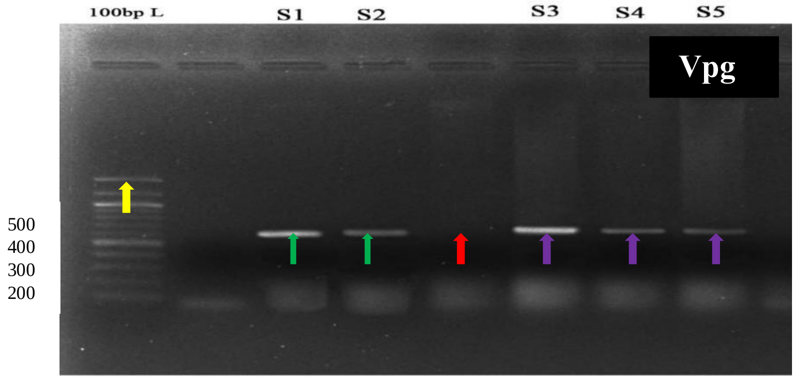


Plate 4.2: Agarose gel: (A) Lane 1 (yellow) 1000 bp NEB DNA ladder, Lane 3;4/S1;S2 (green) Agrobacterium, DNA Lane 5 (red); non-transformed plant, Lane 6;7;8/S3;S4;S5(purple) plant genomic DNA showing VPg 576 bp segment.

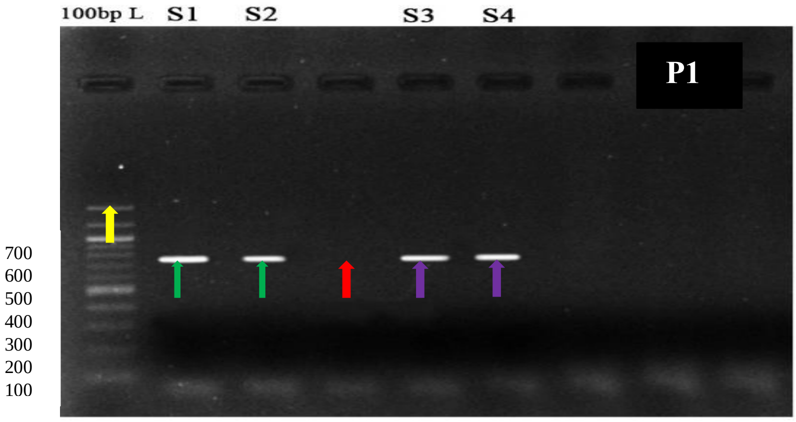


Plate 4.3: Lane 1 (yellow) 1000 bp NEB DNA ladder, Lane 2; 3/S1; S2 (green) Agrobacterium DNA, Lane 4 (red); non-transformed maize, Lane 5;6/S3;S4 (purple) plant genomic DNA showing P1 705 bp band.

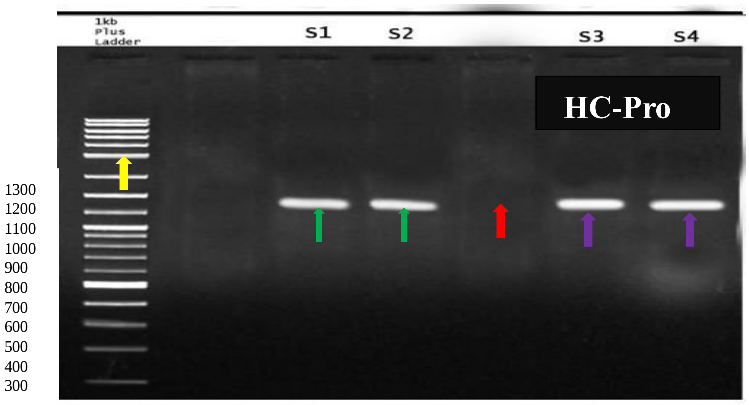


Plate 4.4: Lane 1 (yellow), 1 Kb plus NEB DNA ladder Lane 1 1000 bp, Lane 3; 4/S1; S2 (green) Agrobacterium, DNA Lane 5 (red); non-transformed plant, Lane 6; 7/S3; S4 (purple) plant genomic DNA showing HC-Pro 1389 bp.

Total genomic materials obtained from the given maize samples could not be analyzed due to the presence of unwanted sequences. However, Kadri *et al*. (2019) indicates that through PCR it is possible to clone and purify specific DNA targets; in this case the VPg, P1, HC-Pro DNA and the positive control (similar genes amplified from *pMDC35*). Therefore, PCR was important for the generation and acquisition of VPg, P1, HC-Pro DNA and the positive control fragment through the use of specific primers under specific thermocycler conditions. The VPg, P1, HC-Pro and the positive control DNA were able to move across the gel to the anode due to their negative charges upon the application of an electric field. The different pore sizes of the agarose gel enabled the creation of different banding leading to DNA characterization.

Similar to this study, Asad *et al*. (2023) used electric field of 130V to transit DNA through agarose gel. The electric field causes the negatively charged DNA fragments to move towards the positive anode. Separation of VPg, P1, HC-Pro and the positive control DNA during gel electrophoresis occurred due to the negative charge at the phosphate backbone. Moreover, Semenov *et al*. (2023) demonstrated that 0.8% concentration agarose gel concentrations fall within the required range for DNA separation. Movement of the VPg, P1, HC-Pro and the positive control DNA across the gel was also dependent on the size of the fragments and agarose concentration.

Location of the VPg, P1, HC-Pro and *Agrobacterium* DNA bands in the gel was achieved by incorporating ethidium bromide and visualizing under the UV trans-illuminator. Pandey *et al*. (2024) indicated that the dye intercalates DNA and upon irradiation under the UV light, it fluoresces showing the DNA banding. Separation and analysis of gel products is important in the identification for the presence of a particular gene or genes of interest Vahdani *et al*. (2024), in this case, the VPg, HC-Pro, P1 and the positive control DNA. Agarose gel electrophoresis is deemed as the most effective analytical tool due to its capability to separate, analyze, identify and purify target DNA fragments based on size Bhoyar *et al*. (2024). However, conventional DNA electrophoresis is time consuming, has a low throughput and require the use of dyes, some of them which have been associated with carcinogenic activities and that also increase experiment costs (Kalendar *et al*., 2023).

Upon restriction digestion, expected band sizes of 303bp and 227bp (Plate 4.5); 535bp and 169bp **(**Plate 4.6) and 1222bp and 163bp fragments (Plate 4.7) were obtained from the VPg, P1 and HC-Pro, respectively. This was a confirmation that the three transgenes were present in the analyzed DNA samples.

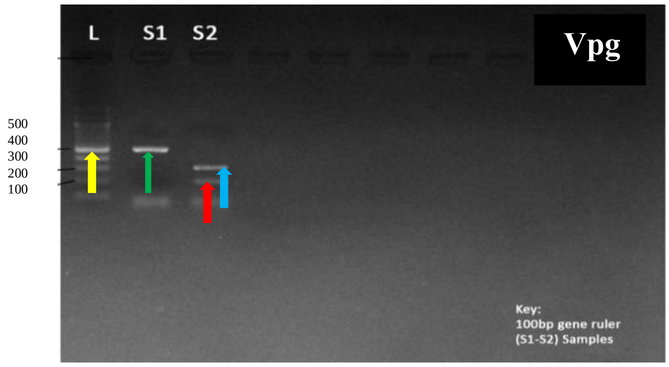


Plate 4.5: Agarose gel: Lane 1/L (yellow), 500 bp NEB DNA ladder, Lane 2/S1 (green) 576 bp unrestricted check, Lane 3/S2 303 bp (red) and 227 bp (blue) restricted products of VPg gene at AscI restriction site.

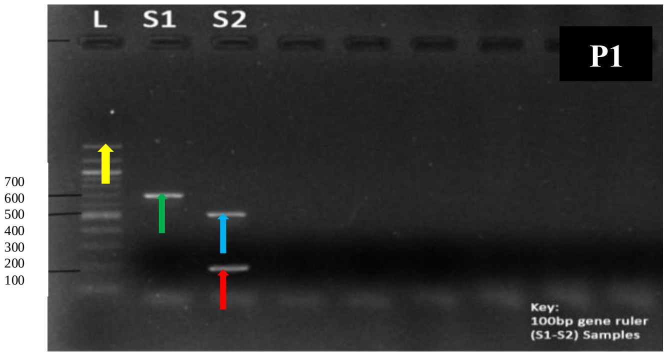


Plate 4.6: Agarose gel: Lane 1/L (yellow), 1000 bp NEB DNA ladder, Lane 2/S1 (green) 704 bp unrestricted check, Lane 3/S2 535 bp (red) and 167 bp (blue) restricted products of P1 gene at BfmI restriction site.

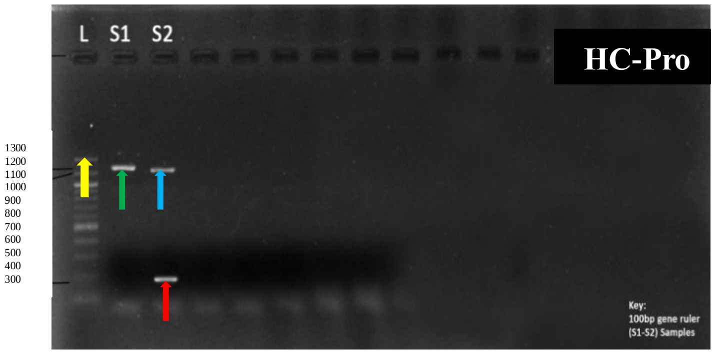


Plate 4.7: Agarose gel: Lane 1/L (yellow), 1000 plus bp NEB DNA ladder, Lane 2/S1 (green) 1389 bp unrestricted check, Lane 3/S2 1222 bp (red) and 163 bp blue restricted products of HC-Pro gene at AsuII restriction site**.**

Restriction digestion has been used to confirm PCR products of ligation cloning. Similar to this study, Szeberényi (2013) used restriction digestion and gel electrophoresis for DNA characterization. The bands were observed due to the ability of the restriction enzymes to cut at the target regions (Lou & Jin, 2017). Restriction endonucleases are specific nucleases that recognize and cut at phosphodiester linkages. Unlike other endonucleases, type II restriction enzymes are the commonly used due to their ability to cut within a sequence in the absence of substrates such as ATP, magnesium ions and Ado met. Upon DNA digestion, the VPg, P1 and HC-Pro fragments were separated in agarose gel that contained ethidium bromide. Ethidium bromide was used for visualization to validate the occurrence of digestion at *AscI*, *BfmI* and *AsuII* sites, for the VPg, P1 and HC-Pro genes, respectively.

