IN VITRO REGENERATION, GENETIC TRANSFORMATION, AND CRISPR/Cas-BASED GENOME EDITING IN YAM (*DIOSCOREA SPP*.)

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DECLARATION

I, Easter Syombua David, declare that this thesis is my original work and has not been presented for a degree or any other award in any university. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person except where due reference is made.

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DEDICATION

To my daughter Olive Katumbi and son Elvis Mutio. You are my pillar of strength; please endeavour to achieve more than I have.

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LIST OF ABBREVIATIONS AND ACCRONYMS

2,4-D	2, 4-dichlorophenoxyacetic acid
BAP	6-Benzylaminopurine
Bp	Base pair
Cas	CRISPR associated
CIM	Callus induction media
CRISPR	Clustered randomly interspaced palindromic repeats
CTAB	Cetyl trimethyl ammonium bromide
DSB	Double-stranded DNA Breaks
EDTA	Ethylene diamine tetra acetic acid
FAO	Food and agriculture organization
FAOSTAT	FAO statistics
FDA	Fluorescein diacetate
FEC	Friable embryogenic callus
FIM	FEC Induction Medium
GA_3	Gibberellic acid
GD	Greshoff and Doy
GFP	Green fluorescent protein
GM	Genetically modified
gRNA	Guide RNA
GUS	β-Glucuronidase
HDR	Homology directed repair
Indel	Insertion-deletion mutations
KM	Kao and Michayluk
LB	Luria Bertoni
MS	Murashige and skoog medium
NAA	α –Naphthalene acetic acid
NHEJ	Non-homologous end-joining
OD	Optical density
ORF	Open reading frame
PAM	Protospacer-adjacent motif
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDS	Phytoene desaturase
RNAi	RNA interference
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase polymerase chain reaction
sgRNA	Single guide RNA
SSA	Sub-saharan Africa
TALEN	Transcription activator-like nucleases
T-DNA	Transfer DNA
X-gluc	5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid
ZFN	Zinc finger nucleases

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ABSTRACT

Yam is a starchy tuberous crop that provides food and income to millions of people in tropical and sub-tropical regions of the world. Despite its importance, several biotic and abiotic constraints beset yam production. Yam improvement by conventional breeding has been hampered by its polyploidy, heterozygosity, and vegetative propagation. Yam genetic improvement will therefore require the development of new techniques that allows direct manipulation of its genome. Targeted genome editing strategies such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the clustered regularly interspersed short palindromic repeats (CRISPR/Cas) system have proven that sequence-specific nucleases are effective tools for use in gene function analysis and crop improvement. Compared to ZFNs and TALENS, the CRISPR system holds more potential due to its simplicity, efficiency, versatility and affordability. Plant genome engineering, however, relies on transformation and regeneration for the recovery of mutants. The production of embryogenic callus is a crucial step in the regeneration of most crops. This study reports a system for CRISPR/Cas9-mediated genome editing in yam. The conditions suitable for somatic embryogenesis, regeneration of friable embryogenic callus, and *Agrobacterium* mediated transformation of two yam species, *D. alata* and *D. rotundata* were determined. Further, a protocol for isolation, purification, and culture of *D. rotundata* protoplasts was established from mesophyll and callus tissues. Various factors, including tissue type, explant age, period of enzyme incubation, enzyme concentration, phytohormone combinations, concentration in the culture medium, were shown to influence the protoplast yield, viability, and regenerative capacity. Two guide RNAs targeting the yam phytoene desaturase (*PDS*) gene were designed, transfected onto a suitable plasmid to generate pCas9-gRNA-gfp-PDS, then to *Agrobacterium* strain EHA 105. The efficacy of the *Cas9-gfp* gene expression in yam was evaluated by agroinfiltration. An optimized agroinfiltration system was developed, consisting of the *Agrobacterium* strain EHA105 harboring pCas9_gRNA-PDS (OD_{600}= 0.75), suspended in infiltration buffer supplemented with 400 μM acetosyringone, infiltered in fully expanded young leaves and heat shock treatment. The CRISPR/Cas9 plasmid was delivered to nodal explants through *Agrobacterium*-mediated transformation, and mutated events were regenerated by organogenesis. Transgene expression of the *gfp* tagged gene in these events was further confirmed by GFP fluorescence under UV light. Eight events were regenerated, among which one was green, while seven showed phenotypes of complete to variegated albinism. Leaves of transgenic plants emitted a bright fluorescence, while wild-type plants did not emit any fluorescence. All putative transgenic plants contained *Cas9*, as confirmed by PCR analysis. All seven mutant events showed indels at both gRNA1 and gRNA2 within 3-4 bp upstream of the PAM sequences. The indels consisted of a mixture of insertions, deletions, and substitutions of 1 to 59 base pairs. As expected, the green plant showed no mutation at either target site. The genome-editing efficiency was 83.3%. The yam regeneration, genetic transformation, and genome editing protocols developed in this study will provide opportunities for yam improvement. Overall, these results demonstrated that the CRISPR/Cas9 system can induce site-specific disruption of the *PDS* gene and generate stable phenotypic changes in yam. The findings reported herein offer new frontiers for gene function analysis and direct manipulation of the yam genome.

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CHAPTER ONE

INTRODUCTION

1.1 Background of the study

Yams (*Dioscorea spp.*) are a multispecies tuber crop that serves as a food staple and an income source for approximately 300 million people in the world (Asiedu & Sartie, 2010). Food yam is also of cultural significance, especially in Melanesian countries and in the 'yam belt' of West Africa, where they are regarded as a fertility symbol (Lebot, 2020). Yam also produces active compounds that can be exploited to produce excipients in the pharmaceutical industry (Salehi *et al.,* 2019).

Yam is the fourth most important food tuber crop in the world after potato, sweet potato, and cassava (FAOSTAT, 2018). Yam cultivation is spread through Africa, Asia, parts of South America, as well as the Caribbean and the Pacific islands. West Africa accounts for 95% of the global yam production, with Nigeria accounting for 68% of the total yam quantities produced in the world (IITA, 2012). Out of the known 600 species of the *Dioscoreaceae* family, *Dioscorea alata*, *D. bulbifera*, *D. cayenensis*, *D. trifida*, *D. esculenta*, *D. opposita-japonica*, *D. nummularia*, *D. pentaphylla*, and *D. rotundata* are the most cultivated *Dioscorea* species (Lebot, 2010; Padhan & Panda, 2020). Of these, *D. rotundata* and *D. cayenensis* are the most predominant both in quantities produced and marketed, and *D. alata* the most widespread species globally. *D. alata* is indigenous to Asia, while *D. cayenensis* and *D. rotundata* are of West African origin (Asiedu & Sartie, 2010). Yam is primarily diploid with a base chromosome number of 20 (2n = 2x = 40). However, numerous cases of polyploid individuals have been reported, including triploids and tetraploids (Denadi *et al.,* 2020; Girma *et al.,* 2018).

