Development of Transgenic Banana and Plantains Using RNAi Approach for Control of Banana Aphids

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DECLARATION

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

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DEDICATION

I dedicate this to my family and friends for their relentless support and love.

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LIST OF ABBREVIATIONS

°C	degrees Celsius
µg	microgram
µl	microliter
µM	micromolar
µm	micrometer (micron)
2, 4-D	2, 4-dichlorophenoxyacetic acid
ANOVA	analysis of variance
BAP	6-benzylaminopurine
BBTD	banana bunchy top disease
BBTV	banana bunchy top virus
BCCM	bacterial co-cultivation medium
Bp	base pair
BRM	bacterial re-suspension medium
CaMV 35S	Cauliflower Mosaic Virus 35S promoter
cDNA	complimentary deoxyribonucleic acid
CTAB	cetyl triethylammonium bromide
DIG	digoxigenin
DNA	deoxyribonucleic acid
dsRNA	double stranded RNA
E. coli	*Escherichia coli*
ECS	embryogenic cell suspension
EDTA	ethylenediaminetetraacetric acid
FDA	fluorescein diacetate
FEC	friable embryogenic callus
g	gram
GUS	beta-*glucuronidase*
h	hour

IAA	indole-3-acetic acid
LB	Luria Bertoni
LSD	least significant difference
min	minute
mM	millimolar
mRNA	messenger RNA
MS	Murashige and Skoog
NAA	α-Naphthalene acetic acid
NOS	gene encoding nopaline synthase
OD	optical density
ORF	open reading frame
PCR	polymerase chain reaction
pH	potential of hydrogen (measure of hydrogen ion concentration)
RM	rooting medium
RNA	ribonucleic acid
RNAi	RNA interference
RSS	RNA silencing suppressors
RT-PCR	Reverse transcriptase polymerase chain reaction
SCV	settled cell volume
siRNA	small interfering RNA
spp	species
ss	single-stranded
Taq	*Thermus aquaticus*
T-DNA	transfer DNA
Tween 20	polyoxyethylene (20) sorbitan monolaurate
UV	ultra violet
WT	wild type
X-gluc	5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid

LIST OF PUBLICATIONS

Some of the work that has been published:

- Jekayinoluwa T., Tripathi J.N., Obiero G., Muge E., Tripathi L. (2020). Developing Plantain for Resistance to Banana Aphids By RNA Interference. MDPI *Proceedings* 2019, 36, 54. https://doi.org/10.3390/proceedings2019036054 (Abstract)
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ABSTRACT

Banana and plantain are consumed by over five hundred million people around the world. Despite their market value and importance in ensuring global food security, several pests and diseases threaten their yield. Banana bunchy top disease (BBTD) is one of the world's most destructive diseases of banana and plantain causing up to 100 % yield loss in severe cases. Transboundary exchange of infected planting materials and banana aphid (*Pentalonia nigronervosa*) are the two transmission modes of the disease. Banana aphid harbouring banana bunchy top virus (BBTV), is the sole vector and an efficient method of transmission of the virus from infected to healthy plants. Controlling the spread of BBTD has been very challenging since there is no known banana germplasm containing an endogenous gene that could confer absolute resistance to BBTV. Therefore, the pre-eminent way of controlling the virus starts with controlling the vector. Biotechnological strategies via RNA interference (RNAi) could be used to target banana aphid as well as BBTV to reduce virus-associated banana and plantain yield losses. The aim of this study was to generate transgenic plantains using RNAi as a strategy to control banana aphids. Plant tissue culture techniques such as somatic embryogenesis and genetic transformation offer a valuable tool for genetic improvement. Identification and quantification of phytochemicals found in banana and plantain are essential in optimizing *in vitro* activities for crop improvement. Total antioxidant capacity, flavonoids, tannins and phenol were determined with varying concentrations in the root, pseudostem, leaf explant and in *in vitro* samples of plantain and banana cultivars. Embryogenic cell suspension (ECS) was developed for three farmer-preferred plantain cultivars, Agbagba, Obino l'Ewai and Orishele. Both Murashige and Skoog (MS) and Gamborg (B5)-based culture media supported the development of friable embryogenic callus (FECs) in plantain cultivars while MS culture media supported the proliferation of fine cell suspension in liquid culture media. Up to 22 ± 24 %, 13 ± 28 % and 9 ± 16 % FECs were obtained for Agbagba, Obino l'Ewai and Orishele cultivars, respectively. To design a reliable synthetic diet for *in vitro* aphid feeding, the type of available sugars in banana and plantain were first analysed and shown to include sucrose, fructose and glucose at varied levels. An optimal synthetic diet containing 7.5 % sucrose supported the survival of banana aphid *in vitro.* The efficacy of acetylcholinesterase as the targeted gene in banana aphid was determined through an *in vitro* feeding assay of banana aphid using dsRNA and siRNA both of which conferred lethal effects on banana aphid. Banana and plantain cell suspension was transformed by *Agrobacterium*-mediated transformation with pNXT-35S-ACE-hp gene construct. The presence and integration of the transgene in elite plants was confirmed by PCR and Southern blot assays. Transgenic events generated were screened for resistance to banana aphid and up to 46.7 %, 75.6 %, and 67.8 % reduction in aphid population was observed in Gonja Manjaya, Orishele and Cavendish Williams respectively compared to those grown on the wild-type plants under glasshouse condition. These results suggest the effectiveness of RNAi targeting an essential aphid gene, could be a useful way of managing the infestation of banana aphid and reducing the spread of BBTD.

CHAPTER ONE

INTRODUCTION

1.1 Background to the Study

Hunger and malnutrition are global threats to the rapidly growing population. The subsistence of tropical crops germplasm is one of the avenues in promoting food security. Tropical crops such as banana and plantain need earnest research attention to contribute in increasing food production globally and especially in sub-Saharan Africa where they are cultivated as a staple food.

Bananas and plantains (*Musa* spp.) are some of the fruit crops important for food and nutritional security in different parts of the world including Asia, America and Africa. They serve as a source of livelihood for over 500 million people around the world including developing countries (FAOSTAT, 2018). In terms of production, it is ranked the world's number one fruit crop (FAOSTAT, 2018). The increasing production of this crop contributes significantly to the economy and food security in developing countries (Bruinsma, 2017). The worldwide production of bananas and plantains in over 141 countries is more than 155 million tonnes with Africa accounting for 43 million tonnes, many of which are grown by smallholder farmers for consumption and as a major source of livelihood (Swennen, 1990; Honfo *et al*., 2011; FAOSTAT, 2018). India is the biggest producer of banana generating over 30 million tonnes, while Central and Western Africa are predominant plantain producers accounting for about 19 million tonnes (FAOSTAT, 2018).

A wide array of products (such as flour, juice, chips, wine), and explants (e.g. pseudostem, fruit peel, root and leaf) derived from bananas and plantains are used as food, feed and for medicinal purposes (Adeniji *et al.*, 2010; Okareh *et al*., 2015). Plantain is a rich source of fibre, carbohydrates, vitamins, nitrogen, phosphorus, calcium, potassium and magnesium (Okareh *et al*., 2015). Ripe plantains are reported to have minimal levels of iron, zinc,

copper, sodium and β-carotene (Baiyeri *et al*., 2011). The pulp is used for culinary purposes while, the bract, peel, leaves, root and, sap can be used as animal feed (Adeolu & Enesi, 2013; Okareh *et al*., 2015), decontamination/purification of effluent (Darge & Mane, 2013; Pandharipande & Deshpande, 2013) and therapeutic purposes (Ighodaro, 2012; Okechukwu *et al.*, 2012).

Despite their market value and importance in global food security, pests (such as bagworm, banana rust thrips, fruit fly, banana rhizome weevil, nematodes, banana lacewig bug, and banana aphids), diseases [like panama disease, black sigatoka, banana *Xanthomona*s wilt, moko disease, banana streak disease and banana bunchy top disease (BBTD)] and other abiotic factors (such as temperature, rainfall and altitude) have been implicated as the causes of their declining yields (Ploetz, 2006; Pérez-vicente, 2014; Calberto *et al*., 2015; Viljoen *et al*., 2016; Chabi *et al*., 2018; Tripathi *et al*., 2019). All these aforementioned constraints have remained a constant hindrance in achieving the full potential of bananas and plantains in supporting global food security.

Banana bunchy top disease (BBTD) has been identified as one of the main biotic and economically important constraints to banana and plantain production worldwide (Dale, 1987). It is one of the world's 100 invasive alien species (Lowe *et al*., 2000) posing a significant negative economic influence on banana and plantain production. The disease is caused by banana bunchy top virus (BBTV) and transmitted by banana aphid *(Pentalonia nigronervosa* Coquerel) and spread through the transboundary exchange of infected planting materials.

BBTV is a circular single-stranded DNA (ssDNA). The ssDNA consists of 6 major encoding protein components which facilitate its pathogenicity; the BBTV DNA-C, -M, -N, -R, -S & -U3 (Harding *et al*., 1991; Burns *at al*., 1995; King *et al*., 2012; Stainton *et al*., 2015). The transmission of BBTV by banana aphid is in a circulative, non-propagative mode (Watanabe *et al*., 2013). Banana aphids acquire and accumulate the virus while

feeding on the phloem cells of an infected plant and eventually transmit the virus to a healthy plant during the subsequent feeding process.

The spread of BBTD could be controlled through the use of resistant cultivars, chemical control or by destroying infected plants (rouging). These control options are however limited because there is no known endogenous gene in the banana/plantain gene pool that could confer absolute resistance to BBTV, coupled with the sterility of the crop, no known chemical can absolutely destroy banana aphids in a banana plant and guarantee no negative impact on the environment inclusive of the ecosystem and the applicator's health, while rouging of infected plants may lead to unwanted spread of infected banana aphids to other surrounding healthy banana/plantain plant(s). Hence, controlling the spread of banana aphids and subsequent transmission of BBTV through advanced methodologies could be a prospective strategy of curtailing the spread of BBTD.

The agricultural revolution brought about the use of plant tissue culture and innovative biotechnological approaches such as the use of RNA interference (RNAi). The former approach is used to generate clean planting materials while the latter can be employed in genetic transformation approach that can be used to inhibit or destroy invading pathogenic viruses and vectors transmitting them into plants.

Plant tissue culture (PTC) is a conglomerate of various methods and systems of growing or maintaining different plant parts, organs or cells under sterile conditions with the potential of sustaining and regenerating to whole plants. This is achieved through the use of essential plant nutrients also known as culture media (usually in synthetic form) with or without the inclusion of required plant growth regulating hormones (Loyola-Vargas *et al*., 2008). PTC has long played a significant role in revolutionizing agricultural practices and thus supporting conventional and molecular breeding for improving crop production. For instance, micropropagation, a PTC technique is used to generate and multiply clean (virus, fungi and bacteria free) plants *in vitro* such as those that have been genetically modified or bred through plant breeding methods (Taşkin *et al*., 2013).

Meristem culture a PTC method has been successfully used alone or in combination with either Thermo-, Chemo-, Cryo- or Electro-therapy to efficiently recover pathogen-free plants by circumventing diseases that may have accrued in stock plants (Meybodi *et al*., 2011; Dugassa & Feyissa, 2011; Alam *et al*., 2013; Neelamathi *et al*., 2013; Vivek & Modgil, 2018). The meristem consists of a group of undifferentiated cells which are found at the active growth site of a plant. Meristem culture is advantageous because the meristematic tissue is devoid of differentiated provascular or vascular tissues which makes it unlikely to be infected by viruses. Other factors include the rapid cell division and multiplication rate which is faster than virus replication (Milosevic *et al*., 2012) as well as gene silencing mechanism which may occur (Foster *et al*., 2002). Another important application of meristem culture is in generating the source material for somatic embryogenesis.

Somatic embryogenesis is an artificial way of manipulating plant tissues to generate somatic embryos. This involves a series of dedifferentiation or redifferentiation of plant tissues transitioning from being somatic cells to becoming embryogenic cells in the presence of plant growth regulators especially the auxins which catalyse the biological processes (Dantu & Tomar, 2010; Loyola-Vargas & Ochoa-Alejo, 2016). Somatic embryogenesis provides the system for studying the developmental stages of plant embryos at the molecular, biochemical and physiological phases (Dantu & Tomar, 2010). It is useful in generating plant stock on large scale, production of synthetic seeds, protoplast isolation and a prerequisite for developing cell suspension cultures.

Plant cell suspension culture is a valuable tool that provides the basis for studying and improving crop plants without the hassle of handling complex plant structures. It is a rich reservoir of many secondary compounds useful for chemical, biotechnological and pharmaceutical assays and production of secondary components/metabolites and other useful products that are of economic importance (Tyagi *et al*., 2010; Imam & Akter, 2011; Upadhyay, 2011; Wadood, 2014).

Biotechnological advances have employed the use of cell suspensions especially in generating transgenic plants vis à vis genetic transformation for crop improvement. Several biotechnological methodologies have been adopted either by conferring genes that improve plant quality or alter specific mechanisms and biochemical or biosynthetic pathways in plants with the aim of developing improved plant varieties for increased quality and quantity. RNAi is one of the biotechnological mechanisms that alter or inhibit gene expression by repressing mRNA. This technique has been successfully used in mediating resistance against green peach aphid, and *Nilaparvata lugens* (Zha *et al*., 2011; Guo *et al*., 2014)

1.2 Problem Statement

Insect pests and diseases is one of the major limitations to banana and plantain production. Also, the genetic similarity of the crop (Jenny *et al.,* 2002), sterility caused by the triploid nature of most plantain cultivars (Ortiz, 2013) which leads to associated difficulty during meiosis due to the uneven chromosome number (Ortiz & Crouch, 1997) prevent the transfer of genes by conventional breeding. Banana aphid (*Pentalonia nigronervosa* Coquerel) is a common insect pest that transmits the banana bunchy top virus (BBTV) causing banana bunchy top disease (BBTD) in bananas and plantains (Hafner *et al.,* 1995) resulting in up to 100% yield reduction (Kumar *et al.*, 2015).

BBTV is acquired by the banana aphids when feeding on an infected plant and transmitted in a non-propagative manner (Watanabe *et al.,* 2013) when the aphids move on to feed on healthy plants. Though the use of chemicals is an option for controlling the aphid population, this strategy is harmful to the environment whereas rouging is not curative. Moreover, there are no known varieties that are immune to BBTV in the commercially cultivated *Musa* germplasm. It is plausible that curbing the transmission of BBTV may be crucial in minimizing the spread and effect of the disease.

An advanced biotechnological approach through the use of RNAi is a potentially viable host-plant resistant option for controlling the disease. To achieve this, there is a need to

establish a platform for recruiting the target gene(s) in farmer preferred plantain cultivars and development of embryogenic cell suspension culture system.

Embryogenic cell suspension culture has been successfully generated in *Musa* species using scalp or immature male flower (Strosse *et al.*, 2003), however, the efficiency of embryogenic response in Agbagba, Obino l'Ewai and Orishele plantain varieties are quite low (Strosse *et al*., 2006). Several factors such as plant species, source/type of tissue, chronological age and physiological state of the tissue, level of endogenous plant hormone, composition of the culture medium and physical culturing conditions such as temperature, photoperiod and pH have been identified to greatly influence plant response to *in vitro* manipulations and the generation of an embryogenic cell suspension (Metwali & Al-Maghrabi, 2012; Boufis *et al*., 2014; Ramakrishna & Shasthree, 2016; Jekayinoluwa *et al*., 2019). As a result, there is a need to optimize the existing protocols and develop an efficient *Agrobacterium*-mediated transformation system for the plantain cultivars. This study focuses on the use of RNAi to target banana aphid for prevention of BBTV transmission and disease spread.

1.3 Justification

Conventional breeding has long played a pivotal role in developing improved crop varieties with different agronomic traits. Through conventional breeding, improved banana cultivars have paved the way for resistance against black leaf streak and Fusarium wilt race 1 (Damodaran *et al*., 2009; Aguilar Morán, 2013; Dale *et al*., 2017), black sigatoka (Vuylsteke *et al*., 1993; Menon *et al*., 2011; Orluchukwu & Ogburia, 2014) and increased crop yield (Tumuhimbise *et al*., 2016). Despite its advantages, potential and contribution of conventional breeding towards crop improvement, it is evident that its accomplishment can be greatly improved if incorporated in other unconventional genetic improvement strategies (Jenny *et al.*, 2002). It is however challenging to develop BBTD resistant plantain cultivars by conventional breeding method. This is because, there is no known banana or plantain cultivar that has been discovered in the banana and plantain

gene pool that is immune to the disease (Niyongere *et al*., 2011). Although, some cultivars have been identified as being less susceptible to BBTD (Niyongere *et al.*, 2011; Ngatat *et al*., 2017) their potential may not be sufficient to confer resistance. Moreover, not all genetic combinations are either efficient or possible for crop improvement (Jenny *et al*., 2002). The vegetative mode of propagation is also slow, hence, there is a need to harness advanced biotechnological methods through genetic transformation to eliminate or inhibit the expression of BBTD in plantain cultivars.

RNA interference (RNAi) also known as post-transcriptional gene silencing is one of the potential mechanisms which could trigger silencing or suppression of disease expression by degrading the virus mRNA (Mourrain *et al*., 2000). The RNAi pathway occurs naturally as a regulatory potential to provide defence against invading disease(s). RNAi is sequence-specific and has been used to selectively induce suppression of a specific gene of interest (Younis *et al.*, 2014). It has been successfully used in suppressing the browning effect in apples and potatoes (Waltz, 2015), suppressing *Mal d 1* allergen in apples (Gilissen *et al*., 2005), and repressing caffeine encoding gene for generating decaffeinated coffee (Ogita *et al*., 2003). The robustness of RNAi technology has gained an interest in insect pest and disease management. For instance, the Western corn rootworm is a major devastating corn pest in the United States causing yield loss of about 1billion USD (Hodgson, 2008), RNAi was adopted by targeting and silencing specific genes that are responsible for the insect's reproductive capability. Niu *et al*., (2017) reported a significant decrease in insect's fecundity using RNAi as a control strategy for insect pest. A broad spectrum root-knot resistance was achieved by silencing the root-knot nematode gene using RNAi technologies (Perrier *et al*., 2011). Owing to the robustness of the technology, its practicability has been seen in the field of biotechnology, medicine, insecticides and herbicides.

Furthermore, understanding the role of a specific gene in an organism is crucial in controlling and managing several emerging crop pests and diseases with the view of crop

improvement. It is paramount to identify the suitable gene(s) that could have an insecticidal effect on the target insect pest without having any adverse effect on off-targets organisms. Several genes such as proteasomes (Ulrich *et al*., 2015), ecdysone receptor (Yu *et al*., 2014), reproductive genes (Niu *et al*., 2017) and acetylcholinesterase (Revuelta *et al*., 2009; Malik *et al*., 2016; Ye *et al.*, 2017) have been targeted with varying degree of insecticidal efficiency. Acetylcholinesterase (AChE), is an enzyme that catalyses the breakdown of acetylcholine in insects and plays a significant role in the physiology of aphids (Guo *et al*., 2014), (Fig 1).

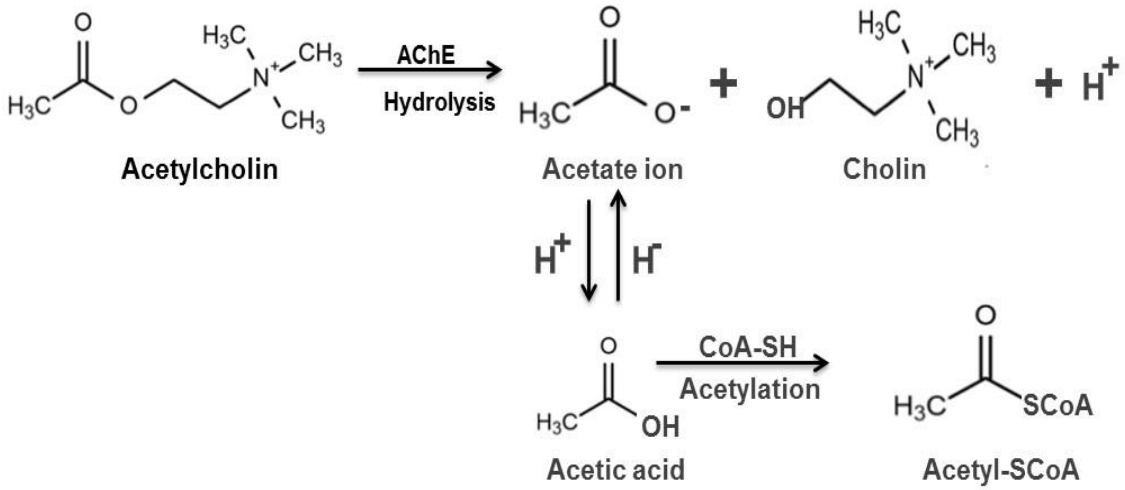


Figure 1.1: Chemical equation of acetylcholine in the presence of acetylcholinesterase (AChE)

This unique role of AChE has led to an exploration of chemical-based insecticides such as the organophosphates and the carbamates in the field of integrated pest management to control insect pests (Villatte & Bachmann, 2002). However, extensive use of chemical-based insecticides is not environmentally friendly, not cost-effective, and may impact negatively on other non-target organisms. Therefore, silencing the *AChE* gene in banana aphids by RNAi could cause growth inhibition and mortality. Hence, a potential way to inhibit the spread of BBTD.

1.4 Objectives

1.4.1 General Objective

To develop transgenic plantain using RNAi as a strategy to control the spread and effect of banana aphids on plantain cultivars.

1.4.2 Specific Objectives:

1. To develop cell suspension system for farmer preferred cultivars of West Africa plantain (Agbagba, Obino l'Ewai and Orishele).
2. To optimize a transformation system for plantain cultivars.
3. To develop transgenic plantain and banana cultivars using RNAi construct targeting the acetylcholinesterase gene and to characterize resultant transgenic plants using molecular tools.
4. To screen transgenic plants for resistance to banana aphids under glasshouse conditions.

CHAPTER TWO

LITERATURE REVIEW

2.1 Taxonomy and Botanical Nomenclature of *Musa*

Plantain and banana are the common names for non-woody herbaceous plants of the genus *Musa* under the family Musaceae (Nayar, 2010). According to the Angiosperm Phylogeny Group III (APG III system, 2009), Musaceae was assigned to the order of Zingiberales which consists of six families of tropical and subtropical perennial plants. Within this order, the Musaceae family which includes the bananas and plantains is of significant importance. The family consists of two genera; *Musa* (plantain and banana) and *Ensete*.

Musa (plantain and banana), are monocotyledonous flowering plants that are native to the tropics of Southeast Asia (Nelson *et al.*, 2006) and grown in the humid tropics and subtropics (Manzo-Sánchez *et al*., 2015). The plants have large herbaceous growth habits with overlapping leave sheaths that form the pseudostem. The pseudostem consists of leaf sheaths springing up from the corm tissue (Karamura *et al*., 2011). The leaf sheaths provide support to the plant and the rachis of the mother plant. The corm is the true plant stem harbouring the meristem at its apex which is close to the soil. The corm tissue gives rise to the plant roots for anchorage and nutrient uptake. It also produces young new plants known as the suckers. The meristem is the active growing site of the plant, producing leaves and inflorescence at maturity. The inflorescence also known as the bunch emerges at the centre of the plant. It consists of flowers attached to the rachis (Swennen & Vuylsteke, 1987). The excrescence on the rachis is the cluster of flowers known as the glomerules which develop into the fruit hand. The fruit finger develops from the female flowers within the range of 10 glomerules of the bunch to produce edible fruit pulp and peel (Vilhena *et al*., 2019). This is followed by the neutral flowers and the male bud at the tip end of the rachis. The male bud is composed of male flowers embedded underneath bract sheaths. The presence or absence of the male bud at maturity depends on the plant

type or class (Swennen, 1990). The classification of banana or plantain type based on the agro-morphological characterization is performed majorly at the maturity stage (Swennen and Vuylsteke, 1987). The plantain fruit is generally used for cooking in contrast to the soft sweet banana. They are bigger than bananas and harder to peel especially when green.

There are about 50 species and 100 sub-species of bananas in existence, each with minor morphological difference from the others. Over 100 plantain cultivars have been identified in West Africa and the Philippines (Swennen, 1990). Bananas and plantains originated from either *Musa acuminata* (A genome) or *Musa balbisiana* (B genome) as well as crosses or backcrosses of both (Nayar, 2017). The major difference between banana and plantain is that banana genome constitutes of either *Musa acuminata* Colla, *Musa balbisiana* Colla or a combination of both (AA, ABB, AAA) while plantain is generally a triploid (AAB) consisting of two *Musa acuminata* Colla and one *Musa balbisiana* Colla genome (Norgrove & Hauser, 2014). Plantains are further divided into four types (False horn, French horn, Horn and French) based on the morphology of the inflorescence. Hybridization of these putative parents has given rise to several varieties of seeded or seedless varieties. Depending on their ploidy level and geographical distribution, *Musa* spp. possess 22, 33 or 44 chromosomes (x = 11). Tetraploid (2n = 4x = 44) clones are not common but diploids are regularly planted in tropical areas for local consumption (Doleel *et al*., 2004).

Diploid (AA) cultivars develop through nuclear restitution during meiosis, to triploid (AAA) cultivars. The usually cultivated clones for commercial purposes are the triploids (2n = 3x = 33) which have more yield than diploids (2n = 2x = 22) (Heslop-Harrison & Schwarzacher, 2007). Today, the AAA group are the main banana cultivars for export (Turner *et al*., 2007). The edible banana, AB, ABB, BBB or AAB are typically cultivated in Northeast and South India, Philippines, Vietnam and Africa (De Langhe *et al*., 2009; Hont *et al*., 2012).

Polyploidy has long been seen as a way to improve crop and thus its potential is being explored to bridge the gap for genetic transmission, fix heterozygosity, increase in plant organ ("Gigas" effect) and stable phenotypic variation that could increase the crop's resilience to environmental changes (Ortiz, 1997; Madlung, 2012; Bharadwaj, 2015; Sattler *et al.,* 2016). Certain agronomic features such as plant size and bunch weight correlate positively to increase ploidy levels in banana (Oselebe *et al.*, 2006). Polyploidization has resulted in the generation of seedless bananas, hence resulting in sterility of some banana and plantain cultivars. However, induced genome duplication could be employed to reverse sterility and restore fertility as in the case of xChitalpa (Olsen & Ranney, 2006; Sattler *et al*., 2016). This has not been reported for triploid plantains.

Edible banana and plantain majorly contain female flowers that develop into seedless fruit by parthenocarpy. The male flowers which are enclosed in the bract may either be sterile or not. The presence or absence of male bud at maturity helps to distinguish cultivars. The parthenocarpy of edible species forms the basis for their vegetative method of propagation. The sterility of flowers is regulated by the genes in the genome of banana and plantain cultivar (Silva *et al*., 2001). Fortescue & Turner (2004) confirmed that pollens of diploid species of *Musa* are more viable than the edible triploids which are sterile. Most cultivated bananas/plantains are triploid and sterile (Nyine & Pillay, 2007). This characteristic of cultivated *Musa* species limits the efficiency of conventional breeding for crop improvement. Despite the disadvantages of triploid cultivars in breeding programs, cultivars have the advantages of better fruit vigour and flavour. The 'A' genome contributes to disease resistance and fruit quality while the 'B' genome enhances the cultivar capacity to withstand environmental influences (Silva *et al.,* 2001).

2.2 Production of Banana and Plantain

Banana and plantain are the world's fourth most important food crops after rice, wheat and maize (Frison *et al*., 2004) and number one fruit crop (Molina *et al.*, 2004; FAOSTAT,

2018). In the world, 52 countries grow plantain annually. In 2017, the total global production of plantain was over 39 million metric tonnes (FAOSTAT, 2017). Asia is the biggest producer of bananas in the world accounting for about 49.7 % of the total world's production while Africa is the largest producer of plantain accounting for about 61.6 % of the total production within a period of three decades (FAOSTAT, 2017).

Plantain is a major staple and a means of livelihood for over 70 million people in West and Central Africa (Swennen, 1990). For instance, in Nigeria, it is the third staple after cassava and yams (Akinyemi *et al*., 2010). In Nigeria and Cameroon, an average of about 265 g and 402 g respectively, of plantain is consumed by an individual per day (Honfo *et al.*, 2011). In 2017, the Democratic Republic of Congo was the largest producer of plantain followed by Cameroon, Ghana, Colombia, Uganda, and Nigeria, respectively (FAOSTAT 2017). In Africa and especially Nigeria, plantains are mostly produced by smallholder farmers mainly for local consumption (Swennen, 1990; Honfo *et al.*, 2011). Despite the enormous effort in increasing banana and plantain production to feed the rapidly growing population, the amount produced is not sufficiently available to many.

2.3 Importance of Banana and Plantain

Plantain and banana are relevant food security crops and contribute significantly to the world's economy. It is especially a source of livelihood to smallholder farmers in sub-Sahara Africa and other developing countries. In terms of market value, banana and plantain are ranked fourth world's most important food crop (Frison *et al.*, 2004; Tripathi *et al*., 2009) and first as a fruit crop (Molina *et al.*, 2004). In Africa and Asia, it ranks second or third most important crop (Persley & Langhe, 1986; Dzomeku *et al.*, 2011; Bill & Melinda Gates Foundation, 2013). Banana production generates an estimated income value of about USD 8 billion per year (FAO, 2019). Value addition through postharvest processing as food and feed has greatly influenced the socioeconomic value of the crop. High-quality flour, beer and several other processed and packaged products are used either in the pharmaceuticals, food industry or consumed as snacks (Surendranathan *et al.*, 2004;

Adeniji *et al.*, 2010; Bornare *et al.*, 2014). Plantain biomass is a potential source of natural fibre which needs to be explored as a probable alternative to reduce the dependence on cotton as a sole raw material for textile (Loos *et al*., 2018). Although the world production of banana and plantain has increased significantly within the last 30 decades (1986 – 2016) at about 164 % and 48 % respectively, their production has not met the demand of consumers. The banana and plantain production industry holds a significant prospect in contributing to food security at the local, national and international level as it serves as a vital source of earnings to many countries.

2.4 Uses and Nutritional Composition of Banana and Plantain

Banana and plantain fruits are a major staple and part of the diet of the populace across the globe. Plantain which is consumed either raw or cooked can be consumed either as snack, main meal or a meal accompaniment at any time of the day. Banana and plantain are mainly composed of water, carbohydrates and vitamins. Plantain has higher starch content as compared to soft sweet bananas (Ngo-samnick, 2011; Odenigbo *et al.*, 2013; Annor *et al.*, 2016). All parts of the banana serve a variety of purpose such as food for human consumption, feed for animal and poultry as well as for the medicinal or therapeutic application. The pulp and peel of plantain and banana is a good source of resistant starch, fibre, antioxidant and polyphenols (Agama-Acevedo *et al*., 2016). The proximate composition of the plantain bract reveals its suitability as resourceful animal feed (Adeolu & Enesi, 2013) while the male bud is a potential source of dietary fibre to control obesity and diabetes (Florent *et al*., 2015). The peel or stalk of unripe plantain can be used as a feedstock for ethanol production (Waghmare & Arya, 2016) and antimicrobial agent in the pharmaceutical industries (Okorondu *et al.*, 2012).

The antibiotics obtained from banana has the potential to act against gram-positive and negative bacteria, mycobacteria and fungi (Chabuck *et al*., 2013; Ehiowemwenguan *et al*., 2014; Kapadia *et al*., 2015; Prakash *et al*., 2017). The antifungal compounds in the unripe peel and pulp can be used to control the fungal disease of tomato (Kumar *et al*., 2012).

Besides the fruit pulp and peel, the leaf sap can be used to treat wounds, burns, digestive disorders, epilepsy, fever and insect bite (Kumar *et al*., 2012); while other organic compounds such as norepinephrine, dopamine and serotonin are useful for several other therapeutic purposes (Kumar *et al*., 2012).

Bananas are consumed when ripe as desserts while plantains are boiled, steamed, fried, roasted or baked when ripe or unripe. Plantain can be processed into storable products such as flour, jellies, beer, wine and jam (Abiodun-Solanke & Falade, 2011). Plantain is a good source of vitamins, minerals such as potassium, phosphorus, calcium and magnesium. Other elements include iron and zinc. It is worthy to note that the levels of these minerals depend on the morphological status of the fruit either ripe or unripe (Anyasi *et al.*, 2013). Potassium found in banana and plantain is an important mineral in human and animal nutrition. It plays an active role in balancing levels of fluids and electrolytes in the body. As a result, it helps to stimulate nerve impulse transmission, normal muscle and skeletal contractions. These contribute actively to maintaining heart function, thus preventing or controlling the risk of heart-related diseases like a heart attack or high blood pressure. Potassium has also been found to reduce the risk of diabetes (Ringer and Bartlett, 2007; WHO, 2012). On the other hand, magnesium is essential in DNA function and repair. Presence of adequate level of magnesium in the body can help to prevent diseases such as; alzheimer, hypertension, diabetes mellitus and migraine headaches (Bhutto *et al.*, 2005; Gröber *et al.*, 2015; de Baaij *et al*., 2015).Vitamins are essential as they possess the properties to regulate body metabolism, improve vision, regulate immune system and reproduction (FAO & WHO, 2001). The chemical composition may vary with cultivars, edaphic and climatic factors (Annor *et al*., 2016).

2.5 Propagation of Banana and Plantain

The morphology and biology of banana and plantain inform their propagation methods. *Musa* spp. is cultivated through a vegetative mode of propagation. Vegetative propagation is a form of asexual propagation in which multi-cellular plant parts or tissues from the

mother plant is detached and when subjected to appropriate conditions, has the capability to develop into a whole plant identical to the parent plant (Ramos-Palacios *et al*., 2012). Suckers or young acclimatized *in vitro* plants are the source material for establishing banana and plantain in the field. *In vitro* plants are generated from shoot-tip or meristem culture; plants generated from the latter technique are usually pathogen-free making it advantageous as source material for planting. Plantain and banana grow well in a warm and humid climate with optimum temperature ranging from 28 °C to 30 °C, minimum of 100-200 mm rainfall and, rich organic soil with pH of 6.5 – 7.0 (Swennen, 1990; Ngo-samnick, 2011). Organic nutrient of the soil can be improved by mulching using plant materials rich in organic matter.

2.6 Macropropagation, Micropropagation, Conservation and Germplasm Exchange of Banana and Plantain

2.6.1 Macropropagation of Banana and Plantain

In an effort to increase the production of banana and plantain, several approaches have been employed to scale up the production and avail good quantity and quality planting materials. Macropropagation is one of the techniques applied to step up production of planting materials. It involves the use of banana/plantain suckers or corm tissues as source material. Plantain/banana sucker is either partially or fully decapitated to induce lateral bud growth by repressing apical dominance of the banana/plantain plant (Ntamwira *et al*., 2017). The excised bud technique is an alternative form of decapitation such that the mother plant is not affected. Soil is piled up around the corm tissue to induce growth of buds which is carefully excised from the mother plant and tended in rich compost soil to generate multiple plants. The detached corm method could either be whole or split corm. The whole corm method involves scarification of the apical meristems while the split corm basically involves splitting the corm into several parts and place them facing down in compost substrate for plants to germinate (Faturoti *et al*., 2002; Baiyeri and Aba, 2007; Staver and Lescot, 2015).

2.6.2 Micropropagation of Banana and Plantain

Micropropagation is an aseptic technique that enables rapid multiplication of plant stock *in vitro* such that plants generated are pathogen-free and identical to the parent plant. Plants generated are acclimatized in a screenhouse or nursery before exposure to the field. Micropropagation has proven to be a commercially viable method for plant production and has acquired acceptance in the world trade because it effectively eliminates the dangers of introducing plant diseases through the transboundary exchange of germplasm. Other significant benefits of plant tissue culture (PTC) include uniformity of plantlets both in appearance and growth habits as well as its availability throughout the year without seasonal influence, hence, providing an avenue for developing medium and long term conservation of plant germplasm (Deventhiran *et al*., 2017). It is also a means of generating large quantities of phytochemicals which may influence *in vitro* and *in situ* responses (Isah, 2017).

Micropropagation can be initiated *in vitro* either by meristem culture or apical shoots (Singh *et al.*, 2011; Asmare *et al*., 2012) as an approach of clonal multiplication of banana/plantain. The mode of initiation of plant material is dependent on the aim and usage. Micropropagation through meristem culture is one of the unique tissue culture methods employed to recoup pathogen-free plants (Al-Taleb *et al*., 2011; Whitehouse *et al*., 2011). Plants, while on the field are exposed to several biotic and abiotic factors which could trigger initiation and accumulation of phytopathogens that could either lead to poor growth and/or decreased production. Meristem culture is a useful approach in generating pathogen-free plants (Quiroz *et al*., 2017). Regardless of the method of introducing germplasm *in vitro*, suckers are collected from healthy plants in the field. Before use, the suckers are reduced to about 1 cm or 2.5 cm for meristem and shoot tip culture respectively. Explants are subjected to surface sterilization using disinfectants such as ethanol and sodium hypochlorite. For meristem culture, the explant is further excised with the aid of the microscope to obtain the meristem. For survival and regeneration, plant tissues are cultured on synthetic nutrient also known as basal medium. Basal medium (e.g.

Murashige & Skoog, 1962) comprising of macronutrients, micronutrients and vitamins in the absence or presence of plant growth regulators (such as cytokinin, auxin, gibberellins etc.) are employed for plant growth. Variation of these constituents can either promote or suppress apical dominance.

Automated systems such as the Temporary Immersion System (TIS) have been successfully used to rapidly multiply banana and plantain planting materials (Roels *et al*., 2005; Jekayinoluwa *et al.*, 2019). Micropropagation through callus culture can produce shoots via organogenesis or somatic embryogenesis. Embryos obtained from somatic embryogenesis can be used in cell suspension culture. Micropropagation via adventitious embryos is another means of propagation. Embryos from explant can be used as source material for clonal propagation (Uma *et al.*, 2011). Organ culture using the male bud as source material for propagation has also been successfully used to establish plantain planting materials (Darvari *et al.*, 2010).

2.6.3 Conservation of Banana and Plantain

Conservation of germplasm is essential for safeguarding genetic material, making it readily available for present and future use in developing improved materials, hence, increasing food production and ensuring sustainable agriculture. The approach for plant genetic resource conservation depends on its nature which can be done *in situ* or *ex-situ* (Shii *et al*., 1994).

In situ conservation provides an ideal platform to conserve clonally propagated germplasm such as banana and plantain in their natural environment where a variation of any sort is minimal or not present. However, this mode of conservation requires large space, besides being labour and cost-intensive (Rajasekharan and Sahijram, 1990). Germplasm is also prone to accumulation of pest and diseases hence, the germplasm can be wiped off in the event of a natural or man-made disaster (Vasile *et al.*, 2011).

Ex-situ conservation involves collecting the germplasm from their natural environment and maintaining them under controlled and sterile conditions (González-Benito *et al.*, 2004). This mode of conservation is time and space-saving as only vegetative explants are preserved *in vitro* at slow growth ambience. As a result, special strategies have been employed to achieve adequate *ex-situ* conservation which could be short, medium or long-term using tissue culture techniques. Short term conservation is used for temporary storage under optimum culture conditions. The medium-term also known as slow-growth culture condition involves keeping germplasm under low temperature while long term conservation uses cryopreservation technique via ultra-low temperature (-196 °C) for storage of germplasm (Engelmann, 1991).

DNA banking is another novel mode of conserving genetic material especially those that are recalcitrant at the *in situ* or *ex-situ* level (de Vicente, 2006; Rice *et al*., 2006). Bananas and plantain germplasm have been successfully conserved in gene banks using either of these conservation strategies (Houwe, 1999; Panis and Thinh, 2001). Successful conservation of plant germplasm provides an avenue for transboundary exchange for research and for crop improvement.

2.6.4 Germplasm Exchange of Banana and Plantain

Exchange of plantain and banana germplasm can be done either at a local level through a transfer of plant suckers from one place to another by smallholder farmers or under specific quarantine regulation by a qualified institution. These institutions screen the germplasm for quarantine important pathogens and ensure that they are safe for exchange. This is usually done using the Standard Material Transfer Agreement (SMTA) which gives details on the germplasm source and health status (FAO, 2001; Verma *et al*., 2014). This approach helps to minimize the spread of plant diseases across the board as compared to transfer by local farmers who lack knowledge and expertise in ensuring the exchange of clean germplasm. The emergence of pest and diseases is posing a great risk to banana and plantain germplasm exchange, hence threatening its yield and production.

2.7 Plant Genetic Transformation

Plant genetic transformation is a process of introducing a gene from an organism to the plant host in an effort to induce transient or stable expression of the desired trait. The ability to transfer genes across different organism has paved the way for crop improvement especially in situations where the genetic diversity is insufficient or not robust. Physical, chemical and biological methods are major ways of introducing a foreign gene into the plant host. These include; microinjection, electroporation, chemical poration, microprojectile bombardment, polyethylene glycol (PEG) mediated gene transfer, pollen tube pathway, sonication, *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* mediated plant transformation (Jouanin *et al*., 1993; Mubeen *et al*., 2016).

Discovery of *Agrobacterium tumefaciens* and its use in plant genetic transformation has gained wide application in plant biotechnology in the development of genetically modified crops, understanding plant gene function and plant-microbe interaction (Hwang *et al*., 2017). Reproducible and efficient *Agrobacterium*-mediated gene transfer has been established for dicotyledonous and monocotyledonous plants (Piers *et al*., 1996).

The biology of *Agrobacterium*-mediated gene transfer employs the transfer and integration of the tumour-inducing (Ti) plasmid region of the *Agrobacterium* into the plant nucleus and/or genome (Hwang *et al*., 2017). The Ti-plasmid consist of the T-DNA and the virulence (*vir*) region which mediates efficient processing, transfer and integration of the T-DNA into the plant cell (de la Riva, *et al*., 1998; Hwang *et al*., 2017). The T-DNA sequence can be replaced with the sequence of the target gene harbouring the trait of interest.

For a successful plant transformation, the choice and source of explant is very crucial in order to allow efficient regeneration of the transgenic plant. Friable embryogenic calli, plant cell suspension, meristem and leaf are some examples of explant choice (de la Riva, *et al*., 1998; Bull *et al*., 2009; Iantcheva *et al*., 2014; Yau *et al*., 2017).

Regeneration capability of the explant of choice determines the success of plant genetic transformation. For instance, successful *Agrobacterium* transformation and regeneration of transgenic banana was achieved using cell suspension as explant (Tripathi *et al*., 2015).

Advancement in biotechnological tools such as RNAi exploits the use of *Agrobacterium* transformation in unravelling gene function as well as plant-microbe interaction (Miki & Shimamoto, 2004; Crane & Gelvin, 2007; Toprak *et al*., 2014).

2.8 Threats to Banana and Plantain Production

2.8.1 Abiotic Factors

The optimum potential of banana and plantain production is threatened and limited by several abiotic factors. Temperature, altitude, soil pH and nutrients, rain and wind are some of the abiotic stresses that could impair adequate growth, development and yield of plantain/banana. Plantain and banana thrives well in a warm, humid climate with adequate rainfall (Swennen, 1990). However, low temperature could adversely affect the yield of plantain/banana either causing chilling or heat injury (Turner & Lahav, 1983). High altitude correlates to a low temperature which in turn poses a negative effect on the growth and yield of plantain in eastern Democratic Republic of Congo (Sivirihauma *et al*., 2016). According to the work of Salau *et al*., (2016) optimum temperature for plantain yield is about 26 °C. Higher or lower temperature impacts negatively on plantain production in the region. In the study, it was also observed that low rain level marked by drought conditions and excess rainfall reduced the yield of plantain. Extreme weather conditions could as well lead to the emergence or repression of certain crop pests and diseases (Ortiz, 2013; Rusu & Moraru, 2015).

2.8.2 Biotic Factors

Biotic factors are other major threat affecting banana and plantain production. These include a wide array of microorganisms, pathogens, weeds, parasites and pests. Nematodes are some of the prime parasites of *Musa* plants. They hamper the root systems

leading to deranged anchorage and destruction of the plant (Gowen *et al*., 2005; Chitamba et al., 2013). Other natural enemies include banana fruit and leaf scarring beetle (Mishra *et al*., 2015), banana stem weevil (Tiwari *et al*., 2006), banana rust thrips (Hara *et al*., 2002) and many more. Of utmost significance are insect pests and the diseases they cause. It is noteworthy that some plant diseases are endogenous while exogenous diseases are mostly transmitted by pest and/or insect vectors.

2.9 Pests and Diseases of Plantain and Banana

Pests, as well as fungal, bacterial and viral diseases, are a predominant threat to the production of bananas and plantains in many parts of the world. A variety of pests and diseases such as; Black sigatoka, banana bacterial wilt (BBW), banana weevils and nematodes account for 25 - 100 % reduction in banana production (Gold *et al*., 2001; FAO, 2013; Tripathi *et al.*, 2015).

2.9.1 Panama Disease

Panama disease caused by the fungus *Fusarium oxysporum* is a widely known soil pathogen found in all banana-producing countries. There are four strains of this fungus which are classified based on their morphology; race 1, race 2, race 3 and race 4, all of which are pathogenic and show no symptomatic variation (Pérez-vicente *et al.*, 2014). Regardless of the race, the devastating effect of the fungus causes a significant reduction in banana/plantain production. This pathogen infects the banana root system and spreads to different parts of the plant. The spores of this fungus can survive in the soil for a long time and susceptible clean plants can be easily infected. Soil treatment stands as a better option but this approach is not environmentally friendly, thus, making it difficult to control. The banana trade was significantly affected by the outbreak of this disease which almost wiped out *Gros Michel* a major export cultivar (Ploetz 2000; Daly 2006).