Besides the integration of PCR and restriction digestion, other molecular methods have been deployed for DNA characterization. For instance, Northern blotting has gained popularity in the case of T-DNA detection and determination of copy number. However, this tool is highly dependent on factors such as origin of plant material, the quantity of digestion enzymes and DNA used and the restriction duration. Moreover, Northern blotting is highly reliant on the quality of DNA used, factors that have heavily constraint its applications (Li *et al*., 2013). Assays including qRT-PCR can also be applied for transgene detection; however, the level of RNA in a sample does not reflect protein expressions or functional activities of the corresponding enzymes (Wong *et al*., 2005). In addition, RNA induced transgenic resistance is sometimes associated with the surge in antisense RNA of the transgene or the accumulation of transgene-derived small RNAs (siRNA) in transformed plants. However, Northern Blot and deep sequencing assays of siRNAs techniques have indicated that transgenic siRNAs are absent in the transgenics and that disease resistance does not occur through the digestion of the virus RNA via the homologous induced mechanism. The absence of siRNA could be due to the fact that siRNAs might not be evenly distributed throughout the host plant. The accumulation of specific siRNAs in resistant plants was also not found to have a significant different from their susceptible counterparts. Hence, siRNA accumulation should not be used as an indicator of validation whether an organism is resistant or susceptible to a certain disease (Jiang *et al*., 2011). Moreover, Southern blotting, DNA sequencing and qRT-PCR techniques are expensive, time consuming and require advanced laboratory skill set limiting their applications. Therefore, conventional PCR coupled with restriction digestion would be ideal options as these tools have been found to be user friendly.

4.5 Conclusion and recommendations

T_0 transgenic plants were successfully obtained and confirmed through both PCR and restriction digestion leading to the rejection of the null hypothesis that there is no transformation whose transgene integration in putative transgenic maize has been formed. Both conventional PCR and restriction digestion would be an epitome of transgene detection, particularly in the African continent that is experiencing endless challenges with DNA blotting and sequencing.

CHAPTER FIVE

EVALUATION OF THE GENERATED TRANSGENIC MAIZE FOR RESISTANCE TO MAIZE LETHAL NECROSIS DISEASE

Abstract

Evaluation for disease resistance is commonly done in greenhouse or fields. However, these types of screening methods require large space and are prone to accidental spillover, which increase the likelihood of pandemic exacerbation. Detached leaf assay (DLA) assay buffers against the aforementioned constraints. However, this method can be influenced by the age of the seedling, inoculum quality, quantity, and environmental conditions. Screening for plant disease resistance has shown success with diseases including fusarium head blight, angular leaf spot disease and maize lethal necrosis disease (MLN). The objective of this study was to screen the transgenic maize inbred line for resistance to MLN through detached leaf assay. The experiment was laid in a completely randomized design with three replicates. Leaves of 21-day-old T_2 transgenic plants were surface sterilized in 50% NaClO and 0.1% TWEEN20®, sectioned into 2 cm squares and braised with a sterile needle. MS media was prepared by incorporating 20 mg/L gibberellic acid, 10 mg/L kinetin and 30% sucrose and pH adjusted to 5.8. Media was autoclaved and dispensed into petri plates. Four sterile leaf sections were cultured on MS media in each plate with the abaxial surface in contact with MS media. Exactly 20 µL of filter-sterilized inoculum containing maize chlorotic mottle virus (MCMV) and sugarcane mosaic virus (SCMV) was applied on the injured leaf sections using sterile ear buds. Non-transformed plant leaf sections were also inoculated as susceptible controls. Plates were placed in the growth chamber at 26-28˚C with 16-hour light and 8-hour dark photoperiod for 50 days. Analysis of variance revealed that the area under disease progress curve and MLN severity scores was significantly different across the tested regimes. There was no leaf chlorosis in the tested leaf sections obtained from the VPg transgenics which recorded a severity score of 1. The P1 and HC-Pro transgenics had severe chlorosis with a score of 4, while the susceptible control was completely necrotic with a severity score of 5. Based on the area under disease progress curve; the VPg, P1, HC-Pro and the susceptible control scored 6.44%, 17.48%, 22.43% and 23.13%, respectively. This was an indication that the VPg transgenic offered maximum protection against MLN.

5.1 Introduction

Maize lethal necrosis disease (MLN) has ravaged crops and is threatening livelihoods in the East and Central African region. The disease is caused by two viruses; maize chlorotic mottle virus (MCMV) and any virus of the potyvirus family however, in common cases, the sugarcane mosaic (SCMV). The synergistic action with the potyvirus increases the MCMV viral load hence, increasing disease severity. Some of the notable MLN symptoms are leaf yellowing and plant drying, premature plant death, stunted growth and low grain setting in the infected plants (Wamaitha *et al*., 2018).

Screening for MLN resistance is important towards the identification of susceptible and resistant plants. Routinely screening for disease resistance in crops takes place in greenhouses or fields however; these two screening approaches are associated with enormous challenges. The environmental conditions in fields are less optimal in imparting the required disease pressure and the occurrence of simultaneous non-target infections is possible in these conditions, which could compromise the results (Annan *et al*., 2023). Differentiation virus infections from symptoms of abiotic constraints such as nitrogen, phosphorus, sulfur and droughts remain a challenge. Greenhouse screening is reliant on seedling age, inoculum quality, quantity, and environmental conditions. Greenhouse/field screening is laborious and human error prone. Further, whole plant screening presents a higher likelihood of accidental pathogen 'spillovers' that are likely to increase chances of emergence or re-emergence of epidemics. Thus, there is need for better screening approaches such as DLA (Rezene *et al*., 2018).

Detached leaf assay (DLA) is the most appropriate measure of safeguarding against accidental disease 'spill-overs'. It is convenient for screening large numbers of genotypes and in multiple pathogens interaction screening. The DLA method requires less screening space rendering it cost effective (Kubota *et al*., 2017). Detached leaf assay has demonstrated correlations with greenhouse screening results, rendering it highly successful. Literature shows that MLN Tonui *et al*. (2018) has been successfully screened via DLA. Based on successful reports of DLA on resistance screening, the same method was also adopted in the present study. DLA was preferred due to biosecurity concerns of accidental disease spill over. This study was also conducted at the Biosafety Level II (BSL II) Plant Transformation facility in Kenyatta University as per the requirement of the National Biosafety Authority on experiments pertaining genetically modified organisms. The facility has automated doors to ensure restricted entries. The laboratory greenhouse also constitutes mesh screens of 680 microns pore size to exclude potential transgene vectors.

5.2 Materials and methods

5.2.1 Inoculum preparation

The inoculum was prepared according to Gowda *et al.* (2018) with modifications. Infected leaves were collected. The leave materials were washed with tap water to dislodge particulate matter. Leaves were rinsed 3 times in sterile water, with each rinse taking 3 min. Whole leaves were further cut into small pieces using a scalpel. The pieces were ground in a mortar and pestle in 10 mM potassium phosphate buffer at pH 7.0. The extract was sieved in a 4-layer sieve sterile cheesecloth. The eluent was centrifuged for 2 min at 12,000 g. Exactly 0.02 g/mL of carborundum was added to decant the sap eluent. Exactly 2 mL of the decanted extract was aliquoted into a 2 mL Eppendorf tube in the laminar flow cabinet and stored at -4˚C for downstream use. Activities involving virus extraction were conducted at 4˚C.

5.2.2 Media preparation, surface sterilization and disease inoculation

MS media was amended with 20 mg/L gibberellic acid, 10 mg/L kinetin and 30% sucrose. Media pH was adjusted to 5.8 followed by the addition of 3 g/L Gelrite and autoclaving at 121˚C for 15 min as described by Tonui *et al.* (2018). Accurately, 30 mL of warm media was dispensed into 100 × 15 mm petri plates under sterile conditions in the laminar flow chamber.

For production of materials for disease severity test, seeds from T_1 transformed CML444 were sown in 25×50 cm plastic pots containing peat moss. Pots were kept under an anti-insect net, Optinet 50 mesh with (0.26×0.83) pore size in the glasshouse. Maize plants were constantly checked for MLN infection with SCMV ImmunoStrip® KIT (Agdia, USA).

Leaf tissues of the VPg, P1 and HC-Pro transgenics were collected. Sterile gloves were won and the kit opened in a quiet place, with no wind. All the reagents were dispensed into the plastic box. The VPg, P1 and HC-Pro transgenics leaves were held by a tweezer and cut a sterile scalpel. The leaves were weighed into 0.1 g for every transgenic and separately placed into the plastic bag enclosed in the kit. The tweezers were disinfected in 70% ethanol. Leave extract was prepared as per section 5.2.1. Transgenic materials were tested as per the kit instructions SCMV ImmunoStrip® KIT (Agdia, USA).

A PCR assay as per section 4.2.6 followed 21 days of sowing to confirm transgenes in T_2 plants. Twenty-one day old maize plants that tested positive for the transgene and free of SCMV were used downstream for disease resistance evaluation.

Twenty-one-day old leaves were harvested from the T_2 transgenic plants and washed in cold running tap water to remove adsorbed sand and particulate matter. The leaves were surface sterilized in 50% (v:v) NaClO and 0.1% drops of TWEEN20® solution for 5 minutes under the laminar flow chamber. Rinsing was done 3 times with cold autoclaved distilled water with each rinse taking 3 min. Leaves were cut into 2×2 cm square sections using a sterile scalpel under the laminar flow hood. The leaf sections were transferred into sterile Whatman® filter papers for drying. Injuries were inflicted on the leaf sections using a sterile needle.

Four sterile leaf sections were placed in each plate with MS media with the abaxial surface in contact with media. Exactly 20 µL of filter-sterilized inoculum was applied on the injured leaf sections using sterile ear buds. This was carried out on the VPg, P1, HC-Pro transgenics and the non-transformed leaf sections as experiment controls. Plates were sealed in a parafilm and transferred to the growth chamber at 26-28°C with 16-hour light and 8-hour dark photoperiod for 50 days. Fluorescent tubes (PHILIPS-TL-D 18W/54-765) were used for illumination. Petri plates with excess water were occasionally opened in the laminar flow chamber to allow off excess water. The experiment was laid in a completely randomized design in three replicates.

5.2.3 Assessment of disease severity

The detached leaf sections were monitored daily for manifestation of chlorosis after every 5 days for 50 days. Leaf chlorosis was scored based on the MLN-severity chart (CIMMYT, 2014). Where 1= No maize lethal necrosis disease symptoms, 2 = Fine chlorotic streaks, 3 = Sever chlorotic mottling, 4 = Excessive chlorotic mottling, 5 = Complete plant necrosis (Plate 5.1). Area under disease progress curve (AUDPC) for 50 days was computed according to CIMMYT (2008).

Plate 5.1: Maize lethal necrosis disease (MLN) chlorosis scale; 1-no maize lethal necrosis disease (MLN) symptoms, 2-fine chlorotic streaks, 3-severe chlorotic mottling, 4-excessive chlorotic mottling and leaf necrosis and 5-complete plant necrosis.