Yam plays a significant role in the food security and income status of smallholder farmers within the yam belt of West Africa, stretching across Benin, Ivory Coast, Ghana, Nigeria, and Togo (Mignouna *et al.,* 2008). Several attributes of the yam plant, such as diversity of maturity periods, tuber dormancy when growth conditions are not favorable, and ability to adapt to a broad range of agro-ecological zones, make it important in ensuring the continued availability of food throughout the year (Asiedu & Sartie, 2010). Additionally, yam tubers are rich in vitamin C, essential minerals, dietary fiber, and starch (Chandrasekara & Josheph Kumar, 2016). Despite these attributes, the cultivation of yams is plagued by several biotic and abiotic constraints. Biotic constraints include susceptibility to pest (insects and nematodes) and disease (viral, fungal, and bacterial) infestations. The abiotic and agronomical impediments to yam production include inadequate planting materials, decreasing soil fertility, labor cost for land (heap) preparation, and low yield potential (Reuben & Barau, 2012). Also, the storage of fresh yams is challenging due to substantial quality deterioration during storage, rendering them unsuitable for human consumption (Maalekuu *et al.,* 2014). Yam production is further threatened by changing climate patterns, causing increased tuber prices, and reduced productivity, profitability and employment losses (Oluwatayo & Ojo, 2016).

Genetic improvement of the yam germplasm through classical breeding methods has been hampered by the dioecious nature of the plant, its polyploidy, poor seed set, heterozygosity, and a prolonged breeding cycle (Mignouna *et al.,* 2008). Yam crop improvement therefore, requires the use of new approaches that allows direct manipulation of its genome (Syombua *et al.,* 2020). Targeted genome editing strategies could be exploited to complement

conventional breeding and transgenic methods for crop improvement and gene function analysis. Additionally, plants generated by DNA-free genome editing are not distinguishable from those generated via classical mutagenesis approaches such by use of radiation or chemicals; hence may bypass the strict regulatory regimes of genetically modified organisms (Kanchiswamy *et al.,* 2015).

Modern genome editing tools such as Zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) and CRISPR/Cas (clustered regularly interspaced short palindromic repeats – CRISPR associated proteins) - based approaches, have revolutionized crop improvement and gene function analysis (Bao *et al.,* 2019). These systems rely on sequence-specific nucleases to create double stranded breaks (DSBs) on a precise locus in the target gene, which is then repaired by the endogenous DNA repair mechanisms. The DSB repair is achieved by either Non-homologous end joining (NHEJ) or homologous recombination (HR). Non-homologous end joining, which is the most preferred repair mechanism in eukaryotic cells, is error-prone, often creates indels at the target site resulting in gene knockout (Puchta, 2005). Among the three genome editing systems, CRISPR/Cas system developed from the adaptive immune system of *Streptococcus pyogenes* is the most preferred due to its high targeting efficiency, binding specificity, low cost, and simplicity of design (Malzahn *et al.,* 2017). The versatility of this for site-directed crop mutagenesis has been demonstrated in vegetatively propagated crops (Cai *et al.,* 2018; Tripathi *et al.,* 2019; Wang *et al.,* 2019), cereal crops (Liang *et al.,* 2017; Shi *et al.,* 2017; Sun *et al.,* 2016), and horticultural crops (Li *et al.,* 2018; Malnoy *et al.,* 2016; Nishitani *et al.,* 2016; Tian *et al.,* 2018).

Genome engineering relies on plant transformation and regeneration to recover transformed events (Altpeter *et al.,* 2016). So far, there is only one published report of stable genetic transformation of food yam, where transformed events were recovered by organogenesis (Nyaboga *et al.,* 2014). Regeneration of transformed cells via shoot organogenesis is, however, prone to the production of chimeric plants hence the need to explore other methods of regenerating transformed cells. There are very few reports of regeneration of yams via somatic embryogenesis, and in most of the reports, the regeneration frequencies are low (Belarmino and Gonzales, 2008; Suárez Padrón *et al.,* 2011). Previously, (Manoharan *et al.,* 2016) reported a robust regeneration system for *D. rotundata* based on somatic embryogenesis. However, the system led to a mixed regeneration where some shoots were produced from somatic embryos while the rest were of adventitious origin. This system needs to be optimized further to eliminate adventitious shoots, which are prone to production of chimeric plants, when used to regenerate transformed cells. Moreover, somatic embryogenesis protocols are highly genotype-dependent hence the requirement to validate the protocol to suit each genotype of interest (Manoharan *et al.*, 2016).

Plant protoplasts are totipotent plant cells whose cell walls have been removed by either mechanical disruption or enzymatic digestion. These fragile wall-less structures provide versatile tools for application in various research fields, including functional gene characterization, somatic hybridization, cybridization, and genetic transformation. However, the ability to isolate high quantities of viable protoplasts and regenerate them into whole plants forms the primary bottleneck in applying these totipotent systems in both research and crop improvement (Davey *et al.,* 2005). Plant protoplasts also provide

excellent targets for Cas9/sgRNA-mediated mutagenesis (Sun *et al.,* 2017; Xing *et al.,* 2014). At present, successful protoplast isolation has been demonstrated in both monocotyledonous and dicotyledonous crops, including sweet potato (Guo et al., 2006), grapevine (Bertini *et al.,* 2019), lettuce (Sasamoto & Ashihara, 2014), tomato (Horváth, 2009), banana (Wu *et al.,* 2020) and soybean (Wu & Hanzawa, 2018a) and apple (Malnoy *et al.,* 2016). However, protoplast based studies in yam are lacking. Tor *et al.* (1998) first reported yam protoplast isolation and DNA uptake by polyethylene-glycol (PEG) mediated transformation, but no attempts were made to regenerate the protoplasts. Therefore, it is paramount to develop more efficient systems for isolating huge quantities of viable protoplasts and subsequent regeneration of callus cultures.