2.9.2 Banana *Xanthomonas* Wilt

Banana *Xanthomonas* wilt (BXW) is caused by Gram-negative bacterium *Xanthomonas campestris* pv. *musacearum*. The disease was first reported on *Ensete* a close relative of

banana in Ethiopia in the late 1960s, Uganda in 2001 and has spread to other banana producing regions. The disease results in wilting and death of the plant. Rouging (either manually or through the use of 2, 4-Dichlorophenoxyacetic acid) and timely de-budding of the male flower immediately after the last hand of the banana bunch is formed are cultural practices in managing the spread of the disease. The bacteria is not only pathogenic to the plant but also causes a soil-borne disease that may persist in soil for over 6 months. Bacterial diseases are not easy to control once they are established. BXW is even a more peculiar case because there is no known banana variety that is completely resistant to the disease. Sterility of banana limits the use of conventional breeding in developing resistant varieties (Biruma *et al*., 2007; Ahimelash *et al*., 2008; Tripathi *et al.*, 2009). However, an advance biotechnological strategy is needed to improve the plant defence mechanism. Transgenic banana resistant to BXW has been developed and tested under field conditions in Uganda (Tripathi *et al.*, 2010; 2015)

2.9.3 Moko Disease

A similar disease to the banana wilt is the Moko disease. This is another deadly disease of banana and plantain caused by a bacterium *Ralstonia solanacearum*. It is a soil pathogen found especially in tropical and subtropical lowland regions (Blomme *et al*., 2017). This disease caused huge losses in banana and plantain production in Guyana, Trinidad, Central, South and Latin America, Colombia, Peruvian Amazon, Brazil, Belize, Costa Rica, Ecuador, El Salvador, Granada, Guatemala, Honduras, Mexico, Nicaragua, panama, Peru, Suriname, Venezuela and Jamaica (Buddenhagen 1986; Thurston 1989; Eyres *et al*. 2005; Elizabeth *et al.*, 2015).

2.9.4 Nematodes and Weevils

Plant-parasitic nematodes and banana weevil (*Cosmopolites sordidus*) are economically important pests of banana and plantain which attack the root and corm system of the plant (Daneel & De Waele, 2017; Twesigye *et al*., 2018). Highland varieties and plantains are mostly affected by these pests (Gold *et al*., 2004; Poeydebat *et al*., 2017; Twesigye *et al*.,

2018). The major parasitic nematode that affects banana/plantain production includes; root-knot (*Meloidogyne* spp.), spiral (*Helicotylenchus* spp.), burrowing (*Radopholus* spp.), and lesion (*Pratylenchus* spp.) nematodes (Daneel & De Waele, 2017). Banana weevil and nematode cause severe yield loss to banana and plantain. Over 40 and 50 % yield losses have been attributed to the effect of banana weevil and nematode damage respectively to banana and plantain production (Rukazambuga *et al*., 1998; Roderick *et al*., 2012).

2.10 Banana Viruses

Plant viruses are other major constraints to banana improvement and production. These can either be endogenous viruses or viruses transmitted by pathogenic insects from one plant to the other.

2.10.1 Banana Streak Virus (BSV)

Banana Streak Virus (BSV) is endogenous to some bananas and plantain having the 'B' genome and when integrated into the plant genome can give rise to episomal infection. BSV can as well be transmitted by mealybugs (Lockhart, 1995). Other examples of banana viruses are cucumber mosaic virus, banana mild mosaic virus, banana bract mosaic virus and banana bunchy top virus. Nevertheless, the banana bunchy top virus has been identified as one of the most severe viruses of banana causing a significant loss to its production.

2.10.2 Banana Bunchy Top Virus (BBTV)

BBTV causing BBTD is a major threat to banana and plantain causing a serious reduction in their production. In severe cases, it may result in 100 % loss in production. BBTV is a complex circular single-stranded DNA virus which multiplies within the phloem tissue of a host plant (Mandal, 2010). BBTV was initially classified as a *Luteovirus* and later reassigned to the *Nanoviridae* family (genus *Babuvirus*). The genome of BBTV is multipartite, comprising of six circular components with an approximate size of 1.1 kb each (Harding *et al*., 1991, 1993; Burns *et al*., 1995) and are known to promote the

pathogenicity of the virus. They were initially named as DNA 1-6 but recently named as DNA-R, -U3, -S, -M, -C and -N respectively (Harding *et al*., 1991; Wu *et al.*, 1994; Abdel-Salam *et al.*, 2012). The six components are encapsidated within separate virions, each about 18–20 nm in diameter (Harding *et al*., 1993). All the six integral components have a common genome organization comprising of a major common region (CR-M), stem-loop common region (CR-SL), potential TATA box 3' of the stem–loop, at least one open reading frame (ORF) for a major gene in the virion sense and polyadenylation signals associated with each gene (Burns *et al.*, 1995). The major component of BBTV, DNA-R, encodes two open reading frames and other components each encoding one protein (Burns *et al.*, 1995; Beetham *et al.*, 1997). Five of the six components have a large open reading frames in the virion sense and a stem-loop structure in the noncoding intergenic region (Burns *et al.*, 1995). The stem-loop initiates replication of viral proteins.

DNA-R encodes a replication initiation protein (Rep) responsible for initiating viral DNA replication, DNA-S encodes the coat protein (CP), DNA-C encodes the cell cycle link protein (Clink), DNA-M encodes the movement protein (MP), DNA-N encodes the nuclear shuttle protein (NSP), while the function of DNA-U3 is unknown (Burns *et al*., 1995; Wanitchakorn *et al*., 1997, 2000; Abdel-Salam *et al* 2012). BBTV DNA-R, -U3, -S, -M, -C and -N (DNA-1 to -6) have been consistently linked with BBTV isolates globally, indicating they are essential components of the BBTV genome. BBTV is widely distributed with confirmed infections in Africa, Asia, Australia, and South Pacific islands but significantly not the Americas. There are two broad groups of BBTV isolates identified based on nucleotide sequence differences between their genome components and geographical delineation (Karan *et al*., 1994; Kumar *et al.*, 2011). The 'South Pacific' group comprises isolates from Australia, Bangladesh, India, Myanmar, Pakistan, Sri Lanka, Fiji, Western Samoa, Tonga, Hawaii and Africa, while the 'Asian' group comprises isolates from China, Indonesia, Japan, Philippines, Taiwan, Thailand and Vietnam (Karan *et al*., 1994; Kumar *et al.*, 2011). Both groups differ from each other with an average of

9·6 % (DNA-R), 11·86 % (DNA-S) and 14·5 % (DNA-N) over the entire nucleotide sequence. However, the difference in the major common region (CR-M) between the two groups is about 32 % (DNA-R), 38·6 % (DNA-S) and 27 % (DNA-N) (Karan *et al*., 1994).

BBTD was first reported in Fiji in 1889 and has since been spreading to several other countries in the world including Western, Central and Southern Africa, Oceania, Asia and South Pacific (Khalid *et al.*, 1993; Xie & Hu, 1995; Kenyon, 1997; Kagy *et al.*, 2001; Kumar *et al*., 2011; Oben *et al.* 2009; Lokossou *et al.*, 2012; Adegbola *et al*., 2013; Jooste *et al.*, 2016) (Fig 2.1). In sub-Saharan Africa, the disease has to-date spread to 17 countries and affected about 12 million smallholder farmers who depend on banana/plantain production for their livelihood (Kumar *et al.*, 2011). In Central and Southern Malawi, BBTD accounted for 80 % decrease in Cavendish banana production (Kumar *et al.*, 2013). In India, the disease caused a drastic reduction in hill banana production from 18000 ha to 2000 ha (Elayabalan *et al*., 2015).

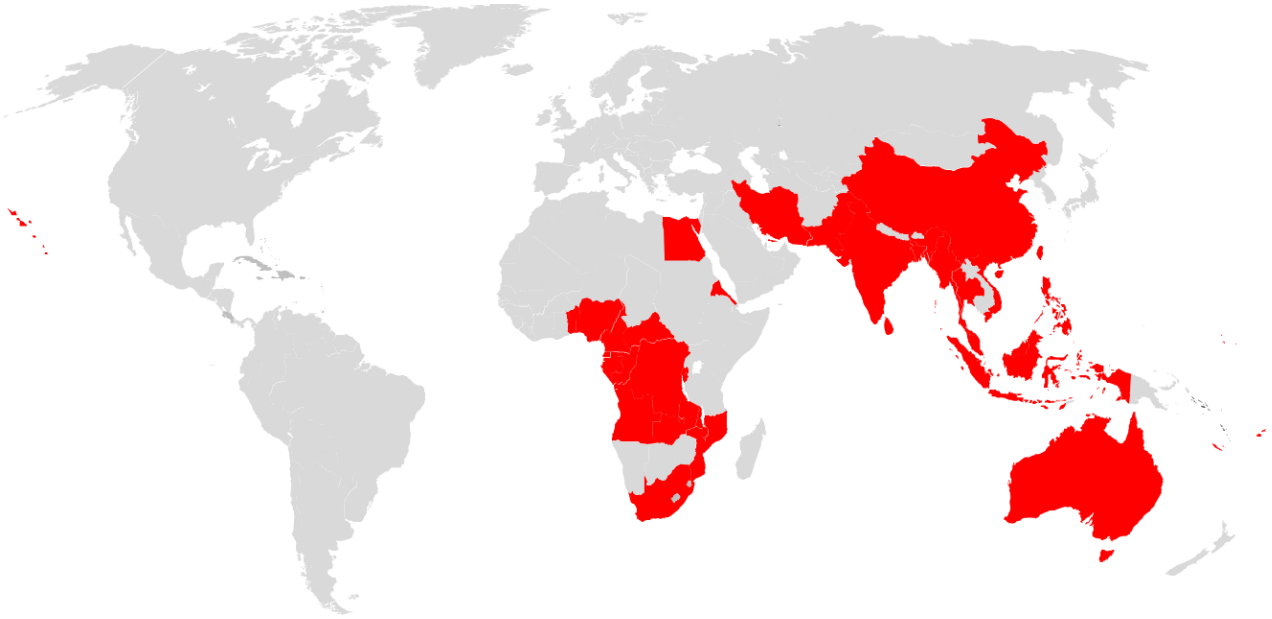


Figure 2.1: Geographical distribution and occurrence of BBTD. Areas highlighted in red represent where BBTD has been reported

2.11 BBTV Vector: *Pentalonia nigronervosa* Coquerel

Banana aphids (*Pentalonia nigronervosa*) is found on banana, Musaceae species as its preferred hosts (Robson *et al*., 2007). The life cycle of banana aphids is from the nymph to adult stage with no egg stage and are prolific in nature. It takes 8 to 16 days from the nymph cycle to the adult stage while the adult aphids can stay for 8 to 26 days. The nymphal stage is categorized into four stages: Newborn nymphs are oval in shape, creamish in colour, about 0.004 inches in length and lasts for 2 to 4 days (Rajan, 1981). The second nymph stage lasts for 3 to 4 days, they are similar to the new-born but bigger and brownish in colour and are about 0.028 inches in length, the third nymph stage is more mature and bigger measuring about 0.036 inches in length (Rajan, 1981). At this stage the physiology of the aphids is defined and prominent, the compound eyes are more noticeable and the antennae are segmented into five (Rajan, 1981). At the fourth nymphal stage, the size is about 0.04 inches long with 6 segmented antennae. The third and the fourth nymph stage last for 2 to 4 days each. The size of an adult banana aphid ranges from 0.08 to 0.04 inches long with six-segmented antennae (Rajan, 1981).

The adult aphid gives birth to young ones a day after reaching maturity and they can reproduce up to 4 aphids per day (Ronald, 1994). Colonies of banana aphids are found beneath the leaf sheaths and at the base of the pseudostem. Banana aphids found on banana plants may either be viruliferous or non-viruliferous. Non-viruliferous aphids are mainly found on non-infected banana/plantain plants. On the other hand, viruliferous aphids acquire the virus while feeding on an infected banana/plantain plant. This could be transmitted when these aphids migrate within the field and feed on a healthy plant, thus transmitting the virus and infecting the healthy plant. Viruliferous aphids cannot be distinguished physically from the non-viruliferous aphids, it requires specific diagnostics on the symptomatic banana plant as well as banana aphid(s) feeding on it to determine BBTV carrier-aphids.

Besides transmitting the virus, the banana aphids acquire sugars while feeding on the nutrients from phloem cells of a banana/plantain plant, and excrete honeydew as the substrate which ants feed on and promotes the growth of sooty mould fungi (Lindsay, 1998; Sarwar, 2011). This could hinder photosynthetic activities of the plant and could as well lead to wilting especially for younger plants (Fig 2.2).

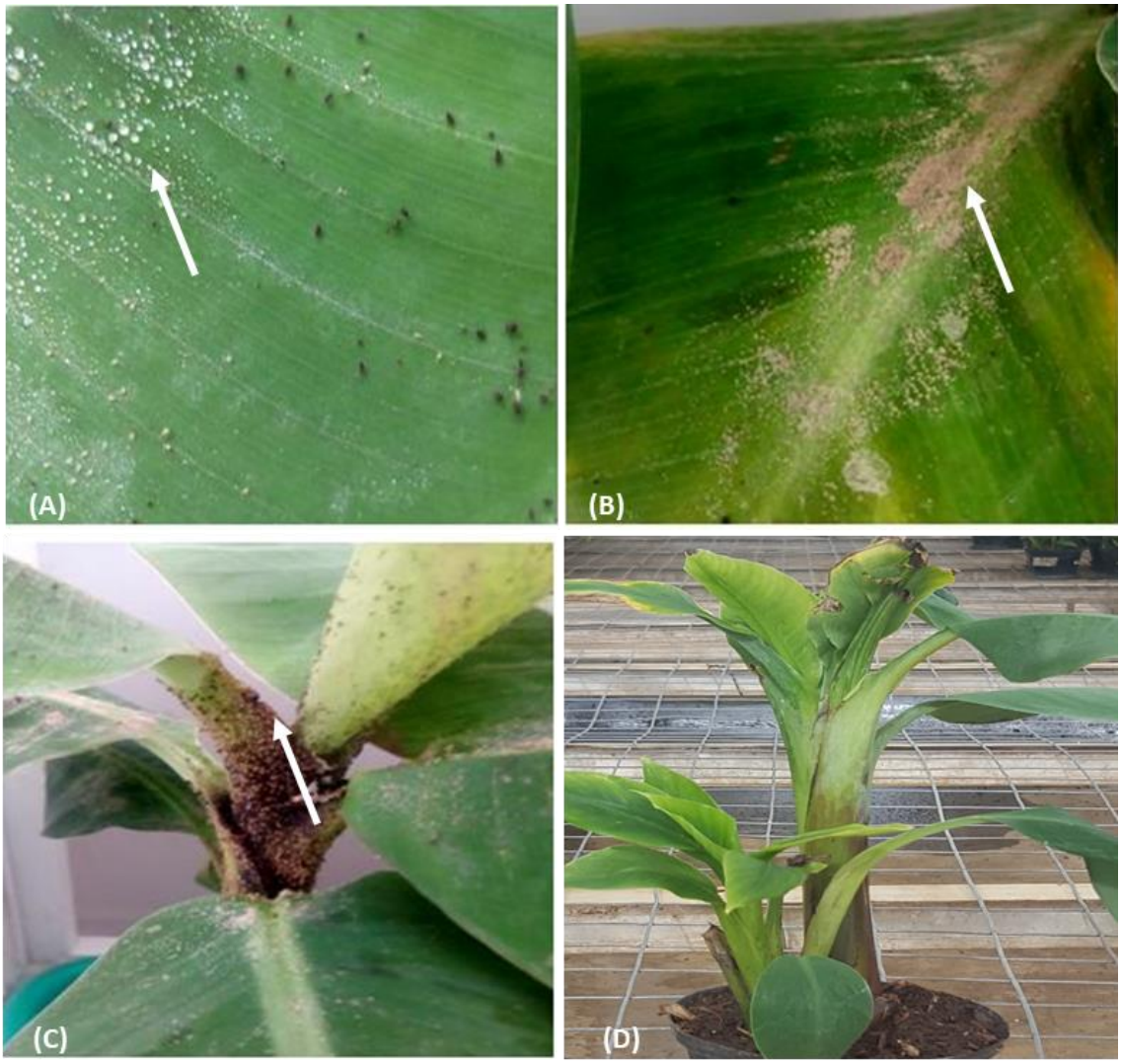


Figure 2.2: Effects of banana aphids on *Musa* spp. A: honeydew excreted by banana aphids B: C: Sooty mould fungi on banana plant D: BBTD symptomatic banana plant (stunted growth).

2.11.1 Features of Banana Aphid

Banana aphid is slightly oval or elongated with six antennae. It is characterized by a shiny reddish-brown, dark brown or black colour. Other features include; head, antenna, stylet, salivary glands, midgut, hemolymph, and fat body cell.

2.11.1.1 The Head*:* It is the anterior part of the aphid carrying the primary sense organ. This includes the antennae for sensing, the mouth part for feeding and the compound eyes for sight.

2.11.1.2 The Antenna: It is an important sensory organ of aphids composed of 3 major segments: the scape, pedicel and flagellum. The scape and pedicel are made up of well-developed muscles while the flagellum consists of non-muscularized flagellomeres. The flagellum consists of sensory hairs for detecting several external features thus coordinating their movement. Many insects are known to use their antennae to detect changes in the level of humidity in their environment (Krause & Dürr, 2004; Elgar *et al*., 2018). The Proprioreceptors located in and outside the scape and pedicel controls the mobility of the antenna and may play a crucial part in the movement of aphids as well as its adaptation in relation to its surrounding climatic conditions (Krause & Dürr, 2004; Elgar *et al*., 2018) hence, providing signals for reproduction.

2.11.1.3 The Stylet: It is the mouthpart of aphids which enables its piercing, sucking and feeding mechanism (Ronald *et al* 1994; Walker, 2009). The needle-like stylet of banana aphid is about 710-1040 µm long (Noordam, 2004). The common feature of a sucking-mouth part includes a sucking pump and a sucking tube both of which play the role of drawing liquid from plant tissues. For hemiptera, the sucking tube is formed from the mandibles, maxillae and labium while the sucking pump is formed from the cibarium. The movement of the stylet is fairly rapid within the sheath formed when aphids secrete saliva on plant tissues and it extends beyond the sheath during ingestion of food material (Sastry, 2013).

***2.11.1.4 Salivary Glands*:** This play a key role in the physiological status of an insect. It is associated with the mouthpart, produces saliva which mixes with food during feeding and is ingested along with food as well (Walker, 2009). Certain digestive enzymes such as amylase, invertase, proteases and lipases are known to be present in the saliva of insects (Walker, 2009). During feeding of hemipterans, the saliva produced may either be in sheath or watery form. A gel-like sheath consisting of lipoproteins is formed shortly after secretion of the saliva; this allows the stylet tips to have contact with the plant tissues. While the watery-like saliva doesn't gel but consist of various enzymes which either help in dissolving the plant cell wall and/or prevents absorption of secondary metabolite which the plant may secrete as a defence mechanism response (Walker, 2009).

2.11.1.5 The Midgut: It is part of the alimentary canal of an insect where digestion of food takes place (Lehane & Billingsley, 1996). The digestive system of hemiptera is divided into different sections due to the absence of true peritrophic membrane (PM), a film-like structure made of chitins, proteins and most importantly, peritrophins (proteoglycans) that separates food from the midgut tissue (Elvin *et al*., 1996; Terra, 2001). The peritophins are glycoproteins essential for connecting the "*dots*" between different cells, taking an active part in the binding of ions (such as Na^+, K^+, Ca^{2+}) of plant sap, water and regulating the movement of molecules through the membrane (Silva *et al*., 2004). The PM prevents the lining of the alimentary canal from food abrasion and microbial damage. The PM of insect vector has been found to affect the survival and virulence of pathogenic organisms. It also functions to determine the ultra-filter features of the membrane (Elvin *et al*., 1996).

The classification of the digestive system which is as a result of the absence of the PM aids the digestion of polymeric molecules. However, an extra-cellular lipoprotein membrane known as the perimicrovillar membrane (PMM) which encloses the microvilli of the midgut cells is present in hemipterans (Silva *et al*., 2004; Gutiérrez-Cabrera *et al*., 2015). The microvillar membrane transports and regulates the concentration of potassium ions from the PMM which would be used to enhance the absorption of proteoglycans into

the midgut cells. It has also been observed that α-glucosidase bounded to the PPM enhances the breakdown of sucrose in the ingested sap (Gullan and Cranston 2010). Using the scanning electron microscope (SEM), Damasceno-Sa *et al*., (2007) observed that PPM were virtually absent in midgut of starved insect and degraded 48 hours after pulse feeding.

***2.11.1.6 Haemolymph*:** It is composed of fluid analogous to blood in insects which functions as a solvent and a nutrient transporter. In banana aphid, it is red in colour (Watanabe *et al* 2013). One of the major components of the hemolymph is the disaccharide trehalose, a type of carbohydrate which acts as metabolic fuel for many insects (Wyatt & Kalf, 1957) and an osmoprotectant in the cytosol. The α-glucosidase in the PPM of aphids may likewise help in the breakdown of trehalose.

***2.11.1.7 The Fat Body Cell*:** This serves as an energy reservoir for insects and makes it readily available for use. Energy is stored in the form of glycogen (carbohydrates) and triglycerides (lipids). Lipids are an important source of energy than carbohydrates because lipids contain more energy per gram than carbohydrate and lipids also contains the water of hydration needed by the insect especially during dry conditions to sustain its life (Hoshizaki, 2013). During starvation, the fat body cell is actively involved in the degradation and recycling of macromolecules in order to ensure continued growth and survival of the insect. The fat body cell comprises of loose tissue found all over the insect body parts including the gut and reproductive organs (Arrese & Soulages, 2010; Hoshizaki, 2013). Fat body cells are closely related to several cells and metabolic pathways within the insect body parts having close contact with the haemolymph to enable metabolite exchange. It is actively involved in lipids, carbohydrate, protein, amino acids and nitrogen synthesis. It also helps to synthesize lipophorins for the lipid transport or vitellogenins for egg maturation (Arrese & Soulages, 2010; Hoshizaki, 2013). During feeding, conservation of lipids increases and reduces during starvation.

Several insect hormones are housed within the fat body cells to enable and/or provide physiological changes needed for reproduction and proper development of the insect. There are 3 major types of fat bodies found in different insect; the adipocytes, urocytes and mycetocytes. The adipocytes are the most predominant and are the basic cell type; urocytes are a less common type of fat body type but are known to conserve uric acid as the end product of nitrogen metabolism in a terrestrial insect (Gullan and Cranston 2010).

The mycetocyte fat body type is found in some hemiptera which includes the aphids. The mycetocyte is known to harbour microorganisms that exist as symbionts in the vacuole of the insects and provide nutrients and support the survival of the insect (Gullan and Cranston 2010). Several symbiotic organisms exist in the aphid mycetocyte which may either be obligatory or secondary. However, *Buchnera aphidicola* and *Wolbachia* sp. are the two known obligatory symbiotic organisms in *Pentalonia nigronervosa*. There are also other secondary symbiotic organisms informally known as the R-type (PASS), S-type (PAR), T-type (PABS) and U-type (PAUS) which are not universally present in all aphids unlike the *Buchnera aphidicola* and *Wolbachia* sp. but they can be transmitted as well (Haynes *et al*., 2003). Detailed information on the importance of aphids to the secondary symbiotic organisms and vice versa are not fully understood. However, it has been observed that R-type bacteria in aphids may contribute to the tolerance of *Acyrthosiphon pisum* to high temperature (Montllor *et al*., 2002). *Buchnera aphidicola* and *Wolbachia* sp. existing as a co-obligatory endo-symbiotic organism found in the fat body cells of banana aphids provides essential nutrients that the insect could not obtain from the phloem of the plant. *B. aphidicola* is also known to supply riboflavin and lysine to the banana aphid (De Clerck *et al*. 2015). The presence of *Wolbachia* in *P. nigronervosa* is 100 % (De Clerck *et al*. 2015). The importance of the symbiotic association of the bacteria and insects is essential for the survival and reproduction (Wu *et al*., 2006). It was noted that aphids whose symbiotic microbe (*Wolbachia* sp.) was removed were friable and had a short life span and did not reproduce (De Clerck *et al* 2015). Understanding the biochemistry of fatty acids will help to understand the intricacies of aphid physiology and

how this can be manipulated to control the vector involved in the transmission of banana bunchy top disease.

2.11.2 Factors Essential for Banana Aphid Transmission and Acquisition

Temperature, drought, age, vector life stage and plant access time have an impact on the survival, multiplication and transmission of banana aphid. Wardlaw (1961) observed a decrease in the number of aphids during drought. Anhalt & Almeida (2008) observed that mature aphids transmitted the virus more effectively than third instar nymphs while Watanabe *et al*. (2013) observed that the nymph had a higher efficiency in transmitting the virus compared to the adult aphids and that the symptomatic effect varied with temperature and age of the plant (Watanabe *et al*., 2013). It has been observed that the membrane barriers of aphid gut and accessory salivary gland membranes play a significant role in circulating the virus (Gray & Gildow, 2003).

In a transmission and acquisition experiment, a longer plant access time and a dormant period favoured acquisition and transmission of the virus. The optimum temperature for aphid transmission is at 25 and 30 ºC, this may explain the differences in BBTV epidemics in different regions as cooler regions may have a lower level of BBTV incidence than warmer regions (especially tropical and sub-tropical regions). Inoculation and acquisition efficiency was in the range of 18-24 h (Anhalt and Almeida, 2008). Understanding the factors essential for banana aphid transmission and acquisition can pave the way towards controlling the spread of the virus.

2.12 Control and Management of BBTD

The continuous spread of BBTD in banana/plantain producing areas calls for immediate and adequate control strategies to minimize and/or eliminate the spread of the disease. Commercial banana cultivars are susceptible to the disease leading to an increased decline in production in affected regions. Several control strategies such as the use of chemicals, biopriming, rouging and use of tissue cultured plants have been employed but are limited in controlling BBTD (Hooks *et al*., 2009).

The use of chemicals is harmful to the environment, it is not cost-effective and has a short term effect in preventing the effect in reducing aphid population (Robson *et al.,*2006). Rouging involves cutting and removal of the infected plant. However, if rouging is not done properly, the surrounding healthy plants could be affected as aphids could migrate to these non-infected plants within the field and transmit the disease. Tissue culture is a useful strategy in generating clean planting materials (Ramos & Zamora, 1990) but does not guarantee the complete elimination of BBTD as clean plants can be re-infected if planted in an infected field. Colonies of aphids can be found at the base of the pseudostem and some inches below the soil surface (Thomas, 2008), it is not unlikely that clean tissue culture banana/plantain planted close to an infected plant may be vulnerable to infection especially if a viruliferous aphid feeds on it.

Biopriming is an alternative treatment to the use of pesticides, it involves treating the infected plants with beneficial microorganisms. Although, there was a reported reduction in BBTV incidence under field conditions, it was not able to provide absolute resistance to BBTD (Kavino *et al.*, 2007).

Consequently, host-plant resistance is considered potentially viable and a promising option for control of this viral disease, however, resistant varieties are lacking (Hooks *et al.,* 2009) and most plantain/banana cultivars are sterile. Conventional breeding has long been a useful tool for developing disease-resistant materials. Its success in breeding depends largely on the availability of disease-resistant cultivar(s) which may be used to develop improved banana/plantain varieties. However, it is a limited approach because there is no known germplasm that is immune or resistant to BBTD. Although there have been some observable relative susceptibility levels among some cultivars in the banana germplasm (Hooks *et al.,* 2009; Ngatat *et al*., 2017), this, however, may not be potent as breeding is greatly influenced by several environmental and biological factors.

Recent advances in biotechnological approaches offer a pathway in improving and/or activating the defence mechanisms of the plant to diseases. RNA interference (RNAi) is

a natural control mechanism in which a plant defence mechanism can be activated to silence invading RNA viruses. Activation of RNAi can be achieved through the use of hairpin RNA expressing vectors, particle bombardment, virus-induced gene silencing (VIGSs) and *Agrobacterium*-mediated transformation. RNAi mechanism regulates the gene transcription level when RNA construct that is complementary to the RNA of interest is introduced. It can either lead to silencing or activation of sequence-specific RNA degradation (Agrawal *et al*., 2004). This has been demonstrated for a number of viruses and insect vectors (Whyard *et al*., 2009; Shekhawat *et al*., 2012; Guo *et al*., 2014).

2.13 Mechanism of RNA Interference (RNAi)

RNA is essential and responsible for translating genetic information and regulating cell function and expression through various developmental stages. RNAi as its names implies is a mechanism that interferes with RNA pathway or processes either by inhibiting transcription or by activating degradation of specific mRNA (Agrawal *et al*., 2004; Fig 2.3).

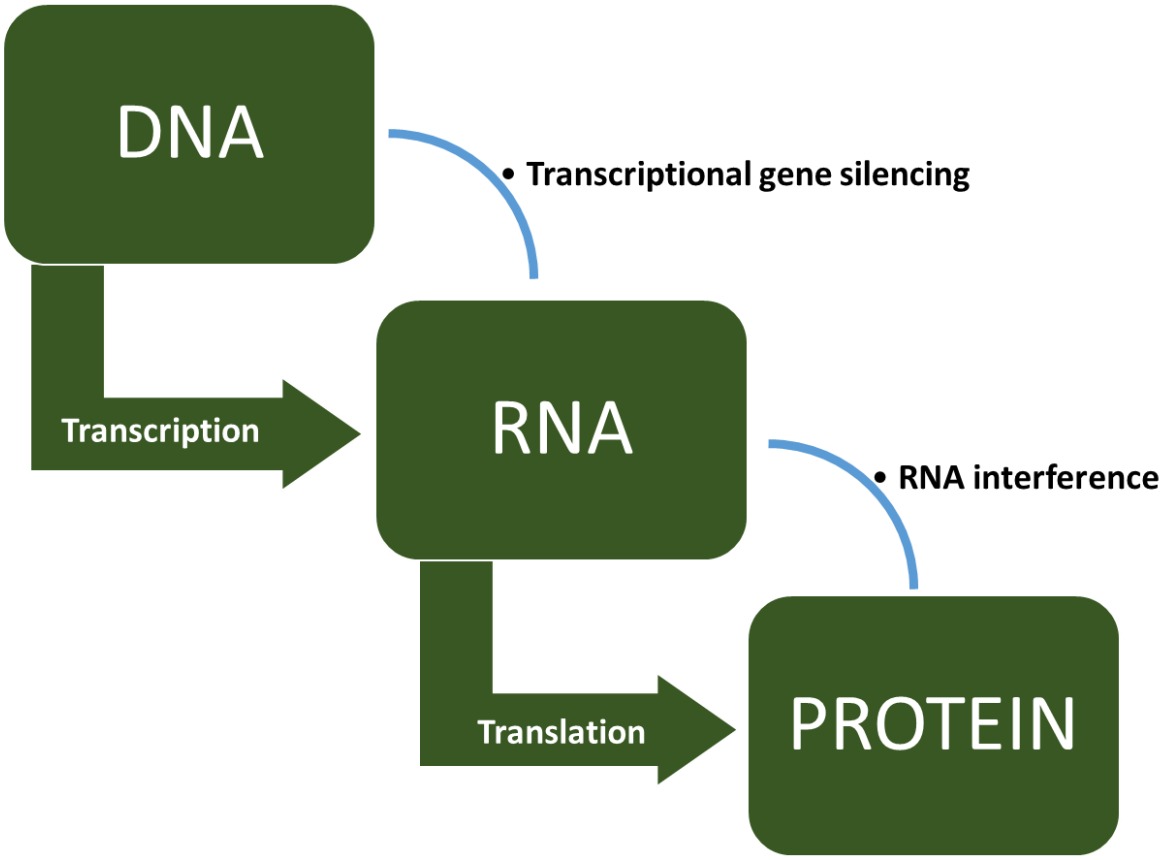


Figure 2.3: RNA pathway in relation to gene silencing mechanism

RNAi is initiated when dsRNA enters the cytoplasm. The dsRNA is chopped with the aid of dicer into small bits of about 20-25 nucleotide long known as the short interfering RNA (siRNA) or microRNA (miRNA) depending on the length of the originating dsRNA (MacFarlane & Murphy, 2010; Schroeder *et al.*, 2010). A population of siRNA is produced and a single strand of the siRNA known as the guide RNA is incorporated into the RNA induced silencing complex (RISC). The RISC cleaves the mRNA, hence preventing the expression or degradation of the mRNA (Agrawal *et al.*, 2004; Das *et al.*, 2011; Fig 2.4).

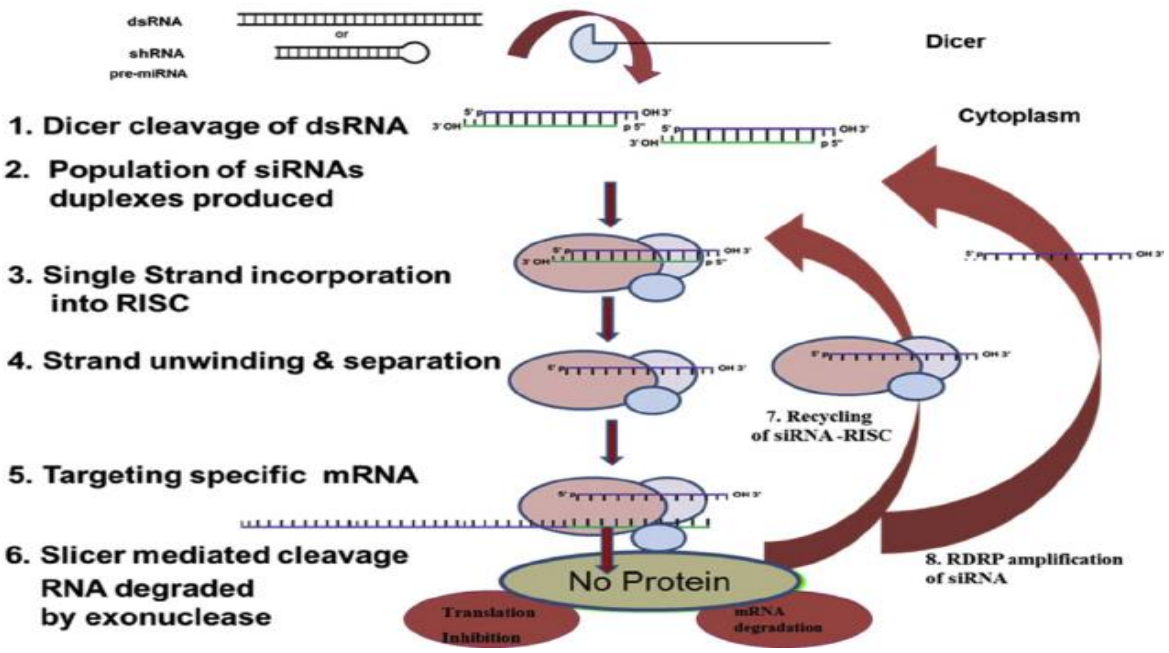


Figure 2.4: RNAi pathway (Sherman *et al*., 2015)

2.14 RNAi Based Approaches for Virus Control

RNAi targeting the viral genes is one of the potential immune responses employed by plants to silence the expression of viruses upon attack. This mechanism alongside other associated processes have been studied and applied in developing disease-resistant crops. RNAi acts by suppressing transcriptional process i.e. transcriptional gene silencing (TGS) or activating degradation of sequence-specific RNA i.e. post-transcriptional gene silencing (PTGS) (Agrawal *et al*., 2004).

The presence of double-stranded RNA (dsRNA) is a potent trigger and end product of RNAi which keeps the cycle of silencing active in biological systems. When dsRNA is induced, an enzyme known as the dicer recognises and cleaves the dsRNA into smaller RNA fragments of about ≈ 21 to ≈ 25 nucleotides known as the small interfering RNAs

(siRNA) (Agrawal *et al*., 2004). The population of siRNA fragments are incorporated into a nuclease containing complex referred to as the RNA induced silencing complex (RISC) where it is bound to the argonaute proteins. The siRNA unwinds and one strand is discharged to reinitiate the process while the other binds the homologous target mRNA and degrades or silence its expression (Waterhouse & Helliwell, 2003). The stoichiometry of the siRNA accumulation and the homologous mRNA specific-sequence influences the efficacy of the RNAi mechanism.

Another factor that influences the effectiveness of RNAi is the presence of suppressors which are encoded as proteins in plants and plant viruses (DNA and RNA viruses). They either prevent the production or accumulation of siRNA and downstream degradation of viral RNA (Tenllado & Diaz-Ruiz, 2001). Several proteins such as the Tomato yellow leaf curl geminivirus encoding V2 protein (TYLCV-Is V2), a *Nicotiana benthamiana* calmodulin-like protein (Nbrgs-CaM), and a triple gene block protein (TGBp1) encoded by the DNA satellite associated with the Tomato yellow leaf curl China virus, a cysteine-rich protein encoded by the potato virus M (CRP), helper component-proteinase encoded by potyviruses (HC-Pro), viral genome-linked protein (VPg) encoded by Turnip mosaic virus and Tobacco etch potyviral genomic RNA encoding a P1 helper-component proteinase and a part of P3 (P1/HC-Pro), a multifunctional protein encoded by turkey herpes virus (HVT063), a P1 protein encoded by sweet potato mild mottle virus (SPMMV P1), CP and P6 encoded by Olive mild mosaic virus (OMMV) are RNA silencing suppressors (RSS) that have been identified to inhibit RNA silencing (Anandalakshmi *et al*., 1998; Zrachya *et al*., 2007; Jing *et al*., 2011; Senshu *et al*., 2011; Li *et al*., 2014; Kenesi *et al*., 2017; Cheng & Wang, 2017; Varanda *et al*., 2018).

The coat protein (CP) and movement protein (MP) of BBTV encoded by DNA-S and DNA-M have been identified as suppressors of RNAi (Niu *et al*., 2009). It was demonstrated that CP and MP act as suppressors at different steps in the RNAi silencing pathways. MP was found to be a stronger suppressor of RNAi compared to CP. Further,

Amin *et al*., (2011) detected MP and the cell-cycle link protein (Clink) encoded by DNA-C as suppressors of RNA silencing. MP could be considered a potential candidate for developing resistance against BBTV using RNAi approach Besides acting as a suppressor of RNA-mediated gene silencing, MP allows efficient cell to cell propagation, by dodging the host cell wall barrier. BBTV is shuttled out of nucleus by nuclear shuttle protein (NSP) and MP transports the DNA-NSP complex to cell plasmodesmata and facilitates further movement across the cell wall. Therefore, inhibition of MP can provide strong resistance to BBTV.

Certain underlying phytochemicals have however been identified to attenuate the activity of the RSS. For instance, Violaxanthin deepoxidase protein of maize, *ZmVDE* decreased the accumulation of *Sugarcane mosaic virus* in *Nicotiana bentamiana* by reducing the RSS activity of SCMV HC-Pro (Chen *et al.,* 2017). Therefore, identifying other RNA silencing suppressors of BBTV, understanding the cross-talk between the host-pathogen, biochemical components and subsequently interfering with the suppressors can improve the efficiency of RNAi for virus control in host plants.

Nevertheless, RNAi has proven to be a useful approach in preventing the expression of viruses in plants. It has been successfully used in conferring substantial varying level of resistance to plant viruses such as Mungbean yellow mosaic India virus, Papaya ringspot virus, Soybean mosaic virus, Cucumber mosaic virus, and Sri Lankan cassava mosaic virus (Ntui *et al*., 2014, 2015; Thu *et al*., 2016; Jia *et al*., 2017; Kumar *et al*., 2017).

Similar promising results have been obtained in suppressing the expression of BBTV. Krishna *et al*., (2013) targeted four viral BBTV component through RNAi and was able to achieve partial resistance or tolerance to BBTV in transgenic Grand Nain banana under controlled conditions. Likewise targeting DNA-R in hill banana, resulted in symptomless plants with suppressed BBTV (Elayabalan *et al*., 2013; Elayabalan *et al*., 2017). The generation of BBTV resistant plants using RNAi and intron-hairpin-RNA (ihpRNA)

transcripts corresponding to the DNA-R, established the efficacy of RNAi mechanism in developing BBTV resistant plants (Shekhawat *et al*., 2012).

The prospect of RNAi relies on a comprehensive understanding of the multipartitism of BBTV and its synergistic interaction with its host and downstream biochemical machinery which would elucidate how to improve the specificity of RNAi for virus transmission control. Table 2.1 shows the various applications of RNAi in virus control.

Table 2.1: Application of RNA interference in the control of various crop viruses

Virus	**Family**	**Target viral gene**	**Crop**	**Phenotype**	**Reference**
Potato virus Y	*Potyviridae*	*Protease*	*Nicotiana tabacum*	Immunity	Waterhouse *et al*. , 1998
Bean golden mosaic virus	*Potyviridae*	*AC1*	*Phaseolus vulgaris*	Resistance	Bonfim *et al*., 2007
Cucumber mosaic virus	*Bromoviridae*	*RD1, RD6*	*Arabidopsis thaliana*	Immunity	Wang *et al*., 2010
Rice stripe Tenuivirus	*Phenuiviridae*	*AGO 18*	*Oryza sativa*	Resistance	Wu *et al*., 2015
Banana bunchy top virus	*Nanoviridae*	*DNA-R, BBTV viral genome*	*Musa spp.*	Resistance/ tolerance	Shekhawat *et al*., 2012; Krishna *et al*., 2013; Elayabalan *et al*., 2013, 2017
Rice dwarf virus	*Reoviridae*	*Pns12*	*Oryza sativa*	Resistance	Shimizu *et al*., 2009
Turnip yellow mosaic virus, Turnip mosaic virus	*Tymoviridae, Potyviridae*	*amiR-P69 159; amiR-HC-Pro 159*	*Arabidopsis thaliana*	Resistance	Niu *et al*., 2006
Africa cassava mosaic virus	*Geminiviridae*	*AC1*	*Manihot esculenta*	Resistance	Vanderschuren *et al*., 2009
Cassava brown streak virus, Cassava brown streak Uganda virus	*Potyviridae*	*Coat protein*	*Manihot esculenta, Nicotiana benthamiana*	Resistance	Patil *et al*., 2011; Yadav *et al*., 2011
Alfalfa mosaic virus, Bean pod mottle virus, Soybean mosaic virus	*Bromoviridae, Secoviridae, Potyviridae*	*Replicase genes*	*Glycine max*	Systemic resistance	Zhang *et al*., 2011
Cucumber mosaic virus	*Bromoviridae*	*CMV-O*	*Solanum tuberosum*	Resistance	Ntui *et al*., 2013
Sri Lanka Cassava mosaic virus	*Geminiviridae*	*SLCMV*	*Manihot esculenta*	Resistance	Ntui *et al*., 2015

2.15 RNAi Based Approaches for Insect Vector Control

BBTV is very difficult to manage and not much success has been achieved towards developing host plant resistance through breeding or transgenic approach. Therefore, controlling virus transmission by aphids represents a new alternative for management of aphid-borne BBTD. One approach could be to block the virus transmission by aphids through interfering with aphid–virus interactions to inhibit virus acquisition by aphids.

The efficiency of virus acquisition is one critical parameter in determining aphid transmission efficiency. The aphid transmission can be blocked by impairing the virus acquisition at the gut epithelial cell barrier via direct interference with aphid–virus protein interactions. The receptors and/or virus-binding protein(s) present on the gut and accessory salivary gland promote the transmission specificity (Brault *et al*., 2010). The vector-virus protein interaction could, therefore, be a potential target for RNAi by blocking the vector's ability to transmit the virus (Heck and Brault, 2018). The cuticular proteins play a key role in plant virus transmission by influencing the structure of cuticles, influences virus-vector interaction, virus entry into the gut and prevent virus degradation in the insect hemolymph (Deshoux *et al*., 2018). Deshoux *et al*., (2020) identified a cuticular protein, receptor RR-1, at the surface of the acrostylet in pea aphid. RR-1 proteins are associated with circulative virus transmission. The interaction between the nuclear shuttle protein encoded by DNA-N and cuticular proteins in aphid's stylet may facilitate virus transmission. However, deactivating the helper factor and/or receptors by targeting RR-1 genes in banana aphid and DNA-N using RNAi could be a potential way to abolish the spread of the virus by the vector.

Another approach of controlling the aphid-transmitted virus is to reduce the vector population. To achieve this would require disrupting the feeding mechanism of vector because it is a phloem feeder and it infects the host plant during feeding. Managing the

effect of these insect pests on plants via RNAi would call for identifying the key gene(s) essential for the insect survival and/or adaptation on host plants.

Table 2.2: Application of RNA interference in the control of aphids

Insect vector	**Plant disease transmitted by vector**	**Target gene**	**Crop**	**Mode of action**	**Reference**
Sitobion avenae	Barley yellow dwarf	*Laccase 1*	*Triticum avestivum*	Iron metabolism and immunity	Zhang *et al*., 2018
Diuraphis noxia	Barley yellow dwarf, barley yellow moaic	*Alkaline phosphatase*	*Triticum avestivum*	Aphid-plant interaction	Cooper *et al*., 2010, 2011
Sitobion avenae	Barley yellow dwarf	*Structural sheath protein (shp)*	*Hordeum vulgare*	Aphid-plant interaction and reproduction	Abdellatef *et al*., 2015
Myzus persicae	Potato virus X (PVX), Potato leafroll virus	*Mp10, Mp42*	*Nicotiana benthamiana , Nicotiana tabacum*	Fecundity	Bos *et al*., 2010
Acyrthosiphon pisum	Bean yellow mosaic	*C002*	*Vicia faba*	Fecundity /survival	Mutti *et al*., 2008
Myzus persicae	Potato virus X (PVX), Potato leafroll virus	*Acetylcholine sterase*	*Nicotiana tabacum*	Resistance	Guo *et al*., 2014
Toxoptera citricida	Citrus tristeza virus	*KAT, Pepck, Gp*	*Citrus sinensis*	Wing development	Shang *et al*., 2016
Myzus persicae	Potato virus X (PVX), Potato leafroll virus	*FAD7*	*Lycopersicon esculentum*	Resistance	Li *et al*., 2018
Myzus persicae	Potato virus X (PVX), Potato leafroll virus	*C002, Rack1*	*Nicotiana benthamiana , Arabidopsis thaliana*	Fecundity	Pitino *et al*., 2011
Sitobion avenae	Barley yellow dwarf	*Chitin synthase 1 (CHS1)*	*Triticum avestivum*	Fecundity	Zhao *et al*., 2018
Myzus persicae	Potato virus X (PVX), Potato leafroll virus	*MpC002, MpPlntO2 and Rack 1*	*Arabidopsis thaliana*	Celluar process, aphid-plant interaction	Coleman *et al*., 2015
Acyrthosiphon pisum	Bean yellow mosaic	*Ap-crt and Ap-cath-L*	*Pisium sativum*	Developmental stage	Jaubert-possamai *et al*., 2007

2.16 Targets for RNAi-Mediated Silencing in Insect Vector Control

A crucial step towards plant disease management is controlling its vector. This requires a specific but targeted approach to keep the vector population in control. Achieving this could require interfering with the feeding machinery of the vector as most are phloem feeders and infect the host plant during feeding. RNAi is a probable way of inducing a specific insecticidal effect on an insect vector without affecting off-target organisms. RNAi has been efficiently applied in pests in the orders coleoptera, lepidoptera and hemiptera (Baum *et al*., 2007; Zha *et al*., 2011; Laudani et al., 2017; Poreddy *et al*., 2017; Knorr *et al*., 2018; Yoon *et al*., 2018). The midgut genes of the insect were successfully knocked down when nymphs were fed on rice plant materials expressing dsRNA. RNAi was initiated and this was marked by lethal phenotypic effects. Zha *et al* (2011) targeted genes encoding proteins that may be involved in the RNAi pathway in a hemipteran insect *Nilaparvata lugens*.