Source: CIMMYT (2014)

5.3 Data analyses

Data on disease severity and AUDPC was transformed through square root transformation and subjected to the general linear model (GLM) for mean separation using SAS software version 8.2 based on the following models;

$Y_{ijk} = \mu + G_j + \varepsilon_{ijk}$……………………………………………………………… Equation 1

Where; Y_{ijk}= observation, μ=overall mean, G =effects due to j^{th} gene, ε_{ijk}=random error.

5.4 Results and discussion

5.4.1 Disease severity

Effects due to the transgenics were significant ($p \leq 0.001$) for areas under disease progress curve and MLN severity scores (Table 5.1). However, plate, number of leaf sections and the interaction of gene × number of leaf sections were not significant on the AUDPC and severity scores.

Table 5.1: Mean squares for area under disease progress curve (AUDPC) and disease severity in the three transformation regimes

		AUDPC				**Severity**			
Source of variation	**df**	**Sum of squares**	**Mean Square**	**F Value**	**Pr > F**	**Sum of squares**	**Mean Square**	**F Value**	**Pr > F**
Plate	2	0.79	0.40	0.36	0.6981	0.03	0.02	0.33	0.72
Gene	3	1704.09	568.03	520.41	<.0001	79.51	26.50	525.05	<.0001
No. of leaf sections (NL)	3	3.00	1.00	0.92	0.4447	0.17	0.06	1.10	0.36
Gene × NL	9	7.28	0.81	0.74	0.6689	0.37	0.04	0.82	0.60
Error	30	32.74	1.09						
C.V (%)		6.45				6.34			
R^2		0.98				0.98			

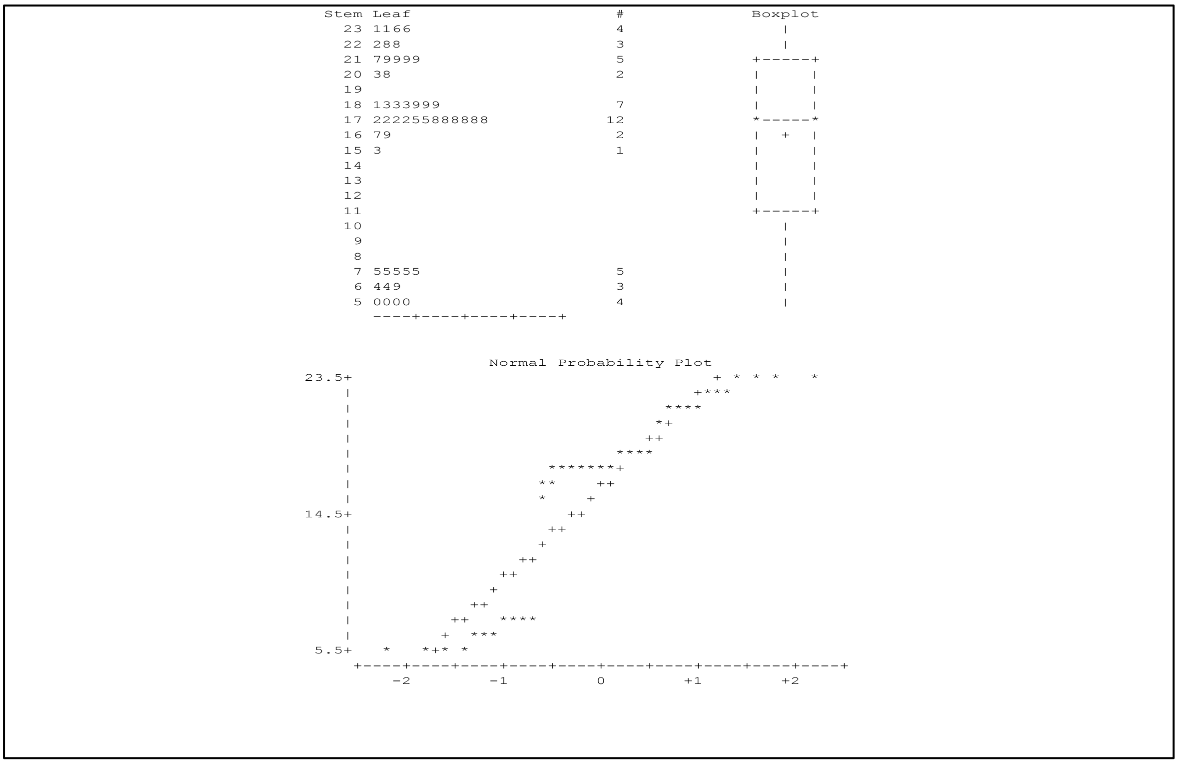
***= Significant at $p \leq 0.001$, and CV=co-efficient of variation.

The normality test from Shapiro wilk test (**W**=0.831923) and normal probability plot showed that the data was normally distributed (Table 5.2; Figure 5.1).

Table 5.2: Shapiro wik test for Area under disease progress curve

Tests for Normality_AUDPC				
Test	**Statistic**		**p Value**	
Shapiro-Wilk	W	0.831923	Pr < W	<0.0001
Kolmogorov-Smirnov	D	0.259046	Pr > D	<0.0100
Cramer-von Mises	W-Sq	0.597376	Pr > W-Sq	<0.0050
Anderson-Darling	A-Sq	3.35748	Pr > A-Sq	<0.0050

Normality test from Shapiro wilk test (**W**=0.829634) and normal probability test showed that the data was normally distributed (Figure 5.2; Table 5.3).



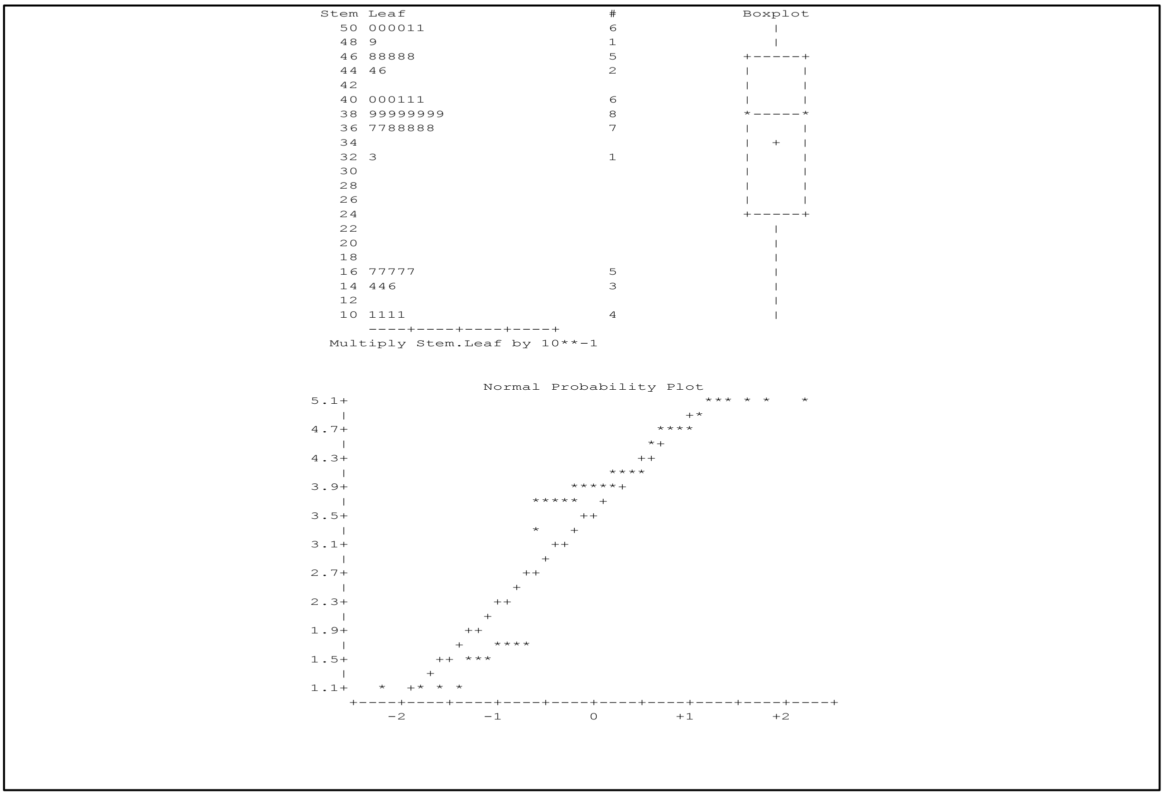
```
  Stem Leaf                      #            Boxplot
    23 1166                      4               |
    22 288                       3               |
    21 79999                     5            +------+
    20 38                        2            |      |
    19                                        |      |
    18 1333999                   7            |      |
    17 222255888888             12            *------*
    16 79                        2            |  +   |
    15 3                         1            |      |
    14                                        |      |
    13                                        |      |
    12                                        |      |
    11                                        +------+
    10                                           |
     9                                           |
     8                                           |
     7 55555                     5               |
     6 449                       3               |
     5 0000                      4               |
       ----+----+----+----+


                   Normal Probability Plot
23.5+                                     + * * *   *
    |                                   +***
    |                                ****
    |                               *+
    |                              ++
    |                           ****
    |                    *******+
    |                   **    ++
    |                   *    +
14.5+                      ++
    |                    ++
    |                   +
    |                 ++
    |               ++
    |              +
    |            ++
    |          ++   ****
    |         +  ***
 5.5+   *   *+* *
     +----+----+----+----+----+----+----+----+----+----+
         -2       -1         0        +1        +2
```

Figure 5.1: Probability plots showing normality for Area under disease progress curve

Table 5.3: Shapiro wilk test for disease severity

Tests for Normality_Severity				
Test	**Statistic**		**p Value**	
Shapiro-Wilk	W	0.829634	Pr < W	<0.0001
Kolmogorov-Smirnov	D	0.26556	Pr > D	<0.0100
Cramer-von Mises	W-Sq	0.604656	Pr > W-Sq	<0.0050
Anderson-Darling	A-Sq	3.394415	Pr > A-Sq	<0.0050



```
 Stem Leaf                     #             Boxplot
   50 000011                   6                |
   48 9                        1                |
   46 88888                    5             +-----+
   44 46                       2             |     |
   42                                        |     |
   40 000111                   6             |     |
   38 99999999                 8             *-----*
   36 7788888                  7             |     |
   34                                        |  +  |
   32 3                        1             |     |
   30                                        |     |
   28                                        |     |
   26                                        |     |
   24                                        +-----+
   22                                           |
   20                                           |
   18                                           |
   16 77777                    5                |
   14 446                      3                |
   12                                           |
   10 1111                     4                |
      ----+----+----+----+
  Multiply Stem.Leaf by 10**-1


                  Normal Probability Plot
5.1+                                     *** * *   *
   |                                   +*
4.7+                                ****
   |                               *+
4.3+                              ++
   |                           ****
3.9+                       *****+
   |                   *****  +
3.5+                        ++
   |                   *   +
3.1+                     ++
   |                    +
2.7+                  ++
   |                 +
2.3+               ++
   |              +
1.9+            ++
   |           +   ****
1.5+         ++ ***
   |        +
1.1+   *  +* * *
    +----+----+----+----+----+----+----+----+----+----+
        -2        -1         0        +1        +2
```