1.2 Problem Statement

The demand for food yam always exceeds the actual supply. This trend is projected to continue, particularly due to population increase (Asiedu & Sartie, 2010). The current tuber yield estimates of yam are less than 10 t/ha, significantly lower than the potential yield of 50 t/ha (FAOSTAT, 2014). The low productivity is attributed to various biotic and abiotic factors, including diseases and pests, low yield potential, inadequate planting material, and decreasing soil fertility (Korada *et al.,* 2010). These challenges are further compounded by up to 30% tuber losses during storage (Mignouna *et al.,* 2014). Efforts towards yam improvement by conventional breeding are ongoing to produce high-yielding, pest and disease-resistant yam varieties (Arnau *et al.,* 2016). However, these efforts are faced with numerous challenges due to the long breeding cycle of yams, dioecious nature, poor flowering, polyploidy, vegetative propagation, and heterozygous genetic background (Darkwa *et al.,* 2020).

Genetic engineering has consistently proven to supplement conventional breeding towards the improvement of many crops, including vegetatively propagated crops such as cassava (Ntui *et al.,* 2015), sweet potato (Magembe *et al.,* 2019), and banana (Tripathi *et al.,* 2019b). To date, however, no yam variety has been improved by the transgenic approach. This delayed progress is primarily due to the lack of yam regeneration and transformation systems.

1.3 Study justification

Considering the challenges encountered in yam production, consumption and improvement,, immense efforts are needed to develop multidimensional approaches towards yam improvement, such as *in vitro* regeneration, genetic transformation, and genome engineering (Syombua *et al.,* 2021). Notably, the ability to regenerate whole plants from particular organs or tissues is affected by the crop genotype, tissue type and age, culture conditions (temperature, photoperiod, and pH), and media components (carbon source, phytohormones, amino acids, and vitamins). Therefore, this study optimized the appropriate combination of parameters for yam regeneration by somatic embryogenesis, since this forms a pre-requisite to *in-vitro* plant regeneration and transformation (Sugimoto *et al.,* 2019).

Though an effective complementary approach to conventional breeding, genetic engineering is challenged by the lack of global approval and immense regulations on genetically modified organisms (Komen *et al.,* 2020). The recent advances in genome engineering, particularly the CRISPR/ Cas9 system, have created a paradigm shift by providing the option of generating DNA-free genome-edited crops. Therefore, the non-

GMO genome-edited crops might alleviate the technical and regulatory barriers currently associated with genetically modified crops (Afzal *et al.,* 2020).

The functional system of the CRISPR/ Cas9 system consists of a cas9 endonuclease and a 20nt single guide RNA (sgRNA) with sequence complementarity to the target region (Tripathi *et al.,* 2019). A number of bioinformatics-based tools, including Cas OFFinder (Bae *et al.,* 2014), CRISPR Tools (Hsu *et al.*, 2013), COSMID (Cradick *et al.,* 2014), Cas Online Designer (Hsu *et al.,* 2013), etc., have been developed to aid gRNA selection and design. However, these tools only assess the theoretical efficiency and specificity of the target loci (Arndell *et al.,* 2019). Besides, bioinformatics-based efficiency prediction is frequently inaccurate, and most of the available online tools were developed and validated using data from non-plant species (Naim *et al.,* 2020). Most importantly, yam transformation methods are technically demanding and time-consuming, taking several months to regenerate putative events (Nyaboga *et al.,* 2014). With these challenges, the development of a quick, efficient, and cheap system of validating the efficiency of sgRNAs is of great significance.

For proof of concept studies in genome editing, it is practical to target genes whose mutation results in an easily identifiable phenotype, such as the phytoene desaturase (*PDS*) gene. Phytoene desaturase is a key enzyme in the carotenogenic pathway that catalyzes a rate-limiting step in carotenoid biosynthesis (Chamovitz *et al.,* 1993; Qin *et al.,* 2007). Disruption of this gene causes albinism and dwarfing by impairing chlorophyll, carotenoid, and gibberellin biosynthesis. As such, the *PDS* gene has been used as a feasible indicator for CRISPR/Cas9-mediated gene knockouts in various crops, including banana (Ntui *et al.,* 2020a), cassava (Odipio *et al.,* 2017), grapes (Wang *et al.*, 2018), petunia (Zhang *et al.,*

2016), apple (Nishitani *et al.*, 2016), populus (Fan *et al.*, 2015) and tomato (Pan *et al.*, 2016).

1.4 Significance of the study

This study established a yam genome-editing system through *Agrobacterium*-mediated delivery of CRISPR/Cas9 reagents into the nodal-explants to regenerate whole plantlets. This optimized genome editing protocol will act as valuable tool for transfecting the *Dioscorea* species with CRISPR/ Cas9 expression cassettes targeting genes of agronomic importance.

1.5 Objectives

1.5.1 General objectives

To develop a CRISPR/Cas9 based toolkit that can be used to improve the value of yam as a crop.

1.5.2 Specific Objectives

i. To optimize a protocol for *in vitro* regeneration and genetic transformation of *D. alata* based on somatic embryogenesis and organogenesis.
ii. To develop a system for regeneration of friable embryogenic calli in *D. rotundata*.
iii. To establish a protocol for protoplast isolation and culture in *D. rotundata*.
iv. To generate CRISPR/Cas9 mutants with a knockout on the endogenous phytoene desaturase gene.

1.6 Study hypotheses

i. *Dioscorea alata* is not amenable to *in vitro* regeneration and genetic transformation somatic embryogenesis and organogenesis

ii. The development and regeneration of friable embryogenic calli is not feasible in *D. rotundata*.

iii. Protoplast isolation and callus development are not feasible in *D. rotundata*

iv. The yam genome is not amenable to CRISPR/Cas9-based modification.

CHAPTER TWO

LITERATURE REVIEW

2.1 The origin and distribution of yams

The major food yams have been independently domesticated in the tropical regions of three different continents, including Africa, Southeast Asia, and South America (Coursey, 1967). The water yam (*D. alata*) was first domesticated in Southeast Asia and is now the most extensively cultivated yam species in the world. The spread of *D. alata* occurred 2000 years ago, and it reached Africa around 1000 AD. From there, *D. alata* was taken to tropical America by Portuguese and Spanish travelers in the 16th century (Mignouna & Dansi, 2003).

In West Africa, yam domestication began as early as 5000 AD, but true yam-based agriculture in the region began at around 3000 BC. The *D. rotundata* and *D. cayenensis* species are native to West Africa (Ayensu & Coursey, 1972). The *D. trifida* species is the only food yam native to tropical America that has gained significance as a food crop. Its production is, however, still limited to the West Indies (Siqueira, 2011).

2.2 Global economic impact and production of yams

The yam is an important staple food for millions of people worldwide, particularly in Africa, Asia, the Caribbean, and Tropical America. In terms of global production of tuber crops, yam ranks fourth after potato, cassava, and sweet potato. Its global production in 2008 was estimated at 51.8 MT, of which 93% (48.1 MT) was from West Africa (FAOSTAT 2018). The dominant yam production zone in Africa is the yam belt which includes West Africa and Central Africa. Traditionally, yams have been produced in East

and Southern Africa, although the production levels are very low (Wanyera *et al.,* 1996). Between 1992 and 2012, yam production in West Africa increased from approximately 27 to 54 million tons due to the use of traditional landraces and an increase of the acreage under yam cultivation (FAOSTAT 2018).