Essential systems such as the central nervous system, midgut and fatty body in the insect, necessary for their survival could be targeted to knock down their viral transmission capacity. The hemipteran insects are phloem feeder or sap-sucking and includes the stink bugs, plant bugs, whiteflies and aphids (Chougule & Bonning, 2012). They damage the plant during feeding and act as vectors for transmitting viruses to host plants. Managing the effect of these insect pests on plants via RNAi would call for identifying the key gene(s) crucial for the insect's survival or adaptation on the host plant.

The salivary gland of aphids (hemiptera) host a wide range of enzymes such as amylase, pectinase, cellulase and protease that promotes its adaptive features on host plants (Cooper *et al*., 2010). The salivary secretions produced during the feeding of aphids on plants may help in degrading the plant cell wall to facilitate penetration of the stylet or proboscis, digestion of phloem nutrients like carbohydrates and breakdown or detoxification of defensive compounds (polyphenol oxidase, peroxidases and oxidoreductases) produced by plants (Cooper *et al*., 2011; Darvishzadeh *et al*., 2014; Van Bel & Will, 2016; Boulain

et al., 2018). A salivary effector protein (C002) was found to be crucial in the feeding and survival of pea aphids on fava beans and suppresses plant defences (Mutti *et al*., 2008) by detoxifying plant induced secondary metabolite like phenols, while Mp10 and Mp42 are known to suppress reproductive potential of aphids as well as trigger plant defenses (Bos *et al*., 2010). GroEL, a protein found in the watery saliva of aphids, originating from *Buchnera aphidicola* also seem to induce plant defense response (van Bel & Will, 2016). Similarly Angiotensin-converting enzyme (ACE) 1 and 2 identified in the saliva of pea aphid is essential for its feeding and survival (Wang *et al*., 2015). Other enzyme encoding genes include but not limited to MIF1, Armet, ACYPI39568 (a cysteine-rich protein) and glutathione *S*-transferase 1 (Guo *et al*., 2014; Naessens *et al*., 2015; Wang *et al*., 2015; Zhang *et al*., 2017; Kang *et al*., 2019).

Alkaline phosphatase is widely distributed in insect's alimentary canal, storage tissue, reproductive system and glands (Day, 1948). It was the first salivary enzyme identified in a Russian wheat aphid saliva and was universally found in five different aphids pointing to its significance in aphid penetration and feeding mechanism (Cooper *et al*., 2011). Silencing the expression of salivary sheath protein (*shp*) required for ingestion of phloem sap led to decreased growth, fecundity and survival rate of grain aphid (Abdellatef *et al*., 2015).

Further work on grain aphid revealed several categories of salivary proteins with defined and unknown functions. This ranged from calcium ion binding proteins, odorant-binding proteins, effector inducing or suppressing plant defences to digestive and detoxifying enzymes. Some examples of protein transcripts includes: beta-mannosidase which helps in degrading the cell wall to enhance probing and feeding by aphids, cytochrome oxidases, glutathione S-transferases 1, esterase FE4 and esterase E4 are responsible for degrading toxic secondary metabolite expressed by the plant to modulate defence mechanism upon insect attack, beta-glucosidase an effector that initiates plant defence response, glucose oxidase is known to suppress plant defence, regucalcin, reticulocalbin and calcumenin are

identified calcium ion binding proteins that interfere with the signaling pathway of inducing plant defence response (Zhang *et al*., 2017).

Besides salivary protein which has been studied extensively, other areas responsible for aphid survival such as the nervous system, midgut and fatty body cells can be targeted by RNAi. Lipids and Fatty acids are crucial in the biology of insects. The common forms of fatty acids that are found and can be synthesised by all insects include palmitic, stearic and oleic acids (Stanley-Samuelson *et al*., 1988).

The composition of fatty acids influences the functional level and physiological status of the insect. The diet, environmental conditions and temperature of the insect influence their fatty acid profile (Stanley-Samuelson *et al*., 1988). It helps to synthesize relevant proteins such as lipophorins for the lipid transport or vitellogenins for egg maturation (Arrese & Soulages, 2010). There are different groups of fatty cells that are essential for the survival of an insect. One of such type of fatty cells found in insects such as aphids is *mycetocyte a*lso referred to as a bacteriocyte. It is specialized in storing energy as fat for the development and survival of the insect. Proline is an amino acid found in the haemolymph of aphid and it is necessary during flight for most insects. Proline is released into the haemolymph following its synthesis in the fat body from acetyl-CoA and alanine.

The importance of *mycetocyte* was further confirmed by Douglas in 1998. *Mycetocyte* is a form of fatty body that contains endosymbiotic bacteria like *Buchnera* species. These provide essential and nonessential amino acids such as proline and other chemicals to their host and are maternally transmitted via the aphid ovary (Douglas, 1998). It was stated that some aphids that do not possess these bacteria have retarded growth and produce few or no offspring. In effect, *Buchnera* is highly dependent on their host for survival and proline is continuously provided from the host to the bacteria. Also, *Buchnera* does not exist without the aphid, thus pointing to the fact that there is a nutritional basis for the association. In the article by Douglas, (1998) it was clear that the synthesis of the fatty acid is done by the aphid itself and not the bacteria.

Flight activity largely depends on the metabolism of lipids. Fatty acids are bound by the intracellular fatty acid-binding protein (FABP) which increases its solubility (Haunerland, 1997). This shows that fatty body cells are crucial to the aphid life cycle regardless of the associated microorganism. Alteration to, or shut down of the biochemical pathway of fatty acid production may reduce its fecundity or impact its mortality. Silencing of 3-Ketoacyl-CoA thiolase, Phosphoenolpyruvate carboxykinase and glycogen phosphorylase-like isoform 2 genes via RNAi impacted wing development in a citrus aphid (Shang *et al*., 2016). Loss of function of abnormal wing discs (*awd*) gene caused lethality in *Drosophila* (Yang *et al*., 2014). This explains the genetic basis of the significance of lipids and fatty acids in mobility and dispersal of aphids. In addition, oxidation of fatty acids in plants induce changes in lipid composition and could modulate resistance or susceptibility to biological or environmental stresses.

Fatty acid desaturase 7 (FAD7) is a common naturally occurring desaturase in plants. An alteration of the function of FAD7 in *spr2* mutant tomato conferred resistance to potato aphid. A mutation in the same gene in *Arabidopsis thaliana* also conferred resistance to green peach aphid (Li *et al*., 2018). Oxidation of fatty acids such as oxylipins is responsible for the resistance of wheat variety, Tugela DN wheat to the Russian wheat aphid (Berner & Van Der Westhuizen, 2015). However, Nalam *et al*., (2012) identified that LOX5-synthesised oxylipins increased infestation of green peach aphids on *Arabidopsis* foliage.

Acetylcholinesterase (AChE) is another key enzyme that has been identified in aphids. It belongs to carboxylesterase group of enzymes and it is found mainly at the neuromuscular junctions and in cholinergic synapses. It takes part in the breakdown of acetylcholine and some other choline esters that function as neurotransmitters (Dvir *et al*., 2010). Inhibition of AChE can either be reversible or irreversible. Irreversible inhibition of AChE leads to long term accumulation of acetylcholine and in turn, may lead to paralysis, stunting or death. The effect of inhibiting *AChE* in aphids was brought to the limelight in the work of

Xiao *et al*., (2014). The study was based on understanding the effect of pirimicarb (carbamate), a commonly used insecticide for control of wheat aphids. Besides the mortality caused by the insecticide (pirimicarb) on the wheat aphids; low lethal concentration of pirimicarb resulted in reduced activity of *AChE* in wheat aphids. Depletion of orthologous or paralogous *AChE* was observed in the green peach aphid, cotton bollworm and German cockroach (Revuelta *et al*., 2009; Guo *et al*., 2014; Saini *et al*., 2018). Furthermore, RNAi was successfully used to silence the expression of acetylcholinesterase (Mp*AChE*2) in green peach aphid (Guo *et al*., 2014), midgut genes (*NlHT1*, *Nlcar* and *Nltry*) in *Nilaparvata lugens* (Zha *et al*., 2011) as well as lipid and glycogen genes in brown citrus aphid (Shang *et al*., 2016).

Flavonoids and polyphenols are some of the major phytochemicals found in plants which play a significant role in attracting or deterring insects from plants (Mierziak *et al*., 2014). Quercetin an endogenous flavonoid prominent in resistant lines of cowpea (*Vigna unguiculata* L. Walp.) has inhibitory potential to aphid's reproduction rate, hence, contributing to the defence mechanism against cowpea aphids (Lattanzio *et al*., 2000). Goławska *et al*., (2013) also confirmed that naringenin and quercetin (flavonoids) hindered aphid probing and feeding. Phytochemicals such as terpenoids, nitrogen-containing alkaloids and sulphur-containing compounds have been reported to have the potentials of providing plant defence during plant-insect interactions (Harrewijn *et al*., 2000; Adhikari *et al*., 2012; Hagenbucher *et al*., 2014; Kozłowski *et al*., 2016). Tannins produce a bitter taste that prevents insects from feeding. It also binds metal ions and induces lesions in the midgut of insects (Barbehenn & Constabel, 2011).

Despite several strategies by plants to protect themselves, insects have also devised mechanisms to sidestep or sequester secondary metabolites produced by plants (War *et al*., 2012). Faba bean contains L-DOPA, a toxic compound to other types of aphids. However, *A. pisum* was able to sequester this compound and use it as a protection against UVA radiation (Zhang *et al*., 2016).

Several genes signalling biochemical reactions could circumvent insect infestation and plant defence mechanism. Laccase 1 (Lac 1), a type of polyphenol is essential for the survival of grain aphid by taking an active part in its iron metabolism and immunity responses. Silencing Lac 1 by RNAi reduced the survival rate of grain aphid (Zhang *et al*., 2018).

Furthermore, efficacy of dsRNA has also been linked to the mode of uptake of dsRNA into insect pest. This could be through injection, ingestion or a recently modified topical application of dsRNA using nanoparticle technology to form a stable and sustained dsRNA delivery to Green peach aphid (Basnet & Kamble, 2018; Knorr *et al*., 2018; Mitter *et al*., 2017; Worrall *et al*., 2019). This new approach enabled the inhibition of Bean common mosaic virus (BCMV) transmission by aphids. Table 2.2 provides a summary of some examples of RNAi applications in aphid control.

In view of the efficient potential targets for RNAi as stated in scientific literature, this research will focus on using the approach as a strategy to control the spread and effect of banana aphids on West Africa preferred plantain cultivars: Agbagba, Obino l'Ewai and Orishele.

CHAPTER THREE

GENERAL MATERIALS AND METHODS

3.1 Source of Plant Material

The study was carried out at the Plant Transformation laboratory of the International Institute of Tropical Agriculture (IITA), hosted by Biosciences Eastern and Central Africa (BecA) hub, ILRI, Nairobi, Kenya. Three farmers preferred cultivars of plantain (AAB) were obtained from IITA Genetic Resources Centre of the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. These varieties are; Agbagba, Obino l'Ewai and Orishele. Plantain cultivars were indexed and certified as free of quarantine important viruses including BBTD by Germplasm Health Unit, IITA-Nigeria. The clean *in vitro* cultures of plantain cultivars were then imported to IITA-Kenya.

3.2 Preparation of Stock Reagents and Culture Media

Stock components were weighed using an analytical balance and transferred quantitatively and made up to mark in a volumetric flask (Table 3.1).

Table 3.1: Stock solutions for various culture media

Chemical	Quantity (mg)	Final volume (ml)	Solvent	Final concentration	Storage
6-Benzylaminopurine (BAP)	100	100	NaOH	1 mg/ml	4 °C
2, 4-Dichlorophenoxyacetic acid (2, 4-D)	100	100	Ethanol	1 mg/ml	4 °C
Trans-zeatin	100	100	NaOH	1 mg/ml	4 °C
Naphthalene acetic acid (NAA)	100	100	NaOH	1 mg/ml	4 °C
Indole butyric acid (IBA)	100	100	NaOH	1 mg/ml	4 °C
Ascorbic acid	1000	100	Water	10 mg/ml	4 °C
NaOH	4000	100	Water	1 M	RT
Acetosyringone*	784.8	10	DMSO	400 mM	-20 °C
Indole acetic acid (IAA)	100	100	NaOH	1 mg/ml	4 °C
Fluorescein diacetate (FDA)	2	2	Acetone	1 mg/ml	-20 °C

*, filter-sterilized using 0.2 µm filter

Media constituents were measured, transferred quantitatively and sterilized by autoclaving for 15 min, at 121 °C and 15 psi (Table 3.2).

Table 3.2: Plant media composition and quantity

Component (/L)	ZZ1	ZZ2	ZZ3	PM2	PM1	RM	P4	RD1	MA4	MA3
Macro (10×) (ml)	_	50	_	_	_	_	_	50	100	_
Micro (100×) (ml)	_	10	_	_	_	_	_	10	10	_
Iron (100×) (ml)	_	10	_	_	_	_	_	10	10	_
MS vitamins (200×) (ml)	_	5	_	_	_	_	_	5	_	5
Moral vitamins (200×) (ml)	_	_	_	_	_	_	_	_	5	_
SH Salt premix (g)	_	_	_	_	_	_	_	_	_	3.2
Gamborg B5 medium (g)	_	_	3.6					_	_	
MS basal salt with vitamins (g)	4.4	_	_	4.4	4.4	4.4	4.4	_	_	
Ascorbic acid (mg)	10	10	10	10	10	10	10	10	_	60
2,4 D (mg)	1	1	1	_	_	_	_	_	_	
Zeatin (mg)	0.219	0.219	0.219	_	_	_	_	_	_	0.1[ɬ]
IAA (mg)	_	_	_	0.180	0.180		0.180	_	2	_
IBA (mg)	_	_	_	_	_	_	1	_		_
BAP (mg)	_	_	_	4	2.5		22	_	0.5	_
Glutamine (mg)	_	_	_	_	_	_	_	_	_	100
Malt extract (mg)	_	_	_	_	_	_	_	_	_	100
Biotin (mg)	_	_	_	_	_	_	_	_	_	1
Proline (mg)	_	_	_	_	_	_	_	_	_	230
Citric acid (mg)	_	_	_	_	_	_	_	_	_	60
L-cysteine (mg)	_	_	_	_	_	_	_	_	_	400
NAA (mg)	_	_	_	_	_	_	_	_	_	0.2
2ip (mg)	_	_	_	_	_	_	_	_	_	0.2
Kinetin (mg)	_	_	_	_	_	_	_	_	_	0.2
Lactose (g)		_	_	_	_	_	_	_	_	10
Sucrose (g)	30	30	30	30	30	30	30	30	30	45
Gelrite* (g)	3	3	3	3	3	3	3	3	3	3
pH	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8

*, Omitted for liquid culture media; [ɬ] , filter-sterilized using 0.2 µm filter and added to the autoclaved medium; ZZ1: full strength MS medium supplemented with 1 mg/l 2,4-D and 0.219 mg/l zeatin, ZZ2: half-strength MS medium supplemented with 1 mg/l 2,4-D and 0.219 mg/l

zeatin, ZZ3: Gamborg B5 medium supplemented with 1 mg/l 2,4-D and 0.219 mg/l zeatin, (ZZ1, ZZ2 and ZZ3 are used for callus induction when gelrite is added to the medium and are used to initiate and maintain embryogenic cell suspension without the addition of gelrite); PM2: proliferation medium supplemented with 4 mg/l BAP; PM1: proliferation medium supplemented with 2.5 mg/l BAP; RM: rooting medium; P4: MS media supplemented with 22 mg/l BAP for inducing scalps with tiny meristems; RDI: embryo maturation medium; MA3: embryo development medium; MA4: embryo germination medium; MS: Murashige and Skoog; SH: Schenk and Hildebrandt; IAA: Indole-3-acetic acid; BAP: 6-Benzylaminopurine; NAA: Naphthalene acetic acid; 2ip: 2-isopentenyl adenine; IBA: Indole-3-butyric acid; 2,4-D: 2,4-Dichlorophenoxyacetic acid.

3.3 Initiation of Plantain Cultivars via Meristem Culture

Plantain cultivars were introduced *in vitro* using meristem culture procedure as described by Gueye *et al*., (2012). Screening for endophytic microbes was done using a protocol as described by Strosse *et al*. (2003).

3.4 Generation of Transgenic Plantain through *Agrobacterium*-Mediated Transformation of Embryogenic Cell Suspension

Callus induction and the embryogenic cell suspension were initiated and generated using modified Tripathi *et al*., (2012) protocol. Modification was in the use of full-strength MS medium (ZZ1), half-strength MS-medium (ZZ2) and Gamborg B5 medium (ZZ3) (Table 3.2). The cell suspensions were transformed using *Agrobacterium tumefaciens* using a modified protocol by Tripathi *et al*., (2015) (Fig 3.1). Modification was in the use of varied concentration of acetosyringone and in the duration of co-cultivation.

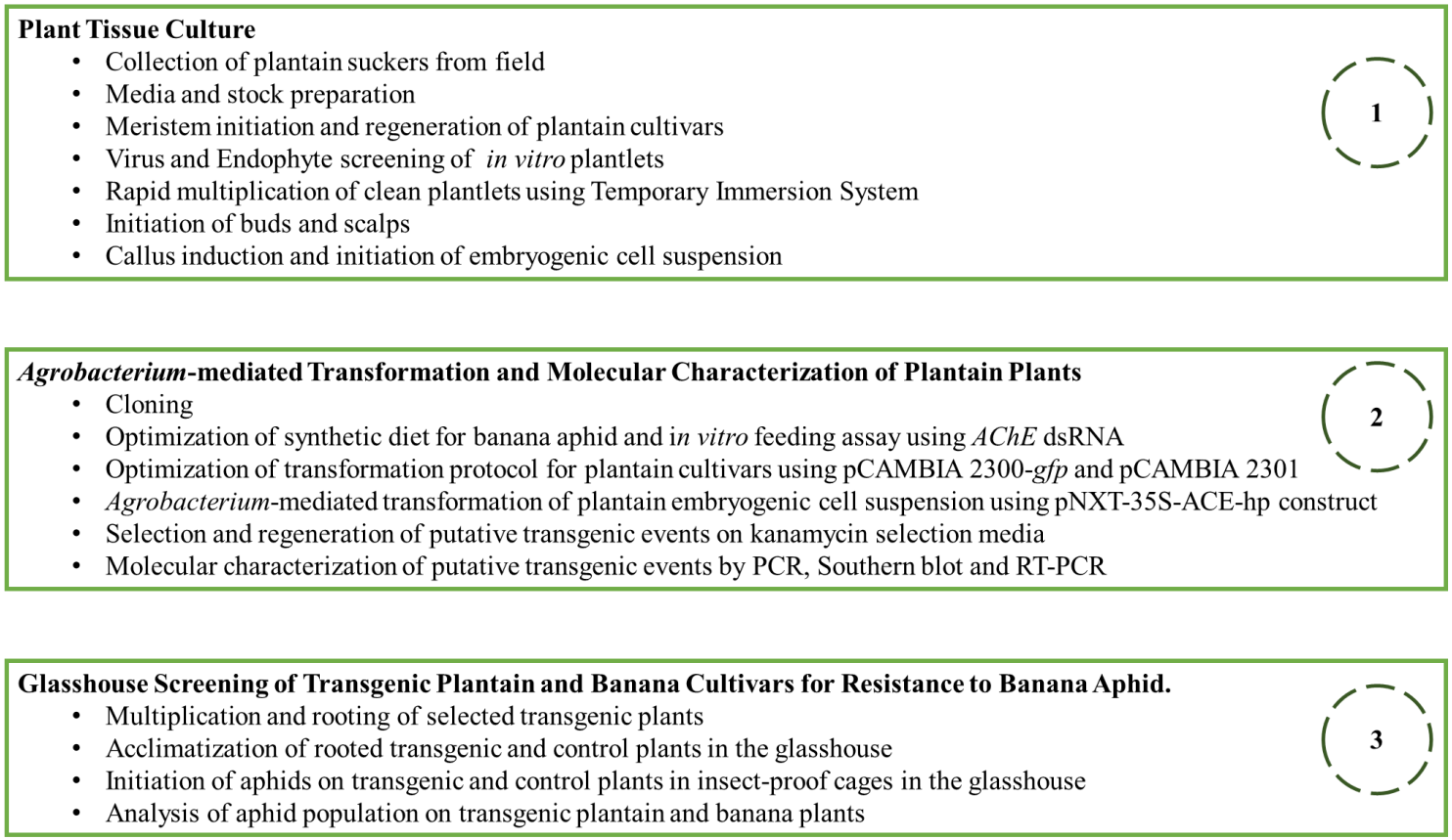


Figure 3.1: Flow chart indicating the general methodology for initiation of plantain cultivars via meristem culture, generating embryogenic cell suspension, *Agrobacterium*-mediated transformation, molecular characterization of putative transgenic events and glasshouse screening of transgenic events.

3.5 Molecular Characterization of Transgenic Plantain Plants

Following the confirmation of the effectiveness of the RNAi construct in *in vitro* feeding assay, plantain and banana cell suspensions were transformed using the RNAi construct harbouring *AChE* gene (pNXT-35S-ACE-hp). The presence and integration of the transgene in plantain and banana cultivars were confirmed by PCR and Southern blot analysis, respectively (Tripathi *et al.,* 2012) and expression was checked by reverse transcription-polymerase chain reaction (RT-PCR).

3.6 Glasshouse Analysis of Transgenic Plantains for Resistance to Aphids

Transgenic events regenerated were acclimatized in a confined glasshouse where they were challenged with non-viruliferous aphids. The population of aphids were counted and compared with the control plants.

3.7 Data Analysis

The data on shoots, embryogenic callus, phytochemical analysis, *in vitro* feeding and screening of transgenic plants were subjected to Excel computer program to generate bar charts and generalized linear model (PROC GLM and PROC GENMOD) in Statistical Analysis System (SAS V9.3) to obtain the variance components. The least significant mean (LSMEANS), least significant difference (LSD) or Duncan's multiple range test (DMRT) was used to compare the means across different cultivars. The comprehensive data analysis for each experiment is described in detail in their respective chapters.

CHAPTER FOUR

DEVELOPMENT OF EMBRYOGENIC CELL SUSPENSION OF PLANTAIN USING FARMER PREFERRED CULTIVARS FROM WEST AFRICA

4.1 Introduction

Somatic embryogenesis (SE) is a reliable tissue culture tool that is essential for plant genetic modification and trait improvement, mass propagation of plant stock, production of synthetic seeds and a preeminent step for generating cell suspension (Nic-Can *et al*., 2015). The process involves regenerating a plant from a somatic cell or somatic embryo. This remodelling process of the somatic embryos can either occur naturally under certain environmental conditions as in the case of Kalanchoë or artificially induced via the use of synthetic plant growth regulators (PGR) (Garcês & Sinha, 2009; Guan *et al*., 2016).

Artificially induced SE could be achieved via direct regeneration to whole plant (organogenesis) or indirect process through the initiation of undifferentiated cells known as callus. The callus undergoes several restructuring to generate embryos and subsequent generation of a whole plant (Méndez-hernández *et al.*, 2019). The embryogenic potential of a plant/explant is highly influenced by several endogenous and exogenous factors such as age and physiology of explant, the stoichiometry between endogenous and exogenous PGR, type of PGR, quantity and ratio of cytokinin and auxin, temperature, pH, phytochemical/secondary metabolites, heavy metal ions, culture media, gelling agent, as well as other cellular and molecular programs such as methylation and transcriptional factors (Zimmerman, 1993; Teng *et al*., 1994; Filonova *et al*., 2000; Pasternak *et al*., 2002; Santos & Fevereiro, 2002; Leljak-Levanic *et al*., 2004; Zheng *et al*., 2013; Smertenko & Bozhkov, 2014; De-la-peña *et al*., 2015; Bahmankar *et al*., 2017; Magnani *et al*., 2017; Méndez-hernández & Ledezma-rodríguez, 2019).

Clonally propagated crops like banana and plantain produce several phytochemicals that occur at different explant and propagation stages. Most of these phytochemicals do not

only influence the developmental stages of SE features and regeneration into complete plantlets, but also have applicable in the pharmaceutical industries and the production of bio-pesticides (Tyagi *et al*., 2010; Imam & Akter, 2011; Upadhyay, 2011; Wadood, 2014). Phenolic compounds, flavonoids, tannins and antioxidants are some of the majorly produced phytochemicals in *Musa* spp. (Onyema *et al*., 2016). Some of these compounds could promote somatic embryogenesis, somatic embryo development and germination of embryos (Lorenzo *et al*., 2001; Kouadio *et al*., 2007; Reis *et al*., 2008). A positive correlation was observed with increased antioxidant activity in promoting regeneration of *Cucumis anguria* L. (Thiruvengadam & Chung, 2015).

The type and concentration of phytochemicals needed to confer positive response on a plant tissue are difficult to determine because the influences that could trigger the production of phytochemicals vary from plant to plant and is also genotype-dependent. Besides, metabolic products of secondary metabolites may inhibit, support or have non-lethal effects in establishing cultures *in vitro* and somatic embryogenesis (Anthony *et al*., 2004; Wang *et al*., 2016). Availability of a broad spectrum of these chemicals and their identification could serve as a basis for crop genetic improvement (Ibarra-Estrada *et al*., 2016), targeted strategy for handling and scaling-up plant tissue culture procedures for optimum output, and an understanding of insect-pest interaction for improved crop health.

Somatic embryogenesis also provides the basis of protoplast isolation for several economically important crops. The success of plant cell suspension is dependent on a wide array of factors such as explant type, cultivar, physiological age of plant, culture conditions, and culture media. Besides all of these influencing factors, plant cell suspension procedure is cultivar dependent (Strosse *et al*., 2004), therefore, there is a continued need to develop procedures and /or manipulate conditions to suit each cultivar(s) that may not respond favourably to existing protocols. This study focuses on optimising procedures for the development of cell suspension for plantain cultivars such as Agbagba, Obino l'Ewai and Orishele, preferred by West African farmers. Besides, this

study also reports on the quantity of targeted phytochemicals present in these cultivars inclusive of other plantain cultivars, Gonja Manjaya and dessert banana cultivar, Cavendish Williams which are of economic value.

4.2 Materials and Methods

4.2.1 Preparation of Stock Solutions and Culture Media

Each chemical was weighed, transferred quantitatively and dissolved in the respective solvent and topped up to final volume in a volumetric flask and stored until use (Table 3.1). Culture media was prepared, adjusted to pH 5.8 and sterilized at 121 °C, 15 psi for 15 min (Table 3.2). This was then allowed to cool to approximately 45-50 °C prior to dispensing and use. Freshly prepared culture media were used for all procedures.

4.2.2 Plantain Meristem Culture

The suckers and corm were reduced to about 1×1 cm by removing the surrounding leaf sheath in such a way that the meristem area was not damaged. The plantain explants were placed in individual clean, labelled baby food jar and rinsed thoroughly first under running clean water and then under sterilized distilled water to remove any debris or soil particles. To the plantain explants, 70 % of ethanol was sufficiently added to cover the samples. Samples were left under the laminar hood for 5 min with intermittent shaking to ensure adequate contact with the sample. Ethanol was decanted and freshly prepared 20 % sodium hypochlorite laced with few drops of Tween-20 was added to the plantain explant for 10 min with intermittent shaking every 2-3 min. After 10 min, sodium hypochlorite was decanted and 10 % sodium hypochlorite laced with few drops of Tween-20 was added for 20 min with intermittent shaking every 2-3 min. Explants were rinsed with sterilized distilled water until hypochlorite was cleared off. With the aid of a dissecting microscope (Luxeo 4D stereo zoom microscope, 4145000 Rev A, USA), sterile forceps and size 11 sterile surgical blade, the meristem was carefully excised and cultured on proliferation media (PM 1) (Table 3.2) (Fig 4.1). A culture identification number and initiation date were assigned to individual meristem culture and kept in the culture chamber at 26 ± 2

°C, 16/8 hr photoperiod for 4 weeks. Afterwards, explants were subcultured on fresh proliferation medium supplemented with 4 mg/l BAP (PM 2) (Table 3.2) for 3-4 cycles before transferring into rooting medium (RM) (Table 3.2) for 1 month and returned on proliferation medium supplemented with 2.5 mg/l BAP (PM1) (Table 3.2) for another 4-6 cycles before re-initiation of germplasm.

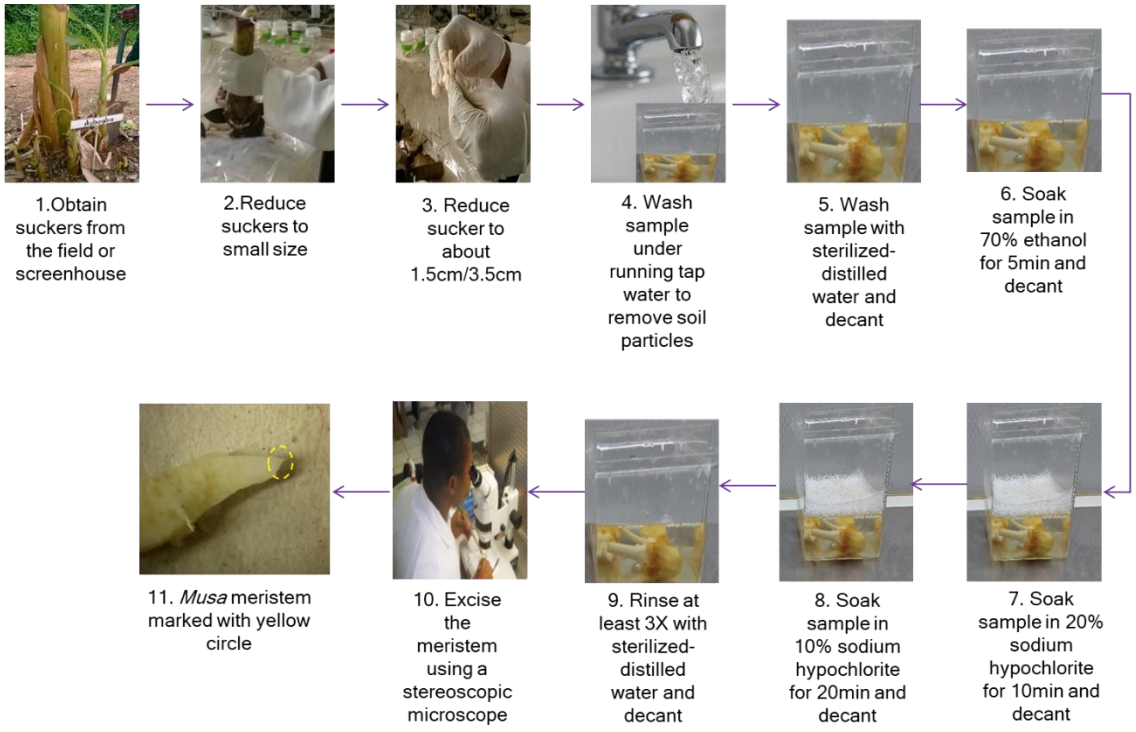


Figure 4.1: A step-wise plantain/banana meristem excision process

4.2.3 Virus Indexing and Screening for Endophytic Bacteria in Plantain Samples

The presence of some endogenous microbes like viruses and bacteria in banana and plantain could hinder downstream experiments and maintenance of embryogenic cell suspension. Therefore, it is essential to screen regenerated *in vitro* plantlets for quarantine important viruses and endophytic bacteria before proceeding with germplasm use and conservation. More so, clean plantlets are essential for generating cell suspension culture. Fresh leaf samples of each line of plantain cultivars were isolated in 1.5 ml eppendorf

tubes. Samples were labelled, placed on ice and sent for virus indexing at the Germplasm Health Unit, IITA. Samples were screened for quarantine important virus of banana including BBTV. Endophyte bacteria screening was done following the procedure described by Strosse *et al*. (2003). Culture medium comprising of 8 g Casein hydrolysate, 10 g sucrose, 4 g yeast extract, 2 g potassium dihydrogen phosphate and 0.15 g magnesium sulphate heptahydrate was dissolved in 1 L of distilled water. The pH of the culture media was adjusted to 6.9 before adding 8 g of agar. The medium was autoclaved for 15 min at 121 °C and dispensed in petri-plates in the laminar flow hood. With the aid of a scalpel, the base of each plantlet was thinly cut or scrapped and cultured on the media. The cultures were incubated for 2-10 days at 24 °C in an incubator. Plant samples with microbial contamination were discarded and the only plant whose explant did not exhibit any form of contamination after 10 days of incubation were assumed to be clean, maintained and used for subsequent experiments.

4.2.4 Multiplication of Plantlets and Induction of Multiple Buds

Clean *in vitro* plantlets were rapidly multiplied using the Temporary Immersion System in RITA bioreactor with immersion time of 15 min every 2 hours (Jekayinoluwa *et al*., 2019) and further multiplied on PM semi-solid medium (Table 3.2). The culture medium was adjusted to pH 5.8 before addition of gelrite (2.5 g/l) and sterilized at 121 °C for 15 min.

Multiple buds were induced by injuring the apical meristem as described by Vuylsteke (1998). This involves cutting through the meristem region of the explant while keeping the base of the plant intact. The dominance of the apical meristem is suppressed to induce the development of axillary buds. After 10 to 21 days of explant in culture, axillary buds were observed around the base of the plantlet. Individual buds were excised with the aid of sterile forceps under the laminar flow hood and cultured on P4 medium (Table 3.2).

4.2.5 Determination of Proliferation Potential of Plantain Cultivars

Suckers were obtained, sterilized and meristems isolated as described in Section 4.2.2.

Each meristem explant was cultured on PM2 media and sub-cultured every 4 weeks. The number of shoots generated per meristem per cultivar (while repressing apical dominance by injuring the apical meristem to induce axillary shoots) were recorded for 3 subculture cycles.

4.2.6 Plantain Scalp Induction

The P4 medium containing 22 mg/l of 6-Benzylaminopurine (BAP) was used to induce a conglomerate of tiny multiple meristems called scalp. Individual bud excised from *in vitro* plantain plantlet was cultured on P4 medium and subcultured every 4 weeks. During subculture, the base of the explant was carefully cleaned to enable absorption of nutrient by explant. The bud subculture underwent 2 to 9 cycles of monthly subcultures to generate fine scalp comprising of several tiny meristems with minimum leaves. The number of cycles to obtain fine scalp depends on a couple of factors such as cultivar type, the viability of buds and culture conditions.

4.2.7 Initiation of Plantain Friable Embryogenic Callus

Somatic embryogenesis (SE) was initiated by an indirect method through callus induction. Callus was induced using a modified protocol of Tripathi *et al.* (2012). The modification involved the use of full-strength MS (ZZ1), half-strength MS (ZZ2) and Gamborg B5 (ZZ3) media. Scalps were thinly excised using a pair of forceps and size 11 scapel to achieve a thickness of about 1.0 - 2.0 mm and placed on the callus induction media; ZZ1, ZZ2, ZZ3, respectively (Table 3.2) with the cut part having contact with the media. The explant was not submerged into the media in order to prevent the tiny meristem on the surface of the scalp from touching the media. Five explants (scalps) were cultured in a 60 mm petri plate. The treatment was optimized for the plantain cultivars and was routinely used in the establishment of friable embryogenic callus (FEC).

A total of 1919, 749 and 1220 scalps for Agbagba, Obino l'Ewai and Orishele, respectively were cultured, labelled, sealed with cling film and kept in the dark at 26 ± 2 °C for 3 – 5 months without refreshing the media or subculturing. The number of friable

embryogenic callus, the yellowish non-embryogenic callus (pro-embryo) and dead explants were recorded.

The effect of ascorbic acid on development of FEC from scalps of plantain was tested by culturing the scalp explants of Agbagba at subculture number 6 and 7 (S6 and S7) on ZZ3 medium supplemented with 0, 10, and 20 mg/l ascorbic acid at 26 ± 2 °C in dark. A total of 80 scalps were used for each treatment at S7, while at S6, a total of 85, 90 and 85 scalps were subjected to 0, 10, and 20 mg/l ascorbic acid, respectively.

4.2.8 Initiation and Maintenance of Plantain Cell Suspension

Friable embryogenic callus (FEC) for each treatment was first observed under the microscope (Luxeo 4D Stereo Zoom Microscope, 4145000 Rev A, USA) to determine the quality of embryogenic callus (EC). Friable embryos were isolated and cultured in 50 ml conical flask containing about 5-10 ml of liquid ZZ1, ZZ2 and ZZ3, respectively. All cultures were assigned an identification number, edges of the conical flask and aluminium foil (serving as a seal) were thoroughly flamed, sealed with cling film and kept on a rotary shaker at 90 rpm continuously at 26 ± 2 °C and in the dark. Culture media was refreshed every 14 days for about 1 month, afterwards, media was refreshed every 10-12 days. During the process of refreshing the media, about a quarter (¼) of the old media was left and topped up with the fresh media. Proliferating cells were sub-cultured into new flasks and plated on embryo development media (MA3). Embryogenic cell suspension (ECS) was routinely initiated and maintained for about 10-12 months in culture. Initiation of EC in liquid culture was done on a continuous basis to ensure availability of fresh ECS.

4.2.9 Viability testing of ECS

Viability of ECS was determined by the cell growth curve, regeneration efficiency and staining with fluorescein diacetate (FDA).

4.2.9.1 Cell Suspension Growth Curve

Cell suspensions were sieved using a 1000 µm sieve in order to obtain homogenous cells. In order to determine the proliferation rate of the cell suspension generated, the growth curve was assessed. Three replicates of 0.2 ml settled cell volume (SCV) of each cell line was used as a starting material and the SCV of actively growing cell suspension was measured to determine the growth for 12 days at intervals of two days (0, 2, 4, 6, 8, 10, 12).

4.2.9.2 Staining of Plantain Embryogenic Cells Using Fluorescein Diacetate Dye

Suspension cells were sieved using a 40 µm filter in order to obtain single cells. One millilitre of the cell was aliquoted in a 1.5 ml tube and centrifuged (Eppendorf centrifuge 5810 R, Germany) at 1500 rpm for 5 min. The supernatant was discarded and the pellet re-suspended in 100 µl Phosphate-buffered saline (PBS). An aliquot of 30 µl was dispensed in new 1.5 eppendorf tube. Fluorescein diacetate (FDA) staining solution (2 µl) was added to the cell, mixed by pipetting and allowed to incubate at room temperature for 15 min. This was followed with washing with PBS buffer and re-suspension in 200 µl PBS and acquired using flow cytometry to measure the number of cells that fluoresced a green colour. The experiment was done in triplicate. The data was analysed using Flowjo version 5.4 + and SAS version 9.4.

4.2.9.3 Regeneration of Complete Plantlets from Embryogenic Cell Suspension

Suspension cells for each cultivar were sieved through a 1000 µm sterile metal sieve. About 0.5 ml settled cell volume (SCV) of each cell line of Agbagba, Orishele and Obino l'Ewai were measured in sterile 15 ml falcon tube and diluted with liquid callus induction (ZZ) medium to 10 ml volume. With the aid of a pipette, homogenized cells were dispensed onto 5 sterile mesh placed on a sterile soft tissue to absorb the liquid. The mesh containing the plant cells were placed on embryo development media (MA3) in petri dishes (Table 3.2). The petri dishes were sealed, labelled and kept in the dark at 26 ± 2 °C. The culture medium was refreshed every 2 weeks for 8 weeks, and transferred onto

embryo maturation medium (RD1) (Table 3.2) for proper development. After 1 month on RD1 media, matured embryos were cultured on embryo germination medium (MA4) (Table 3.2) to enable germination of embryos into small plantlets. The embryo regeneration capacity was determined by the number of plantlets per ml of SCV. Plantlets were cultured on PM2 media (Table 3.2) for full regeneration to the whole plantlet. Viability of embryogenic cell suspensions was routinely checked by plating for regeneration.

4.2.10 Determination of Phytochemical Content of Plantain and Banana Cultivars

Five cultivars of plantain and banana (Orishele, Obino l'Ewai, Agbagba, Gonja Manjaya and Cavendish Williams) were obtained from the Plant Transformation Laboratory and research field of the International Institute of Tropical Agriculture (IITA), Nairobi Kenya (Table 5) located on Latitude: -1.270224, longitude: 36.723712, and altitude: 1823.000000.

Table 4.1: Accession number, genome and cultivar names of plantain and banana

Accession number	Cultivar	Genome
TMp 59	Agbagba	AAB
TMp 28	Obino l'Ewai	AAB
TMp 70	Orishele	AAB
TMp 54	Gonja Manjaya	AAB
TMb 20	Cavendish Williams	AAA

4.2.10.1 *In vitro* Culture of Plantain and Banana Cultivars

Shoot tips of *Musa* spp. (Table 4.1) were initially cultured on proliferation medium and kept in a growth chamber at 26 ± 2.0 °C, light intensity of 38 µmol /m^2s^{-1} and 12-hour photoperiod. Plantlets were subcultured every 4 weeks and rooted in hormone-free MS media.

4.2.10.2 *In vitro* Plantain and Banana Sample Preparation

Composite samples were obtained from 15 representatives of *in vitro* plants per cultivar obtained from ascorbic-acid free culture media and field explants (root, young leaf and pseudostem) from 3 representatives of field grown plants of banana and plantain cultivars. The samples were collected separately in a perforated, labelled sample bags, kept overnight in -80 °C and freeze-dried (Christ Alpha 2-4LSCplus, Germany) for 72 hours. Dried samples were pulverized to a fine powder with the aid of a grinder (Waring commercial blender 8010ES, model HGBTWTS3, USA) in a fume hood. Three replicate samples per explant were subjected to the extraction process depending on the phytochemical to be determined.

4.2.10.3 Preparation of Stock Reagents for Phytochemical Analysis

All reagents used were of analytical grade. Reagents were weighed using an analytical weighing balance (Mettler Toledo classic plus. SNR1123271237, Switzerland) and transferred quantitatively into appropriate volumetric flasks and made up to mark with appropriate solvent. Freshly prepared stock solutions were used for this study and kept away from white light (Table 4.2).

Table 4.2: Summary of stock solution preparation

Component	Concentration	Solvent	Quantity of solute	Quantity of solvent	Final volume	Storage
Methanol	80 %	DW	800 ml	200 ml	1000 ml	RT
Folin-Ciocalteu phenol	0.2 N	DW	10 ml	90 ml	100 ml	-18 °C
Sodium Carbonate	7 %	DW	3.5g	400 ml	500 ml	RT
Sodium Nitrite	5 %	DW	5g	100 ml	100 ml	RT
Aluminium chloride	10 %	DW	10 g	100 ml	100 ml	RT
Sodium hydroxide	2 M	DW	80 g	900 ml	1000 ml	RT
Gallic acid (≥ 99.0 %)	1 mg/ml	Methanol	0.100 g	90 ml	100 ml	-18 °C
Catechin hydrate (≥ 98.0 %)	1 mg/ml	Methanol	0.101 g	90 ml	100 ml	-18 °C
Trolox	1 mg/ml	Methanol	0.103 g	90 ml	100 ml	-18 °C
2,2-diphenyl-1-picrylhydrazyl (DPPH)	0.20 %	Ethanol	0.2 g	90 ml	100 ml	-18 °C

*RT: room temperature; DW: distilled water

4.2.10.4 Determination of Total Tannin

The tannin content in banana and plantain cultivars was determined using a modified Folin-Denis protocol (Price & Butler, 1977; Terrill *et al*., 1992; Saxena *et al*., 2013). The modification was in the reaction volume used to fit the microtiter reader. About 50 mg of each sample was weighed in triplicate in sterile 50 ml falcon tubes and labelled appropriately. A volume of 7.5 ml distilled water was added to each sample and heated gently at 85 °C in a water bath for 1 hour. The volume of each sample was made up to 10 ml with distilled water. An aliquot of 1 ml extract was placed in a clean 1.5 ml Eppendorf tube and centrifuged (Eppendorf Centrifuge 5424 R, Germany) for 10 min at 14, 000 rpm. Using a microtiter plate as outlined in Table 4.3, samples, standards and Folin-Denis solution were pipetted in respective wells and mixed gently by priming (Table 4.3 & 4.4). After 5 min, 7 % Na_2CO_3 was added to the whole mixture and mixed gently by priming. The plate was covered with aluminium foil and the reaction allowed to incubate at room temperature for 30 min. Using a microtiter plate spectrophotometer reader (Biotek Synergy HT Gen5 1.11, USA), the absorbance of the reactions was read at 700 nm.