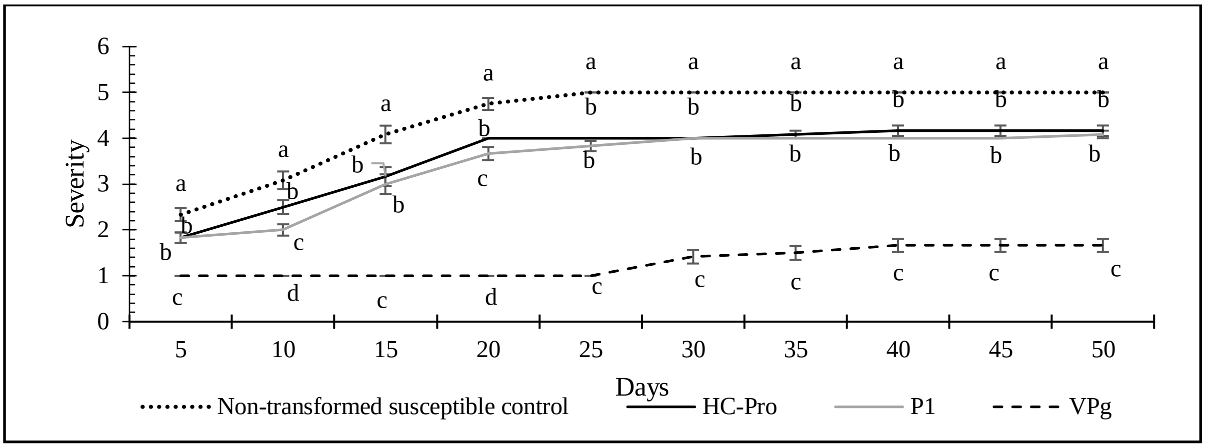
Figure 5.2: Probability plots showing normality for disease

Disease severity progressed rapidly in the tested transgenic leaf materials throughout the assay period except in the VPg transgenics. At 5 dpi the tested Vpg transgenic materials had a severity score of 1 and 2 for the rest of the P1, HC-Pro and the susceptible control. There was a rapid increase in chlorosis between 5-20 dpi in the rest of the tested materials except the VPg. There was no significant increase in severity from 20-50 dpi in all the tested materials. At the end of the assay period, the VPg transgenic materials displayed no MLN symptoms with a score of 1, both the P1 and HC-Pro transgenic materials were excessively chlorotic with a score of 4 while the susceptible control was completely chlorotic with a score of 5 (Table 5.4; Figure 5.3; Appendix vii; ix).

Table 5.4: Means for MLN severity for transformants and their susceptible controls

Gene	**Mean severity**		
Non-transformed susceptible control	5	±	0.04a
HC-Pro	4	±	0.08b
P1	4	±	0.06b
VPg	1	±	0.07c
LSD $_{0.05}$	0.19		

HC-Pro=Transgenic, P1=Transgenic, VPg=Transgenic. Letters indicate statistical difference (LSD $_{0.05}$=2.07) test at $p \leq 0.05$.



Scale: 1-no MLN, 2-fine chlorotic streaks, 3-severe chlorotic mottling, 4-excessive chlorotic mottling and leaf necrosis and 5-complete plant necrosis

Figure 5.3: Disease progression on leaf detached assay for a period of 50 days. C: Non-transformed susceptible control. Each point represents the average and standard error of 4 leaf sections and three biological replicates. At each time point, letters indicate statistical difference (LSD 0.05=2.07) test at p ≤ 0.05.

5.4.2 Area under progression curve for maize lethal necrosis

The VPg transgenic recorded the lowest AUDPC value of 6.44 as compared to 23.13 for its susceptible control. An AUDPC value of 17.48 and 22.08 was recorded in the P1 transgenics and its susceptible control, respectively. While the HC-Pro transgenic had an AUDPC value of 18.38 as opposed to 22.43 for its susceptible control (Figure 5.5). Pathogenesis was varied across the tested materials characterized by a linear disease. The disease was intense in the control experiments, which was comparable to the transgenics (Plate 5.1).

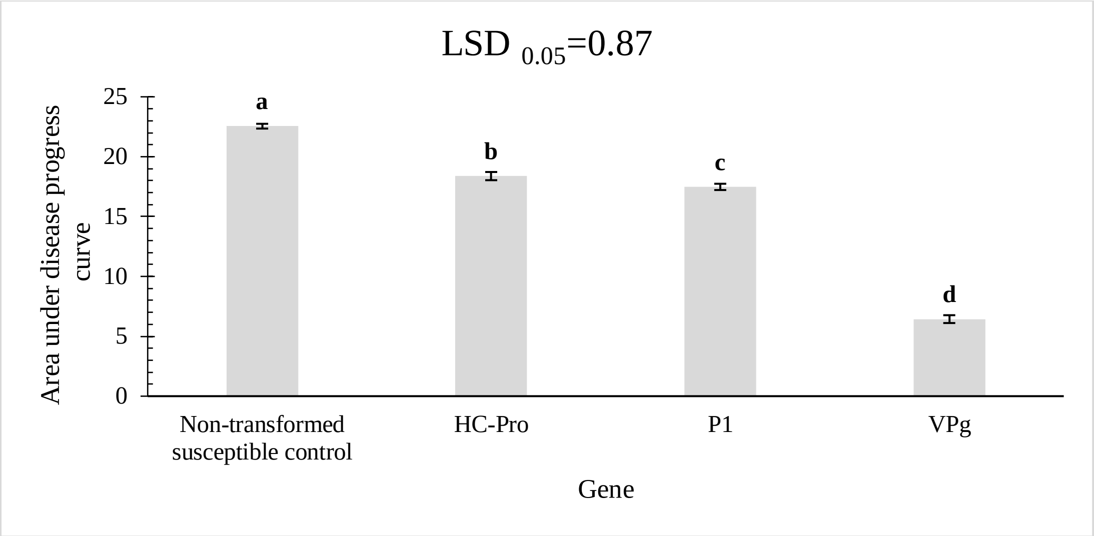


Figure 5.4: Area under disease progress curve at 50 dpi. Values correspond to the last time point in figure 2. Letters indicate statistical difference at $p \leq 0.05$ (LSD 0.05=2.07).

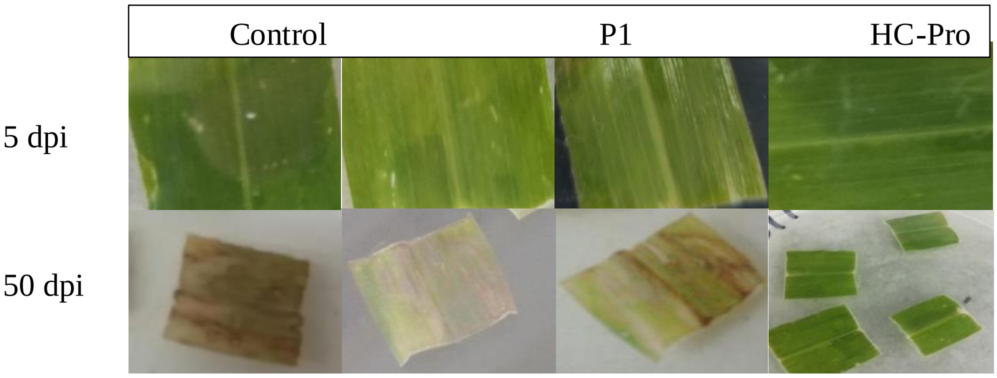


Plate 5.14: Leaf detached assays showing disease progress in the leaves of maize plants transformed with SCMV VPg, P1 or HC-Pro. Leaves of non-transformed plants were used as susceptible control. Representative pictures were taken at 5 dpi and 50 dpi.

There was linear disease progression in the tested P1 and HC-Pro transgenic materials as compared to the VPg transgenic material. Disease progression is as a result of the ability of the pathogen to accumulate and hinder chlorophyll synthesis leading high disease progression and intense leaf chlorosis. Literature demonstrates that several factors might have led to the witnessed MLN progression in the tested materials including poor expression of transgenic induced RNA of the P1 and HC-Pro genes to confer SCMV silencing.

The P1 proteins of the potyviruses act primers in enhancing genome amplification. The HC-Pro is a multifunctional protein tasked with a number of key functions such as elevating viral pathogenicity through countering the endonuclease activities of antiviral defense proteins of the host plants to enhance systemic translocation (Plisson *et al.*, 2003). The HC-Pro in synergistic with the VPg proteins also aids in the production of virus suppressors RNA (VSR) for the suppression of the host RNA silencing inducing complex to accelerate systemic invasion of MLN causing viruses (Rajamäki & Valkonen, 2009).

The variation in AUDPC and severity values as observed in the VPg, P1, and HC-Pro transgenics was in agreement with Liu *et al*. (2009) where transgenic plants of CP1, CP3, CP6 and CP7 showed high resistant ability while others such as transgenic plants of CP5 expressed high susceptibility, which was attributed to the ability of the transgenes to express high level proteins. Patil *et al*. (2011) also indicated that varied levels of disease resistance could be associated with transgene expression levels. According to their findings, transgenes with high levels of detectable transcript produced significant quantities of siRNA which improved disease resistance. However, Germundsson and Valkonen (2006) demonstrated that expression of similar resistance patterns is also possible as in the case of P1, and HC-Pro transgenics.

Based on Hu *et al*. (2020) and Shamandi *et al*. (2015), low severity and AUDPC values could have been due to the base pairing of the viral VPg genes with the VPg transgenic proteins triggering destruction of the anomaly via the RISC/DICER pathway. Destruction of the Vpg double strand structures would have scuttled the translation of SCMV proteins, an event that antagonized the production of Viral RNA Suppressors (VSRs) that are responsible for the suppression of the small interference RNAs (siRNAs). The siRNAs function to impart immunity during virus infections

According to Zhu *et al*. (2014), the destruction of the VPg complex might have acted to inhibit the formation of the invading virus SCMV VPg protein-maize Elongin C (ZmElc) complexes thus, reducing disease severity and disease progression. Awata *et al*. (2019) indicated that the VPg-ZmElc complexes function as primers for SCMV replication, elevating its accumulation. Therefore, prevention of the complex formation could have functioned to reduce the virus replication hence decreasing MLN severity and disease progression. Silencing of the invading SCMV VPg proteins might have inhibited the formation of ZmeIF4E-VPg complex which also enhances MCMV replication and translation leading to reduced pathogenicity. According to Zhu *et al*. (2014), the ZmeIF4E-VPg complex functions as mRNA translation initiation complexes for recruitment of SCMV translation factors. Formation of the VPg transgenic proteins to SCMV VPg proteins complexes and its subsequent destruction could have hindered the formation of the mRNA translation initiation complex leading to no MLN expression. Unbound ZmELC could have also acted to influence the expression of ZmeIF4E proteins that are responsible for the initiation of local and systemic MLN defenses.