The yield of yams in West Africa is about 11 tonnes per hectare. Nigeria is the leading yam producer with 34 million tonnes, followed by Cote d'Ivoire, Ghana, and Benin, which produce 5, 3.9, and 2.1 million tonnes respectively. Ethiopia (174,000 tonnes) leads the production in East Africa, followed by Sudan (137,000 tonnes). The major yam producers in South America are Columbia and Brazil, with yields of 333,000 and 230,000 tonnes, respectively, while Japan is the leading producer in Asia with an average yield of 204,000 tons (IITA, 2012). Yams are equally of importance in the Caribbean and the south pacific islands. Its average consumption per capita per day is highest (364 kcal) in Benin, followed by Cote d' Ivoire (342 kcal), Ghana (296 kcal,) and Nigeria (258 kcal). Ghana is the largest exporter of yams to the international market, with quantities of approximately 12,000 tonnes annually (IITA, 2012).

2.3 Uses of yams

2.3.1 Food value of yams

Yams are principally grown for their starchy tuberous roots that are a rich source of calories for majority of people living in the tropical regions. Yam tubers have an immense ability to store food reserves and, therefore, enrich the food base (Ferraro *et al.,* 2016). The tubers bring food security to food deficient low-income countries, providing approximately 200 kilocalories daily. Yams are comparatively more nutritious, providing several vitamins, minerals, and dietary protein. The dietary content of proteins in yams is approximately

4.6%, which relates well with maize (4.7%) (Chandrasekara & Josheph Kumar, 2016). Socioeconomic studies carried out in Nigeria showed a positive elasticity of demand for yams at all spending levels. Research towards increasing the supply of yams will escalate the amounts consumed by low-income earners in sub-Saharan Africa (Asumugha *et al.*, 2009).

Generally, the protein contents of roots and tubers ranges from 1 to 2% of the tuber dry weight basis, with potatoes and yams having the highest protein quantities (Chandrasekara & Josheph Kumar, 2016). Differences exist in the nutritional qualities of the different *Dioscorea* species. *Dioscorea dumetorum* has the highest protein and mineral values (Siadjeu *et al.,* 2018), while *Dioscorea alata* has a lower content of lipids, higher protein, and vitamin C than other yam species. Yam tubers should be cooked a few days following harvest, failure to which huge post-harvest losses are incurred because the tubers become hard and inconsumable (Medoua *et al.*, 2007).

Yams are mainly eaten as freshly prepared dishes, although the tubers of some species can be consumed raw. The common method of yam preparation in West Africa involves reconstitution of yam flour to a thick glue which is then consumed accompanied by some soup. Alternatively, yam tubers are fried, roasted, boiled, or cooked with proteins such as soy flour (Adepoju & Thomas, 2012).

2.3.2 Non – food uses of yams

In addition to consumption as food by the household, yams contribute to the cash income of families, especially in West Africa. The profitability of yam production, its significance in local trade, and the revenue from its export to European and Northern American markets

are often undervalued (Verter & Bečvářová, 2015). In Nigeria, for instance, yams constitute up to 32% of gross earnings from annual cropping. In 2008, Ghana's yam exports amounted to approximately 21,000 metric tons, valued at 14.89 million USD (Mignouna *et al.,* 2008).

Tubers and other parts of the yam plant produce a range of pharmacologically active secondary metabolites. These include alkaloids, diterpenoids, and saponins that have been documented to possess anti-fungal, anti-mutagenic, anti-oxidative, hypoglycemic, and immunomodulatory effects (Salehi *et al.,* 2019). Dioscorine, the most abundant compound in yams, is medicinally a heart stimulant (Obidiegwu *et al.,* 2020). These secondary metabolites have also found use as ingredients of cosmetic products and dietary supplements (Kumar *et al.,* 2017).

The waste from yam consumed at the household level could be utilized as domestic fodder (Kume *et al.*, 2019). The peel, for instance, is a good source of calories for sheep, although the high lignin concentration inhibits digestion of the protein. Yam is also currently being evaluated to provide industrial starch (Ye *et al.,* 2018).

Yams play a ritual and socio-cultural significance. Across the yam belt, yam ownership and cultivation are attributed to many cultural, religious, and social significance. Several traditional beliefs and taboos have been associated with the planting, harvesting, and consumption of yam. For instance, pounded yams are food for festivities, royalty, and special guests (Obidiegwu & Akpabio, 2017). Also, in various parts of Oceania, several customs and traditions are integral to yam production (Levin, 2019).

2.4 Constraints to yam production

The demand for yams by consumers in sub-Saharan Africa is increasing, but its production is declining due to biotic and abiotic stresses. These include susceptibility to attack by pests and diseases, high cost of propagation material, declining soil fertility, and low yield potential (Adegbite *et al.,* 2008).

2.4.1 Biotic constraints

2.4.1.1 Diseases associated with yam

Yam viruses

Yams are vegetatively propagated from seed tubers, and farmers obtain planting material from their farms or surplus material from their neighbors. This practice promotes pathogen accumulation and dissemination, particularly viruses from the infected low-quality material. Subsequently, farmers record substantial yield losses and a reduction in the quality of yam crops (Mantell & Haque, 1978). Besides, yam viruses impede the international exchange of yam germplasm. Yams are infected by viral pathogens belonging to six different genera, including Potyvirus (Yam mosaic virus, YMV, Yam mild mosaic virus, YMMV and *Dioscorea alata* potyvirus, DAV), Badnavirus (Dioscorea bacilliform viruses, DBVs), Cucumovirus (Cucumber mosaic virus, CMV), Potexvirus, Macluravirus, Fabavirus, Comovirus, Carlavirus and Aureusvirus (Seal *et al.,* 2014; W *et al.,* 2013).

Among these genera, viruses from the potyviridae family, particularly YMV, are the most widespread and economically significant within the yam belt of West Africa (Thouvenel & Fauquet, 1979). The nucleotide sequence of YMV consists of a single-stranded, positive-sense RNA genome of 9608 nucleotides encapsidated by ca. 2000 copies of a34 kDa coat

protein. Notably, the virus has been identified in all yam growing areas, including the the South Pacific, Latin America, and Africa. Infection with YMV is characterized by stunted growth, mottling, necrosis, leaf distortion, and subsequent yield losses of up to 60% (Silva *et al.,* 2015). YMV is particularly spread through the planting of infected materials and is transmitted in a non-persistent manner by several aphid species (Odu *et al.,* 2004). As such, the use of resistant varieties remains one of the most effective strategies for controlling the spread of YMV (Silva *et al.,* 2015).