Table 4.3: Tannin content determination

Sample ID	Concentration of Tannic acid calibration standards (µg)/ml	Volume of calibration standards (µl)	Volume of Folin-Denis solution, 20 % (µl)	Volume of Na_2CO_3, 7 % (µl)
C-001(Methanol)	0	50	50	100
C-002	20	50	50	100
C-003	40	50	50	100
C-004	60	50	50	100
C-005	80	50	50	100
C-006	100	50	50	100
Sample		50	50	100

Table 4.4: Tannic acid, gallic acid and catechin calibration standards

S/N	Standards (µg/ml)	Volume of standards (µl)	Final volume (µl)
1	0	0	25
2	10	0.25	25
3	20	0.5	25
4	40	1	25
5	60	1.5	25
6	80	2	25
7	100	2.5	25

4.2.10.5 Determination of Total Flavonoid, Phenol and Antioxidant Activity

Sample extraction was done by addition of 10 ml of 80 % methanol to 50 mg of pulverized sample in a 50 ml falcon tube and placed on a mechanical shaker (New Brunswick Scientific, EDISON, N.J., USA) for 24 hours at 25 °C. Afterwards, the sample mixture was centrifuged (Eppendorf Centrifuge 5810 R, Germany) at 4000 rpm for 10 min and supernatant aliquoted to determine total flavonoids, phenol and antioxidant activity in plant samples. Total phenol content was determined using a modified Folin-Ciocalteu protocol (Singleton and Rossi, 1965). The modification was in the reduction of reaction volume to fit the microtitre plate reader. Reagents were added sequentially as shown in Table 4.5 and mixed by priming. The reaction was covered with aluminium foil and allowed to incubate for 90 min at room temperature. Absorbance was read at 725 nm in a microtiter plate spectrophotometer reader (Biotek Synergy HT Gen5 1.11, USA).

Flavonoid content in extracts of *Musa* explants was determined using aluminium chloride colourimetric method (Zhishen *et al*., 1999). Samples and reagents were pipetted step-wise as outlined in Table 4.6 with 5 min interval after the addition of $NaNO_2$ and mixed by priming. Samples were covered with aluminium foil and allowed to incubate at room temperature for 30 min and reading was done at 510 nm on a microtitre spectrophotometric plate reader (Biotek Synergy HT Gen5 1.11, USA).

The total antioxidant activity of the extracts was determined by assaying the scavenging activity of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) (Table 4.8). Samples were diluted 40 times before use. The reaction mixture was prepared by pipetting components as stated in Table 4.7 in a microtiter plate, incubated for 30 min at room temperature and absorbance read at 515 nm in a spectrophotometer plate reader (Biotek Synergy HT Gen5 1.11, USA).

Table 4.5: Reaction mix for determination of total phenolics

Sample ID	Concentration of Gallic acid calibration standards (µg)/ml	Volume of calibration standards (µl)	Volume of Folin-Ciocalteu Phenol solution, 0.2 N (µl)	Volume of Na_2CO_3, 7 % (µl)
C-000 (Methanol)	0	20	100	80
C-001	10	20	100	80
C-002	20	20	100	80
C-003	40	20	100	80
C-004	60	20	100	80
C-005	80	20	100	80
C-006	100	20	100	80
Sample		20	100	80

Table 4.6: Reaction mix for determination of total flavonoids

Sample ID	Concentration of catechin acid calibration standards (µg)/ml	Volume of calibration standards (µl)	Volume of water (µl)	Volume of 5 % $NaNO_2$ (µl)	Volume of 10 % $AlCl_3$ (µl)	Volume of 2 M NaOH (µl)
C-000 (Methanol)	0	20	80	10	10	80
C-001	10	20	80	10	10	80
C-002	20	20	80	10	10	80
C-003	40	20	80	10	10	80
C-004	60	20	80	10	10	80
C-005	80	20	80	10	10	80
C-006	100	20	80	10	10	80
Sample		20	80	10	10	80

Table 4.7: Reaction mix for the determination of total antioxidant activity

Sample ID	Concentration of Gallic acid calibration standards (µg)/ml	Volume of calibration standards (µl)	Volume of 60 mM DPPH (µl)
C-000 (Methanol)	0	50	50
C-001	5	50	50
C-002	10	50	50
C-003	20	50	50
C-004	30	50	50
C-005	40	50	50
C-006	50	50	50
Sample		50	50

Table 4.8: Trolox calibration standard solution

S/N	Concentration of standard (µg/ml)	Volume of standards (µl)	Final volume (µl)
1	0	0	25
2	5	0.125	25
3	10	0.25	25
4	20	0.5	25
5	30	0.75	25
6	40	1.0	25
7	50	1.25	25

4.3 Results

4.3.1 Determination of Proliferation Potential of Plantain Cultivars

All the three plantain cultivars tested showed the ability to regenerate to the whole plant from meristem cultures. The proliferation potential among the plantain cultivars varied significantly with the type of cultivar ($p < 0.01$) and subculture number (SN) ($p < 0.001$) (Table 4.9). There was no significant variation for the mean number of shoots among the three plantain cultivars at first subculture (S1). However, Orishele had the highest mean shoots value of 2.5 ± 0.7 at S1 (Fig 4.2). At S2 and S3, the mean number of shoots of the three cultivars varied significantly ($p \leq 0.001$) with Agbagba having the highest mean number of shoots of 119.0 ± 42.2 shoots per explant at S3 (Fig 4.2).

Table 4.9: The proliferation potential of plantain cultivars at different sub-culturing stage

Cultivar	Number of Shoots Per Explants at Various Subculturing Stage		
	S1	S2	S3
Agbagba	1.6 ± 0.5^{ns}	$29.4 \pm 8.2^{***}$	$119.0 \pm 42.2^{***}$
Obino l'Ewai	2.0 ± 1.1^{ns}	$20.4 \pm 11.5^{***}$	$49.4 \pm 25.9^{***}$
Orishele	2.5 ± 0.7^{ns}	$25.0 \pm 1.4^{***}$	$79.5 \pm 7.8^{***}$

Note. Data is presented as mean and standard deviation of number of shoots per explant. ns indicates non-significant ($p > 0.05$) difference in number of shoots per explant among different cultivars at subculture S1. ***indicates highly significant ($p \leq 0.001$) difference in number of shoots per explant among different cultivars at subculture S2 and S3.

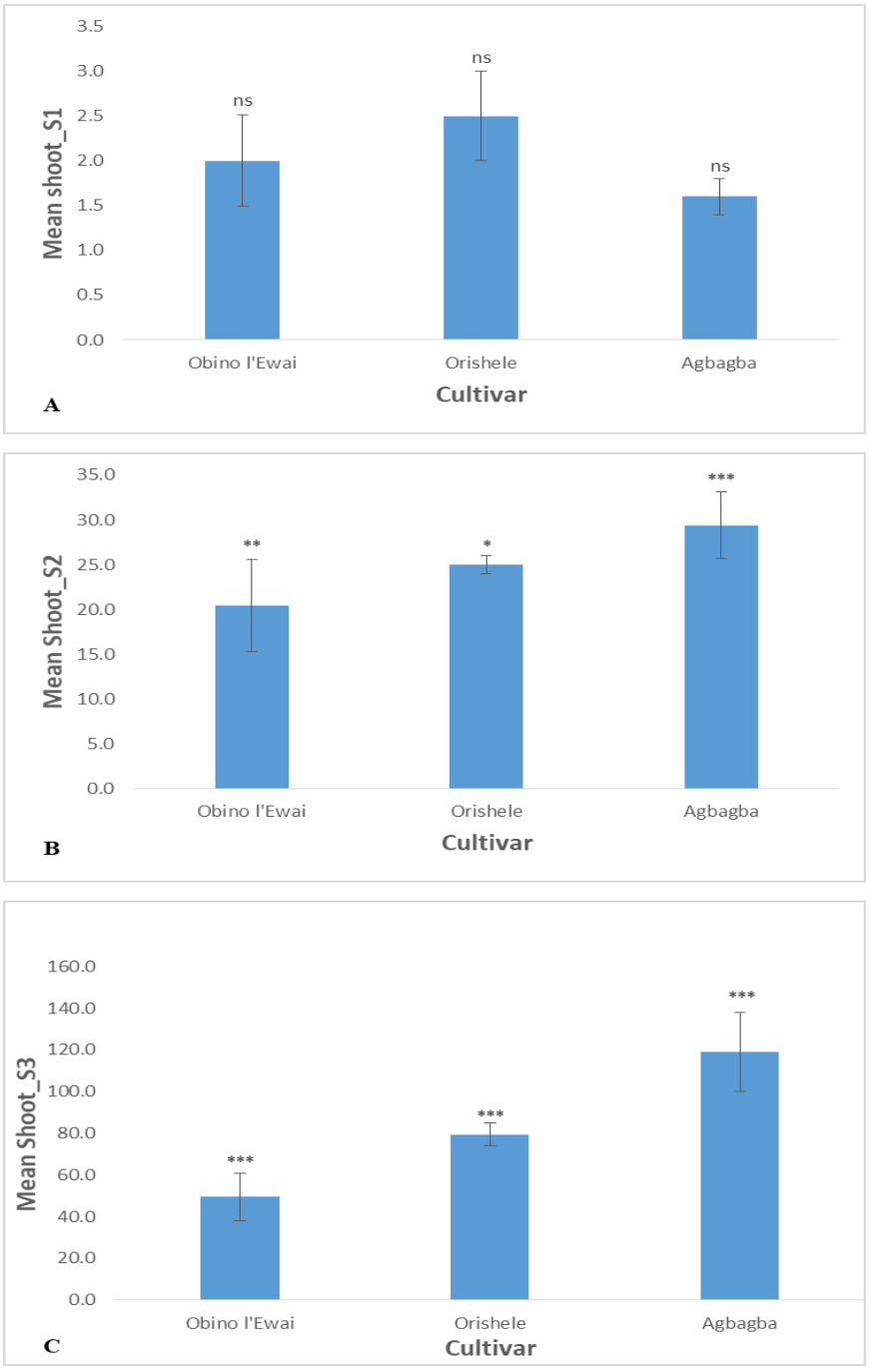


Figure 4.2: Proliferation potential of plantain cultivars *in vitro* as measured by the mean number of shoots at three subculture stages. A: first subculture (S1) B: second subculture (S2) and C: third subculture (S3)

4.3.2 Embryogenic Callus Induction from Scalps of Plantain

Fine scalps with tiny meristems were initiated on embryogenic callus medium at various scalp stages (marked by subculture cycle; S2, S3, S4, S5, S6, S8, and S9) and were subjected to 3 different treatments (ZZ1, ZZ2, ZZ3). Following the initiation of scalps for callus induction, three types of responses were observed across the treatments and cultivars. These include; friable embryogenic callus (FEC), yellowish non-embryogenic callus (NEC) and dead scalps (DS) (Fig 4.3). The number of FEC, NEC and DS was counted and recorded per plate. The response of the scalps to embryogenic development varied significantly ($p \leq 0.001$) across the 3 plantain cultivars, subculture number and treatment used (Table 4.10 a & b).

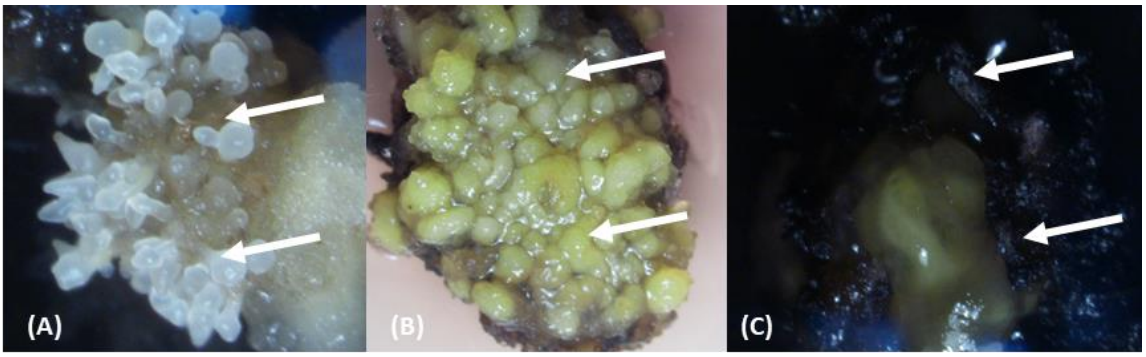


Figure 4.3: Responses of plantain scalps to embryogenic development. A: arrows are indicating friable embryogenic callus (FEC) B: arrows are indicating yellowish non-embryogenic callus (NEC) and C: arrows are indicating dead scalp (DS)

A comparison between the scalp subculture numbers (SSN) showed a significant difference ($p < 0.0001$) between S3 and S4, S4 and S5, and S4 and S6 for FEC development (Table 4.10c). The cultivar influence varied significantly between Agbagba and Obino l'Ewai ($p = 0.05$), Agbagba and Orishele ($p < 0.0001$), and no significant difference was observed between Obino l'Ewai and Orishele with respect to their response to generating FEC (Table 4.10d). Although all the treatments used had the potential of inducing FEC, a significant difference was observed between ZZ1 and ZZ2 ($p = 0.007$),

ZZ1 and ZZ3 ($p < 0.0001$) and no significant difference was observed between ZZ2 and ZZ3 (Table 4.10e)

Table 4.10a: Analysis of variance for embryogenic callus of plantain cultivars

Source of Variation	DF	Chi-Square	Pr > ChiSq
Cultivar	2	31.96	<.0001
SSN	3	65.12	<.0001
TRT	2	17.84	0.0001

Table 4.10b: Analysis of likelihood parameter estimates of plantain cultivars to scalp subculture stage and treatment

Parameter		**DF**	**Estimate**	**Standard Error**	**Likelihood Ratio 95% Confidence Limits**		**Wald Chi-Square**	**Pr > Chi Sq**
Cultivar	Agbagba	1	1.1023	0.2204	0.6875	1.5548	25.02	<.0001
Cultivar	Obino l'Ewai	1	0.5433	0.3031	-0.0598	1.1355	3.21	0.073
Cultivar	Orishele	0	0	0	0	0	.	.
SSN	S3	1	0.271	0.3009	-0.307	0.8799	0.81	0.3677
SSN	S4	1	1.3329	0.2642	0.8398	1.8812	25.46	<.0001
SSN	S5	1	0.0078	0.2966	-0.5609	0.6095	0	0.9791
SSN	S6	0	0	0	0	0	.	.
TRT	ZZ1	1	-0.836	0.2061	-1.25	-0.4399	16.46	<.0001
TRT	ZZ2	1	-0.2689	0.1695	-0.6027	0.0628	2.52	0.1127
TRT	ZZ3	0	0	0	0	0	.	.

Table 4.10c: Multiple comparisons between scalp subculture number of plantain cultivars for FEC development

Differences of SSN Least Squares Means

Adjustment for Multiple Comparisons: Tukey-Kramer

SSN	_SSN	Estimate	Standard Error	z Value	Pr > |z|	Adj P
S3	S4	-1.0619	0.2098	-5.06	<.0001	<.0001
S3	S5	0.2633	0.2493	1.06	0.2909	0.7162
S3	S6	0.271	0.3009	0.9	0.3677	0.8044
S4	S5	1.3251	0.2032	6.52	<.0001	<.0001
S4	S6	1.3329	0.2642	5.05	<.0001	<.0001
S5	S6	0.00776	0.2966	0.03	0.9791	1

Table 4.10d: Multiple comparisons between plantain cultivars for FEC development from scalps

Differences of Cultivar Least Squares Means

Adjustment for Multiple Comparisons: Tukey-Kramer

Cultivar	_Cultivar	Estimate	Standard Error	z Value	Pr > |z|	Adj P
Agbagba	Obino l'Ewai	0.559	0.2449	2.28	0.0225	0.0582
Agbagba	Orishele	1.1023	0.2204	5	<.0001	<.0001
Obino l'Ewai	Orishele	0.5433	0.3031	1.79	0.073	0.172

Table 4.10e: Multiple comparisons between treatments of plantain cultivars for FECs development from scalps

Differences of TRT Least Squares Means Adjustment for Multiple Comparisons: Tukey-Kramer						
TRT	**_TRT**	**Estimate**	**Standard Error**	**z Value**	**Pr > |z|**	**Adj P**
ZZ1	ZZ2	-0.5671	0.2105	-2.69	0.0071	0.0193
ZZ1	ZZ3	-0.836	0.2061	-4.06	<.0001	0.0001
ZZ2	ZZ3	-0.2689	0.1695	-1.59	0.1127	0.2515

A varying percentage value of FEC was observed with the three plantain cultivars. At subculture number S2, S3 and S4, the highest number of FECs were observed with treatment ZZ3 for Agbagba. In addition, at S4 and treatment ZZ3, Agbagba recorded the highest value of FEC (22 %) (Table 4.11a). While for Orishele and Obino l'Ewai, the highest value of FEC was 9 % (with treatment ZZ1 at S4), and 13 % (with treatment ZZ2 at S9) respectively (Table 4.11 b & c).

Table 4.11a: Percentage value of embryogenic development from scalps for Agbagba, plantain cultivar

Cultivar	Treatment	Friable Embryogenic Calli (FEC) (%)	Non-Embryogenic Calli (NEC) (%)	Dead Scalp (DS) (%)
	S2_ZZ1	9 ± 11^{bc}	20 ± 16^{g}	71 ± 20^{ab}
	S2_ZZ2	0 ± 0^{e}	97 ± 8^{a}	3 ± 8^{g}
	S2_ZZ3	15 ± 21^{ab}	25 ± 18^{g}	60 ± 26^{bc}
	S3_ZZ1	2 ± 6^{cde}	50 ± 30^{ef}	48 ± 29^{c}
	S3_ZZ2	5 ± 13^{cde}	90 ± 21^{ab}	5 ± 11^{g}
	S3_ZZ3	8 ± 11^{bcd}	67 ± 22^{cde}	26 ± 23^{def}
	S4_ZZ1	8 ± 15^{bcde}	49 ± 30^{ef}	$42 \pm {}^{32cde}$
Agbagba	S4_ZZ2	15 ± 20^{a}	81 ± 20^{bc}	4 ± 8^{g}
	S4_ZZ3	22 ± 24^{a}	53 ± 33^{ef}	25 ± 24^{ef}
	S5_ZZ1	0 ± 0^{e}	26 ± 31^{g}	74 ± 31^{a}
	S5_ZZ2	7 ± 11^{bcde}	72 ± 26^{cd}	22 ± 27^{f}
	S5_ZZ3	5 ± 9^{cde}	64 ± 30^{cde}	31 ± 32^{def}
	S6_ZZ1	8 ± 14^{bcde}	37 ± 20^{fg}	56 ± 22^{bc}
	S6_ZZ2	2 ± 7^{cde}	80 ± 21^{bc}	18 ± 21^{fg}
	S6_ZZ3	1 ± 5^{de}	55 ± 38^{def}	44 ± 37^{cd}
***p* value**		***	***	***

Note: S: Subculture number, ZZ1: MS-based medium, ZZ2: Half-strength MS-based medium, ZZ3: B5 based medium. Data is presented as percentage mean and standard deviation of friable embryogenic calli (FEC), non-embryogenic calli (NEC) or non-responding explants as dead scalps (DS) initiated from scalps (n = 1919) with different subculture number cultured on various media. Different letters in the same column for each cultivar indicate differences in FEC, NEC, or DS. ns, non-significant ($p > 0.05$), ** very significant ($p \leq 0.01$), *** highly significant ($p \leq 0.0001$).

Table 4.11b: Percentage value of embryogenic development from scalps of Orishele, plantain cultivar

Cultivar	Treatment	Friable Embryogenic Calli (FEC) (%)	Non-Embryogenic Calli (NEC) (%)	Dead Scalp (DS) (%)
	S2_ZZ1	0 ± 0^{c}	57 ± 29^{cd}	43 ± 29^{bcd}
	S2_ZZ2	7 ± 10^{ab}	93 ± 10^{a}	0 ± 0^{f}
	S2_ZZ3	0 ± 0^{c}	60 ± 42^{cd}	40 ± 42^{bcd}
	S3_ZZ1	0 ± 0^{c}	39 ± 28^{de}	61 ± 28^{ab}
	S3_ZZ2	4 ± 12^{abc}	89 ± 19^{ab}	7 ± 10^{ef}
	S3_ZZ3	4 ± 8^{abc}	53 ± 25^{dc}	44 ± 27^{bcd}
	S4_ZZ1	9 ± 16^{a}	49 ± 34^{cd}	42 ± 37^{bcd}
Orishele	S4_ZZ2	2 ± 6^{bc}	69 ± 23^{bc}	29 ± 24^{cde}
	S4_ZZ3	2 ± 6^{bc}	57 ± 24^{cd}	42 ± 26^{bcd}
	S5_ZZ1	1 ± 4^{bc}	50 ± 44^{cd}	49 ± 44^{bc}
	S5_ZZ2	0 ± 0^{c}	86 ± 24^{ab}	14 ± 24^{ef}
	S5_ZZ3	2 ± 6^{bc}	58 ± 27^{cd}	41 ± 29^{bcd}
	S6_ZZ1	0 ± 0^{c}	26 ± 27^{e}	74 ± 27^{a}
	S6_ZZ2	3 ± 7^{bc}	70 ± 33^{abc}	27 ± 34^{de}
	S6_ZZ3	1 ± 5^{bc}	59 ± 26^{cd}	40 ± 28^{bcd}
***p* value**		**	***	***

Note: S: Subculture number, ZZ1: MS-based medium, ZZ2: Half-strength MS-based medium, ZZ3: B5 based medium. Data is presented as percentage mean and standard deviation of friable embryogenic calli (FEC), non-embryogenic calli (NEC) or non-responding explants as dead scalps (DS) initiated from scalps (n = 1220) with different subculture number cultured on various media. Different letters in the same column for each cultivar indicate differences in FEC, NEC, or DS. ns, non-significant ($p > 0.05$), ** very significant ($p \leq 0.01$), *** highly significant ($p \leq 0.0001$).

Table 4.11c: Percentage value of embryogenic development from scalps of Obino l'Ewai, plantain cultivar

Cultivar	Treatment	Friable Embryogenic Calli (FEC) (%)	Non-Embryogenic Calli (NEC) (%)	Dead Scalp (DS) (%)
Obino l'Ewai	S3_ZZ1	0 ± 0^{b}	89 ± 10^{ab}	11 ± 10^{de}
	S3_ZZ2	5 ± 9^{ab}	95 ± 9^{a}	0 ± 0^{e}
	S3_ZZ3	8 ± 15^{ab}	77 ± 29^{abcd}	15 ± 29^{cd}
	S4_ZZ1	0 ± 0^{b}	60 ± 25^{bcde}	40 ± 25^{ab}
	S4_ZZ2	0 ± 0^{b}	96 ± 9^{a}	4 ± 9^{de}
	S4_ZZ3	4 ± 9^{ab}	56 ± 17^{efg}	40 ± 14^{ab}
	S5_ZZ1	0 ± 0^{b}	60 ± 19^{defg}	40 ± 19^{ab}
	S5_ZZ2	1 ± 5^{b}	87 ± 20^{ab}	12 ± 20^{de}
	S5_ZZ3	8 ± 21^{ab}	64 ± 30^{bcdef}	27 ± 25^{bc}
	S6_ZZ1	0 ± 0^{b}	43 ± 8^{fg}	57 ± 8^{a}
	S6_ZZ2	10 ± 15^{ab}	90 ± 15^{ab}	0 ± 0^{e}
	S6_ZZ3	0 ± 0^{b}	97 ± 8^{a}	3 ± 8^{de}
	S8_ZZ1	5 ± 10^{ab}	40 ± 33^{g}	55 ± 34^{a}
	S8_ZZ2	5 ± 10^{ab}	85 ± 19^{abc}	10 ± 12^{cde}
	S8_ZZ3	0 ± 0^{b}	90 ± 12^{ab}	10 ± 12^{cde}
	S9_ZZ1	0 ± 0^{b}	92 ± 11^{ab}	8 ± 11^{de}
	S9_ZZ2	13 ± 28^{a}	80 ± 28^{abcd}	8 ± 15^{de}
	S9_ZZ3	0 ± 0^{b}	87 ± 12^{abcd}	13 ± 12^{cde}
***p* value**		ns	***	***

Note: S: Subculture number, ZZ1: MS-based medium, ZZ2: Half-strength MS-based medium, ZZ3: B5 based medium. Data is presented as percentage mean and standard deviation of friable embryogenic calli (FEC), non-embryogenic calli (NEC) or non-responding explants as dead scalps (DS) initiated from scalps (n = 749) with different subculture number cultured on various media. Different letters in the same column for each cultivar indicate differences in FEC, NEC, or DS. ns, non-significant ($p > 0.05$), ** very significant ($p \leq 0.01$), *** highly significant ($p \leq 0.0001$).

The effect of ascorbic acid in the development of FEC from scalps showed that at S6 and S7, there was a significant difference between treatment with and without ascorbic acid. At S6 (p = 0.04) and S7 (p = 0.0003), the highest number of FEC was observed in treatment without ascorbic acid (ZZ3_0) and differed significantly with treatment harbouring 10 mg/l and 20 mg/l ascorbic acid. (Fig 4.4).

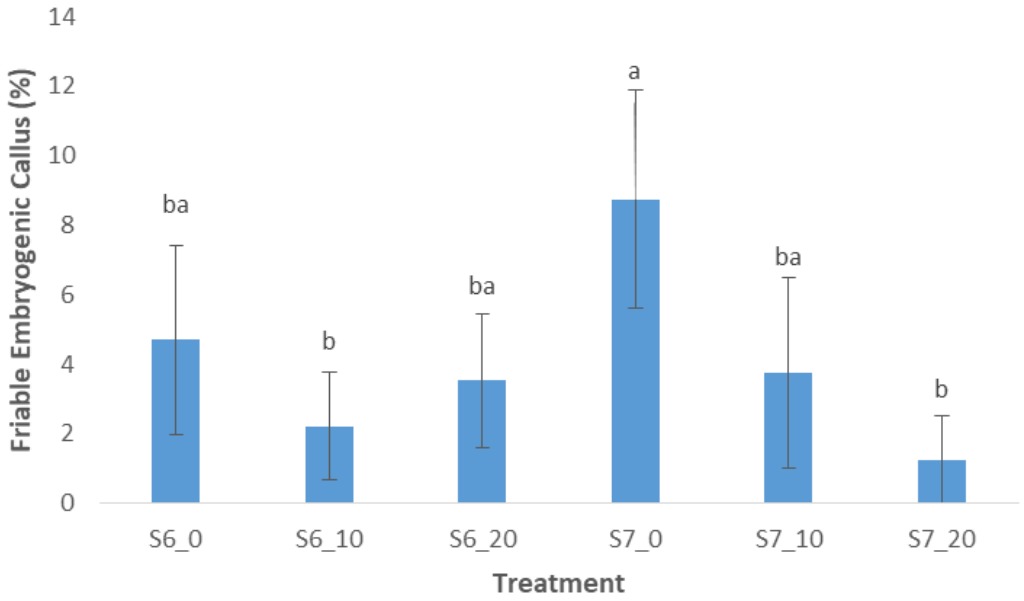


Figure 4.4: Comparing the effect of various concentration of ascorbic acid on FEC development of Agbagba from scalps at subculture number S6 and S7. The results are presented as percentage mean and standard error of FEC obtained from total number of scalps (n ≥ 80) cultured at each subculture number (S6 or S7) in ZZ3 medium (ZZ3_0, ZZ3_10, or ZZ3_20) supplemented with 0, 10, and 20 mg/l ascorbic acid. Different letters (a,b) indicate significant differences in treatment at $p \leq 0.05$.

4.3.3 Cell Suspension Generation and Cell Growth Curve Analysis Based on Settled Cell Volume (SCV)

In order to determine optimum culture media for plantain cell suspension, FEC of Agbagba obtained from ZZ1, ZZ2, and ZZ3 treatments were subjected to the same respective liquid media type (i.e. ZZ1, ZZ2, ZZ3). In addition, the effect of reduced 2, 4-D (0.5 mg/l) was observed for the three treatment types. Cultures were refreshed every 10 days for 3 months when the full proliferation of cells was attained. At three months, the

settled cell volume was measured. The optimum cell volume (3.3 ml) was observed in ZZ2 with 0.5 mg/l 2, 4-D (ZZ2 ½). Although ZZ3 (B5 media type) seemed to enhance cell multiplication to a certain extent, cells that were generated were nodular and not fit for subsequent experiments. However, ZZ1 and ZZ2 (MS media type) either with 1 mg/l or 0.5 mg/l 2, 4-D supported the proliferation of Agbagba in liquid suspension (Fig 4.5).

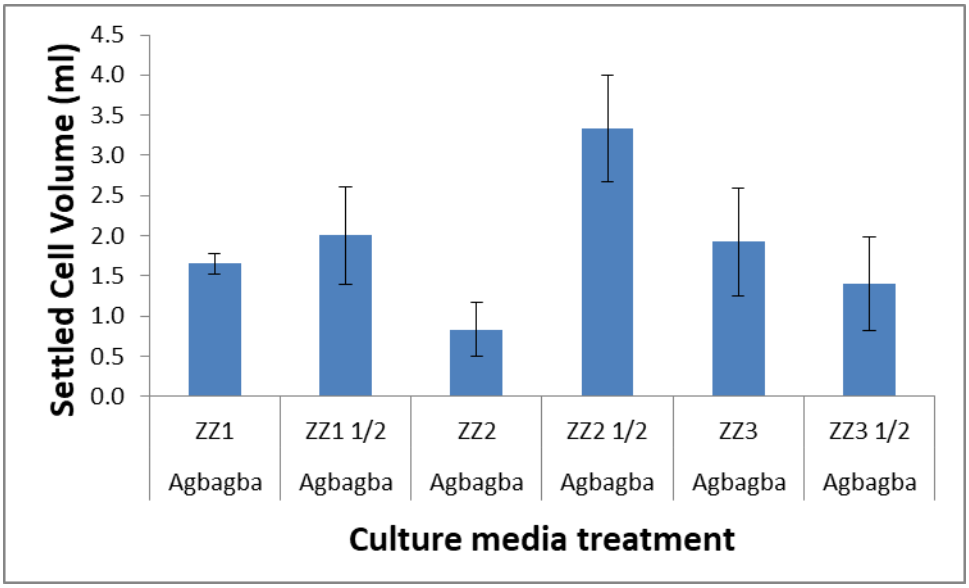


Figure 4.5: Settled cell volume of Agbagba cultivar at 3 months in culture. Data is presented as mean and standard error of settled cell volume.

An aliquot of 0.2 ml settled cell volume (SCV) of actively proliferating cell was further used to monitor cell growth over a period of 12 days by determining the settled cell volume. Cell proliferation was monitored for a period of 12 days in cells cultured in MS (ZZ1) and B5 (ZZ3) media. Although there was a correlation ($R^2 = 0.95$) between the two types of culture media used with respect to cell growth as measured by the SCV, MS medium (ZZ1) produced finer cells while nodular cells were observed in B5 medium (ZZ3) (Fig 4.6).

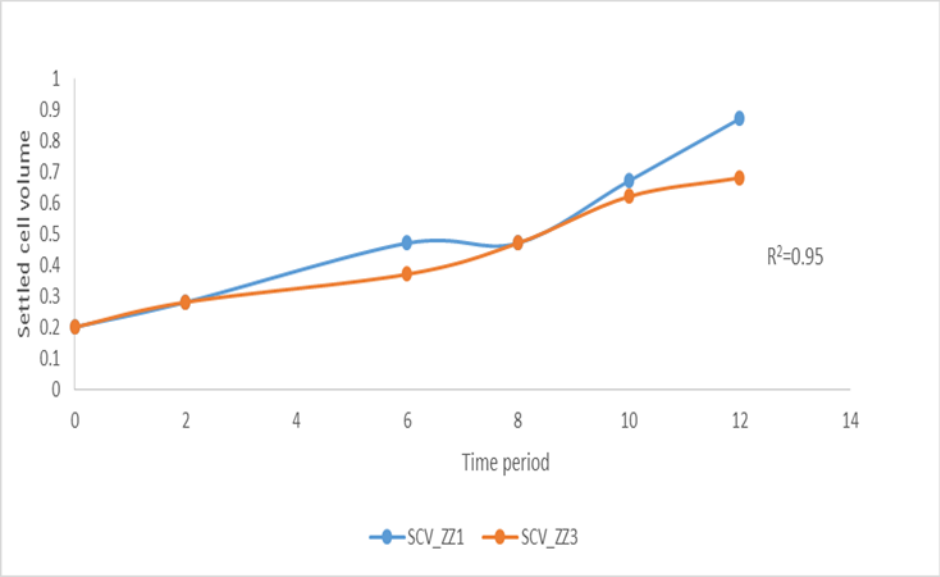


Figure 4.6: Cell growth of actively proliferating cells of Orishele on different media (ZZ1 and ZZ3) over 12 days. Data is presented as mean of three replicates ($n \geq 3$).

4.3.4 Staining of Plantain Embryogenic Cells using Fluorescein Diacetate (FDA)

The viability of plantain cells were estimated by staining with FDA which fluoresces green light when absorbed by live cells using a flow cytometer. The number of available cells in the control (unstained) was also counted using the flow cytometer. Viability of cells was monitored over a period of time in MS (ZZ1) and B5 (ZZ3) media. The value of live cells in both media type varied significantly ($p < 0.0001$) across the time period. However, for cells subjected to media B5 (ZZ3), the highest value of viable cells was observed at day 4 (D4) and at day 8 (D8) for MS. MS medium supports the maintenance of plantain cells in liquid medium while B5 (ZZ3) might not be ideal for plantain cell suspension culture. The number of unstained cells counted on the flow cytometer also showed similar cell proliferation trends (Fig 4.7a&b).

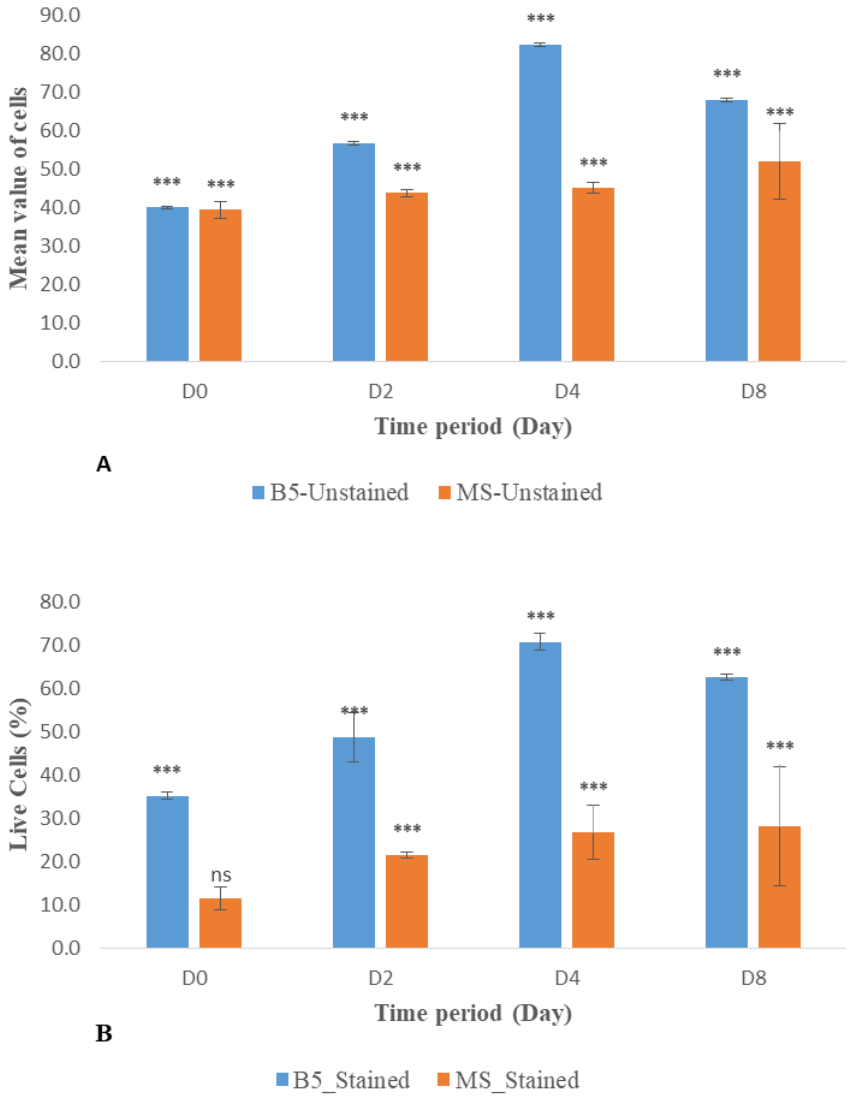


Figure 4.7a: Viability testing for ECS of plantain using FDA. A: mean value of cells counted MS and B5 culture media B: percentage of the live cell on MS and B5 culture media. Data is presented as mean and standard error of stained and unstained cells. ns, non-significant, *** highly significant ($p \leq 0.0001$).

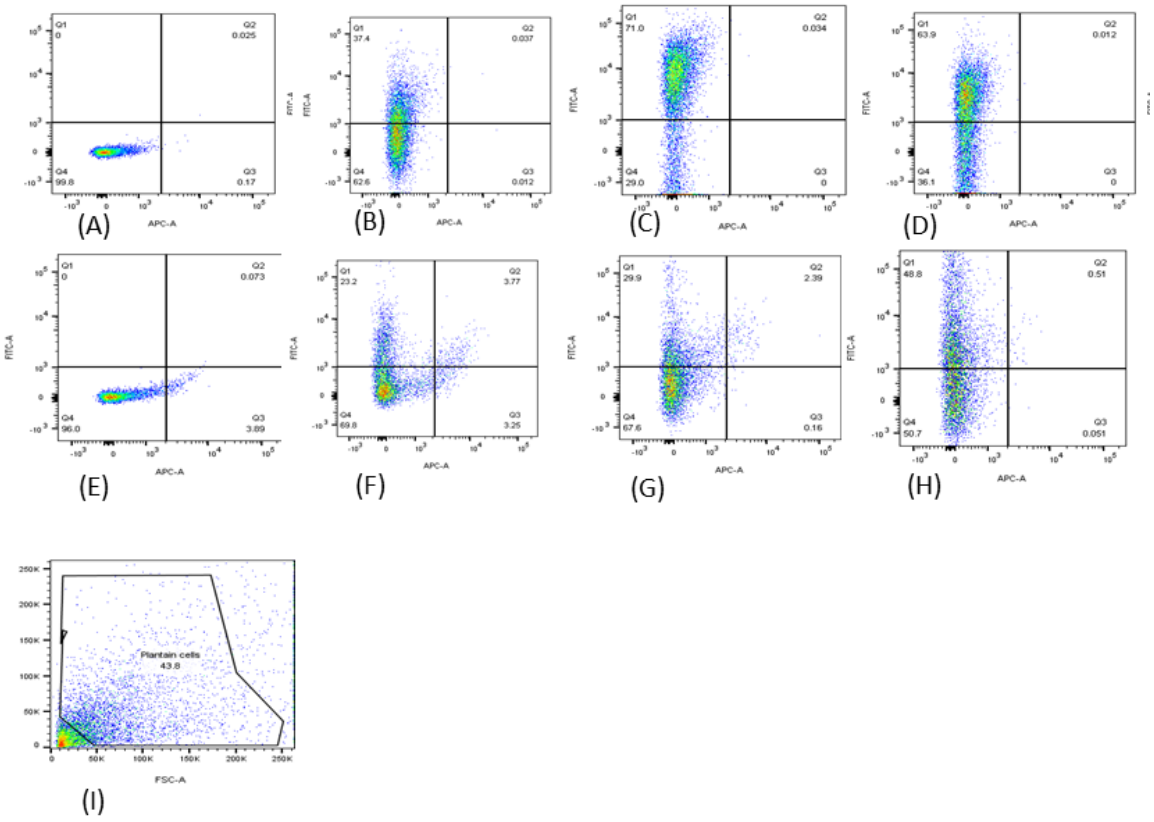


Figure 4.7b: Cell count as determined by the flow cytometer. A-D: cell count on B5 medium at day 0, 2, 4 and 8 E-H: cell count on MS medium at day 0, 2, 4 and 8 I: cell count gate area

4.3.5 Plantain Regeneration Efficiency Test

About 0.5 ml of the settled cell of plantain was plated on MA3 medium and further developed on RD1 medium for 1 month. Developed embryos were germinated on MA4 media for 6 weeks (Fig 4.8). Regenerated plantlets from developed embryos of plantain cells were counted to determine the regeneration potential of the plantain cells. A total of 7942, 11269 and 4082 regeneration capacity was obtained for Agbagba, Obino l'Ewai and Orishele, respectively (Fig 4.9).

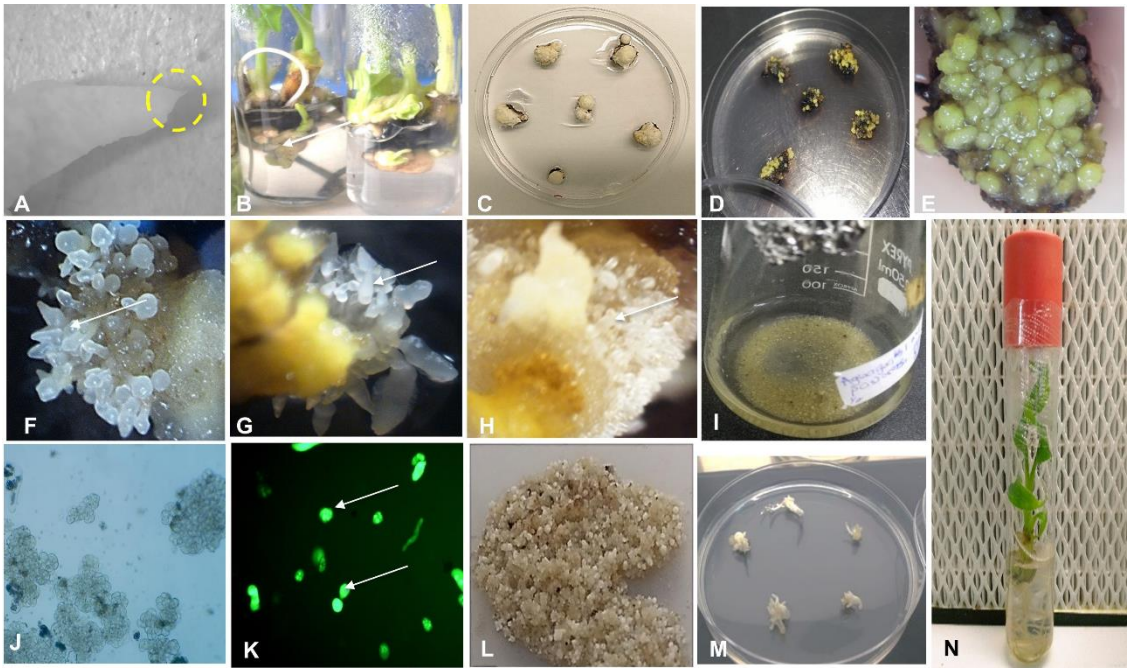


Figure 4.8: Establishment of embryogenic cell suspension and regeneration of complete plantlets of plantain cultivars Agbagba, Obino l'Ewai and Orishele. A: Apical meristem (yellow circle showing the portion of shoot tip having apical meristem under 10× magnification); B: Axillary buds (arrow indicating axillary buds); C: Scalps with tiny multiple meristems; D: Callus induced on the scalp; E: Yellowish non-embryogenic callus; F—H: Friable embryogenic callus of Orishele, Obino l'Ewai and Agbagba, respectively (arrows showing the embryos under 10× magnification); I: Embryogenic cell suspension; J: Magnified view of cell suspension (40× magnification); K: Fluorescein diacetate stained viable embryogenic cells (1000× magnification); L: Mature embryos; M: Germinating embryos; N: Complete plantlet.