Based on Packard *et al*. (2024), it is also likely that the absence of chlorosis in the VPg transgenic materials could have been due to the ability of the VPg transgenic proteins to antagonize the formation of virus replication compartments (VRCs) an event that led to zero replication of intruding SCMV particles and MLN absence. In contrast, the HC-Pro and P1 genes could not arrest the VRCs formation leading to potyvirus replication and subsequent MLN occurrence.

The three proteins are important in VRCs formation, a process that converts cell membranes into ‘virus manufacturing factories. The VRCs act as scaffolds for virus protein replication, housing virus RNAs and host proteins necessary for replication (Morozov & Solovyev, 2020). In a separate study, the VPg interaction with other host proteins to promote membrane remodeling in forming virus replication compartment (VRCs) and SCMV cell fusion as reported by Xue *et al*. (2023).

However, the P1 and HC-Pro transformants were not completely necrotic. An indication, there were SCMV suppression activities. Various studies have demonstrated that the two proteins are important in virus replication. For instance, the highly divergent SCMV P1 proteins’ concentration surges in the nucleolus of the infected cells during the initial stages of MLN infection, targeting the dense granular component in the nucleolus. The presence of the protein has also been observed in the cytoplasm of the infected cells where it associates with the ribosomes by binding the 80S and the 60S ribosome subunits an association that is predicted to help in SCMV translation. The P1 protein functions are aided by the nucleolar localization signal (NoLS) and a nuclear export signal (NES). Both the NoLS and the NES are responsible for docking in the nucleolus and transport to the cytoplasm as reported by Martínez and Daròs (2014).

The P1 protein also functions by binding the *VERNALIZATION INDEPENDENCE 3* (VIP3) sub units over the 5′-fragment of RISC-cleaved target RNA antagonizing the functions of RISC. It also interacts with the MOS4 modifier, 2 IMPORTINs, and BIG5, which are involved in RNA splicing and RNA transportation leading to an increase in virus concentrations. The HC-Pro which is a highly conserved protein also has a significant role in post transcriptional gene silencing under the synergistic action of the P1 proteins as reported by Hu *et al*. (2020). The HC-Pro functions by inhibiting the miR171 or miRNA39 nucleolytic activities that are responsible for the cleavage of mRNAs of the *Scarecrow-like* (SCL) transcription factors. The miRNA39 is complementary to the internal regions of SCL mRNAs and upon formation of the complex, digestion of the target mRNA occurs, leading to degradation of the target pathogen. This is an indication that this miRNAs function as siRNAs that are associated with RNA silencing. It is likely that these processes were interfered with leading to the absence of complete chlorosis in the P1 and HC-Pro transgenics.

The absence of chlorosis in the VPg transgenics showed its ability to arrest SCMV proteins from interfering with chloroplast transport. It is also indicated that the maize ferredoxin-5 (FdV) import into the maize bundle-sheath cell (BSC) causes the transfer of electrons of photosystem I to a number of electron acceptors in the leaves. An influx of electrons in leaves reduces $NADP^+$ via a ferrodoxin NADP oxidoreductase (FNR) to create energy. However, during MLN, the SCMV proteins including the HC-Pro and VPg proteins bind the FdV protein hindering its normal functions. Therefore, inadequate or completely no arrest of these proteins in the HC-Pro, P1 transgenics and the susceptible controls might have altered the chloroplast transport via the formation of the P1-FdV/HC-Pro-FdV complexes leading to low ATP synthesis and leaf chlorosis as reported by Cheng *et al*. (2008).

Variation in resistance across the three regimes could have been influenced by the suppression of the marker and reporter genes or the target genes corresponding to the promoter activities. The transgene expression is dependent on the effectiveness of the promoter and the transcriptional efficiency of respective genes leading to the accumulation of more transcripts and subsequent production of sufficient siRNAs. Accumulation of siRNAs to a certain threshold has been found to significantly influence transgenic RNA mediated resistance as reported by Gammelgård *et al*. (2007).

The genetics of MLN resistance has been defined in maize with followed by the identification of various quantitative trait loci (QTLs) or genes responsible for MLN resistance. It has been discovered that MLN viruses can replicate in some maize plants, however, with little systemic impacts leading to non-significant pathogenicity. For instance, susceptibility of maize to MCMV is possible due to lack of even distribution of QTLs that confer resistance to the specific virus. Candidate genes associated with MLN tolerance have been identified in maize. Twenty four single nucleotide polymorphisms (SNPs) responsible for MLN tolerance were identified through Africa association mapping panels (AMP) in the Improved Maize for African Soils (IMAS) and Drought Tolerant Maize. Moreover, 54 SNPs that confer MCMV and MLN tolerance were identified in the IMAS. This demonstrated that SNPs associated with MCMV/MLN resistance were present in maize. Moreover, MCMV tolerant QTLs were identified in maize chromosomes 1, 2, 3, 5, 6, and 10. However, literature indicates that MLN tolerance can be overcome under severe disease pressure (Murithi *et al*., 2021).

In addition, protein analysis has shown that the host expression of disulfide isomerases like protein ZmPDIL-1 and peroxiredoxin family protein ZmPrx5 would increase MCMV susceptibility (Dang *et al*., 2019). The expression of Class I β-1, 3-glucanase (GluI) which is responsible for plasmodesmatal size exclusion, would also be involved in the movement of MLN viruses across the host plant (Iglesias & Meins, 2000). Zhu *et al*. (2014) also demonstrated that host plant expression of maize Elongin C (ZmElc) would enhance SCMV. A majority of potyvirus proteins responsible for MLN infection are also multifunctional. Maize lethal necrosis is controlled by polygenes. There is scanty information on whether controlling a single gene in the polygene family could reduce MLN expression, however, combating the disease through silencing the expression of a single protein in the virus is possible as described below.

A number of virus transgenic proteins have been used successfully in the induction of transgenic RNA-dependent gene silencing. For instance, Petrov *et al*. (2023) targeted the CI-6K2-NIa and HC-Pro proteins of the Potato Virus Y (PVY) and demonstrated that the virus could be effectively managed. The PVY is a potyvirus that infects a number of plant families. Potato plants that successfully received the ds/siRNAs could not contract the PVY. The results indicated that the disease was reduced by 82% in tobacco after successfully inoculating the dsRNAs constructs complementary to the HC-Pro protein. Upon inoculating double strand RNAs (dsRNAs) or single stranded RNAs (siRNAs) containing the same PVY genes, post transcriptional gene silencing was achieved successfully in potatoes. Similarly, infection-free potato plants were obtained upon treatment with constructs containing complementary sequences of the NIb proteins. This was a demonstration that potyviruses can me managed in a similar fashion in other plant species. **In a separate study,** Chen *et al*. (2014) transformed wheat N12-1 genotype with wheat streak mosaic virus (WSMV) Nib8 gene and was able to prevent significant yield losses. It was also discovered that transgenic resistance could be transferred from N12-1 transgenics to other WSMV susceptible commercial genotypes through multiple backcrossing.

Despite the success, transgenic induced RNA silencing is constraint by a number of factors. RNA interference has been tested in field and greenhouse experiments; however, field experiments results deviate significantly from those of greenhouse and resistance also breaks down with time. The breakdown of transgenic induced virus resistance has been reported in a number of studies. For instance, Petchthai *et al.* (2018) reported that resistance of Odontoglossum ringspot virus (ORSV) significantly reduced resistance in the T_3 with subsequent lack of resistance in T_4. Chen *et al.* (2014) also demonstrated that wheat transgenics containing the coat protein of the replication enzyme gene or coat protein genes of the wheat streak mosaic virus (WSMV) had a significant resistance in the greenhouse as compared to the field where loss of resistance was reported. Reduced resistance was attributed to successful invasion of non-target viruses and their subsequent blockage of the digestion of the target virus and the frequent virus mutations. However in a separate study, Chen *et al.* (2014) indicated that rice transformed with complementary protein of the WSMV Nib8 gene offered stable transgene resistance for five years at different experimental sites. Stability in RNA induced transgenic resistance is rare, therefore, this was a reported as breakthrough and was believed to be the first experiment with such significant results. Later, Jiang *et al.* (2023) also demonstrated that the breakdown of resistance in the T_1 generation of PVX, PVY and PSTVd transgenics was insignificant to that of the T_0 generation.

Various studies have highlighted key factors that are likely to contribute to transgenic resistance breakdown. Zhang *et al.* (2012) differently mounted the Potato Virus X (PVY) CP-gene and Rice Stripe Virus (RSV) coat protein (CP) gene mediated resistance in both tobacco and rice. The results were varied indicating that RSV CP-mediated offered the best protection. The study highlighted that the differences could be attributed to species difference, different transgene expression profiles and the differences between the viruses. The PVY contains a single stranded positive sense RNA with a large open reading frame that translates into a large protein. The CP gene is located at the 3’ end indicating that silencing could have occurred in the 5’-3’ direction probably leading to silencing of the entire genome. In contrast, the RSV contains many genomes that include the drug justice complementary strand RNA3 which encode the CP gene. The other genomes; RNA1, RNA2 and RNA4 that also encode the CP protein are hypothesized to be indirect targets of the CP transgenic mediated gene silencing. The siRNA generated from these RNAs also accumulate rapidly in the plant cells a scenario that may have caused RISC/DICER saturation leading to prevention of the CP gene siRNA antiviral activities. Reduced RNA interference in rice could have also been due to poor binding ability of the CP-active site to the siRISC- recognition site as reported in the amiR2b-mediated Cassava Mosaic Virus (CMV) resistance. Non-significant breakdown of resistance in this case could be caused by the asexual reproduction in potatoes that confers stable gene inheritance a scenario that is likely to differ in maize transgenics. Furthermore, transgenic RNA mediated resistance can be constraint mismatches between the transgene RNA products and the target virus RNA. Ossowski *et al*. (2008) also indicated that a mismatch of more than five base pair between the transgenic and the target sequence has been found to reduce the binding ability of the two leading to lack of target pathogen elimination.

5.5 Conclusion and recommendations

This study demonstrated that management of MLN through transgenic RNA induced gene silencing is effective leading to the rejection of the null hypothesis that there is no information on the effect of P1, HC-Pro and VPg transgene expressions’ potential to silence SCMV and evoke resistance against MLN expression. Therefore, RNA bioengineering would be integral in providing timely solutions towards MLN management.