The yam mild mosaic virus (YMMV), genus Potyvirus, is the second most prevalent virus in the West African yam growing regions. The virus preferentially infects *D. alata* but also has a wide repartition of *D. cayenensis* Lam.–*D. rotundata* Poir. and on *D. trifida* L. YMMV has also been reported in French Guyana and the Caribbean islands of Guadeloupe and Martinique (Bousalem *et al.,* 2003). The disease is characterized by green vein banding, leaf distortion, bleaching, and chlorosis. Control of viral perpetuation is achieved via the planting of virus-free material.

Yam badnaviruses are the most prevalent viral infection of yam worldwide (Bousalem *et al.,* 2009; Galzi *et al.,* 2013; Kenyon *et al.,* 2008). The virion of Badnaviruses is composed of non-enveloped bacilliform particles with a width of 30 nm and modal length of ~130 nm (Hull *et al.,* 2007). Yam plants are hosts to various Badnaviruses, which frequently occur as mixed infections and as integrated forms in the genomes of *D. cayenensis-rotundata* (Bömer *et al.,* 2016; Susan Seal *et al.,* 2014; Turaki *et al.,* 2017). Particles with close resemblance to yam Badnaviruses were first reported in the Caribbean in the 1970s associated with a flexuous virus that caused internal brown spot disease in *D. alata* and *D. cayenensis* (Harrison & Roberts, 1973; Mantell & Haque, 1978). Currently, Badnaviruses

are categorized into only two species, namely *Dioscorea bacilliform sansibarensis virus* (DBSNV) (Seal & Muller, 2007) and *Dioscorea bacilliform alata virus* (DBALV) (Briddon *et al.,* 1999). The Badnaviruses genome contains three open reading frames (ORFs). In contrast, ORF1 encodes a small protein of unknown function (Cheng *et al.*, 1996), OFR2 codes for a ~14 kDa virion-associated protein (VAP) that plays a role in virion assembly. ORF3 codes for a polyprotein that is matured into various proteins, including ribonuclease H (RNase H), reverse transcriptase (RT), an aspartic protease (AP), coat protein (CP) and a movement protein (MP) (Geering *et al.,* 2014).

Notably, the host range of Badnaviruses includes both monocots and dicots. The transmission of yam badnaviruses occurs via the planting of diseased propagules and mechanically in a semi-persistent manner by various species of mealybugs (family *Pseudococcidae*) (Bhat *et al.,* 2014; Odu *et al.,* 2004). Badnavirus infected yam plants could either be symptomless or display a spectrum of symptoms depending on the variety, environmental conditions, and virus species. These symptoms include leaf deformation such as puckering, veinal chlorosis, necrotic streaks, reduced internode length, and eventual stunted growth (Kenyon *et al.,* 2008). The masking of symptoms in Badnavirus-infected plants typically occurs during favorable conditions, but these re-emerge and become more severe during abiotic stress conditions, such as nutrient depletion or temperature shifts (Seal and Muller, 2007; Tripathi *et al.,* 2019).

Fungal yam diseases

Anthracnose is the most economically significant field pathogen constraining the production and marketing of the water yam (*D. alata*). Relative to other yam species, the water yam offers various advantages, including early vigor for weed suppression, high

yield potential even in poor dilapidated soils, sustainable production, and storability of tubers (Abang et al., 2003). Anthracnose is a foliar disease perpetuated by several related fungal pathogens of the *Colletotrichum* genus, particularly *Colletotrichum gloeosporioides.* So far, four types of *C. gloeosporioides* have been characterized in Nigeria: Slow-growing Olive, Fast-growing salmon, Fast-growing grey and Slow growing grey (Abang *et al.,* 2002). The disease is characterized by a scorched appearance, dark lesions surrounded by a yellow halo on the leaves, leaf necrosis, shoot dieback of the stem, and tuber dry rot. These factors reduce the effective photosynthetic surface area of the plant, its ability to store food reserves and results in productivity losses of upto 90% in susceptible genotypes (Egesi *et al.,* 2007). In the Caribbean, for instance, the yam anthracnose disease constrained the production of the popular *D. alata* accessions White Lisbon and Pacala and has resulted in a significant decline in yam yields (Ano *et al.,* 2002).

The disease is mainly spread through garden tools, insects, and wind. Traditionally, control of disease spread has been achieved through the monthly or bi-weekly application of chemical fungicides such as mancozeb, chlorothalonil, maneb, and benomyl. However, the frequent use of fungicides is damaging to the environment and could lead to the development of fungicide-resistant strains (Onyeka *et al.,* 2006). As such, host plant resistance and the use of resistant varieties constitute the single most effective strategy for the control of yam anthracnose disease. Since several virulent strains of *C. gloeosporioides* exist, there is a high probability for virus recombination, resulting in new virulent strains that surpass host resistant mechanisms (Abang *et al.,* 2003; Palaniyandi *et al.,* 2011). Thus, management of anthracnose calls for the deployment of new technologies that introgress

farmer preferred *D. alata* varieties with durable host plant resistance against the various strains of *C. gloeosporioides.*

2.4.1.2 Pests associated with yam

Nematodes

The yam crop is susceptible to infection by nematodes of about ten species, including the *Meloidogyne* spp., *Scutellonema* spp., and *Pratylenchus* spp. (Adegbite *et al.,* 2008; Imafidor & Mukoro, 2016). Yam nematodes destructively feed on the tuber tissues of growing yams in the soil, thereby causing a reduction in tuber size and quality deterioration. Besides, nematode infestation of yams predisposes the tubers to attack by various pathogens resulting in dry and wet rot diseases in stored tubers (Kolombia *et al.,* 2017; Kwoseh *et al.,* 2005).

Across all crop production systems, including yam, root-knot nematodes (RKN) of the *Meloidogyne* spp. are considered the most widespread and devastating genera of plant-parasitic nematodes (Jones *et al.,* 2013; Namu *et al.,* 2018). The commonly reported RKNs in yam include *M. incognita*, *M. javanica*, and *M. acronea*. Infection of yam with RKN causes huge economic losses both in the field and during storage. The disease is characterized by the formation of root galls, which damage the root vascular tissues and therefore affect the uptake of water and nutrients. Also, galling increases the susceptibility of the crops to invasion by other pathogens (Onkendi *et al.,* 2014; Ralmi *et al.,* 2016).