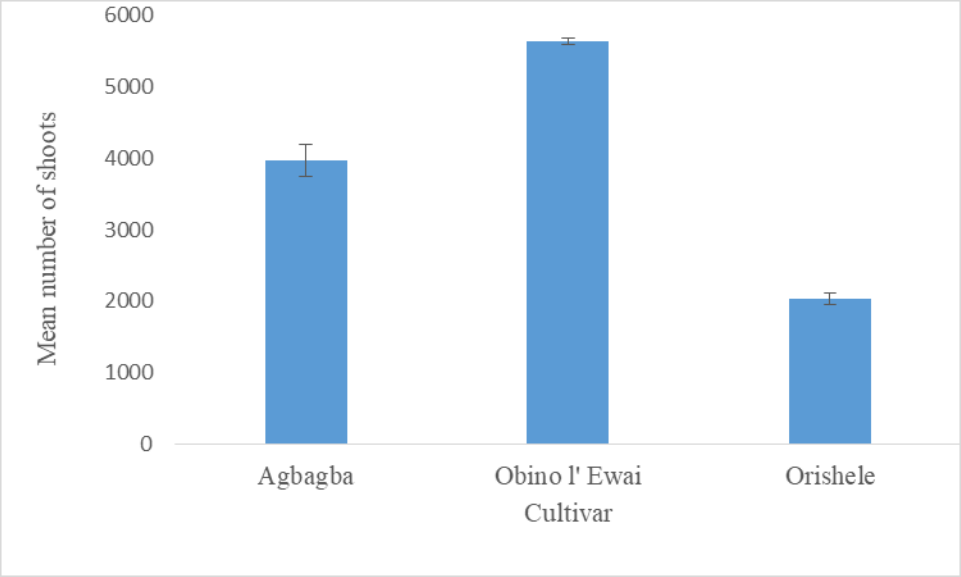
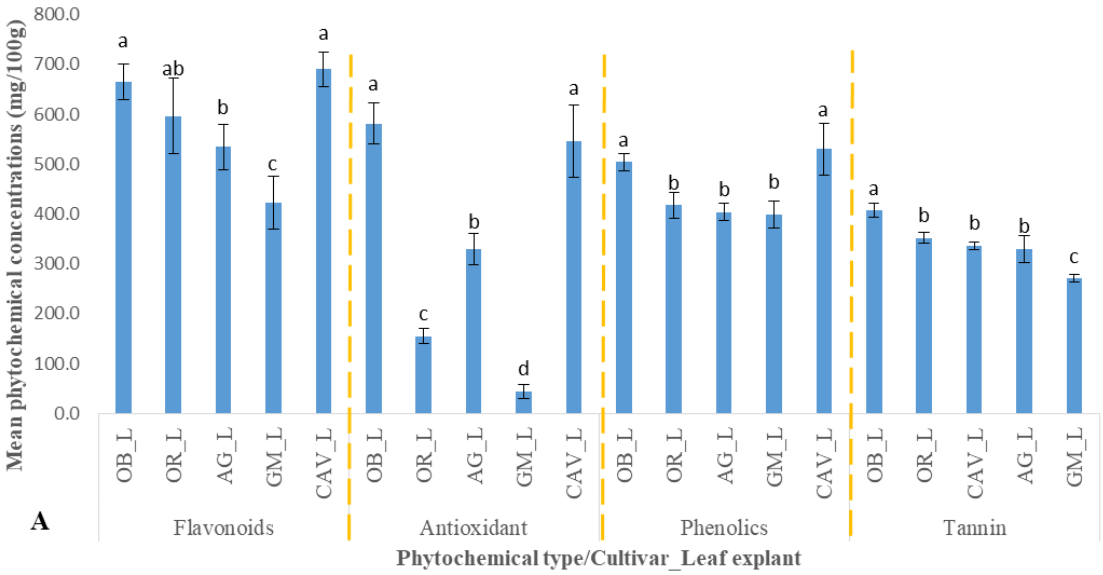


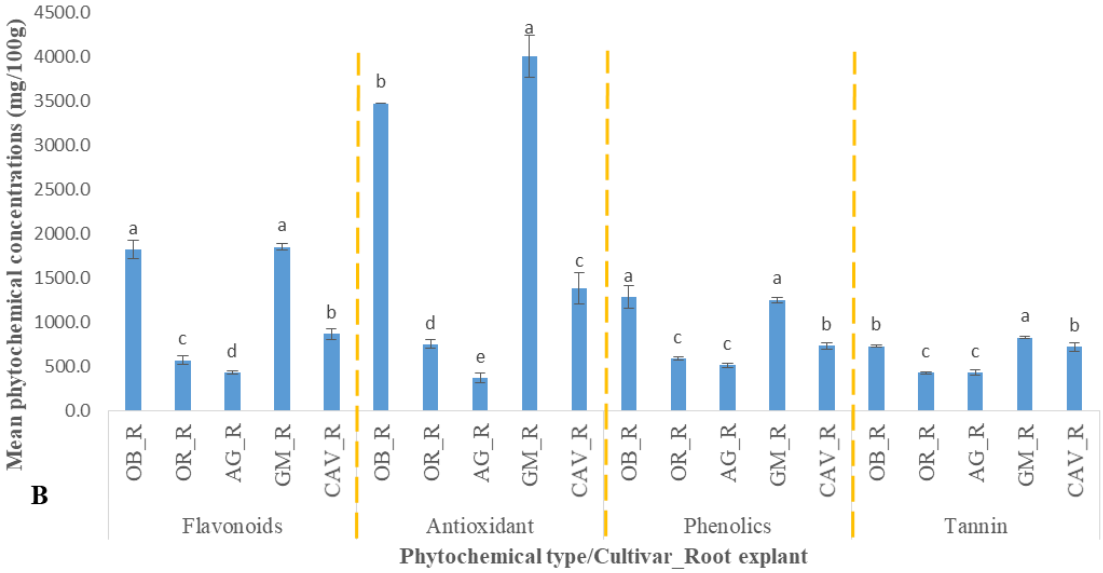
Figure 4.9: Regeneration efficiency of plantain ECS. Data is presented as mean and standard error of SCV

4.3.6 Phytochemical Quantification of Cultivars of Plantain and Banana Explant Extracts

4.3.6.1 Antioxidant Activity

The total antioxidant content was determined by DPPH scavenging capacity as calculated from the calibration curve (R^2 = 0.98) and expressed as mg of Trolox equivalent per 100 g of the sample. The free radical scavenging activity of the total antioxidants varied across the explants and cultivars tested. The highest and lowest antioxidant activity at 4009.5 ± 236 mg/100 g and 44.1 ± 14 mg/100 g were recorded for Gonja Manjaya root and leaf explant, respectively. For *in vitro* samples, the highest concentration of antioxidant capacity was in Cavendish Williams (5142.0 ± 28 mg/100 g) and lowest in Orishele (2595.4 ± 137 mg/100 g). On the other hand, substantial antioxidant content for Orishele was found in the pseudostem (1864.0 mg/100 g). Agbagba produced the least concentration of antioxidant capacity in the root (375.2 mg/100 g) (Fig 4.10, Table 4.12).


Mean phytochemical concentrations (mg/100g)
800.0
700.0
600.0
500.0
400.0
300.0
200.0
100.0
0.0
a
ab
b
c
a
a
c
b
d
a
a
b
b
b
a
a
b
b
b
a
b
b
b
c
OB_L
OR_L
AG_L
GM_L
CAV_L
Flavonoids
Antioxidant
Phenolics
Tannin
A
Phytochemical type/Cultivar_Leaf explant


Mean phytochemical concentrations (mg/100g)
4500.0
4000.0
3500.0
3000.0
2500.0
2000.0
1500.0
1000.0
500.0
0.0
a
c
d
a
b
b
d
e
a
c
a
c
c
a
b
b
c
c
a
b
OB_R
OR_R
AG_R
GM_R
CAV_R
Flavonoids
Antioxidant
Phenolics
Tannin
B
Phytochemical type/Cultivar_Root explant

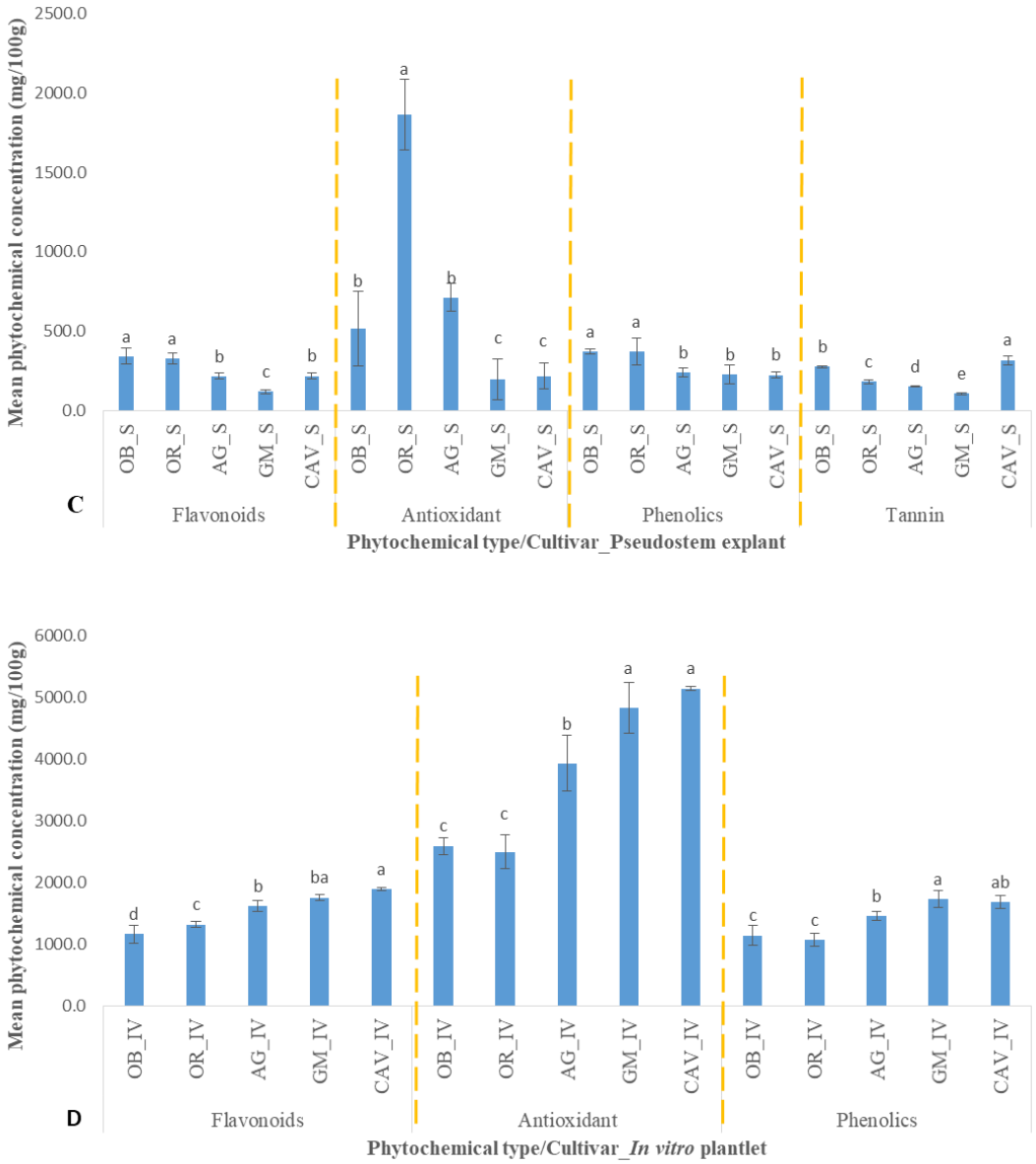


Figure 4.10: **C**oncentration of total flavonoids, total antioxidant, total phenolics and total tannins in explants of field-grown plants and *in vitro* plantlets of banana and plantain cultivars. **(A)** Leaf (L) explant; **(B)** Root (R) explant; **(C)** Pseudostem (S) explant; **(D)** *In vitro* (IV) plantlet; OB (Obino l'Ewai); OR (Orishele); AG (Agbagba); GM (Gonja Manjaya); CAV (Cavendish Williams). The results are means of triplicate samples ($n \geq 3$) and the error bars indicate the standard deviation, values with the same letters are not significantly different, $p \leq 0.05$.

Table 4.12: ANOVA summary of total antioxidant activity in different explants of plantain and banana cultivars

Source of variation	df	Mean square	Explant	Mean antioxidant
Cultivar	4	1875913.1***	Leaf	331.26d
Explant	3	36852108.1***	Root	2002.34b
Cultivar*Explant	12	4099109.4***	Pseudostem	701.88c
Mean		1708.85	*In vitro*	3799.91a
Error		35326.2		
CV		11		

***, *p* value significance at ≤ 0.001; a, b, c, d, samples with different letters of alphabet varied significantly.

4.3.6.2 Phenolic Content

The total phenolic content was calculated from the calibration curve (R^2 = 0.98) as the gallic acid equivalent (GAE) per 100 g sample (Fig. 4.10, Table 4.13). There was a significant ($p \leq 0.0001$) difference in the level of total phenolic content observed in *in vitro* samples for Gonja Manjaya followed by Cavendish Williams, Agbagba and lowest in Obino l'Ewai and Orishele (Fig 4.10). Obino l'Ewai recorded the highest phenolic content in the pseudostem (372.1 ± 14.9 mg/100 g) and root (1290.4 ± 124.1 mg/100 g) explants.

Table 4.13: ANOVA summary of total phenolics content in different explants of plantain and banana cultivars

Source of variation	df	Mean square	Explant	Mean Phenolics
Cultivar	4	174601.26***	Leaf	450.67c
Explant	3	3842711.27***	Root	879.63b
Cultivar*Explant	12	178293.90***	Pseudostem	286.37d
Mean		758.9	*In vitro*	1418.95a
Error		5606.08		
CV		9.87		

***, *p* significance at ≤ 0.001; a, b, c, d, samples with different letters of the alphabet are significantly different

4.3.6.3 Flavonoid Content

The total flavonoid content (R^2 = 0.99) was calculated as catechin equivalent (CE) (Fig. 4.10). The concentration of flavonoids varied significantly ($p \leq 0.001$) across different cultivars and explants used in the study. The flavonoid content was highest and lowest in Gonja Manjaya (1854.5 ± 36.5 mg/100 g) and Agbagba (433.5 ± 18.6 mg/100 g) root explants, respectively. For the *in vitro* samples, the highest and lowest concentrations were in Cavendish Williams (1895.1 ± 28.8 mg/100 g), and Obino l'Ewai (1163.2 ± 139.6 mg/100 g), respectively. There was a significant ($p \leq 0.0001$) interaction between the cultivar type and the explant (Table 4.14).

Table 4:14: ANOVA summary of total flavonoids in different explants of plantain and banana cultivars

Source of variation	df	Mean square	Explant	Mean flavonoids
Cultivar	4	308715.17***	Leaf	581.47c
Explant	3	4985882.88***	Root	1111.70b
Cultivar*Explant	12	476134.19***	Pseudostem	244.09d
Mean		872.06	*In vitro*	1551.00a
Error		3342.44		
CV		6.63		

***, *p*-value significance at ≤ 0.0001, a, b, c, d, varying letters of alphabet showed significant differences

4.3.6.4 Tannin Composition

The total tannin as extrapolated from the calibration curve (R^2 = 0.99) was determined as tannic acid equivalent (TAE) (Fig 4.10). Total tannin concentrations varied significantly ($p \leq 0.0001$) across the cultivars and explants (Table 4.15). Generally, tannin levels were highest in the root explant and lowest in the pseudostem of all cultivars. Gonja Manjaya, Obino l'Ewai and Cavendish Williams recorded the highest levels of tannin in the root (830.4 ± 12.4 mg/100 g), leaf (406.8 ± 13.9 mg/100 g) and pseudostem (317.5 ± 27.5 mg/100 g), respectively. The lowest mean values for the root, leaf and pseudostem plant parts were recorded in Orishele (429.9 ± 13.4 mg/100 g), Gonja Manjaya (270.4 ± 7.8 mg/100 g), and Gonja Manjaya (105.1 ± 4.2 mg/100 g), respectively.

Table 4.15: ANOVA summary of total tannin content in different explants of plantain and banana cultivars

Source of variation	*df*	Mean square	Explant	Mean Tannin
Cultivar	4	53291.62***	Leaf	338.97b
Explant	2	703997.55***	Root	629.97a
Cultivar*Explant	8	40622.64***	Pseudostem	206.46c
Mean		391.38		
Error		384.67		
CV		5.01		

***, *p* values significance at < 0.0001; a, b, c, samples with same letters of the alphabet are not significantly different

4.4 Discussion

The importance of phytochemicals cannot be overemphasized especially in *in vitro* applications and manipulations. The presence and quantity of targeted phytochemicals (total antioxidant, phenolics, flavonoids and tannin) were determined on selected farmer preferred cultivars of banana and plantain namely Cavendish Williams, Orishele, Agbagba, Obino l'Ewai and Gonja Manjaya. The concentration of phytochemicals in explants of the same plants was not uniform. In general, *in vitro* samples in the absence of exogenous antioxidant and plant growth hormone gave the highest levels of total antioxidant capacity compared to field explants in Cavendish Williams (5142 ± 27.6 mg/100 g), Gonja Manjaya (4828.2 ± 413.1 mg/100 g), Agbagba (3934.6 ± 450.3 mg/100 g) and Orishele (2499.4 ± 276.2 mg/100 g). A similar trend was observed in the total phenolic content of these cultivars, this may suggest the influence of micropropagation procedures on increased production of plant biochemicals. Subjecting lowbush blueberry to tissue culture procedure resulted in increased phenolic content and antioxidant activity (Goyali *et al*., 2013).

The root explant of Gonja Manjaya, Obino l'Ewai, and Cavendish Williams contained enormous amounts of phytochemicals while Orishele and Agbagba had higher antioxidant activity in the pseudostem explants. The varying antioxidant potential to reduce DPPH a stable free radical to DPPH-H a non-radical form in different explants tested revealed the diversity among the cultivars, area of localization and their potential in combating the effect of oxidative stress due to increased level of reactive oxygen species (ROS). This is made possible because of the innate ability of plant cell machinery to balance the stoichiometry of free radical flux which is produced when plants are stressed. Chutipaijit (2017), showed the correlation between drought tolerance and antioxidant property in rice. The augmentation of this mechanism in the presence of increased oxidative stress results in the production of phenolics, flavonoids and tannins to confer antioxidant properties (Kasote *et al*., 2015). The ability of natural antioxidant agents to scavenge for free radicals

that are generated through stress imposed on plant tissues and eventual tissue injury is important in *in vitro* and *in situ* activities of plants.

The optimum concentration of certain phytochemicals could influence plant responses *in vitro*. The concentration of total antioxidant and phenolic content in *in vitro* plantlets was highest for Agbagba compared to Obino l'Ewai and Orishele. There was a probable trend observed in the antioxidant capacity, phenol content, multiplication rate and the potentiality to produce FEC in Agbagba compared to Obino l'Ewai and Orishele. It is likely that the stoichiometry of phenol quantity and antioxidant potential during *in vitro* manipulations and/or procedures could have supported the production of FEC and multiplication capacity of Agbagba. However, this could be further investigated.

The proliferation and embryogenic capability of selected plantain cultivars; Agbagba, Obino l'Ewai and Orishele was determined in various culture media type to determine the optimum conditions appropriate for each cultivar. Following initiation of these *in vitro* cultures of cultivars via meristem culture, the three plantain cultivars showed the ability to regenerate to a whole plant. However, their proliferation potential varied significantly among the cultivars. Over time, the highest mean number of shoots was observed for Agbagba at subculture cycle 2 and 3 (S2 & S3) respectively. While the least was observed with Obino l'Ewai at subculture cycle 2 and 3. Similar reports indicated that banana and plantain varieties respond differently to *in vitro* micropropagation (Dagnew *et al*., 2012; Sadik *et al*., 2012). Although the plantain cultivars used are of the same genome (AAB), they obviously exhibited varying proliferation potential.

In vitro manipulations have a strong potential in inducing or triggering the activity of reactive oxidative stress (ROS) in plants. This exhibit both a negative and positive influence on the plant morphogenesis and play a crucial role in cellular functions (Gupta, 2010). High sucrose and nitrogen concentrations are some conditions that affect *in vitro* culture responses (Gupta, 2010). At 500 mg/l NH_4NO_3, maximum antioxidant activity and phenolic production were observed for grape cell suspension (Sae-Lee *et al*., 2014). The

antioxidant potential and phenolic content of an explant or cultivar play a significant role in the cellular development of the plant. The high proliferation rate observed in Agbagba as compared with Obino l'Ewai and Orishele could be the ability of the cultivar to scavenge ROS induced during shoot multiplication and the presence of phenol could have signalled the induction of somatic embryogenesis especially in the presence of 2, 4-D.

A successful development of FEC is a prerequisite to the production of embryogenic cell suspension. Generation of FEC of plantain cultivars varied with the genotype, scalp subculture stage and the type of treatment used. For Agbagba, an optimum treatment using ZZ3 (Gamborg (B5) medium) supplemented with 1 mg/l 2,4-D resulted in the production of higher FEC (22 %) compared to full strength MS (ZZ1) and half-strength MS (ZZ2) culture media. While for Obino l'Ewai and Orishele, half-strength MS (ZZ2) and full-strength MS (ZZ1) gave optimal results. Regardless of the treatment and subculture cycle employed, FECs were generated at varying levels and this result is the first optimal report with increased FEC production in comparison to what was previously published (Strosse *et al*., 2006). Certain factors such as media type, age of explant and genotype are some reported influences on the development of FEC (Jiménez, 2001; Youssef *et al*., 2010). Therefore, optimisation may be done to obtain the best response to suit specific cultivar.

Furthermore, while ascorbic acid is known to improve somatic embryogenesis in some plants (Becker *et al*., 2014), it has been observed to hinder the development of somatic embryos in some other plants (Malabadi & Staden, 2004). In this study, a comparison between scalps of Agbagba at S6 and S7 subjected to media containing 0 mg/l, 10 mg/l and 20 mg/l ascorbic acid indicated a higher production of FEC in medium with no ascorbic acid (Fig 4.4). When *in vitro* cultures are subjected to stress, the plant initiates its innate ability to balance the ROS produced and quenching ability through the use of antioxidants. An imbalance of the two components could affect the plant response (Gupta, 2010). Growth hormones like 2, 4-D are known free radical generators used in inducing somatic embryogenesis in plants. In our study, the development of FECs in the absence

of ascorbic acid could be that the endogenous antioxidant capacity of Agbagba is sufficient to quench the ROS generated or the ROS could have signalled the production of FECs. In addition, a possible counteractive effect of exogenous antioxidant caused by an imbalance in the reaction of ROS and antioxidant quenching ability could have hindered the production of FEC. A similar result in *Arabidopsis* confirmed that deficiency in ascorbic acid improves somatic embryo development (Becker *et al*., 2014). This is an indication that the absence of ascorbic acid does not have any negative influence on FEC development in Agbagba. However, this could be further investigated.

The influence of phenolic compounds on somatic embryogenesis induction is cultivar dependent. Phenol rich compounds have been shown to improve the induction of somatic embryogenesis (SE) in *Feijoa* (Reis *et al*., 2008) whereas, it inhibits SE in *C. canephora* (Nic-can *et al*., 2015). The high level of phenolics in Agbagba as compared to Obino l'Ewai and Orishele may contribute towards its improved response to developing FECs. On the other hand, the response of Obino l'Ewai to FEC development is relatively higher than Orishele. This difference may be attributed to the total phenolic content which is higher in Obino l'Ewai compared to Orishele. The genotypic dependency of SE calls for an evaluation of phytochemicals in genotypes before optimization of SE.

4.5 Conclusion

The present study showed that the proliferation and embryogenic potential of the three plantain cultivars are genotype-dependent. Friable embryogenic callus was formed on 1-22 % of the scalps depending on treatment type and cultivar. These results suggest that ZZ3 was best in generating friable embryogenic callus for Agbagba while and ZZ1/ZZ2 was best for Obino l'Ewai and Orishele. The optimum media type for embryogenic cell suspension in the liquid phase was ZZ1 and ZZ2 with 1 mg/l or 0.5 mg/l 2, 4-D.

The number of shoots generated per subculture cycle varied among the cultivars. Besides, an optimized system for generating FEC and the embryogenic cell suspension was successful for the selected plantain cultivars with improved results. The increase in cell proliferation rate by time period is an indication of cell viability. In addition, the quantification of phytochemicals in the plantain cultivars could provide the basis for their *in vitro* manipulation.

CHAPTER FIVE

OPTIMIZATION OF GENETIC TRANSFORMATION SYSTEM FOR PLANTAIN CULTIVARS FROM WEST AFRICA

5.1 Introduction

Genetic transformation is a process of inserting or removing foreign or detrimental gene(s) in a plant with the sole aim of improving it (Low *et al*., 2018). It entails the processes of altering the genetic makeup of an organism by introducing an exogenous gene through the cell membrane and incorporating it within the genome of the organism (Sharma *et al*., 2005; Mubeen *et al*., 2016). This procedure has gained wide applications in plant genetics for developing improved crop varieties (Nyaboga *et al*., 2014). There are two main methods of transferring a foreign gene into a host plant and these are physical and biological methods. The physical methods of transformation include; the particle bombardment, electroporation, sonication, vacuum infiltration, silicon carbide whisker, agitation with glass beads, laser beams, microinjection and shock wave-mediated genetic transformation while biological methods include; *Agrobacterium rhizogenes* and *Agrobacterium tumefeciens* mediated genetic plant transformation (Rivera *et al*., 2014; Mubeen *et al*., 2016). The physical methods of genetic transformation are either expensive or has low transformation efficiency, however, the biological methods of genetic transformation are able to mitigate this limitation because it occurs naturally and has been explored extensively in crop improvement (de la Riva *et al*., 1998; Hwang *et al*., 2017).

To improve transformation efficiency especially when handling recalcitrant crops, a combination of the biological and physical methods have been employed (Leuzinger *et al*., 2013). The transformation of crops using *Agrobacterium rhizogenes* and *Agrobacterium tumefeciens* takes advantage of the ability of these bacteria to transfer a DNA section (T-DNA) of the tumour-inducing (Ti of *Agrobacterium tumefeciens* or Ri of *Agrobacterium rhizogenes*) plasmid into the nucleus of the plant (host) genome to enable

a transient and stable transformation (de la Riva *et al*., 1998). Similarly, this phenomenal capability of the *Agrobacterium* makes it possible for any foreign gene placed between the T-DNA borders to successfully integrate into the plant genome.

Furthermore, several factors influence the effectiveness of the *Agrobacterium*-mediated gene transformation in plants. These include but not limited to the type of crop, explant, bacteria strain, temperature, sucrose, pH, plant growth regulators, antibiotic type and concentration, acetosyringone concentration as well as co-culture period (de la Riva *et al*., 1998; Amoah *et al*., 2001; Cheng *et al*., 2004; Tripathi *et al*., 2005; Karami, 2008; Liu *et al*., 2017; Ahmed *et al*., 2018). *Agrobacterium*-mediated transformation has been successfully used in both dicotyledonous and monocotyledonous plants such as maize, barley, wheat, sorghum, tomato, potato, soybean, rice, sugarcane, and banana (Gould *et al*., 1991; May *et al*., 1995; Carvalho *et al*., 2004; Tripathi *et al*., 2005; Estiati & Nena, 2009; Hensel *et al*., 2009; Kalunke *et al*., 2009; Liu *et al*., 2017; Reddy *et al*., 2018; Veillet *et al*., 2019). Although several *Agrobacterium*-mediated transformations are available for *Musa* spp., they are targeted at specific varieties because plant genetic transformation is genotype-dependent (Tripathi *et al*., 2015).

In order to optimize and establish an efficient transformation protocol for a specific plant species or cultivar, a preliminary transient gene expression using a reporter gene is recommended. To this end, this study sought to optimize the transformation procedure for three plantain cultivars namely Agbagba, Orishele and Obino l'Ewai (AAB genome) using pCAMBIA 2300-*gfp* and pCAMBIA 2301 containing *neomycin phosphotransferase* II gene (*npt*II/NeoR) as selection marker and *gfp* of *gus*A as a reporter gene, respectively.

5.2 Materials and Methods

Agrobacterium-mediated transformation procedure was employed using *Agrobacterium* strain EHA 105 harbouring pCAMBIA 2300-*gfp* or pCAMBIA 2301 containing *neomycin phosphotransferase* II gene (*npt*II/NeoR) as selection marker and *gfp* or *gus*A as a reporter gene (Fig 5.1) to transfect plantain cells (Chapter 4). The plasmid constructs were obtained from the IITA plant transformation lab, Nairobi, Kenya.

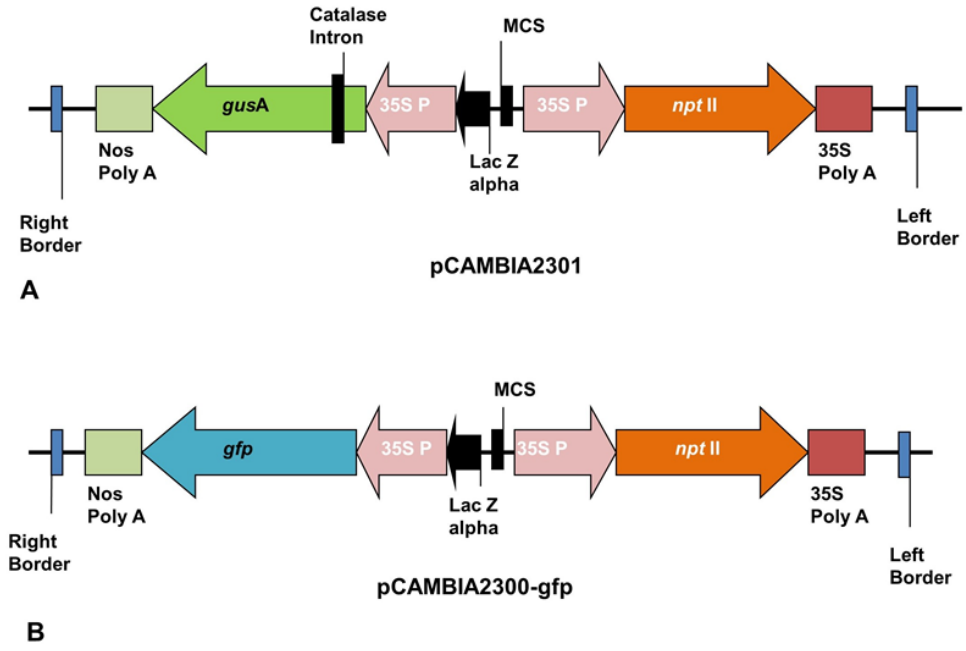


Figure 5.1: Map of plasmid construct. A: pCAMBIA 2301 containing β-*glucuronidase* (*gus*A) gene as reporter gene, *neomycin phosphotransferase* (*npt*II) gene as the selectable marker, Cauliflower Mosaic Virus 35 promoter and NOS as terminator; B: pCAMBIA 2300-*gfp* containing green fluorescent protein (*gfp*) as a reporter gene, *neomycin phosphotransferase* (*npt*II) gene as the selectable marker, Cauliflower Mosaic Virus 35 promoter and NOS as terminator.

5.2.1 Media and Buffer Preparation

Media was prepared with varying acetosyringone concentration of Bacteria co-cultivation medium (BCCM) A (APPENDIX 1) and bacteria resuspension medium (BRM) B

(APPENDIX 4) media. Sterilization was done either by autoclaving or filter-sterilization. The bacteria co-cultivation medium (BCCM) A and B (APPENDIX 2) was prepared separately and mixed together after sterilization. The same was done for resuspension medium (BRM) A and B (APPENDIX 3).

5.2.2 *Agrobacterium*-Mediated Transformation of Cells of Plantain Cultivars

5.2.2.1 Preparation of Bacterial Culture

Agrobacterium strain EHA 105 harbouring pCAMBIA 2301 or pCAMBIA 2300-*gfp* was cultured as described by Tripathi *et al*. (2012). A single colony of *Agrobacterium* harbouring the respective construct (pCAMBIA 2301 or pCAMBIA 2300-*gfp*) was streaked with the aid of a sterile tip under the laminar flow hood. Each distinct colony was inoculated in 25 ml Luria broth (LB) medium containing 25 µl (50 mg/ml) Kanamycin and 25 µl (25 mg/ml) Rifampicin. The cultures were sealed, labelled and incubated for 48 hours at 28 °C and 200 rpm on a rotary shaker (New Brunswick Scientific, Incubator shaker series, I 26, USA). The culture was refreshed by taking 1 ml aliquot of the previously grown culture in 50 ml LB medium supplemented with 50 µl (50 mg/ml) kanamycin and 50 µl (25 mg/ml) Rifampicin in a 100 ml sterile conical flask. Cultures were incubated overnight at 28 °C and 200 rpm on a rotary shaker (New Brunswick Scientific, Incubator shaker series, I 26, USA).

The bacterial cultures grown overnight were transferred into sterile falcon tube and centrifuged (Eppendorf Centrifuge 5810 R) at 4000 rpm for 15 min at 25 °C. The LB medium was decanted, and bacteria pellet re-suspended in 25 ml of bacteria re-suspension medium mix (BRM A (100ml) + BRM B (25ml)). This was allowed to incubate at room temperature on a rotary shaker at 70 rpm. The optical density (OD_{600nm}) of each bacterial suspension culture was adjusted to 0.5 using the BRM medium.

5.2.2.2 Preparation of Plantain Explants for Transformation

Embryogenic cell suspensions of plantain cultivars (Agbagba, Obino l'Ewai and Orishele) were refreshed 5 days prior to the transformation day.

5.2.2.3 Co-Cultivation

On the day of transformation, about 0.5 ml of the settled cell volume of ECS was transferred into a sterile falcon tube. The excess medium was removed and cells incubated in 10 ml of warm (45 °C) cell suspension culture medium (ZZ medium (Table 3.2) for 5 min. The hot medium was removed and about 10 ml of pre-induced *Agrobacterium* harbouring respective pCAMBIA 2301 and pCAMBIA 2300-*gfp* construct was added to the cells and 50 µl of 2 % pluronic F 68 was added. The sample was centrifuged (Eppendorf centrifuge 5810R, Germany) at 900 rpm for 10 min. Cells were then incubated at room temperature for 1 hour on a rotary shaker at 30 rpm.

After 1 hour, the transfected cells were transferred on a sterile mesh, blotted dry and plated on bacteria co-cultivation medium (BCCM) (BCCM A 300 ml + BCCM B 200 ml) and incubated in an incubator (INCUDIGIT, J.P SELECTA, Cod 2001616, Spain) at 22 °C for 2 to 3 days, respectively.

5.2.2.4 Selection and Regeneration of Transformed Embryos

After co-cultivation for 2 or 3 days, the *Agro*-infected cells were washed thoroughly (3 times) with MA3 media containing 300 mg/l cefotaxime (Table 3.2). A portion of these cells was tested for transient expression of reporter gene *gus*A by histochemical GUS assay or GFP through fluorescent microscopy. The remaining *Agrobacterium*-infected ECS were regenerated as described by Tripathi *et al*. (2015). The washed cells were plated on MA3 medium containing 300 mg/l cefotaxime for 2 weeks and subsequently refreshed every 2 weeks on MA3 medium containing 300 mg/l cefotaxime and 100 mg/l kanamycin. Developed embryos after 2-3 months were transferred onto RD1 medium containing 100 mg/l kanamycin (Table 3.2) for one month and transferred onto MA4 medium containing 100 mg/l kanamycin (Table 3.2) for proper germination of embryos. Putative transgenic events were maintained on RM media containing 100 mg/l kanamycin (Table 3.2). The transformation efficiency was calculated based on the number of PCR positive events per ml settled cell volume (SCV).

5.2.2.5 Histochemical GUS Assay

Histochemical GUS assay of *Agro*-infected ECS and leaf explant of transgenic plants was carried out as described by Jefferson *et al*. (1987) and Tripathi *et al*. (2012). Explant of the putative transgenic plant was incubated overnight at 37 °C in GUS assay solution (APPENDIX 5). Plant chlorophyll was washed off with 70 % ethanol. Transformed explants, marked by blue colouration were micrographed using a stereomicroscope (SMZ1500, LH-M100C-1, Japan).

5.2.2.6 Transient Expression of *gfp* Gene

Agrobacterium-transformed ECS and leaf explant of the transgenic plant were exposed to UV stereomicroscope (SMZ1500, LH-M100C-1, Japan) to observe the *gfp* expressions.

5.2.3 Molecular Characterization of Transformed Plants

5.2.3.1 Genomic DNA Extraction

Genomic DNA was extracted from putative transgenic plant events using a modified Cetyl triethylammonium bromide (CTAB) method as described by Tripathi *et al*., (2019). About 500 mg of leaf sample was isolated from the plant, freeze-dried in liquid nitrogen and pulverized to a fine powder. About 5 ml of pre-warmed CTAB extraction buffer (APPENDIX 6) was added to the sample and mixed thoroughly by vortexing. Samples were incubated at 65 °C for 30 min with occasional mixing by inversion for about 2-3 times during the incubation period. An equal volume of chloroform: isoamyl alcohol (24:1) was added to each sample and mixed thoroughly. Samples were centrifuged (Eppendorf Centrifuge 5810 R, Germany) at 4000 rpm for 15 min in order to separate the organic and the aqueous phase. The aqueous layer was transferred quantitatively into a new sterile tube. An equal volume of ice-cold isopropanol was added to the mixture and mixed gently by inversion for about 2-3 times. Samples were incubated at -20 °C for 1 hour and subsequently centrifuged (Eppendorf Centrifuge 5810 R, Germany) at 4000 rpm for 10 min. The iso-propanol was decanted carefully without disturbing the pellet and 2 ml of 70 % ethanol was added to the pellet. The tube was flicked to prevent the pellet from

sticking to the base of the tube. Samples were centrifuged (Eppendorf Centrifuge 5810 R, Germany) at 4000 rpm for 5 min. This washing step was repeated twice. The ethanol was removed and DNA pellet re-suspended in 1 M NaCl and 2 µl of RNase and incubated in a water bath at 37 °C for about 4 hours. Following this, 3 ml of absolute ethanol was added to the solution. The genomic DNA which formed a jelly-like substance was carefully looped out into a sterile 1.5 ml Eppendorf tube. An aliquot of 1 ml 70 % molecular grade ethanol was added and centrifuged (Eppendorf centrifuge 5424, Germany) at 13000 rpm for 2 min at 4°C (this step repeated once). Genomic DNA pellet was air dried and suspended in nuclease-free water. The quantity and quality of DNA were determined using a spectrophotometer (NanoDrop 2000 spectrophotometer, ThermoScientific) and electrophoresis on 1 % (w/v) agarose gel respectively.

5.2.3.2 PCR Analysis of Transformed Plantain Events

The putative transgenic events regenerated on the selective medium were analysed for the presence of the transgene by PCR analysis using primers specific to *npt*II gene. A gradient PCR (95 °C: 15 min, 94 °C: 30 sec, 50-70 °C: 30 sec, 72 °C: 1 min (25 cycles), 72 °C: 5 min, 4 °C: ∞) using plasmid DNA, non-transformed plant and non-template control was done to determine the optimal annealing temperature for the *npt*II primer to be used for the PCR screening and validated using random *npt*II transgenic samples. PCR was performed using *npt*II specific primers (*npt*II_F: GATGGATTGCACGCAGGTTCTC, *npt*II_R: AGAAGAACTCGTCAAGAAGGC) for GFP and GUS lines. A positive (plasmid DNA) and negative (non-transformed plant) controls were included in the experiment. A 20 µl reaction mix containing 10 µl Hot Star Taq master mix, 1 µl (10 µM) each of *npt*II F & R primers, 6 µl Nuclease free water, 2 µl (25 ng) of DNA template was prepared and incubated in a thermocycler using the following PCR conditions to amplify the primer specific sites (95 °C: 15 min, 94 °C: 30 sec, 68.2 °C: 30 sec, 72 °C: 1 min (25 cycles), 72 °C: 5 min, 4 °C: ∞). The amplified PCR product was resolved by electrophoresis on a 0.8 % (w/v) agarose gel stained with 1 × GelRed.

5.2.3.3 Southern Blot Analysis of Transformed Plantain

PCR positive events were selected randomly for Southern blot analysis to determine the copy numbers of the transgene integrated into the plant genome. About 20 µg of genomic DNA, 5 µl *Hind*III enzyme HF, and 3 µl cuttsmart buffer was incubated at 37 °C for 18 hours on a heating block (Block heater, Stuart SBH130DC, UK). Fragments were resolved on gel electrophoresis using 0.8 % (w/v) agarose gel, stained with 3 µl gel red and viewed under the UV to ascertain proper restriction. Marker was prepared by adding 1 µl of DIG (digoxigenin; Roche), 4 µl water and 2 µl of 6x loading dye. The gel was run at 80 V for 45 min and 50 V for 4 hours. The excess gel was trimmed off the gel and its dimension measured. The gel was depurinated by placing in depurination buffer (APPENDIX 7) with shaking on a shaker (Mini See-saw rocker SSM4, Stuart, UK) at 40 rpm for 15 min. Indication for complete depurination is a change of the dye from blue to yellow colour. The gel was rinsed twice with sterilized distilled water and placed in denaturation buffer (APPENDIX 7) for 30 min. The colour changed from yellow to blue and was rinsed twice before placing in the neutralization buffer (APPENDIX 7) for 30 min while shaking at 40 rpm. The gel was washed twice with sterilized distilled water and placed in 20 × SSC buffer (APPENDIX 7) for 10 min at 40 rpm to equilibrate the gel. The DNA was blotted on a pre-treated membrane that was cut to the same size as the gel. The casting tray was prepared in a way as to enable gradient ion exchange through a capillary system. About 1000 ml of the 20 × SSC buffer was added to the tray and a filter (20 × 20cm) paper was placed on the casting tray. Same size filter paper (20 × 20) was halved equally and placed in the buffer to overlap the filter on the casting tray to enable a capillary system. Another filter paper, the same size as the gel was placed on top of the overlapping filter paper and the gel flipped over the filter paper and air bubbles carefully removed. The positively charged pre-wetted nylon membrane in 20 × SSC was placed on the gel and same size filter paper placed on top of the membrane such that the gel was sandwiched in between the filter paper and membrane. Sufficient soft tissue was placed on the filter paper and a mass of about 500 g was placed on it such that the membrane could have adequate contact

with the gel and the buffer to enable adequate blotting. This set up was left at room temperature for 18 hours. The stack of wet tissue was removed and replaced with new ones within the 18 hour transfer period. Subsequently, the stack of tissues was removed and the wells of the gel were traced out on the membrane with the aid of a pencil. The orientation of the gel also marked on the membrane. The membrane was moistened by adding some 2 × SSC buffer. To fix the DNA, the membrane was irradiated two times with UV (Energy = 1200 * 100 µJ/cm^2/30 sec) in a UV crosslinker (UVC 500 Crosslinker, USA). The membrane was carefully removed and placed in the hybridization tube containing the 10 ml warm pre-hybridization buffer (Roche Diagnostics, Germany) and incubated at 42 °C for 1 hour while at 60 rpm in a hybridization oven (Hybridization oven/shaker, Stuart, S130H, UK). The probe label was prepared using *npt*II primers (PCR buffer (vial 3): 5 µl, PCR DIG probe synthesis mix (Vial 2): 5 µl, *npt*II Forward primer: 5µl, *npt*II Reverse primer: 5 µl, Enzyme mix (Vial 1): 0.75 µl, plasmid DNA (p2301): 2 µl, Nuclease free water: 29.25 µl). The same components were constituted for the unlabelled probe with the inclusion of 5 µl dNTP stock solution (Vial 4) with exemption of PCR DIG probe synthesis mix. The samples were incubated in a thermocycler (95 °C: 5 min, (95 °C: 30 sec, 60 °C: 30 sec, and 72 °C: 1 min: 34 cycles), 72 °C: 2 min and 4 °C: ∞). About 10 µl of the labelled probe was added to 40 µl nuclease-free water in a sterile tube, mixed by priming, sealed and placed in boiling water. This was allowed to boil for 10 min and placed immediately on ice for 5 min. After cooling, it was added to the fresh pre-hybridization buffer and incubated at 42 °C for 18 hours at 60 rpm. Afterwards, the membrane was washed twice in low stringency buffer (APPENDIX 7) for 10 min each at 45 rpm. The membrane was washed twice with hot (68 °C) high stringency buffer and incubated at 68 °C for 20 min at 45 rpm in a hybridization oven (Hybridisation oven/shaker Stuart S130H, UK). The membrane was then transferred into the normalization buffer (APPENDIX 7) for 5 min at 45 rpm and then in a 100 ml blocking solution (APPENDIX 7) for 30 min at 70 rpm followed by incubation in a 50 ml blocking solution containing 3 µl anti-dioxigenin-antibody for 30 min at 70 rpm. The membrane

was then washed twice in washing buffer (APPENDIX 7) for 45 min and 60 min respectively each at 70 rpm. The membrane was transferred onto a detection buffer (APPENDIX 7) for 5 min at 70 rpm. The buffer was removed and a mixture of 10 µl CDP star and 990 µl detection buffer used to flood the surface of the membrane for about 2 min under dim light and incubated in the dark for 2 min. The membrane was carefully removed and sandwiched in between cling film while excess buffer and bubbles were carefully removed by swiping gently with a soft tissue. Excess cling film was trimmed off and the membrane fastened on an X-ray cassette. The radiograph was done in the dark by placing an X-ray film on the membrane. After 30 min and 2-4 hours of exposure, the film was washed in developer solution for 2 min, rinsed in water for 30 sec, swirled in a fixer solution for 2 min, rinsed for 30 sec and finally viewed in the light.

5.3 Results

5.3.1 Regeneration of Transgenic Events

The plantain cells of Obino l'Ewai generated was successfully transfected with *Agrobacterium* harbouring pCAMBIA 2301 or pCAMBIA 2300-*gfp* containing *npt*II as selection marker *gfp* and *gus*A gene as a reporter gene (Fig 5.2). The regenerated transgenic events were validated by expression of reporter genes using histochemical GUS assay, UV-stereomicroscopy for GFP, and molecular characterization to confirm the presence and integration of the transgene. Transformed embryos developed from transformed cells on embryo development medium (MA3) supplemented with kanamycin while non-transformed cell turned black. Selected transformed embryos further germinated on proliferation medium and individual plantlet developed into complete rooted plants in the hormone-free medium after one month in culture. A total of 141 of fully developed kanamycin-resistant putative transgenic events of Obino l'Ewai were obtained in a single transformation experiment across all the treatments. For *gfp*-T4-2-day co-cultivation period 16 transgenic plants were obtained out of which 15 events tested positive by PCR. The transformation efficiency of treatment 2 (T2) was improved at 3-day co-cultivation period. The number of plantlets generated for *gus*A transgenic events at 2 and 3-day co-cultivation period for treatment 4 (T4) was 15 and 9 plants, respectively all of which tested positive by PCR (Table 5.1). Although stable expression of reporter gene experiment was done once, more than three experiments were done for transient expressions to confirm the transformability of the plantain cells. However, more experiments for stable expression could be done for future studies.

Table 5.1: Transformation efficiency of ECS of plantain Obino l'Ewai with varying concentration of acetosyringone and co-cultivation period

Construct	Treatment	Co-Cultivation Period	No. of Plant Generated on Selective Medium	No. of PCR Positive Plants Generated from 0.5 mL SCV	Transformation Efficiency Plantlets/mL SCV
pCAMBIA 2300-gfp	T2	2D	30	19	38
	T2	3D	17	14	28
	T3	2D	13	10	20
	T3	3D	2	1	2
	T4	2D	16	15	30
	T4	3D	15	12	24
	T5	2D	11	10	20
	T5	3D	11	8	16
pCAMBIA 2301	T2	3D	2	2	4
	T4	2D	15	15	30
	T4	3D	9	9	18

Note: T2-T5-Treatment with various concentration (50, 100, 200, 400 µM) of acetosyringone, 2D: 2-days co-cultivation period, 3D: 3-days co-cultivation period.