CHAPTER SIX

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

6.1 General discussion

Maize inbred lines CML444 and CML442 were selected for this study as both lines have significant general and specific combining (GCA/SCA) abilities for traits of economic importance (Dhliwayo *et al*., 2009). During breeding, combining abilities are important in the evaluation of gene action. Both GCA and SCA are also central, particularly to population development (Beyene *et al*., 2017). Despite the high GCA/SCA, both lines are highly susceptible to maize lethal necrosis disease (MLN) hence; there is need for engineering for resistance. Establishment of *in-vitro* culture is the pre-requisite to genetic transformation. Plant transformation is important as it provides the platform for integration of beneficial or desirable traits of economic and/or agronomic importance (Carsono *et al*., 2021). Successful induction and regeneration in the laboratory is dependent on the tissue culture components used including the type of basal media, plant growth regulators (PGRs) and their appropriate ratios, genotype and type or the source of the explant. However, incorporation of PGRs in the right quantity is a critical factor to successful regeneration. The commonly used PGRs in *in-vitro* regeneration are auxins and cytokinin. For instance, 2, 4-dichlorophenoxyacetic acid (2, 4-D) and/or naphthalene acetic acid (NAA) are deployed in callus induction in most plant species (Buko *et al*., 2020). 2, 4-dichlorophenoxyacetic acid is preferred due to its ability to stimulate the formation of embryogenic callus although this depends on the type of the explant and genotype. Embryogenic callus are easy to transform, which was our second objective. Somatic embryogenesis is also highly reproducible and maintains genetic integrity as compared to organogenesis. Moreover, diverse explants such as pollens, young leaves and roots, mature and immature embryos and germinating hypocotyls can be used to regenerate somatic embryos (Faisal *et al*., 2021). As reported in Yan *et al*. (2021), incorporation of PGRs in the present study showed significant results towards explant regenerability. Moreover, application of rooting PGR is required in difficult to root regenerants, our choice of IBA for root induction was based on its ability to induct healthier and vibrant adventitious roots (Elmongy *et al*., 2018).

Genetic transformation offers the platform for incorporation of beneficial traits that otherwise are difficult to impart through conventional breeding techniques. For instance, it has become possible to introduce specific genes that confer biotic and abiotic stress resistance in target plants (Carsono *et al*., 2021). Despite the existence of many binary vectors, *Agrobacterium*

GV3101 was preferred due to its ability to enhance transformation efficiency and increase explant regeneration potential as different *Agrobacterium* strains posse varied transformation efficiency (Ondzighi-Assoume *et al*., 2019). Moreover, deployment of *Agrobacterium* alongside highly expressing constitutive vectors is possible, conforming to our choice of *pMDC35* constitutive vector which has a superior *35S* promoter for P1, VPg and HC-Pro transgenes expression. In addition, the *Hygromicin* (*Hygro*) selection marker in the *pMDC35* vector ascertained complete elimination of false positives as *Hygromicin* is a better selection agent as compared to *Kanamycin* (Ontiveros-Cisneros *et al*., 2022). Detection and selection of organisms is vital after genetic transformation. Sensitive and reliable analytical tools are a prerequisite for successful identification of transgenes. Molecular tools inclusive of conventional PCR, QRT-PCR, Southern blotting and DNA sequencing and restriction digestion have been successfully utilized on molecular characterization of transgenes. In the present study, conventional PCR and restriction digestion were preferred. Both methods are cheap, less labour intensive and generate results within a short period of time. Additionally, restriction digestion remains a powerful tool in confirming specific DNA inserts (De Saeger *et al*., 2022)

Determining plant disease response is required post plant transformation for disease resistance. Detached leaf assay (DLA) mode of evaluation for disease resistant has become popular due to a number of factors. The method is rapid and screening can be conducted all year-round. It is less costly, and minimizes risks of pandemic or epidemic spill over. Moreover, optimal disease progression accruing from the highly regulated environment guarantees reliable results as compared to field and greenhouse assays (Deshmukh *et al*., 2018).

6.2 Conclusions

Inbred lines are regenerable leading to the rejection of our null hypothesis that CML444 and CML 442 cannot be generated *in-vitro*. T_0 transgenic plants were successfully obtained and confirmed through both PCR and restriction digestion leading to the rejection of the null hypothesis that transformation and confirmation of transgenes in putative transgenics is not possible. Management of MLN through transgenic RNA induced gene silencing was possible thus, the rejection of the null hypothesis that the tested transgenes have no potential to silence sugarcane mosaic virus (SCMV) and impart MLN resistance.

6.3 Recommendations

1. This regeneration protocol was effective and successful therefore, it could be considered in future transformation studies.
2. PCR and restriction digestion proved effective and could be the solution for molecular characterization of transgenes in the future.
3. Future management of viruses lies in new breeding techniques such as transgenic RNA dependent gene silencing due to their rapidness, ability to concerve the environment through the significant reduction of pesticide application and economic viability.

6.4 Areas for further research

This research has provided information that management of maize lethal necrosis diseases (MLN) through transgenic induced RNA silencing is possible. The study has further provided the new insight into the three genes namely the VPg, P1 and HC-Pro where the VPg candidate gene offered the best MLN protection. Although further research on the gene is important, testing the ability of other genes such as P3, 6K1, CI, 6K2, Nia and Nib of the sugarcane mosaic virus (SCMV) to elicit transgenic RNA mediated gene silencing against SCMV to confer MLN resistance is pivotal.

A majority of potyvirus proteins are multifunctional and their silencing would provide further details on the best approach to combat sugarcane mosaic virus (SCMV) towards MLN management. For instance the 6K1 is responsible for SCMV replication while the 6K2 protein is important for SCMV replication within the endoplasmic reticulum. This event is salient as it causes rapid SCMV multiplication inside the chloroplast leading to significant increase in MLN severity. Other SCMV proteins including the P3, CI, NIa-Pro, and NIb are protagonistic to 6K2-induced virus replication compartments (VRCs), elements that are essential for titer and virulence increase.

Research on silencing maize chlorotic mottle virus (MCMV) proteins would also be important. Two MCMV proteins, the P31 and P32, have been found fundamental for the increase of both the viral load and disease pathogenicity. Therefore, silencing of these proteins through RNA interference would provide information and broaden the avenues on the best way possible for MLN management.

Moreover, the application of gene silencing on host plant genes would be one of the most reliable and sustainable platform for conferring transgenic RNA induced gene silencing towards the reduction of MLN expression. A number of host plant genes such as the ZmELC and ZmeIF4E have been found to interact with MCMV proteins during replication and translation. Silencing of ZmELC and ZmeIF4E in maize would reduce or inhibit replication and translation of MCMV, a virus that amplifies SCMV titers leading to systemic infections and MLN expression.

Whole plant assay would give deeper insights on how individual transgenic plants and their organs respond when challenged with MLN. Whole plant assay would enable for disease incidence analysis rather than disease severity that only shows the impacts of the disease on the affected leaf area. Measurement of disease severity is error prone as compared to disease incidence as assaying for disease severity is dependent on estimations based on reference chats. Disease severity relies on visual judgements which might as well be inaccurate as opposed to disease incidence which can allow for the application of digital technologies which are accurate. Whole plant assay would also give more information on the MLN response of different plant parts and provide further details on the host plant multi-protein interactions and their effect on MLN resistance or severity. It would also provide more insights on the impact of the environment on the expression and activity of the transgenic proteins towards MLN resistance.

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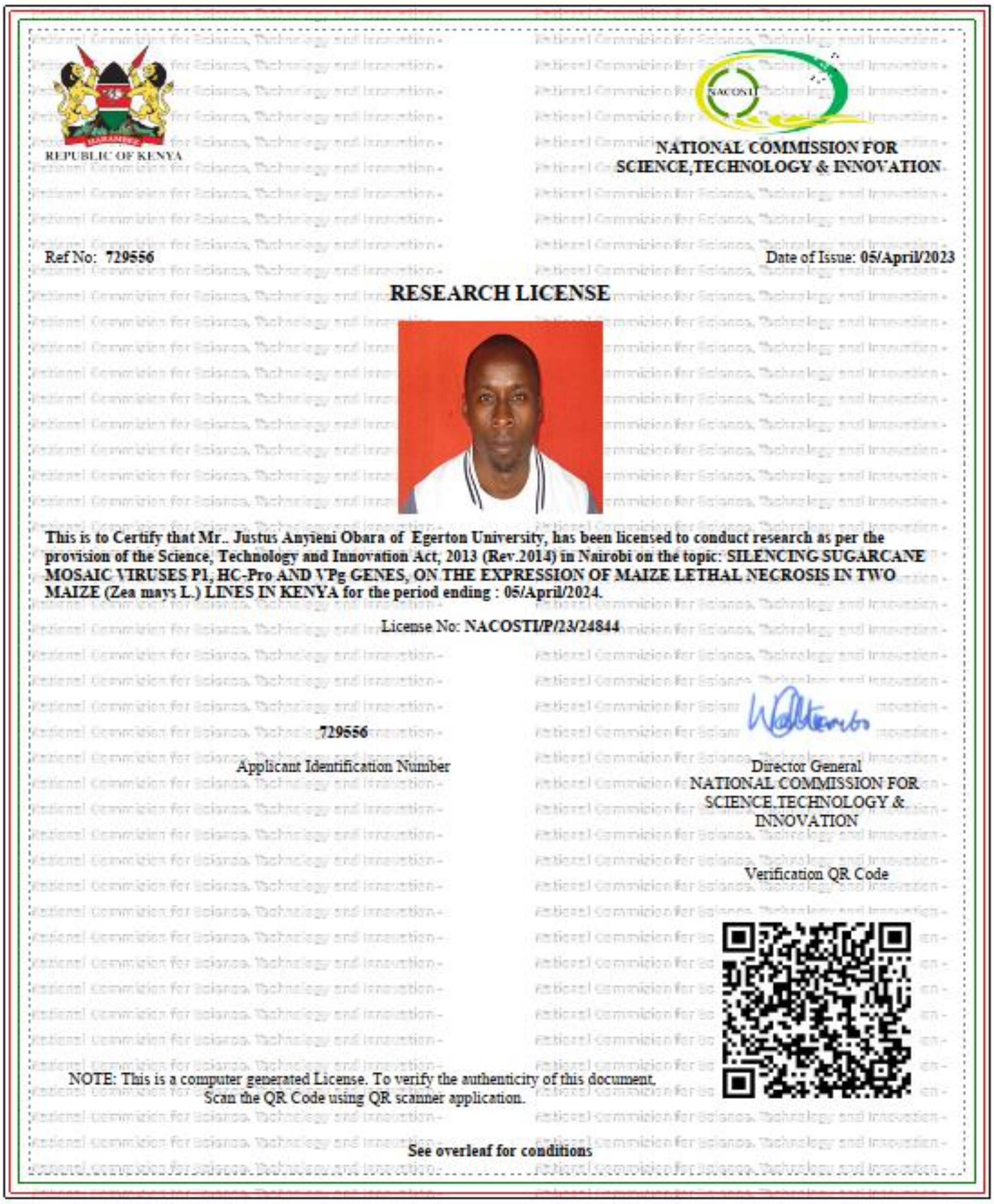
APPENDICES