The *Scutellonema* spp is the second most prevalent yam nematode caused by *S. bradys.* This nematode causes dry rot disease on yam tubers, and its origin has been traced to West and Central Africa. The nematode has also been reported in yam growing regions of the Americas and Asia, where it has been shown to cause severe deterioration of tuber quality

and even total of stored tubers (Coyne *et al.*, 2012; 2016). Yam infection with *S. bradys* manifests as cracking or flaking of the epidermal surface of the tuber, from where the nematodes feed on the sub-peridermal and peridermal layers as a migratory endoparasite. Subsequently, the cells develop necrotic lesions and cavities with a dark-brown layer extending to 1-2 cm into the tuber, which constitutes the yam dry rot disease. The cracked epidermis predisposes the tuber to bacterial and fungal invasion, which translates to wet rot disease. Field and storage yield losses due to *S. bradys* vary depending on nematode population densities, genotype, extend of infestation, and conditions (Kolombia *et al.*, 2017). Several alternate hosts of *S. bradys* such as melon (*Cucurbita melon*), sesame (*Sesamum indicum*) and cowpea (*Vigna unguiculata*), and sweet potato (*Ipomea batatas*) facilitate the survival and reproduction of the yam nematode in soil in the absence of yam (Coyne *et al.*, 2012; Jatala & Bridge, 1990).

Nematode infestation in crop fields is conventionally managed by use of chemical treatments to treat the soil or propagation material before planting. However, these treatments can only reduce the degree of invasion but do not eliminate the nematodes. Besides, some of the potent chemical treatments are costly and contain noxious products that are damaging to the environment (Castagnone-Sereno *et al.*, 1993; Haydock *et al.*, 2006). Subsequently, the only environmentally sustainable and cost-effective strategy for managing plant-parasitic nematodes remains to be the development of resistant varieties (Khanal *et al.*, 2017). Yam crop improvement strategies should, therefore, focus on the development of resistant accessions and promoting the use of nematode-free planting material by farmers (Aighewi *et al.*, 2015).

Insects

Yams are infested by a wide variety of insects belonging to various genera and orders, including Coleoptera, Diptera, Hemiptera, Hymenoptera, Isoptera, Lepidoptera, and Thysanoptera (Korada *et al.,* 2010). These insects include more than seventy-three species that infect yams during field cultivation and approximately 27 insect species that infect during storage (Morse & McNamara, 2014). Among the individual species, mealybugs, yam scales, aphids, termites, and beetles have been reported to cause the most significant losses (Onwueme, 1978). As such, there is a need to adopt insect integrated approaches to maintain the population of these insects in the field and even storage. During storage, farmers should prioritize using biocontrol agents that target the white scales and the mealybugs (Loko *et al*., 2013).

Mealybugs (*Rastrococcus* spp)

Mealybugs are an economically significant yam pest with flattened oval or round shape, whose presence may also attract fungus. These insects have a broad host range that includes ferns, angiosperms, and gymnosperms. The mealybug infestation symptoms include the attraction of ants and the formation of a sooty mold following the colonization of the area surrounded by the sugary honeydew (Shylesha & Mani, 2016).

White Scale insects (*Aspidiella hartii*)

These insects exist globally and fall into various categories, including Coccidae, Pseudococcidae, Diaspididae, Margarodidae, and Eriococcidae. The infestation symptoms of white scale insects include the leaves and the tubers being covered with white scales, both in the field and during storage. Foliage may be attacked hence causing low growth, while the tubers may experience delayed growth during germination or stop altogether. In

cases where the infestation is very severe, the yam tubers may shrivel, thus causing significant yield losses. The most common method for controlling yam pests and diseases involves chemical control (Korada *et al.,* 2010).

2.4.2 Abiotic stress

Various abiotic stress factors, including extreme climatic events, poor soil fertility, and soil salinity, pose serious challenges to sustainable crop production and account for the highest yield losses than any other factor in rain-fed agriculture (Wang *et al.,* 2003). Compared to other food staples such as maize, the yam crop is known to grow in a wide range of agro-ecological zones, including marginalized lands. Subsequently, most farmers cultivate yams in smallholdings under low-fertility conditions, except in some cases where short fallows are incorporated into the cropping systems to revive soil fertility (Yasuoka, 2009). However, yams are high-nutrient-demanding species, and planting in such poor nutrient soils only results in poor yields because of a compromised growth and productivity of the crops (Lebot, 2010). Despite increases in the total land areas under yam cultivation in West Africa, a decline in the yields per unit land area has been reported. Although the potential yields for *D. alata* and *D. rotundata* under optimal conditions are 51 $t\,ha^{-1}$ and 27 $t\,ha^{-1}$ respectively, the yields obtained in West African smallholder systems range between 9 and 10 $t\,ha^{-1}$. The degradation of soil fertility and poor crop nutrition have been identified as key constraints to yam production, but little has been done to mitigate these challenges (Diby *et al.,* 2011; Frossard *et al.,* 2017).

Recently, changes in climatic conditions, altered and unpredictable weather patterns such as floods, drought, and high temperatures have become more prevalent. These variations in weather patterns increase the vulnerability of crops to pests and diseases. Drought

reduces the proportion of arable land and has been reported as the most significant cause of yield losses globally. As the frequency of these extreme climatic conditions become more prevalent, the associated yield losses are expected to intensify (Chen *et al.,* 2010; Takeda & Matsuoka, 2008). The levels of soil moisture grossly affect root development and therefore have a significant impact on the yield of yam tubers. Subsequently, sustainable yam production will require the development of drought-tolerant varieties for environments with reduced water levels. Yam breeders and technology developers will, therefore, need to focus on providing accessions that can flourish in unsuitable weather conditions and in soils with reduced nutrient profiles.

2.5 Milestones achieved towards improvement of the yam crop

The need for increased attention to crop improvement goes beyond the requirement of providing safe, nutritious, and sufficient food that meets the dietary requirements and food preferences of a growing global population (Prosekov & Ivanova, 2018). First, climate change has affected all aspects of food security, including production, availability, access, quality, utilization, and stability. Because of climate change, extreme weather-related disasters such as drought, high temperatures, and floods have intensified, thereby reducing agricultural yields. Second, the pressure to increase food production from limited cropland has resulted in agricultural intensification coupled with poor farm practices (Takeda & Matsuoka, 2008). These efforts have subsequently compromised on environmental sustainability, reduced the available arable land, and promoted land degradation, loss of biodiversity, increased emissions, and reduction of soil fertility because of increased use of fertilizers and pesticides. Third, as petroleum-based energy sources dwindle, the focus

is now shifting on the use of bio-fuel as substitutive energy sources, which will reduce the available arable land for crop cultivation (Farooq *et al.,* 2019; Prosekov & Ivanova, 2018).