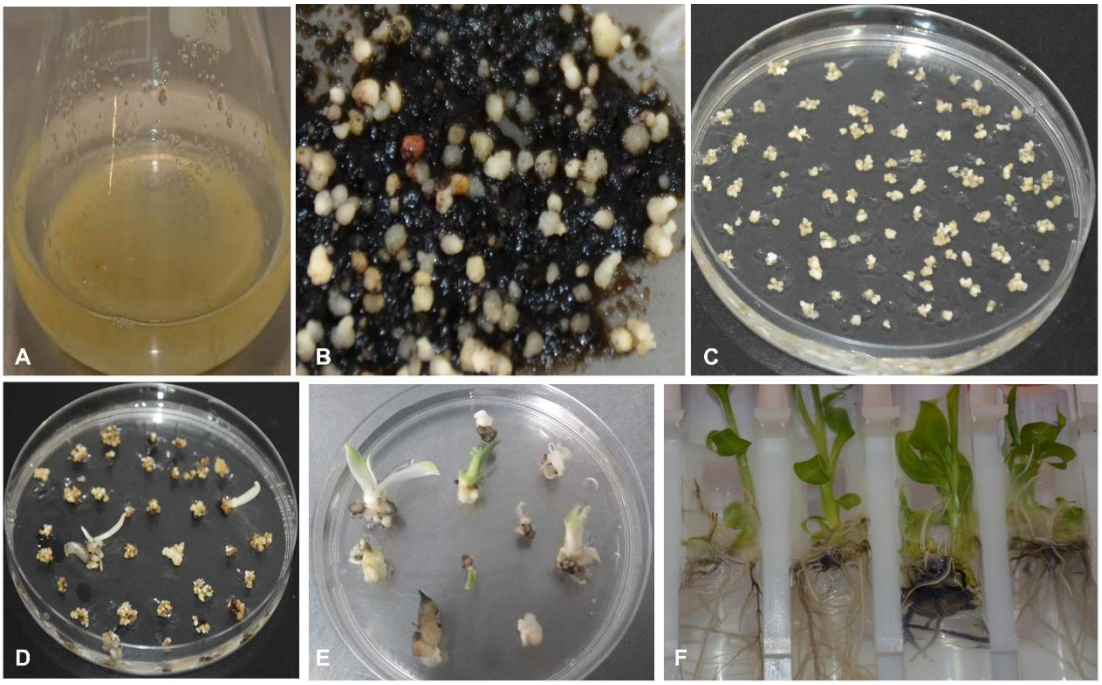


Figure 5.2: Transformation and regeneration of putative transgenic events of plantain cultivar Obino l'Ewai. A: Cell suspension ready to be transformed B: *Agro*-infected cells showing the development of embryos on selective medium containing kanamycin C: Embryo maturation on selective medium D & E: Embryos germinating on selection medium E: Fully regenerated putative transgenic events

5.3.2 Transient and Stable Expression of Reporter Genes

Transient and stable expression of the *gfp* and *gus*A reporter genes confirmed that all the three plantain cultivars were transformable. The blue coloration as the transient expression of *gus*A gene confirmed the transformability of ECS of the three plantain cultivars through *Agrobacterium*-mediated transformation (Fig 5.3 a & b). The blue pigmentation observed when X-gluc reacted with beta-glucuronidase (GUS), an enzyme produced by the *gus*A gene in the transformed ECS, is an indication of expression of the reporter gene in the plant cells. The higher expression of the *gus*A gene was observed in cells with treatment T2-T5 (with acetosyringone) compared to T1 (no acetosyringone) at both 2-days and 3-days co-cultivation of ECS with *Agrobacterium*. The blue coloration and green fluorescence were in embryos and leaf explants of transgenic events (Fig 5.3 a&b).

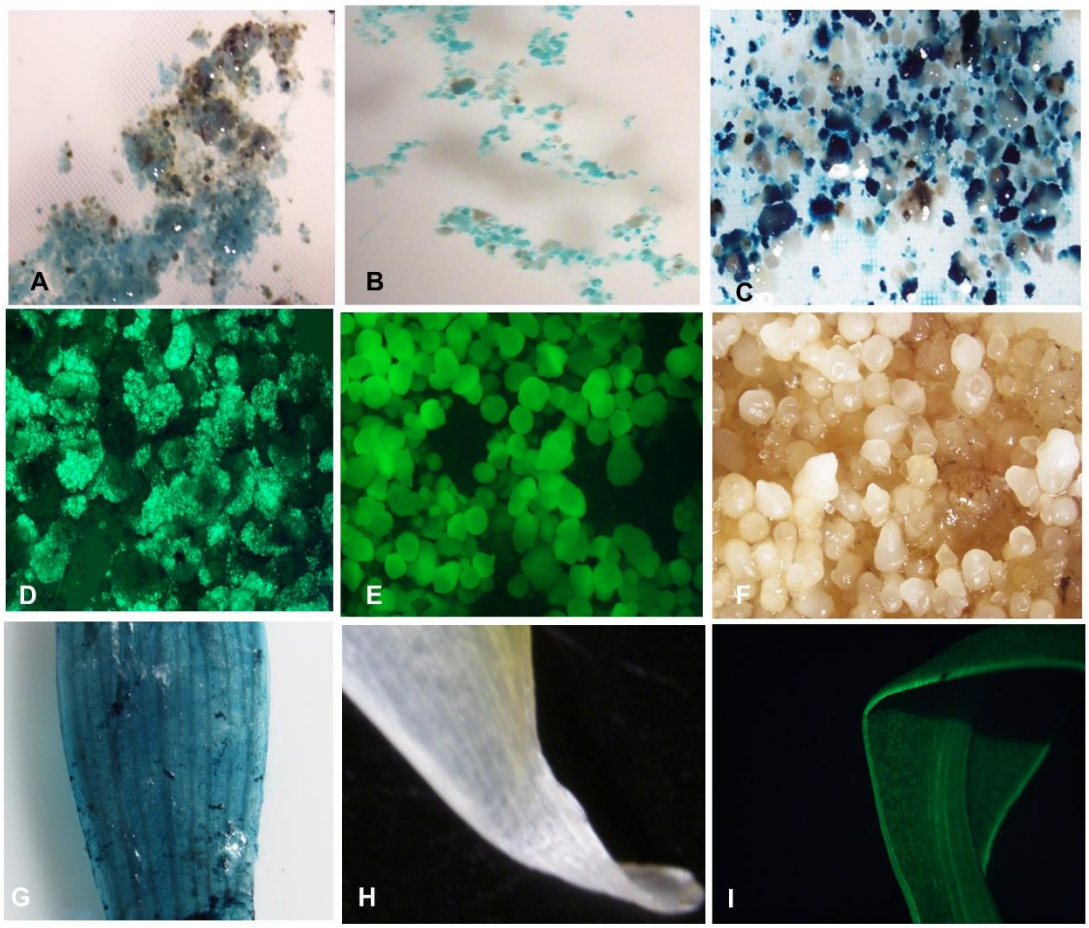


Figure 5.3a: Transient and stable expressions of *gus*A and *gfp* in transformed plantain. A-C: Transient expression of *gus*A gene in Agro-infected embryogenic cell suspension (ECS) of different cultivars, A: Obino l'Ewai B: Orishele C: Agbagba; D: Transient expression of *gfp* gene in the *Agro*-infected ECS of Obino l'Ewai, E: Expression of *gfp* gene in mature embryos of Obino l'Ewai, F: Control non-transformed embryos of Obino l'Ewai, G: Stable expression of *gus*A gene in leaf explant of the transgenic plant of Obino l'Ewai, H: Control leaf from non-transgenic plant of Obino l'Ewai, I: **S**table expression of *gfp* gene in leaf explant of the transgenic plant of Obino l'Ewai. Photographs (A–I) were taken using stereomicroscope (SMZ1500) with 10X magnification.

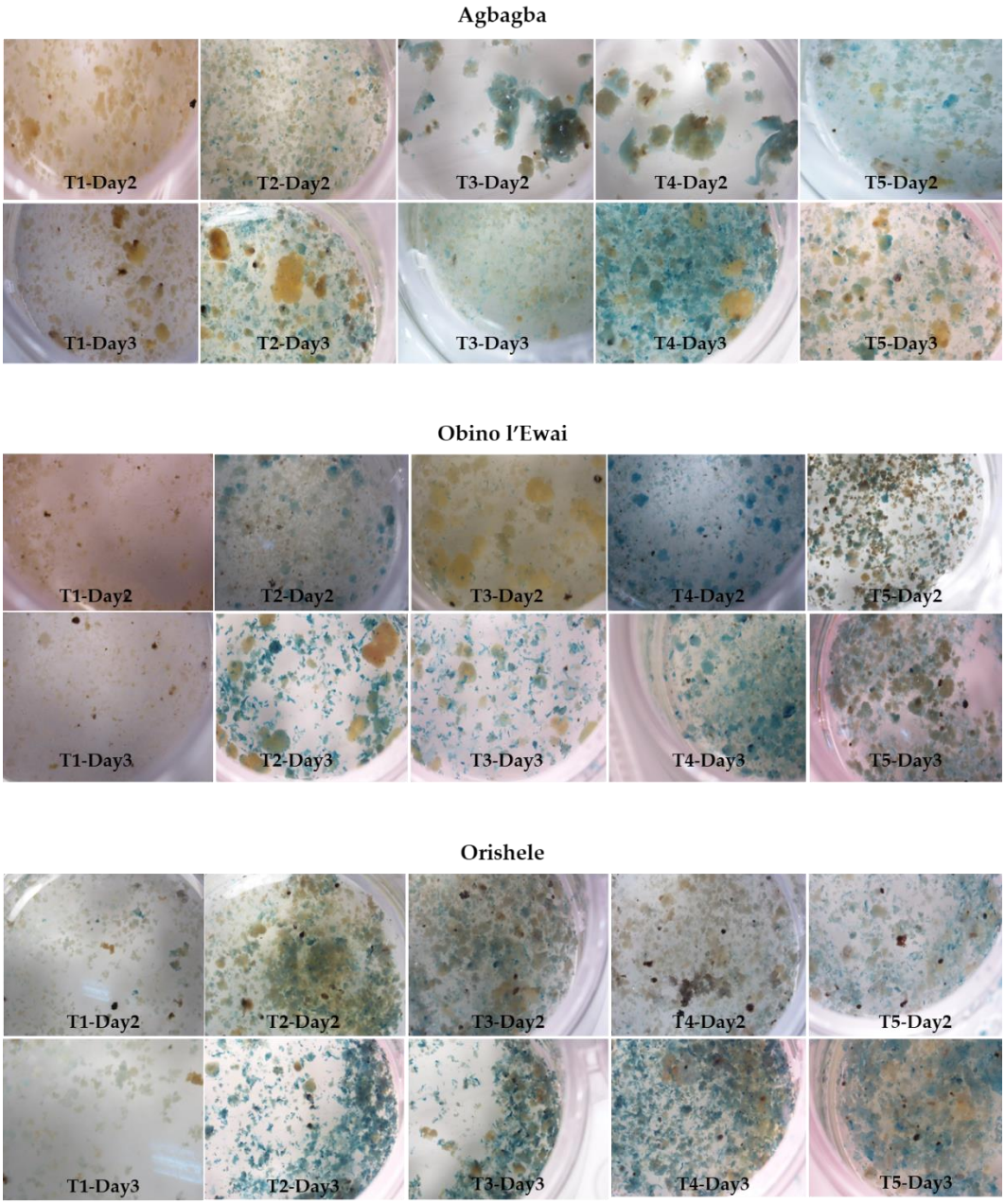


Figure 5.3b: Transient expression of *gus*A gene across the five treatments and two co-cultivation periods for the three plantain cultivars Agbagba, Obino l'Ewai and Orishele. T1-T5: 0, 50, 100, 200, 400 µM of acetosyringone, respectively.

5.3.3 Molecular Characterization of Putative Transgenic Events

5.3.3.1 Genomic DNA Isolation

Genomic DNA was successfully isolated from 141 putative transgenic events using the CTAB method as described in section 5.2.3. The quantification was done using Nanodrop spectrophotometer while the quality was determined by electrophoresis on a 1 % agarose gel (Fig 5.4).

5.3.3.2 PCR Analysis

The amplification of the gradient PCR indicated that at 68.2 °C annealing temperature, a distinct amplicon was observed with the absence of non-specific bands in the non-template control (Fig 5.5). Also, a validation of the PCR conditions with positive controls, wild type (negative control) and random samples confirmed the optimal annealing temperature for PCR (Fig 5.5). A total of 115 transgenic plants were confirmed positive by PCR. The expected amplicon size of 781 bp was obtained in all the PCR positive events confirming the presence of *npt*II genes in the genome of respective transgenic plantain plants tested (Fig. 5.6, Table 5.1).

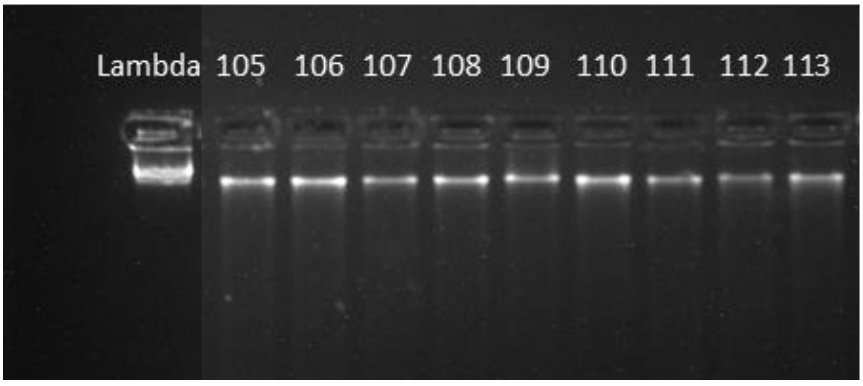


Figure 5.4: Genomic DNA of selected putative transgenic events of plantain cultivar Obino l'Ewai. Lambda: Lambda genomic DNA; samples 105-113 represent transgenic events of Obino l'Ewai

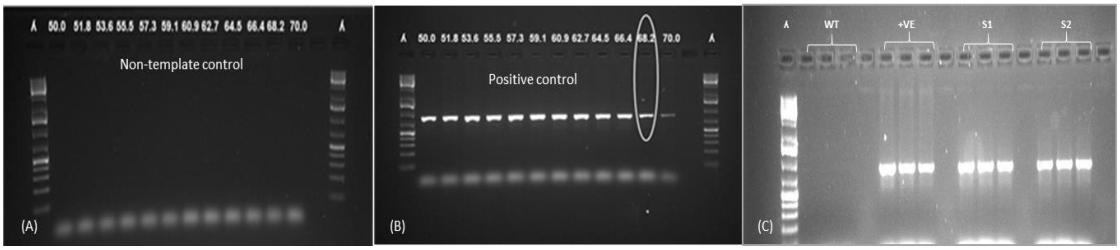


Figure 5.5: Gradient PCR of genomic DNA using *npt*II primers. (A), Non-template control, (B), Positive control (pCAMBIA 2301 plasmid), (C), Validation of *npt*II PCR primers WT: wild type (non-transgenic), +ve: plasmid DNA as a positive control, S1: Transgenic plant sample 1, S2: Transgenic plant sample 2, ʎ: 1kb+ ladder (Thermo Fisher Scientific)

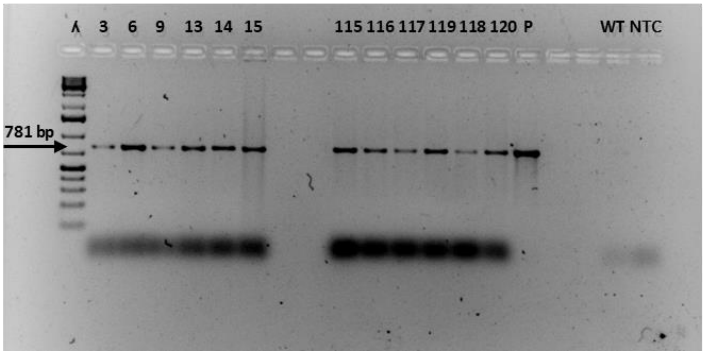


Figure 5.6: PCR analysis of selected transgenic events of plantain cultivars using *npt*II specific primers. ʎ: 1 kb+ DNA ladder (Thermo Fisher Scientific), Samples 3- 15: *gfp* transgenic events, Samples 115-120: *gus*A transgenic events, P: plasmid DNA as positive control, WT: non-transgenic plant, NTC: non-template control

5.3.3.3 Southern Blot

The Southern blot results of the selected events across the treatment tested confirmed the integration of transgenes in transgenic events tested. (Fig 5.7). The *Hind*III restriction enzyme has a single restriction site in the pCAMBIA 2301 and pCAMBIA 2300-*gfp* constructs. The eight transgenic events tested showed positive bands with varying copy number, however no band was observed in non-transgenic control plant (wild type). Irrespective of the concentration of acetosyringone used and the co-cultivation period that the plant cells were subjected to, reporter genes were successfully integrated into the

plantain genome. In addition, the non-transgenic plant (wild type) has no band which is an indication of absence of reporter transgene.

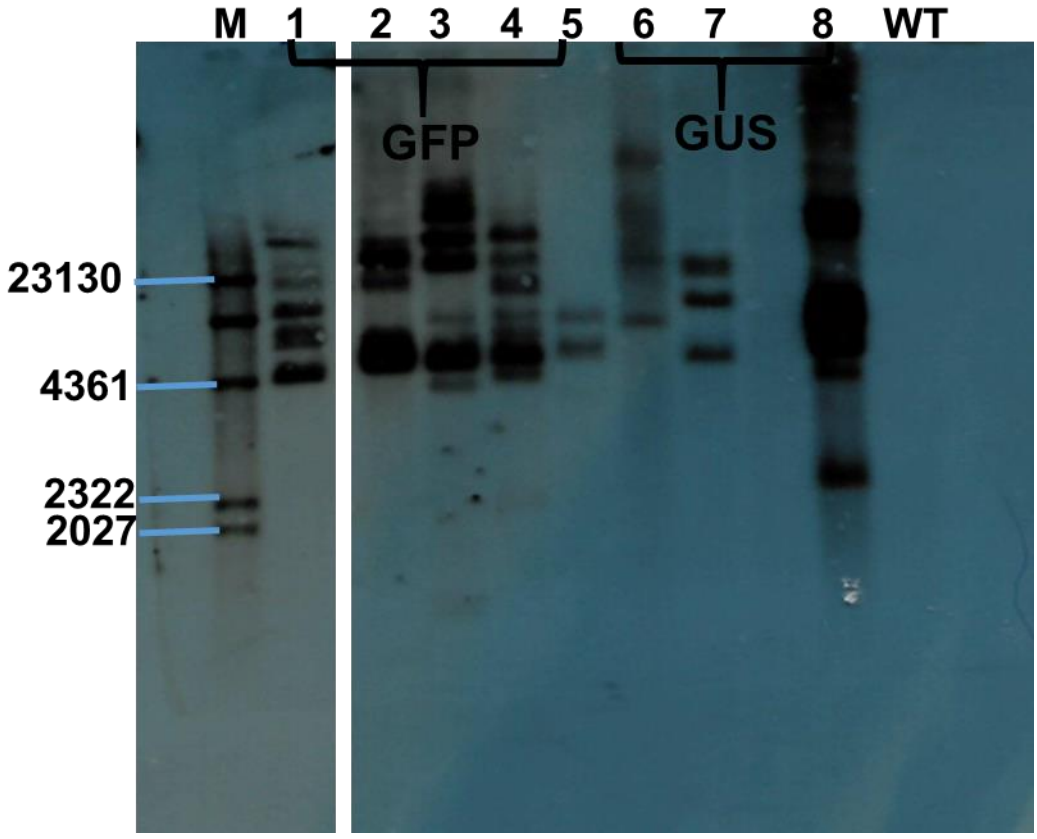


Figure 5.7: Southern blot of selected transgenic events using *npt*II probe to detect *gfp* and *gus*A genes. M: DIG molecular marker (Roche Diagnostics, 0.12-23.1 kbp) Sample 1-8 (GFP 2-2D, GFP 4-2D, GFP 4-3D, GFP 5-2D, GFP 5-3D, GUS 2-3D, GUS 4-2D, GUS 4-3D) WT: wild type

5.4 Discussion

Transformability of the embryogenic plantain cells is essential for downstream experimental procedures especially for crop genetic improvement. This was determined using *Agrobacterium*-mediated transformation procedure with *Agrobacterium* strain EHA 105 harbouring pCAMBIA 2300-*gfp* and pCAMBIA-2301 *npt*II as selection marker and *gfp* and *gus*A as reporter genes respectively. The transient and stable *gfp* and *gus*A expressions indicated the transformability of the plantain cells of Agbagba, Obino l'Ewai and Orishele that were generated (Fig 5.3). This was further confirmed by molecular characterization of the transgenic events (Fig 5.6, Table 5.4) which gave a product size of 781 bp.

The influence of acetosyringone, analogous to the type of phenolic compound secreted when dicot plants are wounded was observed on the transformation efficiency of the plantain ECS. This compound is known to activate the virulence (*vir*) gene in *Agrobacterium* by binding to the receptor of the cell, hence enabling the transfer of the T-DNA into the plant cell (Nakano, 2017). The synthetic form of this naturally occurring compound is employed during *Agrobacterium*-mediated transformation of dicots like cotton (Afolabi-Balogun *et al*., 2014) and monocot plants like rice (Xi *et al*., 2018), wheat (Ahmadpour *et al.*, 2016) and *Musa* spp. (Liu *et al*., 2017) to improve its transformation efficiency. About 100 - 250 µM acetosyringone has been successfully reported for bananas (Tripathi *et al*., 2005; Rao *et al*., 2014; Liu *et al*., 2017).

The result obtained from this study is in line with the existing reports. Although, this work has also shown that a lower concentration of 50 µM acetosyringone for co-cultivation can be used to achieve transformation in Obino l'Ewai. A higher concentration of 200 µM and 400 µM acetosyringone is equally efficient in transforming plantain cells within 2 days of co-cultivation period which is similar to previous studies (Tripathi *et al*., 2012, 2015; Liu *et al*., 2017).

The co-cultivation period also influences the efficiency of *Agrobacterium*-mediated transformation of plantain cells. Although, 2-day co-cultivation period favoured the generation of more transformed plants, 3 day co-cultivation period is also beneficial and doesn't impose any negative influence on transformation efficiency. This similar observation was also confirmed together with optimum co-cultivation of 2-day period in the transformation of tobacco (Uranbey *et al*., 2005) and cucumber (Faisal *et al*., 2015). However, both authors did not state if acetosyringone was used to improve the transformation efficiency. Also, previous work on banana suggested an optimum co-cultivation period of 3 and 4 days (Chee *et al*., 2006; Sreeramanan *et al*., 2006; Tripathi *et al*., 2012, 2015).

Transient expressions by GUS assay showed that acetosyringone is essential for successful transfection of plantain cells. Varying intensity of blue colouration as marked by expression of *gus*A gene showed the influence of acetosyringone and co-cultivation period of *Agrobacterium* with plantain cells studied. As depicted by the transient expression of reporter genes, a transfection period of 3-days may allow more cells to be transformed.

The Southern blot analysis confirmed the integration of both *gfp* and *gus*A gene in the plant as well as the independence of the events generated as confirmed by the varying copy numbers. The result also confirmed that the successful transformation of plantain cell can be obtained either at 2-days or 3-days of co-cultivation period and low concentration (50 µM) of acetosyringone in the co-cultivation medium of plantain transformation.

5.5 Conclusion

In this study, *Agrobacterium*-mediated transformation of three plantain cultivars; Agbagba, Obino l'Ewai and Orishele was successfully established. It was observed that for the plantain cultivars, transformation procedure is optimum at 2 and 3-day co-cultivation period. The viability of the cell also contributes to the efficiency of *Agrobacterium* transformation as cells in the dormant phase may not be susceptible to transfection. In addition, this is the first report for the successful use of 50 µM acetosyringone concentration for co-cultivation in plantain transformation.

CHAPTER SIX

DEVELOPMENT OF TRANSGENIC PLANTAIN AND BANANA RESISTANT TO BANANA APHIDS USING RNA INTERFERENCE

6.1. Introduction

Plantain and banana are one of the major staples, providing food and a source of livelihood to over 500 million people around the world (Tripathi *et al*., 2019). However, several pests and diseases plague the production of *Musa* spp. (Ploetz, 2006; Pérez-vicente, 2014; Viljoen *et al*., 2016; Tripathi *et al*., 2019). Banana bunchy top disease (BBTD) has been identified as one of the economically important diseases that significantly affects banana and plantain production. BBTD is caused by banana bunchy top virus (BBTV) and it is spread by banana aphids (*Pentalonia nigronervosa*). In severe cases, this disease is able to cause up to 100 % loss in banana and plantain production (Selvarajan & Balasubramanian, 2014). Dependency on insecticides for the control of BBTD is not ideal because of environmental pollution, the effect on off-target-organisms, insecticide resistance, etc. Therefore, the availability and potential of developing pest-resistant bananas and plantains through genetic engineering provide a durable way of enabling greater resilience in various climatic conditions (Schuler *et al*., 1998) hence, increasing banana and plantain production.

Agrobacterium-mediated genetic transformation offers a valuable and efficient tool for crop improvement and production (Abalaka, 2011). The availability of robust and reproducible transformation systems makes it possible for the modification of several crop species for agronomic improvement. These facilitates the activation of certain immune response mechanisms such as RNA interference (RNAi) in plants to enable resistance to pests and diseases.

RNAi is a post-transcriptional gene silencing mechanism whose potency to activate innate resistance against viruses and insect pests have been established (Zha *et al*., 2011; Guo *et*

al., 2014) and it could be an alternative strategy to control banana aphids. RNAi has been successfully used in mediating resistance against green peach aphid, coleopteran insect pest, and *Nilaparvata lugens* (Whyard *et al*., 2009; Zha *et al*., 2011; Guo *et al*., 2014). Furthermore, RNAi is a sequence-specific strategy whose effectiveness relies to an extent on the selection of a target gene. It is important to select a gene of crucial function in the aphid life cycle for disruption. This study targeted acetylcholinesterase (*AChE*), an enzyme that plays a significant role in the physiology, fecundity and survival of banana aphids. To determine the effect of *AChE* dsRNA uptake on banana aphid, firstly the available sugar content of banana and plantain plants was determined. An artificial liquid feeding complex containing sucrose and an increasing levels of *AChE* dsRNA was then developed. Aphids fed on the dsRNA-containing media were monitored over time for mortality. Transgenic banana and plantains expressing hpRNA targeting *AChE* gene were then generated by *Agrobacterium*-mediated transformation of cell suspensions. Plants were genetically characterized and transgenic plants screened for resistance to aphids under glasshouse conditions.

6.2 Materials and Methods

6.2.1 *In vitro* Feeding of Banana Aphids with Synthetic Diet Laced with dsRNA and siRNA

6.2.1.1 Rearing of Banana Aphids

Single non-viruliferous banana aphid was isolated from a banana plant in the field (GPS coordinates: Latitude -1.270224 Longitude 36.72312, Altitude 1823.00) at the International Livestock Research Institute (ILRI) Nairobi, Kenya. Aphid was introduced on an eight weeks old potted tissue culture banana plant in an insect-proof cage. The cage was placed in a glasshouse under room temperature and normal light. A single aphid (progeny) was isolated from the plant to initiate a fresh population on another young plant after 4-8 weeks to obtain a pure colony.

6.2.1.2 Optimization of an Artificial Diet for Banana Aphids (*Pentalonia nigronervosa*)

P. nigronervosa diet composition was optimized based on the protocol developed by Dadd and Mittler (1966). The various components as listed in Table 6.1 were dissolved in milli-Q water. Varying concentration (0 g, 5 g, 7.5 g, 15g, 20 g and 30 g) of sucrose was tested for each diet type respectively to determine optimum sucrose concentration for *P. nigronervosa*. The pH of diets was adjusted to approximately 7.5 with 1 M KOH. Each diet was filter sterilized using a 0.2 µm filter in a laminar flow hood. Diet aliquots were stored at -20 °C and thawed when needed. The control diet (Diet 5) containing only milli-Q water was included.

Table 6.1: Diet composition for banana aphid (*Pentalonia nigronervosa*)

Composition	**Quantity (mg)/100 ml diet**			
	Diet 1	**Diet 2**	**Diet 3**	**Diet 4**
Di-potassium hydrogen orthophosphate	750.0	750.0	750.0	750.0
Magnesium sulphate	_	123.0	_	123.0
Magnesium chloride	123.0	_	123.0	_
L-tyrosine	40.0	40.0	40.0	40.0
L-asparagine hydrate	550.0	550.0	550.0	550.0
L-aspartic acid	140.0	140.0	140.0	140.0
L-tryptophan	80.0	80.0	80.0	80.0
L-alanine	100.0	100.0	100.0	100.0
L-arginine monohydrochloride	270.0	270.0	270.0	270.0
L-cysteine hydrochloride, hydrate	40.0	40.0	40.0	40.0
L-glutamic acid	140.0	140.0	140.0	140.0
L-glutamine	150.0	150.0	150.0	150.0
L-glycine	80.0	80.0	80.0	80.0
L-histidine	80.0	80.0	80.0	80.0
L-isoleucine (allo free)	80.0	80.0	80.0	80.0
L-leucine	80.0	80.0	80.0	80.0
L-lysine monohydrochloride	120.0	120.0	120.0	120.0
L-methionine	40.0	40.0	40.0	40.0
L-phenylalanine	40.0	40.0	40.0	40.0
L-proline	80.0	80.0	80.0	80.0

L-serine	80.0	80.0	80.0	80.0
L-threonine	140.0	140.0	140.0	140.0
L-valine	80.0	80.0	80.0	80.0
Ascorbic acid (vitamin C)	100.0	100.0	100.0	100.0
Aneurine hydrochloride (vitamin B)	2.5	2.5	2.5	2.5
Nicotinic acid	10.0	10.0	10.0	10.0
Folic acid	0.5	0.5	0.5	0.5
(+)-pathothenic acid (calcium salt)	5.0	5.0	5.0	5.0
myo-inositol	50.0	50.0	50.0	50.0
Choline chloride	50.0	50.0	50.0	50.0
EDTA Fe(III)-Na chelate pure	1.5	_	_	_
EDTA Zn-Na_2 chelate pure	0.8	_	_	_
$MnCl_2.4H_2O$	0.8	_	_	_
EDTA Cu-Na_2 chelate pure	0.4	_	_	_
$FeSO_4.7H_2O$	_	1.5	_	_
$ZnSO_4.\ 7H_2O$	_	0.8	_	_
$MnSO_4.H_2O$	_	0.8	_	_
$CuSO_4.5H_2O$	_	0.4	_	_
Pyridoxine hydrochloride (vitamin B6)	2.5	2.5	2.5	2.5
D-biotin, crystalline	0.1	0.1	0.1	0.1

6.2.1.3 Preparation of dsRNA and siRNA for *AChE* Gene

6.2.1.3.1 Plasmid Construct Acquisition, Transformation and Isolation

Plasmid (pGEMT-ACE 400) containing the *AChE* gene (400 bp) of *P. nigronervosa* was acquired from the Queensland University of Technology (QUT), Australia. The plasmid construct was transformed into competent cells of *E. coli*. Competent *E. coli* cells (50 µl) was thawed on ice for 15 min before addition of 2 µl of the plasmid construct pGEMT-ACE 400 and mixed by priming in a sterile Eppendorf tube. The tube containing the cell mixture was plunged into a warm water bath at 42 °C for 90 secs and immediately cooled on ice for 2 min. Thereafter, 100 µl LB broth was added to the culture and incubated at 37 °C and at 200 rpm for 1 hour on a rotary shaker (Incubator Shaker Series, New Brunswick scientific, INNOVA 40). About, 100 µl of the culture was plated on LB semisolid media supplemented with ampicillin (50 mg/ml) and incubated overnight at 37

°C. An individual colony was carefully picked using a sterile tip and cultured in LB broth media supplemented with ampicillin and incubated overnight at 37 °C and at 200 rpm on a rotary shaker. The plasmid DNA was isolated from 4ml of the culture using Invitrogen PureLink Quick Plasmid Miniprep Kit (K2100-10 and K2100-11). The culture medium was centrifuged (Eppendorf centrifuge 5424 R, Germany) at 14000 rpm for 5 min, the LB medium removed and the bacterial pellet re-suspended in 250 µl of re-suspension buffer (R3) containing RNase A. To the suspension, 250 µl lysis buffer (L7) was added and mixed by inverting the tubes and incubated for 5 min at room temperature. About 350 µl of precipitation buffer (N4) was added to the solution and mixed immediately by inverting the tubes. The lysate was centrifuged (Eppendorf centrifuge 5424 R, Germany) at 14000 rpm for 10 min. The supernatant was transferred into the spin column and centrifuged (Eppendorf centrifuge 5424 R, Germany) for 1 min at 12000 rpm. The flow-through was discarded and the column placed in the wash tube. About 700 µl wash buffer (W9) containing ethanol was added to the column and centrifuged (Eppendorf centrifuge 5424 R, Germany) for 1 min at 12000 rpm. The effluent was discarded and the column replaced in the wash tube and spurned in the centrifuge at 12000 rpm for 1 min. The spin column was placed in a sterile 1.5 ml Eppendorf tube and 75 µl nuclease-free water was added to the centre of the column. This was incubated at room temperature for about 2-3 min before centrifuging (Eppendorf centrifuge 5424 R, Germany) at 12000 rpm for 2 min. The spin-column was discarded while the purified plasmid DNA was stored at -20 °C.

6.2.1.3.2 Restriction Digestion and Production of dsRNA

The purified plasmid DNA was linearized overnight by restriction digest using two restriction enzymes (*Nco*l and *Sal*I) to produce the SP6 (sense) and T7 (antisense) transcripts respectively. The reaction mix includes *Nco*l (SP6) or *Sal*I (T7): 1 µl Buffer: 3 µl (2.6 µg) pGEMT-ACE 400 plasmid DNA and nuclease-free water to make a total of 30 µl reaction mix. Each of the restriction digest mixture (i.e. SP6 and T7 respectively) was incubated on a heating block at 37 °C overnight. Single-stranded RNA was produced using the SP6 and T7 RNA polymerase (Promega); 5× buffer: 20 µl, 100 mM DTT: 10

µl, RNase inhibitor: 2.5 µl, rNTP mix (2.5 mM each): 20 µl, linear pGEMT-ACE 400 pDNA: 3 µg, SP6 (*Nco*I) or T7 (*Sal*I): 4 µl and Nuclease free water to make a total of 100µl. The reaction mix was incubated on a heating block at 37 °C for 90 min. This was followed by a DNase treatment (Promega) to remove any residual DNA template (11 µl 10 × buffer, 3 µl RQ1 RNase-Free DNase; 100 µl Sample). The reaction was incubated for 15 min at 37 °C. The ssRNA transcripts were purified by addition of 114 µl of chloroform: isoamyl alcohol (24:1) to the reaction mix. It was centrifuged at room temperature (RT) for 2 min at 10,000 rpm. The supernatant was collected in sterile 1.5 ml eppendorf tube before addition of 57 µl of ammonium acetate and 285 µl of ice-cold absolute ethanol and mixed by priming. The mixture was incubated at -80 °C for 30 min and centrifuged for 20 min at 14000 rpm. The pellet was washed with 1 ml ice-cold 80 % ethanol and centrifuged for 5 min at 14000 rpm. The ethanol was discarded and the pellet dried in a laminar flow hood. The pellet was suspended in 30 µl of 1 × annealing buffer (6 mM HEPES, 20 mM NaCl). The solution was combined in a PCR compatible tube and annealed by boiling slowly for 1 hour in a thermocycler (PCR cycles: 95 °C for 2 min, the temperature was decreased by 1 °C every 2 min till it reaches79 °C. The temperature was further decreased by 2 °C for every 2 min till it reached 30 °C, 30 °C for 2 min, and 25 °C for 2 min and 4 °C ∞). The concentration of the dsRNA was determined by spectrophotometry.

6.2.1.3.3 Production of siRNA

The dsRNA was digested to generate siRNA and purified using the Shortcut RNase III protocol (Biolabs, M0245S). A reaction mix containing 40 µl nuclease-free water, 10 µl 10 × ShortCut reaction buffer, 10 µg of dsRNA (30 µl), 10 µl Shortcut RNase III and 10 µl 10 × $MnCl_2$ was mixed by priming and incubated at 37 °C on a heating block for 20 min. About 10 µl EDTA was added to the sample to stop the reaction. The siRNA was purified by adding a one-tenth volume of 3 M NaOAc (pH 5.5), 2 µl RNase-free glycogen and 3 volumes of cold 95 % molecular grade ethanol. The solution was incubated at -70 °C for 30 min or at -20 °C for 2 hours. The sample was centrifuged for 15 min at 14000

rpm. The supernatant was removed and 2 volumes of 80 % ethanol added to the pellet. This was incubated at room temperature for 10 min and centrifuged at 14000 rpm for 5 min. The pellet was air-dried and resuspended in 10 mM Tris HCl, 1mM EDTA. The siRNA generated was quantified on the NanoDrop spectrophotometer.

6.2.1.4 Aphid *In vitro* Feeding Assay

Diet tube was made by cutting 15 ml Falcon tube to about 10 mm × 10 mm. Rough edges were smoothened to prevent it from piercing the parafilm and cause leakage and contamination of diet. Adequate sterile conditions are essential for optimum aphid rearing and bioassay. All items (parafilm, diet tubes, pipette, sterile pipette tips, gloves, soft tissue) except for the diet were sterilized in a UV hood for 30 min. With the aid of the sterile gloves, the parafilm was carefully stretched over one side of the diet tube without touching the sterile surface. About 60 - 100 µl of filtered-sterilized diet was dispensed on the stretched parafilm and covered with another piece of parafilm such that the diet was firmly sandwiched between the two stretched parafilm sterile surfaces (Fig 6.1).

Twenty adult aphids were collected from potted plants and introduced on the piece of banana pseudostem in a petri-plate. After 24 - 48 hours, four first instar aphids were collected and placed on the diet from the open end of the diet tube. The open end was covered by stretching a piece of parafilm over it to secure the aphids in the diet tube. The diet tubes were placed in a controlled conviron chamber (CMP 6050, Canada) at 25 °C, 75 % RH and 12:12 photoperiod.

The artificial feeding assay was first optimized before the feeding experiment to compare the effect of diet type and sucrose concentration on banana aphid. To avoid overcrowding of aphids and competition for nutrients, four aphids were introduced on a single micro-cage and replicated three times. The experiment was repeated until consistent results were obtained for the optimization process.

After optimizing the conditions, the feeding assay experiments were carried out. Four first instar aphids were isolated from the banana plant as described above and placed in a micro-cage, this was replicated thrice and the experiment was repeated twice to ensure reproducibility of results. Aphids were kept under controlled conditions as stated above. This was monitored daily to ensure aphids were feeding on diet and that no leakage or contamination of diet occurred. The number of aphids was counted at 5 and 7-day post-feeding.

Following the artificial feeding assay to determine the optimal diet type and sucrose concentration that promoted the growth and reproduction of banana aphid, the diet was used to carry out further experiments to see the effect of dsRNA and siRNA on banana aphid.

An artificial diet containing a varying concentration of dsRNA was made by adding 0, 100, 200, 300 and 500 ng of freshly prepared dsRNA separately to the selected diet type. This was dispensed on the micro-cages under the laminar flow hood. Four first instar aphids were isolated as earlier described and initiated on the dsRNA laced diet. This was replicated and repeated twice. All experiments were monitored daily and mortality was recorded at 2, 3, and 7-day post-feeding.

Freshly prepared siRNA was added to the optimal diet as earlier described at 0, 50, 100 and 250 ng. The experiment was first optimized by subjecting four first instar aphids to feed on the siRNA diet and a control containing no siRNA was used. Following the optimization, a single experiment with 3 replicates per treatment was done due to the limited availability of siRNA synthesis kit. The result of the siRNA was based on the single experiment and replicates that remained intact at the point of data collection.

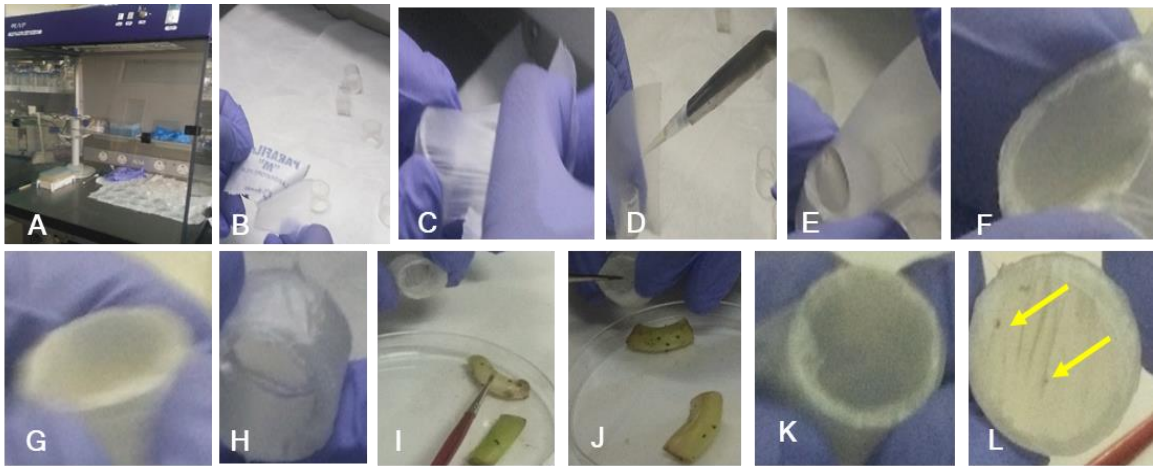


Figure 6.1: Procedure (steps A-L) for making synthetic diet sachets and inoculating aphids in micro-cages. (procedure was adopted from Douglas & van Emden, 2007). A: sterilization of materials under ultra violet (UV) light, B & C: stretching sterilized parafilm over one opening of the micro-cage, D: dispensing sterile diet on parafilm, E: covering the diet with another piece of sterile parafilm, F & G: diet sandwiched between two layers of parafilm on a micro-cage, H: open end of the micro-cage for aphid inoculation, I: isolation of first instar aphid with the aid of a fine tip brush, J: placing isolated aphid on the diet through the open end of the micro-cage, K: sealing the open end with a sterile parafilm to prevent aphid from escaping the cage, L: arrows indicating aphid feeding on diet.

6.2.1.5 RNA Extraction from Aphids and RT-PCR

Five live aphids that were fed on a synthetic diet laced with dsRNA, siRNA and control were collected with the aid of a fine (0.4 mm) tip brush in a 2 ml Eppendorf tube and placed on ice. Aphids were frozen in liquid nitrogen and homogenized with the aid of a sterile pestle. About 500 µl of Trizol reagent solution was added and mixed by priming. The mixture was allowed to incubate at room temperature for 15 min and vortexed intermittently during the incubation period. About 100 µl chloroform was added and left at room temperature for 15 min with occasional mixing. Samples were centrifuged at 10000 rpm and 4 °C for 10 min to separate the organic and aqueous phase. The supernatant was transferred into a new sterile 1.5 ml Eppendorf tube. An equal volume of cold

isopropanol was added to the supernatant and mixed by inversion 5-6 times. Samples were left to incubate at room temperature for 10 min and subsequently, 300 µl of 75 % ethanol was added. The samples were incubated on ice for 5 min before spinning at 7500 rpm for 5 min and pellets dried for 15 min. The pellet was re-suspended with 30 µl nuclease-free water and RNA quantification was done using a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, USA).

The complementary DNA (cDNA) was synthesized using the maxima reverse transcriptase enzyme mix (Thermo Scientific, K1642, R1362). The reaction mix was prepared by adding 1 µg of RNA (extracted from aphid), 4 µl of 5 × buffer, 2 µl enzyme and nuclease-free water to make a 20 µl reaction in a sterile PCR tube. Samples were incubated in a thermocycler at 25 °C for 10 min, 55 °C for 30 min, 85 °C for 5 min and 4 °C ∞.

To measure the RNA expression levels, the reverse transcriptase PCR (RT-PCR) was performed using 1 µg (1 µl) cDNA, 10 µl of the HS Taq premix, 0.2 µl each of the *AChE* specific forward and reverse primer and nuclease-free water was added to make a 20 µl reaction. The samples were incubated in a thermocycler at 95 °C for 15 min, (94 °C for 30 sec, 57 °C for 30 sec, 72 °C for 30: 35 cycles), 72 °C for 5 min and storage 4 °C. A housekeeping control (*Buchnera aphidicola*) primer pair (F: GGTGGAGCATGTGGTTTAATTC; R: CCGATGGCAACAGAGGATAA) was also used as internal control using same PCR conditions but 60.1 °C was used as the annealing temperature. Samples were resolved on 1 % (w/v) agarose gel electrophoresis ran at 70 V for 90 min.

6.2.2 Description and Validation of Plasmid Construct pNXT-35S-ACE-hp

6.2.2.1 Description of Plasmid construct

Plasmid construct pNXT-35S-ACE hp was processed and acquired from the Queensland University of Technology (QUT), Australia. The plasmid contains kanamycin resistance gene (*npt*II) under NOS promoter and *AChE* gene (400 bp inverted-repeat DNA fragment) under the regulation of CAMV 35S promoter (Fig 6.2).

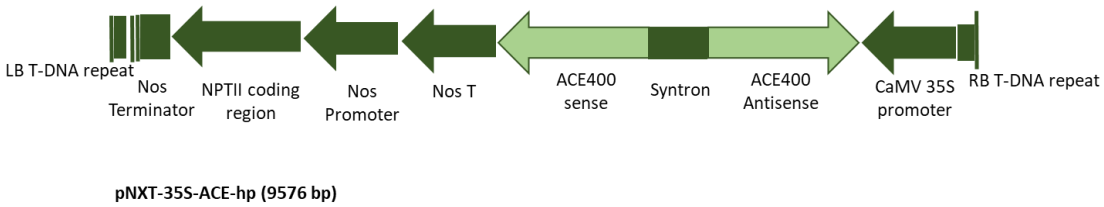


Figure 6.2: Map of plasmid construct of pNXT-35s-ACE-hp. Expression of the *AChE* gene (ACE400) is driven by the Cauliflower Mosaic Virus 35S promoter and the kanamycin resistance gene (*npt*II) as the selection marker under NOS promoter.