Appendix I: NACOSTI Research permit



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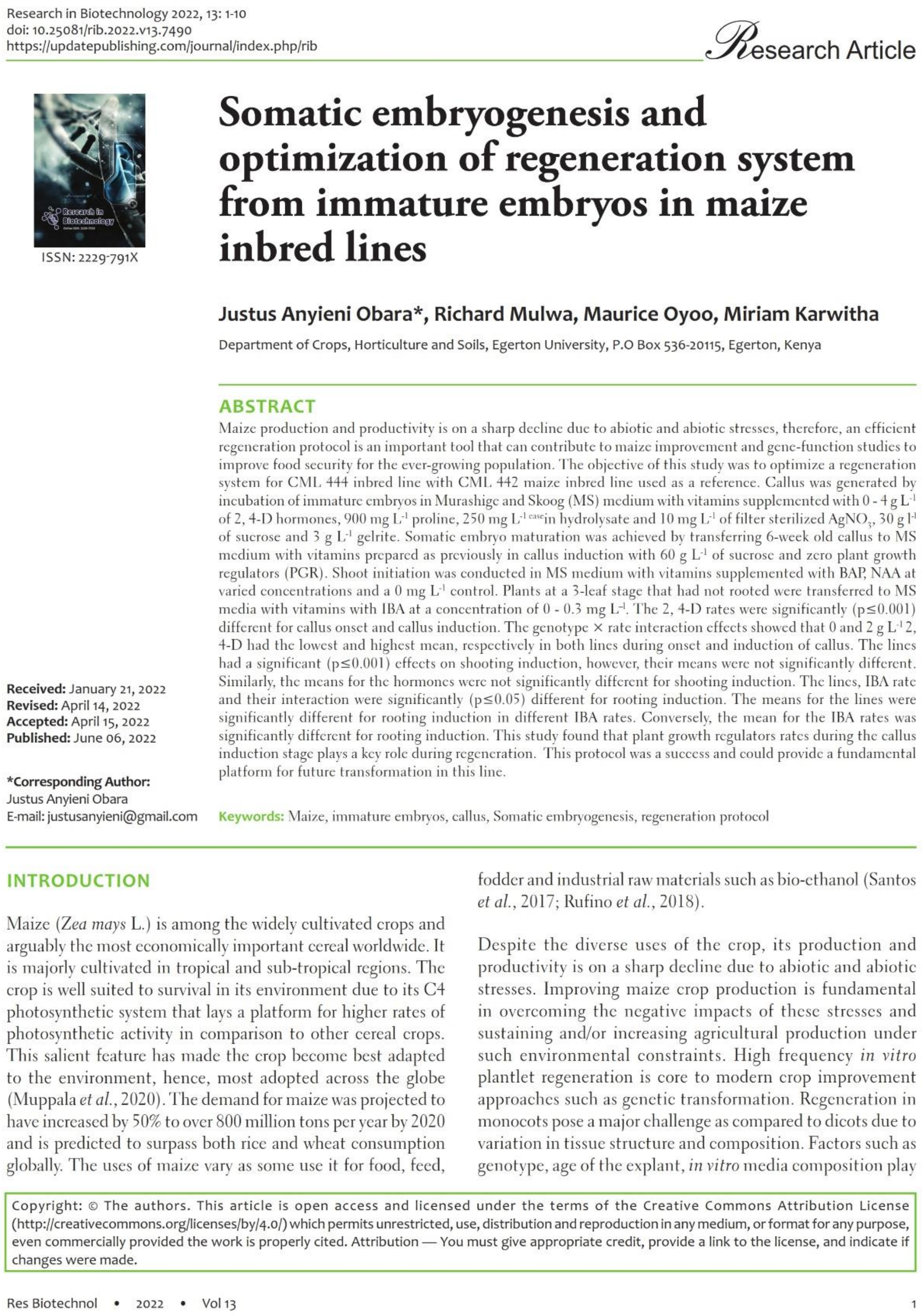
Appendix II: Abstract for somatic embryogenesis and optimization of regeneration system from immature embryos in maize inbred lines



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Somatic embryogenesis and optimization of regeneration system from immature embryos in maize inbred lines

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ABSTRACT

Maize production and productivity is on a sharp decline due to abiotic and abiotic stresses, therefore, an efficient regeneration protocol is an important tool that can contribute to maize improvement and gene-function studies to improve food security for the ever-growing population. The objective of this study was to optimize a regeneration system for CML 444 inbred line with CML 442 maize inbred line used as a reference. Callus was generated by incubation of immature embryos in Murashige and Skoog (MS) medium with vitamins supplemented with 0 - 4 g L^{-1} of 2, 4-D hormones, 900 mg L^{-1} proline, 250 mg $L^{-1\,case}$in hydrolysate and 10 mg L^{-1} of filter sterilized $AgNO_3$, 30 g l^{-1} of sucrose and 3 g L^{-1} gelrite. Somatic embryo maturation was achieved by transferring 6-week old callus to MS medium with vitamins prepared as previously in callus induction with 60 g L^{-1} of sucrose and zero plant growth regulators (PGR). Shoot initiation was conducted in MS medium with vitamins supplemented with BAP, NAA at varied concentrations and a 0 mg L^{-1} control. Plants at a 3-leaf stage that had not rooted were transferred to MS media with vitamins with IBA at a concentration of 0 - 0.3 mg L^{-1}. The 2, 4-D rates were significantly ($p \leq 0.001$) different for callus onset and callus induction. The genotype × rate interaction effects showed that 0 and 2 g L^{-1} 2, 4-D had the lowest and highest mean, respectively in both lines during onset and induction of callus. The lines had a significant ($p \leq 0.001$) effects on shooting induction, however, their means were not significantly different. Similarly, the means for the hormones were not significantly different for shooting induction. The lines, IBA rate and their interaction were significantly ($p \leq 0.05$) different for rooting induction. The means for the lines were significantly different for rooting induction in different IBA rates. Conversely, the mean for the IBA rates was significantly different for rooting induction. This study found that plant growth regulators rates during the callus induction stage plays a key role during regeneration. This protocol was a success and could provide a fundamental platform for future transformation in this line.

Keywords: Maize, immature embryos, callus, Somatic embryogenesis, regeneration protocol

INTRODUCTION

Maize (*Zea mays* L.) is among the widely cultivated crops and arguably the most economically important cereal worldwide. It is majorly cultivated in tropical and sub-tropical regions. The crop is well suited to survival in its environment due to its C4 photosynthetic system that lays a platform for higher rates of photosynthetic activity in comparison to other cereal crops. This salient feature has made the crop become best adapted to the environment, hence, most adopted across the globe (Muppala *et al.*, 2020). The demand for maize was projected to have increased by 50% to over 800 million tons per year by 2020 and is predicted to surpass both rice and wheat consumption globally. The uses of maize vary as some use it for food, feed, fodder and industrial raw materials such as bio-ethanol (Santos *et al.*, 2017; Rufino *et al.*, 2018).

Despite the diverse uses of the crop, its production and productivity is on a sharp decline due to abiotic and abiotic stresses. Improving maize crop production is fundamental in overcoming the negative impacts of these stresses and sustaining and/or increasing agricultural production under such environmental constraints. High frequency *in vitro* plantlet regeneration is core to modern crop improvement approaches such as genetic transformation. Regeneration in monocots pose a major challenge as compared to dicots due to variation in tissue structure and composition. Factors such as genotype, age of the explant, *in vitro* media composition play

Appendix III: Abstract for Transgenic Expression of Sugarcane Mosaic Virus VPg in Maize Inbred Line CML444 Confers Resistance to Maize Lethal Necrosis Disease

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Transgenic Expression of Sugarcane Mosaic Virus VPg in Maize Inbred Line CML444 Confers Resistance to Maize Lethal Necrosis Disease

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Abstract

Maize is the most important crop in Kenya and parts of Sub-Saharan Africa. However, yields are below potential due to abiotic and biotic constraints. One of the major biotic concerns is maize lethal necrosis disease (MLN), which causes up to 100% yield losses. MLN is caused by the synergistic infection of two viruses, Maize chlorotic mottle virus (MCMV) and a potyvirus, commonly Sugarcane mosaic virus (SCMV). Because MLN is dependent on synergism, reduction of infection by either virus is expected to confer resistance or tolerance to MLN. Proteins P1, HC-Pro and VPg are essential for SCMV replication and movement. Pathogen-derived resistance has been used to design antiviral resistance in plants. Here, we hypothesized that transgenic expression of SCMV P1, HC-Pro or VPg confers resistance to SCMV and thus, to MLN. To test this hypothesis, we transformed maize inbred line CML444 with SCMV P1, HC-Pro or VPg genes; confirmed the presence of the transgene in T1 plants and evaluated T2 for MLN resistance using a detached leaf assay. Whole plant assays were not possible due to the legal restrictions of genetically modified plants in Kenya. MLN severity was evaluated on a scale of 1-5 using the chart developed by CIMMYT. Leaves from VPg transgenic plants recorded a severity score of 1.44 representing no MLN. In contrast, leaves from P1 and the HC-Pro transgenic plants had excessive chlorosis with a score of 4.0 and 4.1, respectively, while the susceptible control was completely chlorotic with a severity score of 5.0. Based on the area under disease progress curve, the VPg, HC-Pro, and P1 transgenic had 6.44%, 22.43%, and 17.48%, respectively, while the susceptible control had 23.13%. Analysis of variance revealed that the area under disease progress curve and MLN severity scores were significantly different across the transgenes, with transgenic expression of VPg providing the most protection against MLN. These results show that MLN management can be improved through gene silencing induced by transgenic expression of

Appendix IV: The FASTA format of the *VPg* gene sequence showing *AscI* restriction site at the bolded nucleotide