Therefore, it is urgent to develop multidimensional strategies that ensure food security while maintaining environmental integrity and without negatively impacting agricultural sustainability. The present chapter highlights the progress made towards the enhancement of our scientific understanding and technological capabilities in improvement of the yam crop. It also highlights some potential strategies that could be implemented to achieve sustainable production of the crop and provide maximum nutritional and economic gain to yam farmers and consumers.

For a better understanding of the progress so far attained towards yam crop improvement, this section is divided into five milestones: (1) Identification of useful genetic elements (2) Breeding for resistance (3) Development of regeneration protocols (4) Genetic transformation (5) Genome editing

2.5.1 Identification of useful genetic elements

The core of crop improvement lies in the identification and characterization of useful genetic elements. To date, genes and quantitative trait locus (QTLs) governing complex agronomic traits have been identified in various crops using different approaches such as QTL mapping and traditional molecular mapping, which exploit reverse and forward genetic screens. These genes have been further applied for marker-assisted breeding (MAB) to generate crop plants that are tolerant to various biotic and abiotic stresses and for quality improvement.

Mignouna *et al.* (2001) and Petro *et al.* (2011) identified Amplified fragment length polymorphism (AFLP) markers associated with anthracnose resistance in *D. alata*. Tostain *et al.* (2006) developed simple sequence repeat (SSR) markers from *Dioscorea praehensilis, Dioscorea abyssinica,* and *Dioscorea alata*. These traditional efforts for trait identification have been augmented by novel techniques such as genome sequencing, functional genomics research, and genome editing. In yam, there are limited research efforts that help generate its genomic information and understand its genetics. The existing genomic resources, like genome-wide molecular markers, will quicken the breeding efforts and application of genomic selection in yams. For instance, the recent sequencing of the genomes of *D. alata* (Saski *et al.,* 2015), *D. rotundata* (Tamiru *et al.,* 2017), and *D. dumetorum* (Siadjeu *et al.,* 2020) are expected to speed track the identification of novel traits.

2.5.2 Breeding for resistance

Advancements in yam breeding programs to contemporary levels for designing varieties with tolerance or resistance to biotic and abiotic stresses has been hampered by insufficient information on the crop genomics and genetics (Mignouna *et al.*, 2008). In most crops, plant breeding has greatly influenced their improvement. In the yam crop, for instance, resistance to YMV has been identified in some *D. rotundata* breeding events, and efforts to incorporate this resistance into agronomically useful varieties are ongoing (Darkwar *et al.*, 2020). When parental and the progeny accessions of *Dioscorea alata* were inoculated with an aggressive YMV strain, the resulting offspring showed a resistant phenotype, indicating that some varieties could be having resistance. Therefore, this

technology can be further explored to introgress disease and drought resistance in farmer-preferred yam accessions (Mignouna *et al.*, 2003).

Nevertheless, variability identification for incorporation into new cultivars is becoming more difficult because of declining germplasm resources. Besides, genetic improvement of the yam germplasm through classical breeding methods has been hampered by the dioecious nature of the plant, polyploidy, poor seed set, heterozygosity, and a prolonged breeding cycle (Mignouna *et al.*, 2008). Yam crop improvement will therefore require the development of new techniques that will allow scientists to directly manipulate the yam genome.

2.5.3 Development of regeneration protocols

The *in vitro* manipulation of the yam plant dates back to a report by Mantel et al. (1978), who explored the effects of medium supplementation with sucrose and peptones on the organogenesis of *D. opposite* and *D. alata* . Most of the subsequent studies on yam *in vitro* techniques focused on the use of meristem culture for rapid multiplication and disease elimination. Recently, however, yam tissue culture techniques are being developed for application in genetic transformation (Nyaboga *et al.*, 2014). Attempts have been made towards the regeneration of various yam species from different explants, including tuber tissue, leaf, stem, petiole, and auxiliary bud. Also, yam has been regenerated by shoot organogenesis and somatic embryogenesis, and attempts for regeneration by protoplast and suspension cultures have been made (Anike *et al.*, 2012; Manoharan *et al.*, 2016).

Mantell *et al.* (1980) reported meristem regeneration of *D. alata* on modified Murashige and Skoog (MS) medium. Five years later, Ng and Hahn (1985) reported successful

regeneration of *D. rotundata* plantlets within 24 weeks of meristem culture on MS basal medium containing 0.2 µM gibberellic acid (GA_3), 0.6 µM 6-benzyl amino purine (BAP), and 1 µM NAA naphthaleneacetic acid (NAA). In the elephant yam, Irawati *et al.* (1986) reported shoot regeneration rates of 22% at 24 weeks and 75% at 36 weeks using MS medium supplemented with 5 µM NAA and 0.05 µM kinetin. By modifying the medium described by Ng and Hahn (1985), Malaurie *et al.* (1995) reported 18% success in shoot regeneration of the meristems of *D. praehensilis* and *D. cayenensis- D. rotundata* complex. The modified medium was composed of MS medium supplemented with 0.44 µM BAP and 2.69 µM NAA, and shoot regeneration was achieved within 12 weeks. More recently, it has been shown that various yam species, including *D. alata, D. cayenensis,* and *D. rotundata,* can be regenerated via shoot organogenesis (Adeniyi *et al.,* 2008; Anike *et al.,* 2012). Also, there are reports on micropropagation of *D. rotundata* by shoot organogenesis, which has become the standard system for maintenance and rapid multiplication of yam *in vitro* plantlets (Nyaboga *et al.,* 2014).

Notably, yam regeneration via somatic embryogenesis has also achieved substantial progress. Unlike regeneration by meristem culture and shoot organogenesis, which is of multicellular origin, somatic embryos are formed from single cells and, therefore, offer a reliable system for the recovery of transformed single cells (Mariashibu *et al.,* 2013). As such, somatic embryogenesis will be of great significance in facilitating the rapid improvement of the yam crop via genetic transformation and genome editing. Somatic embryo production in food yam was first achieved by Osifo (1988) using the zygotic embryos of *D. rotundata.* Shortly after that first report, Viana & Mantell (1989) and Nagasawa & Finer (1989) simultaneously reported somatic embryo production in *D.*

composita and *D. cayenensis* and *D. opposita*, respectively. In *D. alata*, plants have been regenerated from petiole explants (Fautret *et al.* 1985), root segments (Twyford & Mantell, 1996), and nodal stem segments (Belarmino and Gonzales, 2008). Respectively, *D. Rotundata* has been regenerated from petiole, and auxiliary bud derived somatic embryos (Manoharan *et al.,* 2016; Suárez Padrón *et al.,* 2011). Table 2.1 below summarizes the adavnces achieved towards developing efficient tissue culture-based regeneration systems in yam.