6.2.2.2 Validation of Plasmid Construct

6.2.2.2.1 Introduction of Plasmid Construct into *E. coli*. Strain (DH5α)

To 2 ml of LB medium, 2 µl of *E. coli* starter culture was added and incubated overnight at 37 °C on a shaker at 200 rpm. The overnight culture was refreshed in the ratio 1: 100 (culture: LB medium) and incubated at 37 °C for a further 3-4 hours at 200 rpm. The culture was placed immediately on ice for 10 min and centrifuged at 6000 rpm for 3 min at 4 °C. The supernatant was removed and the pellet resuspended in 0.1 M $CaCl_2$ and incubated on ice for 60 min. The culture was centrifuged at 4 °C for 10 min at 4000 rpm. The supernatant was removed and the pellet resuspended in 500 µl ice-cold 0.1 M $CaCl_2$ containing 15 % glycerol. An aliquot of 50 µl of the culture was dispensed in a 1.5 ml Eppendorf tube and frozen in liquid nitrogen before storage at -80 °C.

Competent *E. coli* cells (50 µl) was thawed on ice for 15 min before addition of 2 µl of the plasmid construct pNXT-35S-ACE-hp and mixed by priming in a sterile Eppendorf

tube. The tube containing the cell mixture was plunged into a warm water bath at 42 °C for 90 secs and immediately cooled on ice for 2 min. Thereafter, 100 µl LB broth was added to the culture and incubated at 37 °C and at 200 rpm for 1 hour on a rotary shaker (Incubator Shaker Series, New Brunswick scientific, INNOVA 40). About, 100 µl of the culture was plated on LB semisolid media supplemented with kanamycin (50 mg/L) and incubated overnight at 37 °C. An individual colony was carefully picked with a sterile tip and cultured in LB broth media supplemented with kanamycin and incubated overnight at 37 °C and 200 rpm on a rotary shaker. *E. coli* cultures were dispensed in a 2 ml sterile tube and centrifuged at 13000 rpm for 30 seconds at 4 °C. The supernatant was discarded, excess medium removed and transfected cell pellet dried under the laminar hood. Plasmid DNA was extracted from the transfected cell using a QIAGEN kit (QIAprep spin Miniprep Kit Protocol). The quantity was determined using a NanoDrop spectrophotometer.

6.2.2.2.2 PCR Analysis of Plasmid DNA

Plasmid pNXT-35S-ACE-hp was validated by confirming the presence of the target gene (*AChE*) by PCR using gene sequence-specific primers (sense F: GAGCTCAAGTCCAGCGTTCCCTGGA; syntron R: AGAATTGGCGCGCCATTTAAATC; product size: 466 bp). The reaction mixture consisted of 2.5 µl 10 × PCR buffer, 0.5 µl dNTPs, 1.0 µl forward primer (10 µM), 1.0 µl reverse primer (10 µM), 0.2 µl Taq polymerase, (1 µl) 200 ng plasmid DNA, and 18.8 µl nuclease-free water. Samples were mixed by flicking, centrifuged for 5 sec and placed in a thermocycler at 95 °C for 5 min, 94 °C for 30 sec, 60 °C for 30 sec (35 cycles), 72 °C for 1 min and 4 °C ∞. The PCR amplicon was resolved by electrophoresis on a 1 % agarose gel.

6.2.2.2.3 Introduction of Plasmid DNA into *Agrobacterium* Strain (EHA 105)

To approximately 10 ml LB medium, 5 µl (25 mg/ml) rifampicin and 100 µl *Agrobacterium* strain EHA 105 was added. The culture was incubated at 28 °C and at 200 rpm. The optical density (OD_{600nm}) of the culture was adjusted to approximately 0.8.

Thereafter, the culture was chilled on ice for 20 min and centrifuged at 4000 rpm for 10 min at 4 °C. The supernatant was discarded and the pellet resuspended in 10 ml ice-cold 10 % glycerol before centrifuging at 4000 rpm for 20 min at 4 °C. This step was done twice before resuspending the pellet in 1 ml of 10 % glycerol. An aliquot of 50 µl was dispensed in a 1.5 ml Eppendorf tube and frozen in liquid nitrogen before it was stored at -80 °C.

To introduce plasmid pNXT-35S-ACE-hp into *Agrobacterium*, 50 µl of EHA 105 competent cells was thawed on ice for 5 min. About 2 µl of the plasmid DNA was added to the cell and the mixture transferred in an electroporation cuvette. The cuvette was placed in the electroporator and 2800 V passed through the cell (Biorad, GenePulser ×Cell™, USA). After the pulse, 950 µl LB was added to the culture immediately and mixed by priming. The culture was transferred into a 1.5 ml sterile Eppendorf tube and incubated at 28 °C for 3 hours in a rotary shaker. An aliquot of 200 µl of the culture was plated on LB semisolid medium supplemented with 50 mg rifampicin and 50 mg of kanamycin. Plates were sealed and incubated in an incubator (HeraThermo Incubator, ThermoScientific, Germany) at 28 °C for 3 days. A single colony was isolated with the aid of a sterile tip and cultured in 10 ml LB medium supplemented with 10 µl (25 mg/ml) rifampicin and 10 µl (50 mg/ml) kanamycin. Cultures were grown for 48 hours in an incubator at 28°C on a shaker. An aliquot of 500 µl of the culture was refreshed overnight in 50 ml LB broth supplemented with 50 µl (25 mg/ml) rifampicin and 50 µl (50 mg/ml) kanamycin. Plasmid DNA was then extracted from the culture using the QIAGEN kit (QIAprep spin Miniprep Kit Protocol). Thereafter, a PCR validation was done as described in section 6.2.2.2.2 to confirm the presence of *AChE* in the plasmid.

Glycerol stock of the culture was prepared by adding 150 µl sterilized glycerol to 850 µl *Agrobacterium* culture harbouring pNXT-35S-ACE-hp construct. Mixing was done by priming and 1ml was dispensed in a sterile cryovial, plunged for a few seconds in liquid nitrogen and stored at -80 °C for future use.

6.2.3 *Agrobacterium*-Mediated Transformation using RNAi Construct pNXT-35S-ACE-hp

Embryogenic cell suspensions of three *Musa* spp. (Cavendish Williams, Gonja Manjaya and Orishele) were selected for the *Agrobacterium*-mediated transformation using *Agrobacterium* strain EHA 105 harbouring pNXT-35S-ACE-hp construct. Transformation procedure was done as described in Chapter 5 (5.1.2). A three-day co-cultivation period was used. Selection and regeneration of putative transgenic lines were done as described in section 5.1.4.

6.2.4 Molecular Characterization of Transformed Banana and Plantain Plants

6.2.4.1 Genomic DNA Isolation and PCR Analysis

Leaf explant was isolated from each putative transgenic line, wrapped in labelled aluminium foil, snap-frozen in liquid nitrogen and pulverized with the aid of a sterile mortar and pestle. Genomic DNA was isolated using a CTAB protocol as described in section 5.2.3.1. The quality and quantity of the DNA was determined on 1 % agarose gel and NanoDrop spectrophotometer, respectively.

The presence of the *AChE* gene in transgenic events was determined by PCR analysis using the gene-specific primers. The PCR cocktail consisted of 2 µl (50 ng) of the genomic DNA, 2.0 µl 10 × buffer, 0.4 µl dNTPs, 0.2 µl sense forward (10 µM), 0.2 syntron reverse primers (10 µM), 0.2 µl Taq polymerase and 15 µl nuclease-free water. The sample was flicked and spurn briefly before placing in the thermocycler at 95 °C for 5 min, (94 °C for 15 sec, 63 °C for 30 sec, 72 °C for 30 sec at 35 cycles), 72 °C for 2 min, 4 °C ∞. PCR product was resolved by electrophoresis on a 1 % (w/v) agarose gel stained with 1× Gelred.

6.2.4.2 RNA Isolation, cDNA Synthesis and RT-PCR Analysis

RNA extraction was done using the Qiagen RNeasy Plus Mini Kit (cat nos. 74134 and 741360, USA). About 100 mg of fresh leaf sample was collected from 5, 6 and 10 individual events of Gonja Manjaya, Orishele and Cavendish Williams, respectively.

Samples were frozen in liquid nitrogen before pulverisation with the aid of a sterile mortar and pestle. About 450 µl of RLT plus buffer was added to the sample and mixed by vortexing. The sample was centrifuged at 14000 rpm for 3 min. The lysate was transferred into a gDNA eliminator spin column and centrifuged at 14 000 rpm for 1 min. The column was discarded while 600 µl of 70 % ethanol was added to the flow-through and mixed by priming. About 700 µl of the mixture was transferred into a new RNeasy spin column and centrifuged at 10000 rpm for 1 min. The flow-through was discarded and 700 µl of RW1 buffer added to the spin column and centrifuged for 1 min. The flow-through was again discarded and 500 µl RPE buffer added to the spin column and centrifuged at 10000 rpm for 2 min. The spin column was placed in a sterile 1.5 ml Eppendorf tube and 50 µl nuclease-free water added to the centre of the spin column and allowed to incubate for 5 min at room temperature before centrifuging at 1000 rpm for 1 min. Quantification was done using the NanoDrop spectrophotometer and samples were used immediately to synthesise cDNA strand.

The complementary DNA strand was synthesized using the maxima reverse transcriptase enzyme mix (Thermo Scientific, K1642, R1362) as described in section 6.1.1.5. Subsequent gene expression levels were determined by RT-PCR as described in section 6.1.1.5 using *AChE* specific primers (F: CCCTGGAACATCTTCAGTG; R: TAGGCACCCTCGCCCAATTG) and *Musa* 25S housekeeping gene-specific primers (F: ACATTGTCAGGTGGGGAGTT; R: CCTTTTGTTCCACACGAGATT).

6.2.4.3 Southern Blot Analysis

The size and copy number of the genomic DNA was determined using Southern blot analysis as described in section 5.1.5. The restriction enzyme used for the digestion was *Nco*I.

6.2.5 Glasshouse Screening of Plantain Transgenic Events

Transgenic events were selected based on PCR and Southern positive events and rooted in rooting media (RM) (Table 4.1). Three replicates per transgenic event and control were

selected *in vitro* and hardened in the glasshouse for about 8 weeks. Sterile soil and chicken manure were mixed in the ratio 3:1. Plants were carefully removed with the aid of a forceps, ensuring that the roots are not damaged and placed under running water to wash off the synthetic culture media and gelrite. Some gravel was used to base the perforated plastic cups to enable aeration and adequate drainage. Plants were potted in the plastic cups using the soil mixture. Potted plants were sprayed with water and placed in a humidity chamber in the glasshouse at 16/8-hour photoperiod, 25 ± 2.0 °C and watered sparingly every week for about 8 weeks.

Plants were then removed from glasshouse and placed in an insect-proof cage. Replicate number was assigned to each transgenic event. The surface of the soil was covered with soft fabric and fastened to the plant with the aid of masking tape to prevent the aphids from burrowing into the soil. Five first instar aphids were administered on the pseudostem of the banana plant. The cage containing the plants and aphids were placed in a controlled growth chamber at 25 °C, 75 % relative humidity and 12-hour photoperiod. The population of aphids was counted every 7th-day post-initiation for 3 weeks and recorded for analysis. A minimum of three screening trial experiments were conducted. The decrease in aphid population in transgenic plants relative to the aphid population in the control plants was measured as relative resistance to the banana aphid.

Equation 6.1: Percentage relative resistance

$$Relative\ Resistance\ (\%) = \left[\frac{Average\ aphid\ population\ on\ control\ plants - Average\ aphid\ population\ on\ transgenic\ plant}{Average\ aphid\ population\ on\ control\ plants}\right] \times 100$$

6.2.6 Identification and Quantification of Available Sugars in Plantain and Banana Cultivars

Sugar has been identified as a major phagostimulant for banana aphids, this study therefore, sought to determine the presence of sucrose, fructose and glucose in banana and plantain cultivars and their respective concentrations among the cultivars tested (Agbagba, Obino l'Ewai, Orishele, Gonja Manjaya and Cavendish Williams) using the procedure described by Latimer (2012). *In vitro* plantlets and field explants (leaf, pseudostem and root) were sampled in collection bags. Samples were labelled and kept at -80 °C for 24 hours before freeze-drying for 72 hours. Samples were pulverized to a fine powder. About 1 g of each sample was weighed in triplicate in a sterile 50 ml falcon tube and 40 ml of 85 % ethanol added. Samples were heated on a water bath at 85 °C for 1 hour while mixing 2-3 during the incubation period. The samples were then centrifuged at 3500 rpm for 10 min and the extract was filtered into a new tube using a Whatman filter paper (11 µm). The extract was concentrated by evaporation in a water bath to 5 ml. About 1ml of the concentrated extract was dispensed in the HPLC vials for analysis in the HPLC machine (Waters 2695 Separation Module, Waters 2414 Refractive Index Detector, USA). The results were extrapolated from the electropherogram detected after the run.

6.3 Results

6.3.1 *In vitro* Feeding Assay

6.3.1.1 Determination of Optimum Diet for *In vitro* Assay of Banana Aphids

Aphids were successfully reared on the banana plant under glasshouse conditions in an insect-proof cage (Fig 6.3). Banana aphids were fed on a synthetic diet to enable *in vitro* assay procedures. Hence, four diet types (Diet 1, Diet 2, Diet 3 and Diet 4) and a control diet (Diet 5) containing a varying sucrose levels (0 g, 5 g, 7.5 g, 15 g, 20 g and 30 g) were examined to determine an optimum diet type and sucrose level for aphid survival *in vitro*. The results obtained indicated that there is a significant ($p < 0.0001$) interaction between the sucrose level and the diet type that aphids were subjected to. The highest mean aphids and nymph population were observed in diet 2 and sucrose level of 7.5 g at day 5 (3.83 ± 0.5 and 8.44 ± 1.8) and day 7 (4.0 ± 0 and 9.8 ± 1.2), respectively. Furthermore, sucrose level between 5 g - 20 g supported the survival of banana aphid, and the optimal sucrose level for aphid survival and reproduction was 7.5 g (Table 6.2). In contrast, the highest aphid mortality was observed in Diet 5 and sucrose level of 0 g and 30 g across all the diet types (Table 6.2).

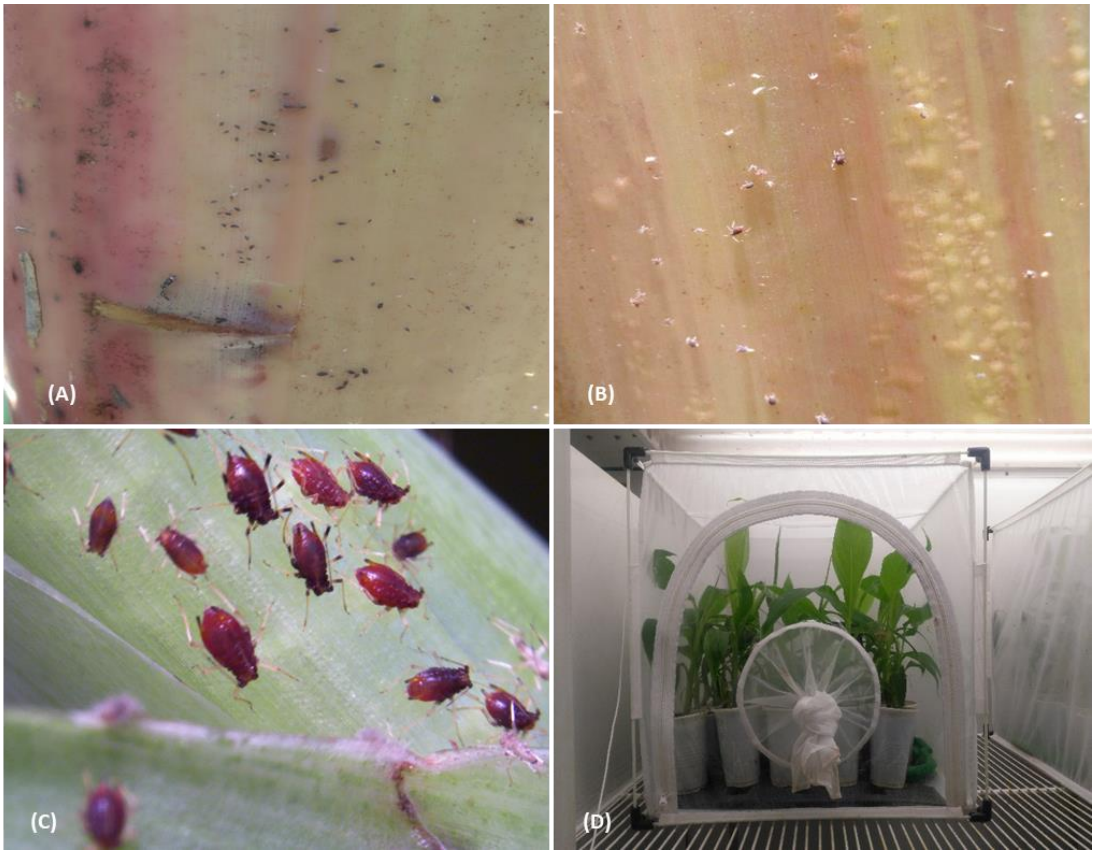


Figure 6.3: Aphid feeding on a banana plant. A-C: Banana aphid on banana plant and D: Rearing of aphids on a banana plant in an insect-proof cage in a conviron chamber.

Table 6.2: Effect of sucrose level and diet type on banana aphid

Day	Sugar Level (g/100 ml)	Diet Type	Dead Aphid	Live Aphid	Nymph
5	0	Diet 1	$4 \pm 0^{***}$	0 ± 0^{ns}	0.08 ± 0.3^{ns}
		Diet 2	$4 \pm 0^{***}$	0 ± 0^{ns}	0.05 ± 0.2^{ns}
		Diet 3	$4 \pm 0^{***}$	0 ± 0^{ns}	0.13 ± 0.4^{ns}
		Diet 4	$3.9 \pm 0.3^{***}$	0.08 ± 0.3^{ns}	0.33 ± 0.7^{ns}
		Diet 5	$4 \pm 0^{***}$	0 ± 0^{ns}	0.08 ± 0.3^{ns}
	5	Diet 1	$2.33 \pm 1.4^{***}$	$1.75 \pm 1.6^{***}$	$3.5 \pm 1.7^{***}$
		Diet 2	$0.8 \pm 1.2^{**}$	$3.2 \pm 1.2^{***}$	$5.73 \pm 2.7^{***}$
		Diet 3	$0.86 \pm 1.1^{**}$	$3.13 \pm 1.1^{***}$	$3.73 \pm 1.0^{***}$
		Diet 4	$0.66 \pm 1.2^{*}$	$3.25 \pm 1.1^{***}$	$3.66 \pm 1.8^{***}$
		Diet 5	$3.83 \pm 0.6^{***}$	0.16 ± 0.6^{ns}	0.25 ± 0.5^{ns}
	7.5	Diet 1	$1.5 \pm 1.5^{***}$	$2.5 \pm 1.5^{***}$	$3.08 \pm 1.8^{***}$
		Diet 2	0.17 ± 0.5^{ns}	$3.83 \pm 0.5^{***}$	$8.44 \pm 1.8^{***}$
		Diet 3	$0.71 \pm 0.9^{**}$	$3.28 \pm 0.9^{***}$	$4.21 \pm 0.9^{***}$
		Diet 4	$1.25 \pm 1.4^{***}$	$2.75 \pm 1.4^{***}$	$3.66 \pm 1.9^{***}$
		Diet 5	$3.33 \pm 1.1^{***}$	$0.66 \pm 1.1^{*}$	$0.83 \pm 1.3^{*}$
	15	Diet 1	$1.5 \pm 1.7^{***}$	$2.5 \pm 1.7^{***}$	$3.08 \pm 1.9^{***}$
		Diet 2	$0.78 \pm 0.7^{**}$	$3.21 \pm 0.7^{***}$	$5.35 \pm 2.2^{***}$
		Diet 3	$1.2 \pm 1.5^{***}$	$2.8 \pm 1.5^{***}$	$3.26 \pm 1.6^{***}$
		Diet 4	$0.66 \pm 0.8^{*}$	$3.33 \pm 0.8^{***}$	$3.0 \pm 1.6^{***}$
		Diet 5	$2.8 \pm 1.3^{***}$	$1.16 \pm 1.3^{***}$	0.75 ± 1.1^{ns}
	20	Diet 1	$2.3 \pm 1.9^{***}$	$1.67 \pm 1.9^{***}$	$2.16 \pm 1.9^{***}$
		Diet 2	$1.4 \pm 1.2^{***}$	$2.6 \pm 1.2^{***}$	$3.0 \pm 1.8^{***}$
		Diet 3	$1.33 \pm 1.3^{***}$	$2.66 \pm 1.3^{***}$	$2.53 \pm 1.5^{***}$
		Diet 4	$1.16 \pm 1.3^{***}$	$2.83 \pm 1.3^{***}$	$2.58 \pm 0.7^{***}$
		Diet 5	$2.66 \pm 1.6^{***}$	$1.33 \pm 1.6^{***}$	0.75 ± 0.9^{ns}
	30	Diet 1	$3.08 \pm 1.7^{***}$	$0.83 \pm 1.6^{**}$	$1.08 \pm 1.3^{**}$
		Diet 2	$3.13 \pm 1.12^{***}$	$0.86 \pm 1.1^{**}$	0.46 ± 0.8^{ns}
		Diet 3	$3.00 \pm 1.5^{***}$	$0.86 \pm 1.2^{**}$	$0.86 \pm 1.3^{**}$
		Diet 4	$3.66 \pm 0.5^{***}$	0.33 ± 0.5^{ns}	0.42 ± 0.7^{ns}
		Diet 5	$3.58 \pm 0.8^{***}$	0.42 ± 0.8^{ns}	0.25 ± 0.5^{ns}
7	0	Diet 1	$4.0 \pm 0^{***}$	0 ± 0^{ns}	0 ± 0^{ns}
		Diet 2	$4.0 \pm 0^{***}$	0.0 ± 0^{ns}	0 ± 0^{ns}
		Diet 3	$4.0 \pm 0^{***}$	0 ± 0^{ns}	0.33 ± 0.5^{ns}
		Diet 4	$3.83 \pm 0.4^{***}$	0.16 ± 0.4^{ns}	0.66 ± 0.8^{ns}
		Diet 5	$4 \pm 0^{***}$	0 ± 0^{ns}	0 ± 0^{ns}
	5	Diet 1	$2.5 \pm 1.5^{***}$	$1.66 \pm 1.9^{***}$	$3.0 \pm 2.1^{***}$
		Diet 2	$1.0 \pm 1.5^{*}$	$3.0 \pm 1.5^{***}$	$6 \pm 3.7^{***}$
		Diet 3	0.5 ± 0.8^{ns}	$3.5 \pm 0.8^{***}$	$4.16 \pm 0.8^{***}$
		Diet 4	$1.0 \pm 1.5^{*}$	$3.0 \pm 1.5^{***}$	$4.0 \pm 1.1^{***}$
		Diet 5	$3.66 \pm 0.8^{***}$	0.33 ± 0.8^{ns}	0.5 ± 0.5^{ns}
	7.5	Diet 1	0.83 ± 1.0^{ns}	$3.16 \pm 1.0^{***}$	$4.5 \pm 1.2^{***}$
		Diet 2	0 ± 0^{ns}	$4.0 \pm 0^{***}$	$9.8 \pm 1.2^{***}$
		Diet 3	$1.16 \pm 1.2^{**}$	$2.83 \pm 1.2^{***}$	$4.33 \pm 1.0^{***}$
		Diet 4	0.66 ± 1.6^{ns}	$3.33 \pm 1.6^{***}$	$4.83 \pm 2.0^{***}$
		Diet 5	$2.83 \pm 1.3^{***}$	$1.16 \pm 1.3^{**}$	$1.5 \pm 1.6^{**}$

	15	Diet 1	0.66 ± 1.2^{ns}	$3.33 \pm 1.2^{***}$	$3.66 \pm 1.6^{***}$
		Diet 2	0.4 ± 0.5^{ns}	$3.6 \pm 0.5^{***}$	$7.2 \pm 0.8^{***}$
		Diet 3	$2.16 \pm 1.8^{***}$	$1.83 \pm 1.8^{***}$	$3.66 \pm 2.0^{***}$
		Diet 4	0.5 ± 0.8^{ns}	$3.5 \pm 0.8^{***}$	$3.66 \pm 1.9^{***}$
		Diet 5	$3.16 \pm 1.3^{***}$	0.8 ± 1.3^{ns}	$1.17 \pm 1.2^{*}$
	20	Diet 1	$2.5 \pm 2.0^{***}$	$1.5 \pm 2.0^{***}$	$3.16 \pm 2.1^{***}$
		Diet 2	$1.16 \pm 1.2^{**}$	$2.83 \pm 1.2^{***}$	$4.5 \pm 2.0^{***}$
		Diet 3	$1.83 \pm 1.2^{***}$	$2.16 \pm 1.2^{***}$	$3.33 \pm 1.0^{***}$
		Diet 4	$1.5 \pm 1.5^{***}$	$2.5 \pm 1.5^{***}$	$2.66 \pm 0.8^{***}$
		Diet 5	$2.16 \pm 2.0^{***}$	$1.83 \pm 2.0^{***}$	1.0 ± 0.9^{ns}
	30	Diet 1	$4.0 \pm 0^{***}$	0 ± 0^{ns}	0.83 ± 1.2^{ns}
		Diet 2	$3.33 \pm 0.8^{***}$	0.66 ± 0.8^{ns}	0.33 ± 0.8^{ns}
		Diet 3	$2.83 \pm 1.5^{***}$	$1.16 \pm 1.5^{**}$	$1.83 \pm 1.5^{***}$
		Diet 4	$3.83 \pm 0.4^{***}$	0.16 ± 0.4^{ns}	0.66 ± 0.8^{ns}
		Diet 5	$3.44 \pm 0.9^{***}$	0.55 ± 0.9^{ns}	0.33 ± 0.5^{ns}
	Main effect *p* value				
	SL		***	***	***
	DT		***	***	***
	Day		ns	ns	***
	Day*SL*DT		***	***	***

Note: SL: sucrose level, DT: Diet type, Diet 5: control. Data is presented as mean and standard deviation of live aphid, dead aphid, and nymph. ns, non-significant ($p > 0.05$), * significant ($p \leq 0.05$), ** very significant ($p \leq 0.01$), *** highly significant ($p \leq 0.001$)

6.3.1.2 Determination of Lethal Dose of dsRNA on Banana Aphids

Following the identification of ideal diet composition for banana aphid survival *in vitro*, the synthetic diet (Diet 2) was supplemented with dsRNA at 100, 200, 300 and 500 µg/µl diet. The results showed a significant ($p < 0.0001$) effect of dsRNA concentration on the survival of banana aphids (Table 6.3). A dose of 500 ng/µl of dsRNA had the highest lethal effect on banana aphids (Table 6.3).

Table 6.3: Effect of varying concentrations of *AChE* dsRNA on banana aphid

Treatment (ng)	Day	Dead Aphid	Live Aphid	Nymph
100	2	0 ± 0^{ns}	$4 \pm 0^{***}$	2.5 ± 0.7^{ns}
	3	0 ± 0^{ns}	$4 \pm 0^{***}$	$3.5 \pm 0.7^{**}$
	7	0.5 ± 0.7^{ns}	$3.5 \pm 0.7^{***}$	$6 \pm 1.4^{***}$
200	2	0.5 ± 0.7^{ns}	$3.5 \pm 0.7^{***}$	2.5 ± 2.1^{ns}
	3	0.6 ± 0.9^{ns}	$3.6 \pm 1.1^{***}$	$3.4 \pm 1.5^{***}$
	7	1.0 ± 1.4^{ns}	$3.0 \pm 1.4^{***}$	$7.0 \pm 4.2^{***}$
300	2	0 ± 0^{ns}	$4.0 \pm 0^{***}$	$3.0 \pm 1.4^{*}$
	3	1.0 ± 0^{ns}	$3.0 \pm 0^{***}$	2.5 ± 0.7^{ns}
	7	1.0 ± 1.4^{ns}	$3.0 \pm 1.4^{***}$	$4.5 \pm 2.1^{***}$
500	2	1.0 ± 1.4^{ns}	$3.0 \pm 1.4^{***}$	1.5 ± 0.7^{ns}
	3	$2.83 \pm 1.8^{***}$	$1.16 \pm 1.8^{**}$	0.66 ± 1.2^{ns}
	7	$3.5 \pm 0.7^{***}$	0.5 ± 0.7^{ns}	$3.5 \pm 2.1^{**}$
Control	2	0 ± 0^{ns}	$4.0 \pm 0^{***}$	$4.0 \pm 0.7^{***}$
	3	0.11 ± 0.3^{ns}	$3.88 \pm 0.3^{***}$	$5.33 \pm 2.5^{***}$
	7	0.43 ± 0.5^{ns}	$3.57 \pm 0.5^{***}$	$8.86 \pm 1.9^{***}$
Main effect *p* value				
Treatment		***	***	***
Day		*	*	***
Treatment * Day		ns	ns	ns

Note: Data is presented as mean and standard deviation of live aphid, dead aphid, and nymph. ns, non-significant ($p > 0.05$), * significant ($p \leq 0.05$), ** very significant ($p \leq 0.01$), *** highly significant ($p \leq 0.001$)

6.3.1.3 Expression levels of *AChE* Gene in Banana Aphids Fed on dsRNA

At 7th-day post-feeding assay, there was an abundant expression of *AChE* transcript in the whole aphid at 100 ng and 300 ng as compared to the control non-treated aphids. The abundance of *AChE* transcript was slightly reduced at 500 ng as compared to the control non-treated aphid. However, a relatively low abundance of *AChE* transcript was observed at 200 ng (Fig 6.4). The lower expression of *AChE* transcript at 200 ng could be due to a possible degradation of the template because the transcript expression of the internal control gene was also impaired. Primers targeting *Buchneria aphidichola* sequence commonly present in banana aphids, used as a housekeeping control was conserved in the treated and non-treated aphid (Fig 6.4). This confirms the authenticity of the biotype of banana aphid that was used in this study.

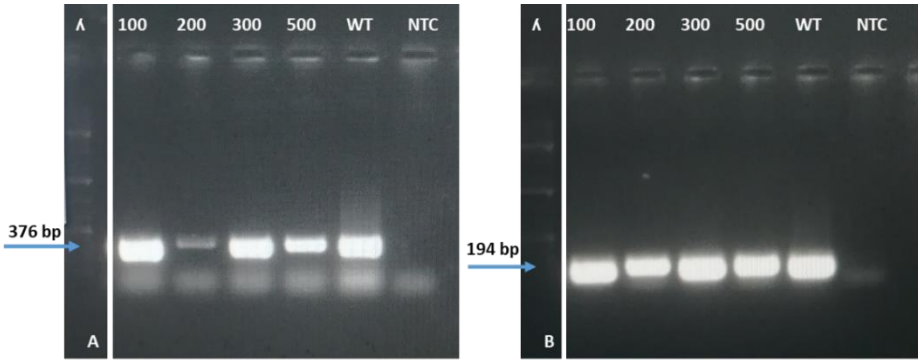


Figure 6.4: The expression level of *AChE* gene by RT-PCR. **A:** Expression level of *AChE* gene in banana aphids fed with 100, 200, 300, and 500 ng dsRNA, WT: Aphid fed on control diet and NTC: non-template control ʎ: 1 kb+ ladder (Thermo Fisher Scientific) **B:** *Buchnera* housekeeping gene as an internal control

6.3.1.4 Determination of siRNA Lethal Dosage for Banana Aphids

Banana aphid fed with siRNA showed no significant difference with the control at 50 and 100 ng. However, relatively higher mortality was observed at 250 ng compared with the control (Table 6.4 a&b).

Table 6.4a: ANOVA summary of siRNA artificial feeding assay

ANOVA Summary_SiRNA				
Source of Variation	DF	Mean Square_DA	Mean Square_LA	Mean Square_Nymph
Treatment	3	1.73ns	1.73ns	3.4ns
Mean		1.9	2.09	3.4ns
Error		0.52	0.52	6.8
CV		38.08	34.77	52.26

Note: ***, **, significance at $p < 0.0001$ and 0.0002 respectively, ns, not significant.

Table 6.4b: Summary of varying concentration of siRNA on banana aphid

Mean aphid population fed with siRNA			
Treatment	DA	LA	Nymph
Control	1.6 ba	2.4 ba	5.8 a
50 ng/µl	1.10 b	3.0a	5.5 a
100 ng/µl	2.50 ba	1.50 ba	3.5 a
250 ng/µl	3.00 a	1.00 b	4.0 a

Note: same letters are not significantly different

6.3.2 Validation of Plasmid Construct

The validation of *AChE* gene in transformed *E. coli*. (DH5α) and *Agrobacterium* strain (EHA 105) was done using gene-specific primers to amplify the target gene in a PCR. This was done to confirm the successful transformation of pNXT-35S-ACE-hp in both bacteria before transformation in an embryogenic cell suspension. An amplicon target size of 466 bp was observed confirming the presence of target gene (Fig 6.6).

6.3.3 Regeneration, Selection and Molecular Characterization of Putative Transgenic Events

Actively proliferating embryogenic cell suspension of Gonja Manjaya, Orishele and Cavendish Williams were transformed using *Agrobacterium* strain harbouring pNXT-35S-ACE-hp plasmid construct of which CaMV 35S promoter was used to drive the *AChE* gene. Only successfully transformed cells developed embryos and subsequently regenerated to plantlets on kanamycin (50 mg/l) selection media. However, cells without the target gene did not develop embryos and turned black. The germinating embryos were further transferred on selection media to enable development to a whole plant after which leaf explant was isolated for molecular characterization (Fig 6.5). A total of 13 transformation experiments were carried out for Orishele and Gonja Manjaya while one experiment was done for Cavendish Williams. Twenty-three, forty-three and fifty-six transgenic events were regenerated for Orishele, Gonja Manjaya and Cavendish Williams respectively. However, a total of 32, 13, and 34 putative transgenic events of Gonja Manjaya, Orishele and Cavendish Williams respectively were subjected to PCR analysis to validate the presence of the *AChE* gene in the plants.

Subsequently, a selection of 18, 8 and 12 PCR positive events of Gonja Manjaya, Cavendish Williams and Orishele were randomly chosen for Southern blot analysis which confirmed the integration of *AChE* as well as the respective copy numbers (Fig 6.7). Expression analysis by RT-PCR also showed varying expression levels (Fig 6.8).

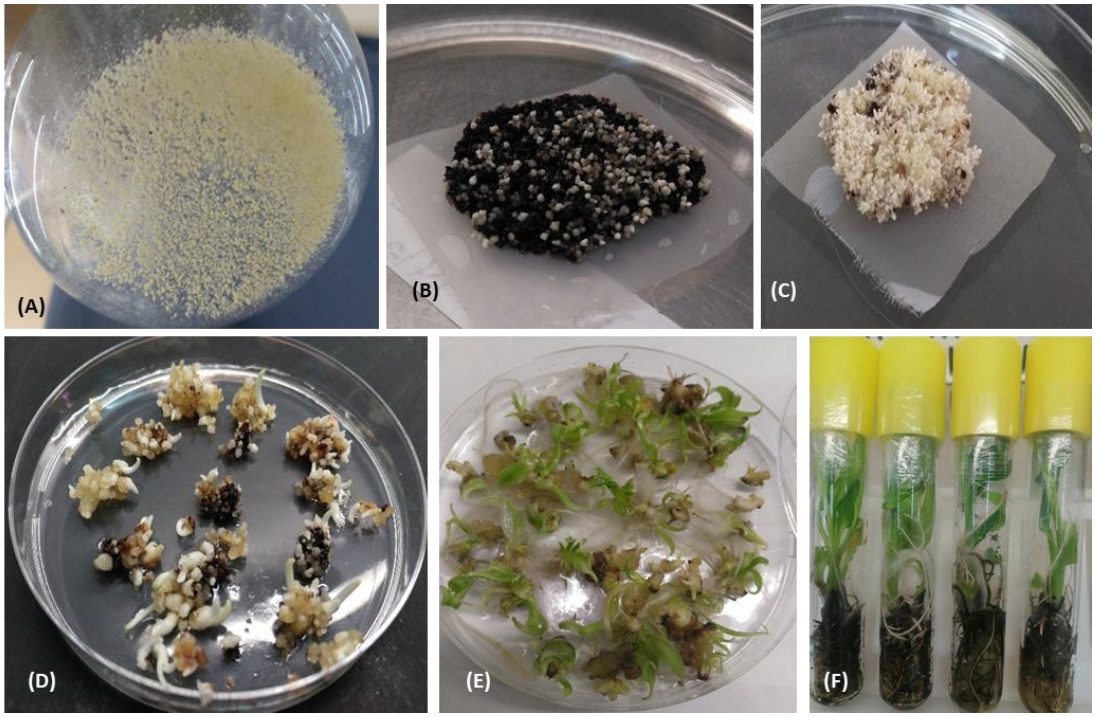


Figure 6.5: Transformation, selection and regeneration of putative transgenic events. A: Proliferating EC, B: Developing embryos from transformed EC on selection media, C: Developing embryos from untransformed EC, D: Germinating embryos, E: Regenerating embryos to plantlets and F: Fully regenerated plant.

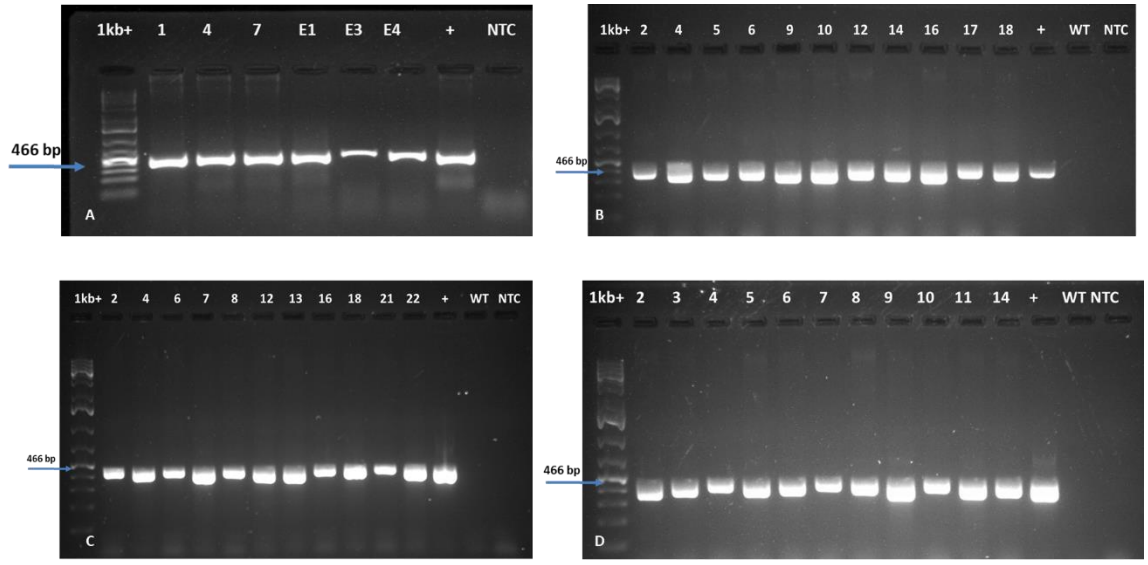


Figure 6.6: PCR detection of *AChE* in *E. coli* (DH5 α), *Agrobacterium* strain (EHA 105) and putative transgenic events. A: DH5 α (lanes1, 4, 7) and EHA 105 (lanes E1, E3, E4), B: Cavendish Williams events C: Orishele events D: Gonja Manjaya events. 1kb+ ladder (Thermo Fisher Scientific), NTC: non-template control, WT: wild type

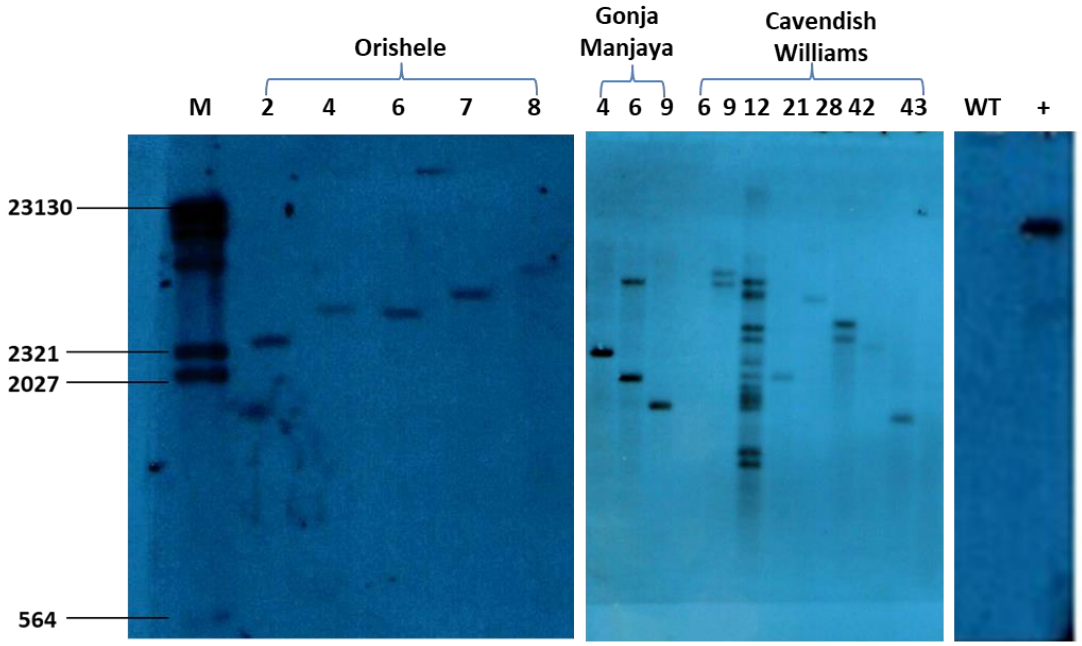


Figure 6.7: Southern blot analysis for plantain and banana cultivars. WT: wild type, +: positive control, M: DIG molecular ladder (Roche Diagnostics, 0.12-23.1 kbp)

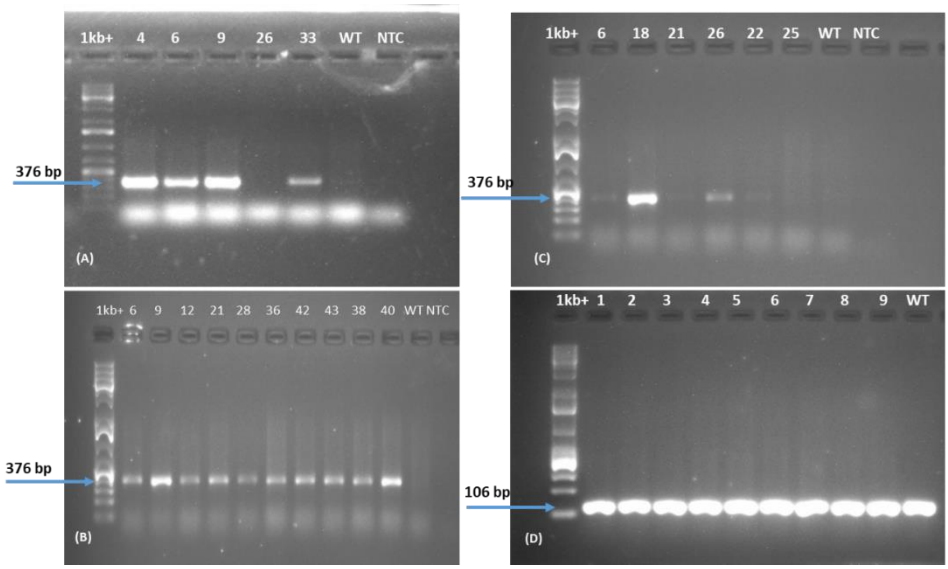


Figure 6.8: Expression levels of *AChE* in selected transgenic events by RT-PCR. A: Gonja Manjaya, B: Cavendish Williams, C: Orishele and D: *Musa* 25S ribosomal endogenous gene as an internal control.

6.3.4 Screening of Transgenic Events

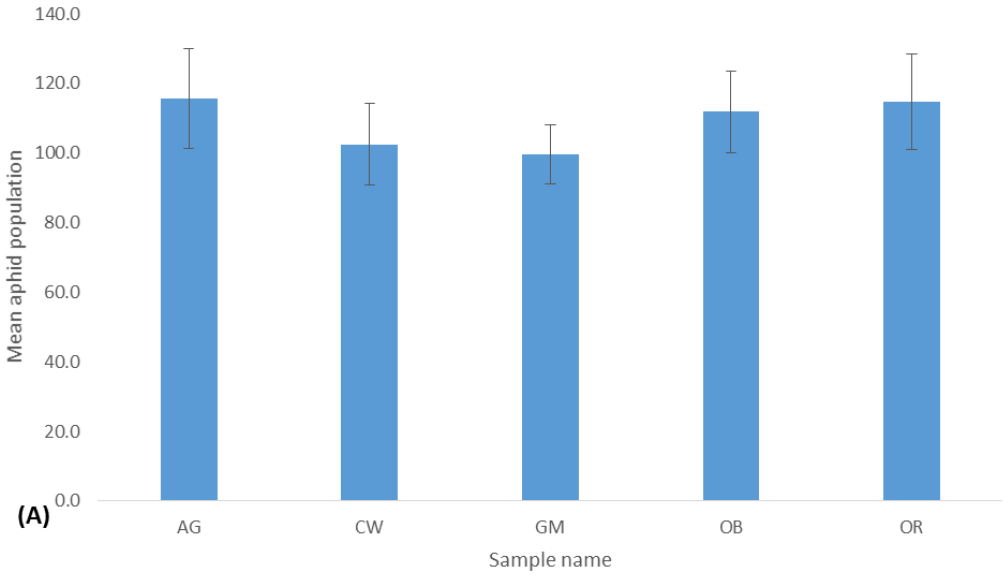
All the transgenic events were screened in insect-proof cages in the glasshouse to determine resistance to the banana aphid. The result showed that the three improved banana cultivars varied in their responses to banana aphids (Table 6.5).