> [316_pMDC32_SCMV_VPg (2).xdna - 10714 bp] - Selection From [506] To [1079]
CACCATGGGGAAAAACAAACGCAGTAGACAAAAACTTCGATTCAAAGAAGCAA
GAGACAACAAATATGCATATGACGTCACAGGATCGGAAGAATGCCTCGGTGAAA
ATTTTGGAACAGCCTACACAAAGAAAGGTAAAGGAAAAGGAACTAAGGTTGGAC
TCGGTGTGAAGCAGCATAAATTCCATATGATGTATGGTTTCGATCCTCAAGAGTA
CAACCTAATTCGGTTTGTCGATCCACTCACGGGAGCAACTCTGGATGAACAAATT
CATGCCGATATGCGTTTAATTCAAGAGCACTTCGCC**G**AAATTCGTGAGGAGGCA
GTGGCTAATGACACAATTGAAAGGCAGCAGATTTACGGCAATTCTGGACTACAA
GCATTTTTCATACAAAATGGGTCAGCAAACGCTCTGAGAGTTGATTTAACACCAC
ATTCACCTACACGAGTTGTCACAGGTAATAACATAGCAGGGTTCCCAGAATATGA
AGGGACACTTCGTCAAACTGGAACAGCTATAACCATACCCATTGGTCAAGTCCCA
ATCGCAAATGAAGCAGGGGTTGCACACGAG

Key: Coloured base=*AscI* restriction site

Appendix V: The FASTA format of the *P1* gene sequence showing *BfmI* restriction site at the bolded nucleotide

> [310_pMDC32_SCMV_P1 (13).xdna - 10843 bp] - Selection From [506] To [1208]
CACCATGGCGGGAACGTGGACCTACGTGACACGTAAGTGGCAGCCAGATGTTAA
CAATGATCGTCACATTAAAAGAGTGATGGAAATGTTTGCAGCAAAACATCAACA
TTACTCAGAAGAACAGCGACTTGCTCATAATATGAAACTATTGAGGAAGGCAAG
TGTTGTAAGCGCTGAACCTGCGAAACCAAAGCAGAAGCAGGCAACTCAACAGAT
GTGGGTTGAGAAATGTGATCACAATCCTGTTGATCACTTAGTATATCCACGACTT
GGAAGATCCGCAAACAAGGTGGAAATGAATATTAAAAGAACATCTGTGAGCAAA
TTAACCAGGGAGGTTTTAGAGATCTCAAAGTCAAGCGGTCTTAAAGTTGAACTAA
TTGATAAACGGAAAAGATCCAAAACACAGTTATCAATCAAAAGGTTCAATGGTA
AAAATTTCCTCCATTGCAAAACGAATCACGAGAACAATTTATTCAAAAGGAGGG
ACATAGCCATTGGACATAAATGGTTTCCAACAATTGAAGCCATTGCTCGAA**G**CTA
TAGCACGATGAATCGAGAGGAACTACAAAGCCTTTGTAGAGGAAGCAGTGGTCT
CACATTCATCCAAAACGAAGAGTTATTCATTGTCAGAGGGAGAATGAATGGTGA
ACTCATTAATAGCTTGCACGAAACAAATCGGGTTTTGGATATTGAGCACTAC

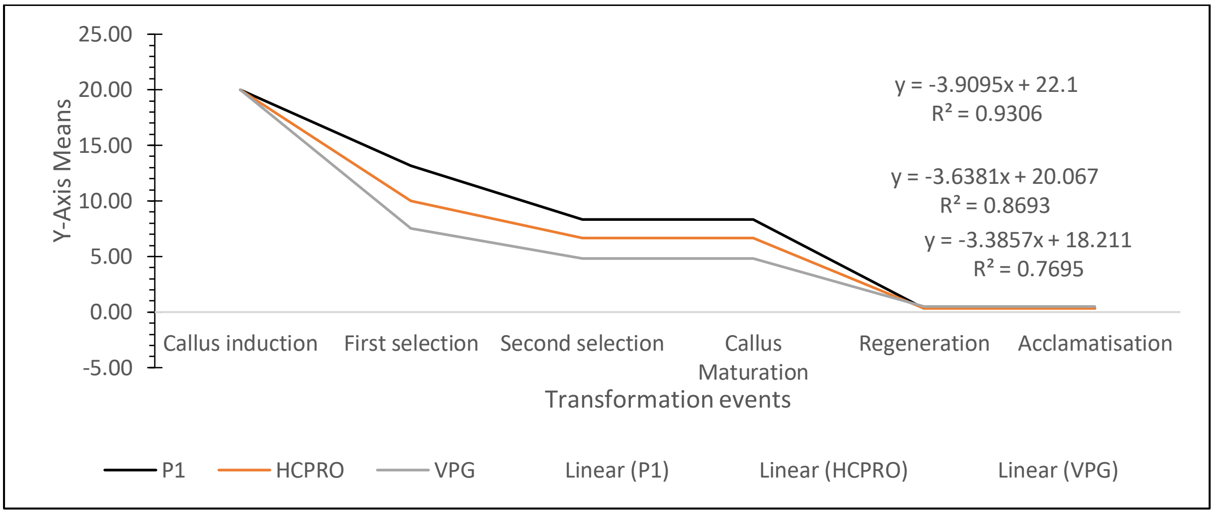
Key: Coloured base=*BfmI* restriction site

Appendix VI: The FASTA format of the HC-Pro gene sequence showing AsuII restriction site at the bolded nucleotide

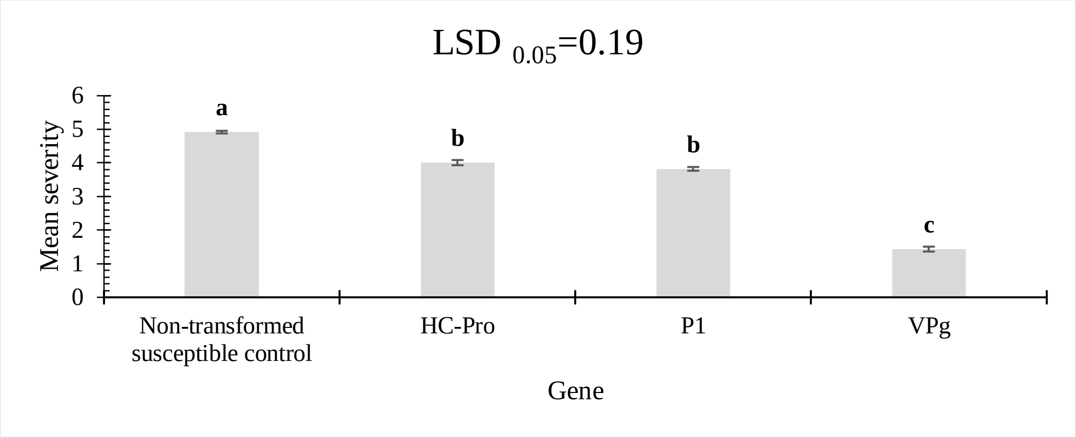
> [311_pMDC32_SCMV_HC-Pro(1).xdna - 11527 bp] - Selection From [506] To [1894]
CACCATGGCAGATCCCCAGGCTAATGATTTTTGGAGGGGATACACAAATGCTTAC
GTAGAGAATCGCAGCATTCCGACCACTCATACAGAGCACACCCCTACAGTCAAT
CTAGAGGAATGTGGAAAACGAATGGCTTTACTTGAAATACTATTTCACTCTACAT
TCG**A**AATTACATGCAAGGCGTGCAACATCGATGATCTTGAATTATCAGATGATGA
ATTTGGAGCCAAACTCTATAAGAATTTGCAACGTATCGAAGAAAAACAAAGAGA
GTACCTTGCAAAGGATCAAAAACTATCCAAAATGATACAATTTATCAAAGAAAG
GTGTAATCCAAAATTTTCGCATCTACCAACGTTATGGCAAGTCGCAGAAACAATA
GGACACTATACTGATAATCAGTCAAAGCAAATAATGGACATTAGCGAAGCGCTC
TTTAAAGTTAATACTTTAACTCCTGACGATGCTATGAAAGCAAGCGCAGCATTAC
TTGAAGTATCGCGATGGTATAAGAACCGTAAGGAGTCGCTCAAAACTGACTCATT
GGAATCTTTTAGAAATAAGATATCACCAAAGAGTACAATAAATGCAGCTTTAAT
GTGCGGTAATCAATTGGATAAAAATGCAAATTTTGTATGGGGTAATAGGGAATA
TCACGCCAAACGATTTTTCGCGAACTATTTTGAAGCAGTGGATCCCACAGATGCA
TATGGAAAGCACGTCACACGGTTTAACCCTAATGGGCAACGAAAGTTATCAATA
GGAAAGTTAGTTATCCCACTGGACTTTCAAAAGATTCGAGAATCATTCGTTGGAC
TTCCGATAAATAGACAACCGCTGGGTAAATGTTGCGTTAGCAAGATCGAAGGAG
GGTATATATACCCATGTTGCTGCGTCACAACAGAATTTGGTAAACCAGCATACTC
TGAGATAATACCTCCAACGAAAGGCCACATAACAATAGGCAATTCTGTTGATCC
AAAGATTGTGGACCTGCCAAACACAACACCACCTAGCATGTACATCGCTAAGGA
TGGGTATTGCTACATCAACATCTTTTTAGCAGCCATGATCAACGTTAACGAAGAA
TCCGCCAAGGATTACACGAAATTTTTGAGGGACGAACTAGTCGAGCGTCTCGGA
AAGTGGCCAAAGCTTAAAGACGTAGCAACAGCGTGTTATGCATTATCCGTAATGT
TTCCAGAAATTAAGAATGCTGAGCTACCTCCAATTTTAGTTGACCATGAAAATAA
ATCAATGCATGTAATCGATTCATATGGTTCACTAAGTGTTGGATTTCACATATTA
AAAGCAAGCACGATTGGTCAATTAATCAAATTTCAATATGAATCTATGGATAGTG
AAATGCGCGAATACATAGTAGGA

Key: Coloured base=*AsuII* restriction site

Appendix VII: Transformation events and their respective means for the three transgenic activities



Appendix VIII: Disease severity at 50 dpi. Values correspond to the last time point in figure 2. Letters indicate statistical difference at $p \leq 0.05$ (LSD $_{0.05}$=0.19)



Appendix IX: Means for MLN severity score in transformants and their controls taken at an interval of 5 days for 50 days

Gene	DAY5	DAY10	DAY15	DAY20	DAY25	DAY30	DAY35	DAY40	DAY45	DAY50
HCPRO	1.83	2.50	3.17	4.00	4.00	4.00	4.08	4.17	4.17	4.17
P1	1.83	2.00	3.00	3.67	3.83	4.00	4.00	4.00	4.00	4.08
VPG	1.00	1.00	1.00	1.00	1.00	1.42	1.50	1.67	1.67	1.67
Control	2.25	3.50	4.50	5.00	5.00	5.00	5.00	5.00	5.00	5.00

HC-Pro=Transgenic, *P1*=Transgenic, *VPg*=Transgenic, *C=Control* (Non-transgenic)

Area under disease progress curve at 50 dpi. Values correspond to the last time point in figure 2. Letters indicate statistical difference at $p \leq 0.05$ (LSD $_{0.05}$=2.07).