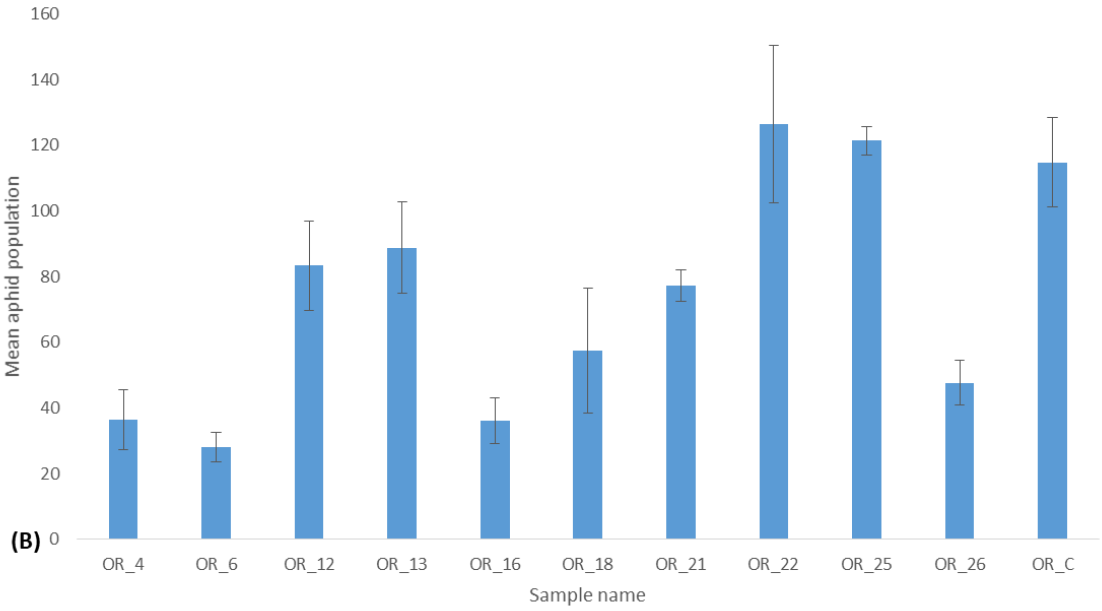
The mean population of banana aphid on transgenic events showed that 15, 4 and 2 events of Gonja Manjaya, Cavendish Williams and Orishele, respectively had an average aphid population more than the control (Fig 6.9).

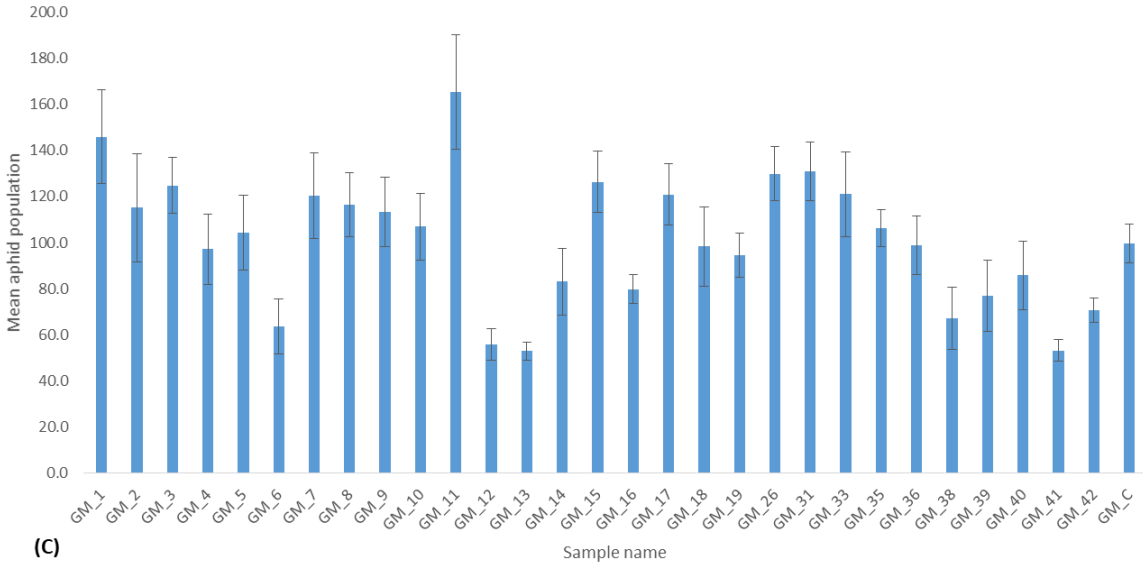
The relative decrease of banana aphid population in comparison to the control as a measure of relative resistance to banana was calculated. The result obtained showed that, in Gonja Manjaya (GM), 14 out of 29 events tested showed 0.7 to 46.7 % decrease in aphid population relative to the control plants. The remaining 15 events of GM had more aphid population ranging from (-4.8 to -65.9 %) relative to the control (Fig 6.10). For Cavendish Williams, only four transgenic events had an increase in aphid population (-0.3 to -67.8 %) relative to the control while 28 events showed a range of 7.7-79 % decrease

in aphid population relative to the control (Fig 6.10). In the case of Orishele, two events tested had a higher aphid population than the control (-5.8 and -10 %) while 8 events showed a range of 22.6 to 75.6 % decrease in aphid population relative to the control (Fig 6.10). The percentage decrease in aphid population in transgenic events varied within and among cultivars.

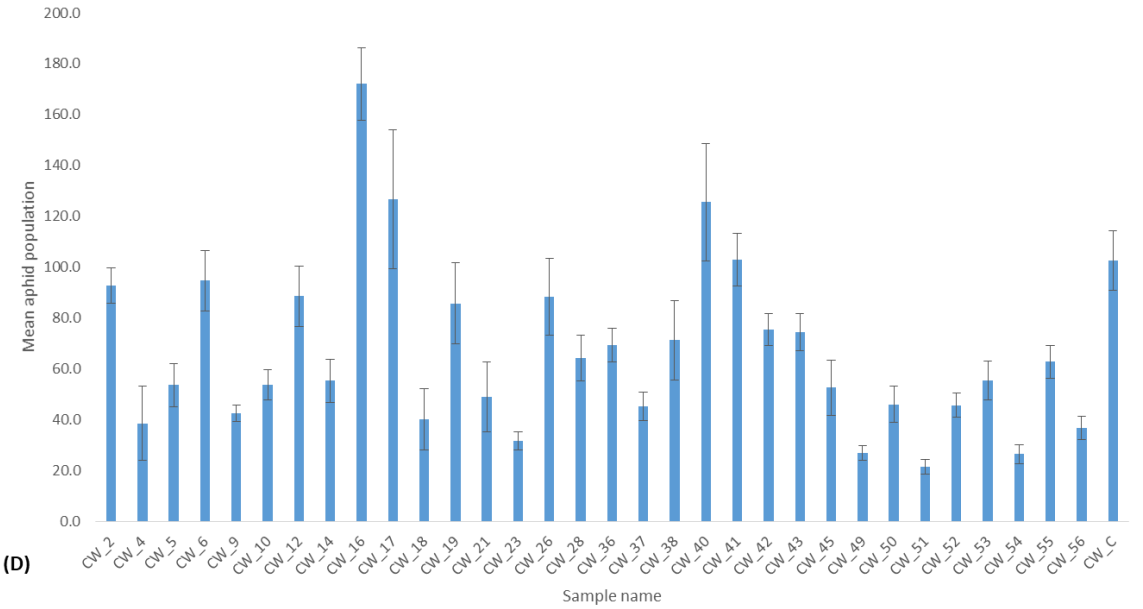
A comparison between aphid population on control plants including Agbagba, Obino l'Ewai, Orishele, Gonja Manjaya and Cavendish Williams confirmed that there was no significant difference in aphid population across the 5 cultivars (Fig 6.9). Although the mean population of banana aphid varied across the cultivars tested, the result was not significant.


140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
Mean aphid population
(A)
AG
CW
GM
OB
OR
Sample name


160
140
120
100
80
60
40
20
0
Mean aphid population
(B)
OR_4
OR_6
OR_12
OR_13
OR_16
OR_18
OR_21
OR_22
OR_25
OR_26
OR_C
Sample name

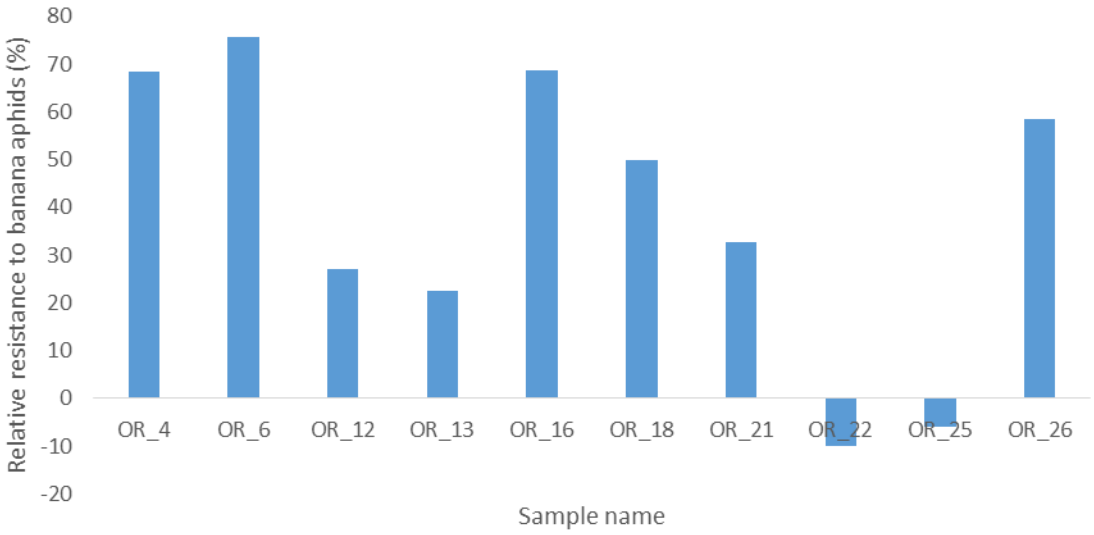


(C)

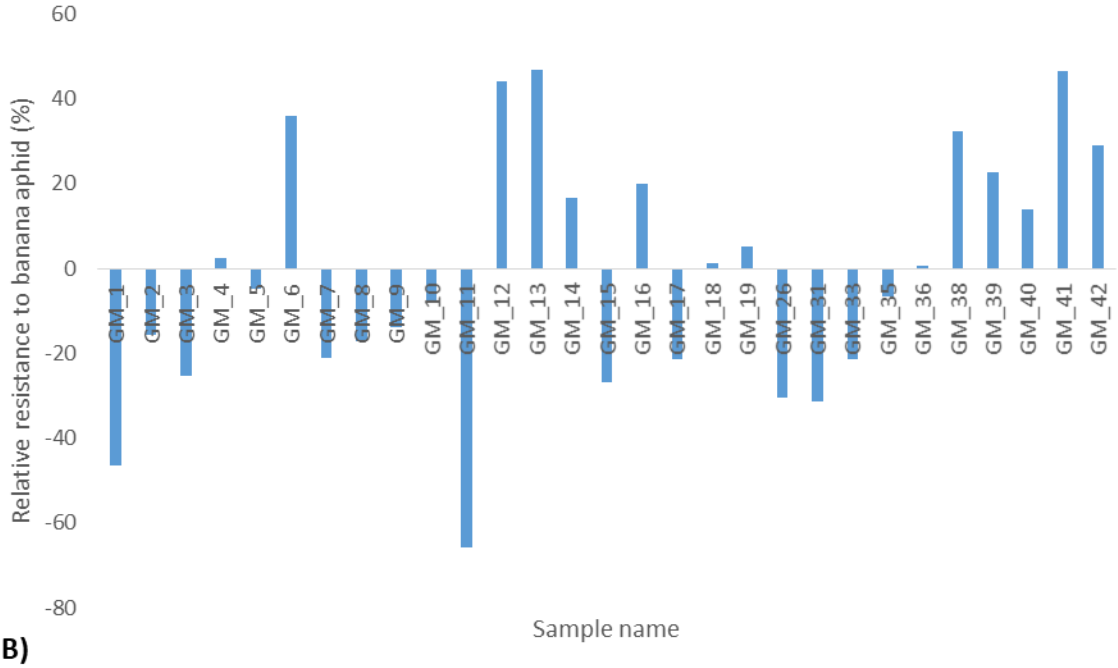


(D)

Figure 6.9: Mean population of aphids on control and transgenic plants. **A:** Control banana and plantain cultivars, **B:** Orishele transgenic and control plants **C:** Gonja Manjaya transgenic and control plants, and **D:** Cavendish Williams transgenic and control plants


Relative resistance to banana aphids (%)
80
70
60
50
40
30
20
10
0
-10
-20
OR_4
OR_6
OR_12
OR_13
OR_16
OR_18
OR_21
OR_22
OR_25
OR_26
Sample name

(A)


Relative resistance to banana aphid (%)
60
40
20
0
-20
-40
-60
-80
GM_1
GM_2
GM_3
GM_4
GM_5
GM_6
GM_7
GM_8
GM_9
GM_10
GM_11
GM_12
GM_13
GM_14
GM_15
GM_16
GM_17
GM_18
GM_19
GM_26
GM_31
GM_33
GM_35
GM_36
GM_38
GM_39
GM_40
GM_41
GM_42
Sample name

(B)

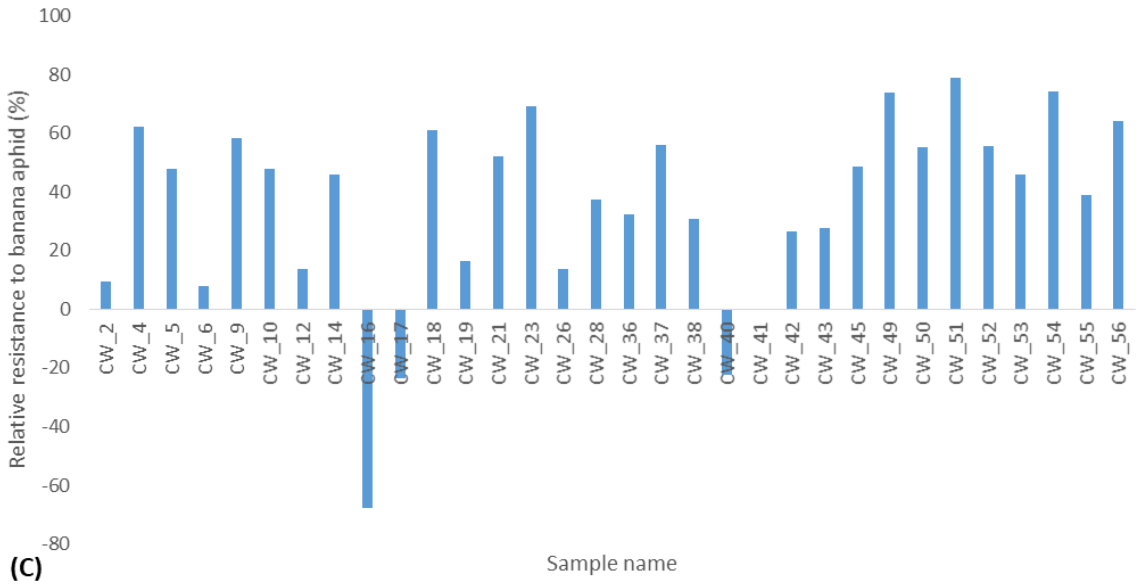
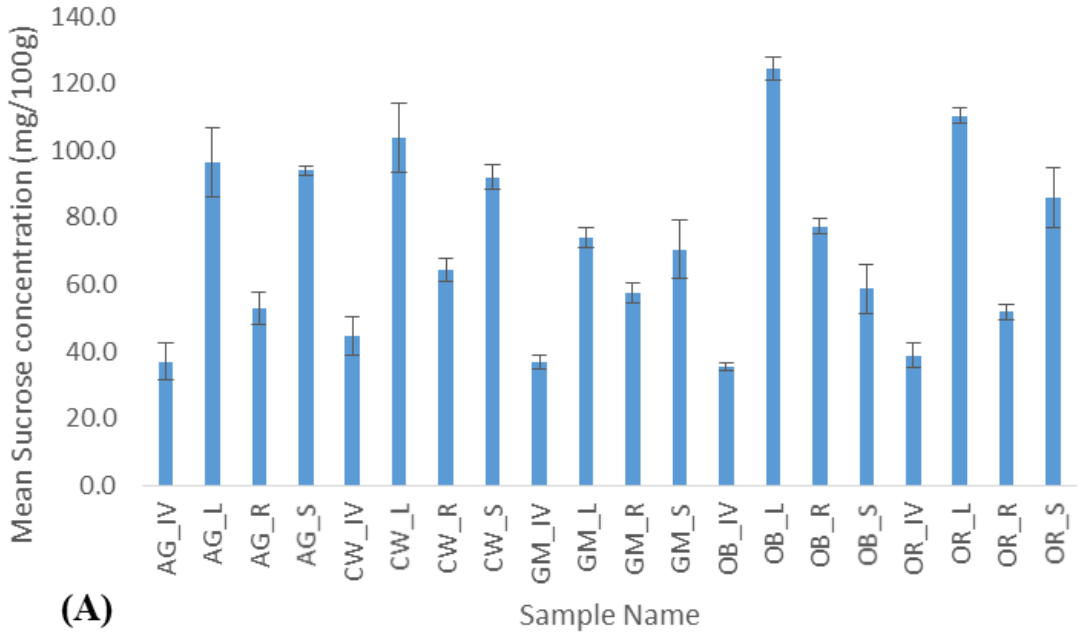


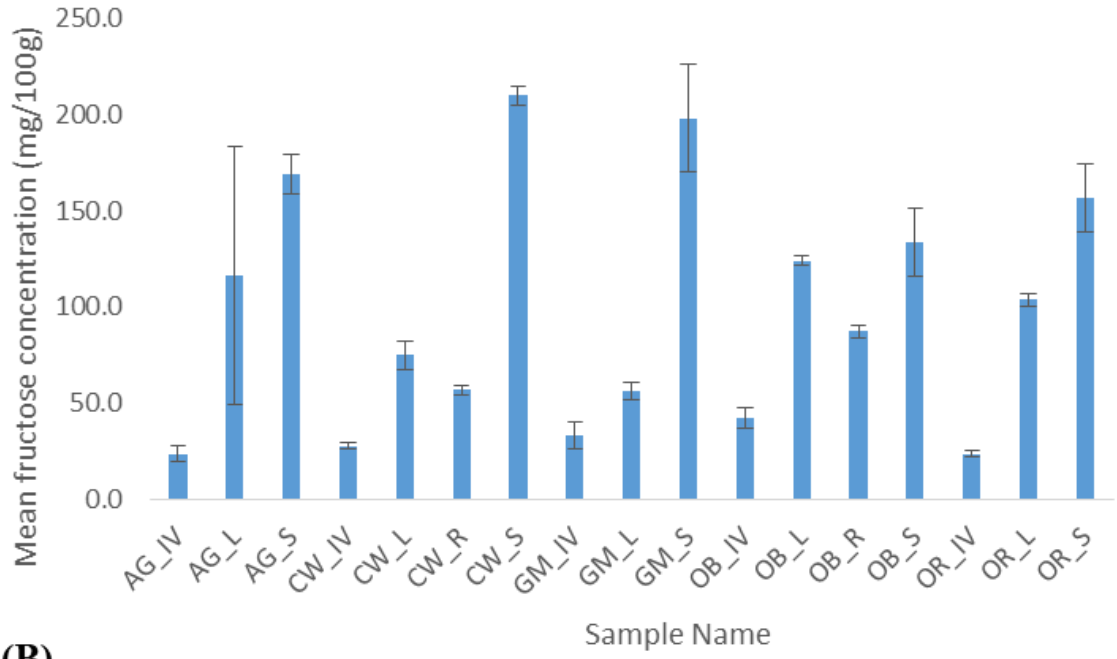
Figure 6.10: Percentage decrease in aphid population in transgenic plants relative to the control plants. **A, B, and C** represent the percentage decrease in aphid population in Orishele, Gonja Manjaya and Cavendish Williams relative to their respective controls

6.3.5 Identification and Quantification of Available Sugars in Plantain and Banana Cultivars

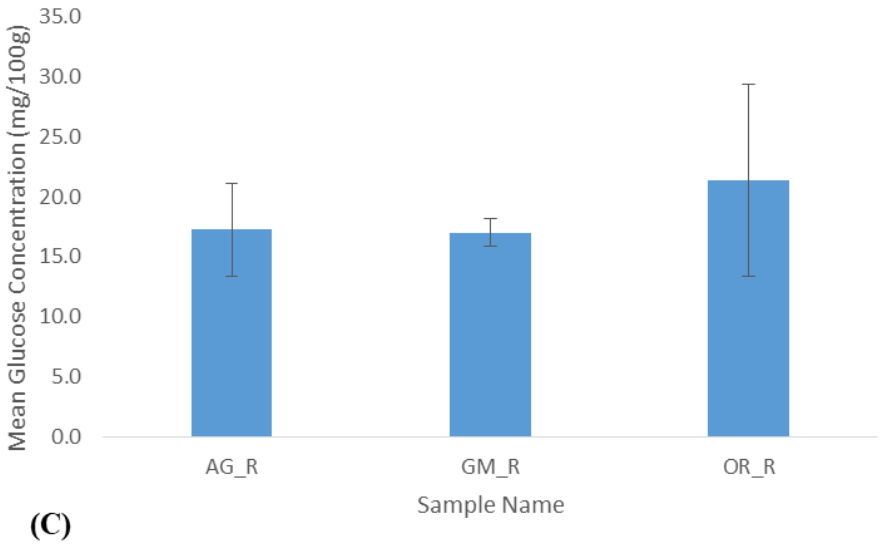
Sugar analysis confirmed the presence of sucrose, glucose and fructose in plantain and banana explants and cultivars. The concentration of sugar was extrapolated from the calibration curve (R^2 = 0.99) and expressed as mg of sucrose, fructose and glucose equivalent respectively per 100 g of the sample. The sucrose level was highest in the leaf explant across all cultivars; Obino l'Ewai recorded the highest level of sucrose content (124.4 ± 3.5 mg/100 g). In the pseudostem, Orishele recorded the highest sucrose content (85.9 ± 9.1 mg/100 g) and Gonja Manjaya the least (70.4 ± 8.7 mg/100 g). Glucose was detected only in the root explant of Agbagba (17 ± 3.9 mg/100 g), Gonja Manjaya (17 ± 1.2 mg/100 g) and Orishele (21.4 ± 8.0 mg/100 g). The availability and concentration of fructose varied across the explant and cultivars; Cavendish Williams gave the highest fructose in the pseudostem (209.8 ± 5.2 mg/100 g) (Fig 6.11).


Mean Sucrose concentration (mg/100g)
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
AG_IV
AG_L
AG_R
AG_S
CW_IV
CW_L
CW_R
CW_S
GM_IV
GM_L
GM_R
GM_S
OB_IV
OB_L
OB_R
OB_S
OR_IV
OR_L
OR_R
OR_S
Sample Name

(A)


Mean fructose concentration (mg/100g)
250.0
200.0
150.0
100.0
50.0
0.0
AG_IV
AG_L
AG_S
CW_IV
CW_L
CW_R
CW_S
GM_IV
GM_L
GM_S
OB_IV
OB_L
OB_R
OB_S
OR_IV
OR_L
OR_S
Sample Name

(B)



(C)

Figure 6.11: Sugar levels in banana and plantain explants. **A**: Sucrose levels in banana and plantain explants, **B:** Fructose levels in banana and plantain explants, **C:** Glucose levels in plantain cultivars. IV: *in vitro*, S: pseudostem, R: root, L: leaf, AG: Agbagba, OR: Orishele, OB: Obino l'Ewai, GM: Gonja Manjaya, CW: Cavendish Williams

6.4 Discussion

Banana bunchy top disease is an economically important disease of bananas and plantains causing a significant loss in yield and production. Banana aphid, a predominant pest capable of transmitting the disease is found in all banana producing areas around the world. The availability of infected planting material infested with insect pest is responsible for the continuous spread of the disease. Several attempts have been made in controlling BBTD via the virus with limited results. Consequently, this calls for a different approach in mitigating the continuous spread of the disease by targeting the sole vector that transmits the virus causing the disease; the banana aphid (*Pentalonia nigronervosa*). To this end, a hairpin construct harbouring an important gene for aphid survival, acetylcholinesterase was designed to degrade this specific gene in the banana aphid.

First, there was a need to determine the efficiency of degrading *AChE* gene in banana aphid before *Agrobacterium*-mediated transformation in plant cells. A synthetic diet optimised from Dadd and Mittler, (1966) protocol was designed to enable successful rearing of aphids for *in vitro* assay. It was evident that a diet with balanced carbohydrate and amino acid is essential for aphid survival and reproduction (Sabri *et al*., 2013). All the four diet types used in this study supported the growth of banana aphids except for Diet 5 which contained only water supplemented with sucrose at varying concentrations where necessary. The highest mortality of aphids was observed in this diet, supporting the fact that aphids are unable to survive or reproduce in the absence of adequate nutrients.

Trace metals like iron, zinc, manganese, and copper are essential for aphid reproduction (Akey & Beck, 1972). The availability of this minerals in chelated form (Diet 1) or sulphate form (Diet 2) supports the development and reproduction of banana aphids. Also, Diets (3 & 4) with sulphate and chloride complexes without the inclusion of trace metals supported the growth of banana aphid for the time period tested. This could be due to the availability of magnesium as well as amino acid-containing compounds in the diet. Work on pea aphid (Mittler & Dadd, 1965; Srivastava & Auclair, 1971) confirmed that the use

of trace elements (Fe, Cu, Mn and Zn) in sequestrate form improved the performance of the aphid. The unavailability of sequesterene form of Manganese led to the use of the sulphate complexes for all the trace metals in diet 2 but the result obtained showed that the growth and reproduction of aphids were not compromised. However, an extended feeding period trials could be done to confirm this.

Carbohydrates such as sucrose, fructose and glucose are known phagostimulants for insects. Sucrose, however, has been identified as a major phagostimulant for some aphids at a concentration range of 10-35 % depending on the aphid species (Auclair, 1969; Srivastava and Auclair, 1971; Douglas *et al*., 2006; Puterka *et al*., 2017; Moon, 2019). In this study, banana aphid survived on sucrose concentration from 5-20 %. Although banana aphid could survive on low sugar concentration, an optimum sucrose level in this study was 7.5 g/100 ml diet.

Sugar analysis of selected banana and plantain used in this study confirmed the presence of sucrose in all the cultivars. Other types of sugars detected include fructose and glucose. Sugars with varying concentrations and types are predominantly found in phloem cells of plants (De Schepper *et al*., 2013) with sucrose being the major transport of assimilates (Ciereszko, 2018).

In our study, low concentration of sugars was observed in *in vitro* plants compared with the field explants suggesting that the age of plant and photosynthetic activities could have influenced accumulation of sugar levels overtime in the field explants. The presence of more sugars in leaf explant has earlier been reported as part of natural plant physiology. This is because the leaves are photosynthetically active and act as a *source tissue* where sucrose is transported to other parts of the plant (Truernit, 2001). It is without a doubt that sugars are essential for naturally occurring aphid-plant interaction. Concepts such as "high-sugar resistance" and "Sweet priming" sheds light on the important role of sugars in signalling plant defence mechanisms (Ferri *et al.*, 2011; Moghaddam & Van den Ende, 2012). Sugars have been reported to enhance plant resistance either by providing a

systemic defence or playing a role in the signalling network for regulating plant immunity (Morkunas & Ratajczak, 2014). An alteration to sucrose-H+ symporter (*StSUT1*) gene led to reduced sucrose in phloem cells and impacted probing by aphid *Macrosiphum euphorbiae* (Pescod *et al*., 2007). The knockdown of sucrose transporter genes (*stp1* & *stp2*) by RNA interference in tomato triggered the reduction of glucose and sucrose but did not affect xylose. This led to reduced infectivity and stylet thrusting of plant-parasitic nematode *Meloidogyne incognita* (Warnock *et al*., 2015). In other instances, glucose or sucrose has been identified to activate the expression of pathogenesis-related protein-coding genes (Morkunas & Ratajczak, 2014). The presence of sugars, as phagostimulants could be one reason why banana aphids are attracted to banana plants. The relative quantity of sugars produced by the plant during the process of feeding may be just enough to sustain banana aphids, or not high/less enough to deter aphids from feeding on banana/plantain plants. Therefore, the chemistry between sugars as well as induction of secondary metabolites when plants are infested on by aphids could vary with plant type and cultivar and could be further investigated. An understanding of this interaction through *in vitro* analysis of infested and non-infested cultivars could unravel the potential trigger for innate defence mechanism in banana/plantain plants.

Acetylcholinesterase is an enzyme that plays a key role in the physiology and survival of all arthropods including banana aphid. AChE is known to activate the breakdown of acetylcholine (ACh) through hydrolysis thereby relinquishing the possible adverse effect of over-accumulation of an impulse generated by this neurotransmitter (Dvir *et al*., 2010). Choline and an acetate ion as by-products of the chemical reaction. Choline reinitiates the production of AChE while acetate ion plays an important role in the synthesis of fatty acids and carbohydrate when it combines with co-enzyme A (Colovic *et al*., 2013). AChE is one of the prime enzymes that can be targeted to control most crop pest like aphids (Kishk *et al*., 2017). The inhibitory effect of AChE on banana aphids using dsRNA harbouring the *AChE* gene as a supplement in an optimised synthetic diet for banana aphids confirmed that silencing of *AChE* could lead to mortality of banana aphids at a

high dose of up to 500 ng/µl. A lethal dose of 125 ng/µl dsRNA harbouring *AChE* led to the silencing of Asian citrus psyllid (Kishk *et al*., 2017) while a dose of 1000 ng/µl *BC-Actin* led to reduction gene expression in *B. cockerelli* (Wuriyanghan *et al*., 2011).

The effect of *AChE* siRNA uptake in banana aphid showed that *AChE* is a prime gene for aphid survival. At 7-day post-feeding, up to 75 % mortality was observed on aphids fed with 250 ng *AChE*-siRNA diet. The impact of siRNA concentration and the level of observed mortality is organism-specific. Borgio (2010) and Upadhyay *et al* (2011) were able to achieve over 80 % mortality in whitefly while significant knockdown was observed using 100 ng/µl in *B. cockerelli* (Wuriyanghan *et al*., 2011).

Agrobacterium-mediated transformation of the *AChE* gene was carried out using embryogenic cell suspension of Gonja Manjaya, Orishele and Cavendish Williams. Only transformed cells regenerated into a whole plantlet on kanamycin (100 mg/l) selection media. These events were selected and subjected to molecular characterization to confirm the presence of the gene by PCR, integration and copy numbers by Southern blot and expression level of genes by RT-PCR. An amplicon size of 466 bp was confirmed positive by PCR in 13, 32, and 34 events of Orishele, Gonja Manjaya and Cavendish Williams, respectively. A random selection of PCR positive events subjected to Southern blot analysis confirmed the integration of the gene in some events with varying copy numbers from 1 to 11. Eight, four and eleven events of Orishele, Cavendish Williams and Gonja Manjaya, respectively had a single gene copy number.

Reverse transcription-polymerase chain reaction (RT-PCR) is a molecular technique that enables the measurement of gene expression at the RNA level (Bell, 1995). Total RNA isolation from selected transgenic events, cDNA synthesis and subsequent PCR amplification of *AChE* gene using *AChE* specific primers were done to confirm the expression levels of *AChE* transcript in the selected transgenic events (Fig 6.8). There was a variation in the expression of *AChE* transcript in the transgenic plants tested. One event of Gonja Manjaya, and three events of Orishele showed absence of *AChE* transcripts while

some Cavendish Williams events showed minimal expression as depicted by their band intensities. However, stable expression of *Musa* housekeeping gene (*Musa 25S* ribosomal endogenous gene) was conserved across the genotypes. A quantitative real time-PCR, Northern and Western blot could give better insight into the expression levels of *AChE* transcripts in transgenic events. This could be considered in future study.

The transgenic events in insect-proof cages in a confined glasshouse were exposed to non-viruliferous banana aphids including the control plants and the resistance to banana aphids was measured relative to the control plants and termed as "relative resistance". For Orishele, almost all the events subjected to Southern blot hybridization had single copy numbers, but this does not correspond to their respective decrease in aphid population. Although transgenic event 18 showed an abundant *AChE* transcript, the reduction in aphid population relative to control non-transgenic plant was 50 % while event 6 with minimal transcript expression showed about 75 % reduction in aphid population and event 22 with a similar expression as event 6 showed more aphid population relative to the control (-10 %). A similar trend was observed in Gonja Manjaya and Cavendish Williams. There was no direct relationship between the copy number of the T-DNA integration in transgenic events and resistance to banana aphid.

There might be low siRNA accumulation in transgenic plants which may limit the efficiency of RNAi trigger in aphids. Also, the type of expression vector may have influenced the low resistance, in this study *AChE*-hp construct was used to transform plants. However, Guo *et al*., 2014 observed that transgenic plants expressing amiRNAs targeting two sites in *MpAChE2* gene showed better resistance to aphids than the *MpAChE2*-hp transgenic plants. The mode of uptake may have enabled other factors such as pH of the vector to degrade the siRNA during feeding. Studies have shown that the pH of an insect or midgut secretion has the potential to degrade dsRNA (Christiaens & Smagghe, 2014; Ghodke *et al*., 2019) and prevent downstream systemic RNAi effect. Future studies could explore gene(s) which may prevent probing by the aphid, use of

multiple gene families, and topical application for probable durable, selective resistance to the banana aphid. Only transgenic events that have shown a significant decrease in aphid population relative to the control should be considered for future field testing or further improvement by targeting the viral gene(s) for silencing by RNAi.

6.5 Conclusion

Controlling BBTD begins with controlling banana aphid, the vector that transmits BBTV causing BBTD. To this end, an i*n vitro* insect assay was designed to rapidly evaluate the *AChE* gene function in banana aphid as well as determining an optimum diet for a successful *in vitro* assay. This study reports the ideal diet for rearing banana aphid *in vitro* as well as the impact of silencing *AChE* in banana aphid through ingestion using dsRNA and siRNA at varying concentrations. Although this is the first report of in vitro assay using synthetic diet for banana aphids, diet compositions were optimised based on pea aphid diet that was previously published. This information provides an insight into the nutritional requirements of banana aphid as a vector that transmit BBTV causing BBTD in banana/plantain. Besides, the *AChE* gene was successfully transformed in embryogenic cell suspension of banana cells via *Agrobacterium*-mediated transformation approach. The integration of the gene was confirmed by molecular characterization. The copy number of the transgenic events does not influence their resistance to banana aphid under glasshouse conditions. This is the first report on the generation of banana/plantain plants that are resistant to banana aphids.

CHAPTER SEVEN

GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATION

7.1. General Discussion

The need to curtail the spread of banana bunchy top disease (BBTD) cannot be overemphasized to achieve food security in the world by mitigating the potential threat to banana and plantain production. The presence of the disease vector, banana aphid in all banana/plantain producing regions, transboundary exchange of germplasm, sterility of the crop and lack of banana/plantain germplasm that is immune to BBTD makes it very challenging to manage the disease.

However, RNAi offers an alternative strategy to mitigate the spread of BBTD by controlling the vector. This study employed this approach to develop banana/plantain cultivars that are resistant to the banana aphid.

Embryogenic cell suspensions (ECS) were developed for farmer preferred plantain cultivars in West Africa; Agbagba, Obino l'Ewai and Orishele. Increased efficiency of up to 22 % friable embryogenic callus was obtained in this study compared to an average of 1.8 % which was previously reported (Strosse *et al*., 2006). The cultivar, media type, phytochemicals, and proliferation potential of *in vitro* plantlets are some factors that influence the development of friable embryogenic callus and embryogenic cell suspension. It is noteworthy that Agbagba recorded the highest proliferation potential *in vitro* and generation of friable embryogenic callus. Identification and quantification of phytochemicals found in banana and plantain cultivars are important for *in vitro* manipulations and optimization for crop improvement. Total antioxidant capacity, phenolics, flavonoids and tannins were determined and reported. Cultivar and explant types determine the variation in phytochemicals. The high level of phenolics in Agbagba compared to Obino l'Ewai and Orishele may have influenced the improved response of the cultivar to generating friable embryogenic calli (FEC). It is therefore important to

understand the basic phytochemical composition of a cultivar before *in vitro* procedure optimizations.

ECS is usually the preferred starting material for *Agrobacterium*-mediated transformation. The successful generation of plantain ECS led to the determination of the transformability of the cell generated. Transient and stable *gus*A and *gfp* expressions confirmed that the plantain cells are transformable. Interestingly, plantain cells were also successfully transformed at 2 and 3-day co-cultivation period. Also, a low concentration (50 µM) of acetosyringone can aid the transformation efficiency of plantain embryogenic cell suspensions.

The ingestion *AChE* gene in the form of dsRNA under *in vitro* feeding conditions conferred lethal effect on banana aphid, thus, confirming the efficiency of *acetylcholinesterase* to aphid survival as well as the effectiveness of RNAi. Several reports have confirmed the efficacy of RNAi on insect orders like the coleopteran, lepidopteran, hemipteran insect pest (Baum *et al*., 2007; Zha *et al*., 2011; Laudani *et al*., 2017; Poreddy *et al*., 2017; Knorr *et al*., 2018; Yoon *et al*., 2018).

Furthermore, *in vitro* feeding assay of banana aphid confirm the importance of sucrose as one of the adaptive components that aphids depend on for survival and obtain from the phloem cell of banana/plantain plants. Also, the absence of a sequestrate form of trace elements should not deter *in vitro* banana aphid assay as sulphate complexes of the trace metals did not compromise banana aphid growth and reproduction.

Plant-insect interaction usually results in the production of biochemicals which may either have a positive or negative influence on the insect pest. On one hand, some of these chemicals have insecticidal effects on the insect pest, and on the other, insect pests have evolved a complex mechanism to detoxify or neutralize the defence chemicals exuded by plants upon attack (Mittapalli *et al*., 2007; Kerchev *et al*., 2012; Nishida, 2014). Phytochemicals such as antioxidants have been identified to confer insecticidal effects on

Red flour beetle (Chuang *et al*., 2018), initiate defence response of pea leaves to *A. pisum* infestation (Morkunas *et al*., 2016), as well as induce flavonoids in groundnut following infestation by *Helicoverpa armigera* and *Aphis craccivora* (War *et al*., 2016). Also, inhibition of insect growth by hydrolysable tannins contributed to plant defence in oak species (Moctezuma *et al*., 2014). It is plausible that phytochemicals contribute to plant defence mechanisms. The work of Moctezuma *et al*., (2014) showed that for durable resistance to an insect pest, a combination of specific phytochemicals rather than the total or individual compounds could play a significant role in the defence mechanism. Once identified, genetic modification approach could be used to either express higher concentration of these compounds or silence the underlying gene that is responsible for detoxification of plant biochemicals in insect pest (Wu *et al*., 2015).

Resistance of transgenic events to banana aphid determined by comparing aphid population on elite lines to the control plants confirmed varying resistance levels. While Gonja Manjaya recorded the least decrease in aphid population relative to the control plants, up to 78 and 79 % resistance was observed in Cavendish Williams and Orishele respectively. Low siRNA accumulation, type of expression vector, mode of uptake, pH of aphid midgut and RNA silencing suppressors encoded as proteins in plants could have limited the efficacy of RNAi in banana aphid.

7.2 Conclusions

- Plant tissue culture techniques and genetic transformation are useful for crop genetic improvement.
- An optimized protocol developed in this study for generating friable embryogenic callus (FECs) and embryogenic cell suspension (ECS) of farmer-preferred plantain cultivars can be adopted to improve other plantain cultivars.
- FECs and ECS generated can be used for mass propagation of clean germplasm, protoplast isolation and genetic modification of plantain.
- An optimized genetic transformation protocol for plantain cultivars developed in this study can be used to introduce genes of important agronomic traits in plantain/banana cultivars.
- An optimal *in vitro* assay for banana aphid developed during this study will be useful for rearing banana aphids and to test gene functionality *in vitro*.
- Transformation of plantain and banana cultivars with pNXT-35S-ACE-hp construct generated elite lines which would be used in future field trials.

7.3 Recommendations

The following could be recommended for future studies:

- Simultaneous silencing of multiple family and/or non-family genes in banana aphid could be an efficient alternative method.
- Topical application of dsRNA using nanotechnology for plant protection could be explored.
- Field trials of elite lines that are resistant to banana aphids should be done.

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APPENDICES

Appendix 1: Bacteria co-cultivation medium (BCCM) A with varied acetosyringone concentration

BCCM A (300 ml)					
Component	**T1**	**T2**	**T3**	**T4**	**T5**
Maltose	15 g	15 g	15 g	15 g	15 g
Proline	150 mg	150 mg	150 mg	150 mg	150 mg
Malt extract	50 mg	50 mg	50 mg	50 mg	50 mg
Biotin (1 mg/ml)	500 µl	500 µl	500 µl	500 µl	500 µl
Ascorbic acid (10 mg/ml)	500 µl	500 µl	500 µl	500 µl	500 µl
Acetosyringone (400 mM)	0 µl	62.5µl	125 µl	250 µl	500 µl
MS vitamins + myo-inositol (200×)	5 ml	5 ml	5 ml	5 ml	5 ml
Sucrose	15 g	15 g	15 g	15 g	15 g
Glucose	5 g	5 g	5 g	5 g	5 g
L-Glutamine	50 mg	50 mg	50 mg	50 mg	50 mg
L-Cystein	200 mg	200 mg	200 mg	200 mg	200 mg
pH	5.3	5.3	5.3	5.3	5.3

Note: filter sterilize media

Appendix 2: Bacteria co-cultivation medium (BCCM) B

BCCM B	
Component (MS)	**200 ml**
Macro (10×)	5 ml
Micro(100×)	5 ml
Iron (100×)	5 ml
Gelrite	5 g
pH	5.5

Note: Autoclave culture media

Appendix 3: Bacteria re-suspension medium (BRM) A

BRM A	
Component	**500 ml**
Macro (10×)	6.25 ml
Micro(100×)	6.25 ml
Iron (100×)	6.25 ml
MS vitamins(200×)	2.5 ml
Sucrose	42.75 g
pH	5.3

Note: Autoclave culture media

Appendix 4: Bacteria re-suspension medium (BRM) B with varied acetosyringone concentration

BRM B (/125ml)					
Component (MS)	**T1**	**T2**	**T3**	**T4**	**T5**
Thiamine (10 mg/ml)	5.63 mg	5.63 mg	5.63 mg	5.63 mg	5.63 mg
L-cystein	250 mg	250 mg	250 mg	250 mg	250 mg
Glucose	22.5 g	22.5 g	22.5 g	22.5 g	22.5 g
Acetosyringone (400 mM)	0 µl	78.13 µl	156.25 µl	312.5 µl	625 µl

Note: pH 5.3, filter sterilize, make aliquots of 25 ml and store at -20 °C

Appendix 5: GUS assay stock solution and buffer

Component	Quantity	Final Volume
Tris (10 µM), NaCl (50 mM) (pH 7.2)	1.21 g Tris, 2.92 g NaCl	1 L
X-Gluc (10 mg/ml)*	100 mg	10 ml
Triton X-100 (10 %)	5 ml	45ml
GUS buffer	890 µl 10 mM Tris/50 mM NaCl, 100 µl X-Gluc, 10 µl 10 % Triton X-100	1 ml

Note: * is dissolved in Dimethylsulphide

Appendix 6: Cetyltrimethylethyl ammonium (CTAB) extraction buffer

Component	Quantity
CTAB (0.8 %)	4 g
N-laurosarcosine (1 %)	5 g
Tris HCL 220 mM (pH 8.0)	110 ml of 1 M Tris HCL
EDTA 22 mM (pH 8.0)	22 ml of 0.5 M Tris EDTA
NaCl 0.8 M	23.36 g
Mannitol 0.14 M	12.75 g

Note: Components were weighed and transferred quantitatively into a sterile bottle and made up to 500 ml

Appendix 7: Southern blot buffer

Buffer Type	Component	Quantity	Final Volume
Depurination	250 mM HCL (37 %)	20.6 ml	1 L
	H_2O	979.4 ml	
Denaturation	500 mM NaOH	20 g	1 L
	1.5 M NaCl	87.75 g	
Neutarlization (pH 7.5)	500 mM tris ultrapure	60.57 g	1 L
	1.5 M NaCl	87.75 g	
	1 mM EDTA	0.3724 g	
20× Sodium Citrate dehydrate (pH 7.0)	300 mM SSC	88.23 g	1 L
	3 M NaCl	175.32 g	
20 % Sodium Dodecyl Sulfate*	20 % SDS	200 g	1 L
Maleic acid (pH 7.5)	100 mM maleic acid	11.6 g	1 L
	150 mM NaCl	8.75 g	
Detection (pH 9.5)	100 mM Tris-HCL	100 ml from 1 M Tris HCL	1 L
	100 mM NaCl	20 ml from 5M NaCl	
Low stringency Wash buffer**	2X SSC	100 ml 20× SSC	1 L
	0.1 % SDS	10 ml of 10 %SDS	
High stringency Wash buffer**	0.1× SSC	5 ml 20× SSC	1 L
	0.1 % SDS	10 ml 10 % SDS	
Wash Buffer**	Maleic acid	1 L maleic acid	1 L
	3 % Tween-20	3 ml Tween-20	
Blocking solution*	Blocking powder	2 g	200 ml

Note: Components were weighed and transferred quantitatively into a sterile bottle and sterilized by autoclaving with the exception of buffers*. Buffer ** were reconstituted with sterilized distilled water.