Table 2.1: Summary of regeneration systems in yam

Species	**Explant**	**Regeneration product**	**Reference**
D. rotundata and D. alata	Node	Plant	Mantell *et al.* (1978)
D. alata	Meristem	Plant	Mantell *et al.* (1980)
D. cayensis	Embryo	Callus and plant	Okezie *et al.* (1983)
D. rotundata	Protoplast from tuber	Isolation and purification	Onyia *et al.* (1984)
D. rotundata	Node	Plant	Ng and Hahn (1985)
D. rotundata	Node	Plant	Arnolin (1985)
D. alata	Petiole	Somatic embryo and plant	Fautret *et al.* 1985
Elephant yam	Meristem	Plant	Irawati *et al.* (1986)
D. rotundata	Zygotic embryo	Embryogenic callus	Osifo (1988)

D. cayensis and *D. composite*	Zygotic embryo	Embryogenic callus	Viana & Mantell, (1989)
D. opposite	Zygotic embryo	Embryogenic callus	Nagasawa & Finer, (1989)
D. praehensilis and *D. cayenensis- D. rotundata* complex	Meristem	Plant	Malaurie *et al.* (1995)
D. alata	Petiole	Somatic embryos and plant	Twyford & Mantell, (1996)
D. alata	Nodal stems	Somatic embryos and plant	Belarmino & Gonzales, (2008)
D. alata	Node	Plant	Adeniyi *et al.* (2008)
D. rotundata	Petiole	Somatic embryos and plant	Suárez Padrón *et al.* (2011)
D. cayensis	Node	Plant	Anike *et al.* (2012)
D. rotundata	Auxiliary bud	Somatic embryos and plant	Manoharan *et al.* (2016)

2.5.4 Genetic transformation

Several studies have developed systems for transient and stable gene expression in yam, including particle bombardment (Tör *et al.,* 1993), polyethylene glycol (PEG) mediated transfection (Tor *et al.*, 1998), and *Agrobacterium* mediated transformation (Nyaboga *et al.,* 2014; Quain *et al.,* 2011). Among these protocols, *Agrobacterium* mediated transformation is the most preferred because it is easily available, facilitates the integration

of large nucleotide segments with negligible rearrangements, allows for the transfer of only a single copy of the gene, and is cheap. In genetic engineering, the traits of interest can be obtained from the same crop, from a different species, or even from a different kingdom (Shibata *et al.,* 2000; Tripathi *et al.,* 2019). Therefore, genetic engineering is a suitable strategy for the improvement of crops that suffer a dearth of desirable traits.

Tör *et al.* (1993) delivered genetic material harboring the *GUS* reporter system into *D. alata* suspension cells via microprojectile bombardment. Transient gene expression was assessed by histochemical GUS assay, while stable integration was confirmed by Southern blot analysis and fluorometric analysis of GUS activity. However, the study did not generate whole transgenic plants. Tor *et al.* (1998) demonstrated for the first time that yam protoplasts could take up foreign genetic material via PEG mediated transfection of *D. alata* and *D. rotundata-cayensis* complex. Regeneration of transgenic plantlets was, however, not feasible. Quain *et al.* (2011) and Nyaboga *et al.* (2014) reported transient and stable *Agrobacterium* mediated transformation of yam, respectively. Despite the availability of a system for stable gene integration in yam, there is currently no report on the integration of agronomically important traits in yam. Therefore, more research is required to harness the value of this technology for the improvement of this crop that forms the basis of food security for millions of people globally.

2.5.5 Genetic transformation systems

Agrobacterium-mediated transformation is the most preferred method of gene introduction in plants because it is less labor-intensive, does not involve the use of sophisticated equipment, and is cheap. Additionally, this system results in a low copy number of the introduced gene (Ziemienowicz, 2014).

For most crops of agronomic importance, particle bombardment remains the only truly genotype-independent protocol for delivering foreign genetic material into the plant. Particle bombardment is a highly physical process in nature, and there is, therefore, no biological hindrance to the delivery of genetic material to the plant (Rivera *et al.*, 2012). The merits of using microprojectile bombardment for plant transformation are that it enables the transformation of organized tissue, fast recovery of transformed events, and transformation of recalcitrant species. The main disadvantages of using particle bombardment for plant transformation are that target tissues can be damaged during bombardment, and the DNA is not transferred into a specific region of the host genome. Additionally, the high cost of particle bombardment equipment restricts application of the technology (Rashid & Lateef, 2016).

2.6 Protoplast technology

Plant protoplasts provide a versatile tool not only for the study of numerous aspects of modern biotechnology but also for crop improvement. For instance, PEG-mediated protoplast transformation facilitated the production of transgenic potato and mandarin plants (Fossi *et al.*, 2019; Omar *et al.*, 2018). In the advent of genome engineering using nucleases, preassembled CRISPR-Cas9 ribonucleoproteins complexes can be delivered into protoplasts. This allows the production of DNA-free genome-edited plants, which may relieve the regulatory issues associated with genetically modified plants (Liang *et al.*, 2017).

Unfortunately for the *Dioscorea* species, very little progress has been reported in the application of protoplast technology for yam improvement. Tor *et al.* (1998) reported protoplast isolation in *D. alata*, but no regeneration was achieved. Using the polyethylene

glycol-mediated gene transfer method, the study also demonstrated the ability of yam protoplasts to take up foreign genes.

2.7 Genome engineering

2.7.1 The evolution of plant genome engineering

Nature differentially allows the reproduction and survival of various crop genotypes within the population and facilitates variations in gene frequencies via natural selection. For over 10,000 years, humans have been domesticating crops based on the artificial selection of wild crops that fit human interest (Flint-Garcia, 2013). Farmers and breeders identified and propagated plant varieties with desirable attributes such as high seedling vigor, high number of seeds per inflorescence, reduced seed dormancy, and altered plant morphology like reduced branching/ tillering and compact/dwarf growth (Fernie & Yan, 2019). The modern-day corn, for instance, evolved from its wild ancestor teosinte courtesy of artificial selection. It was estimated that artificial selection has resulted in the domestication of approximately 2500 plant species (Dirzo & Raven, 2003). The quest for crop improvement saw breeders introduce genetic variation into crop varieties through intergeneric, interspecific and intraspecific crosses then selecting the best genetically recombined progeny; this was, however, not feasible for vegetatively propagated crops. (Meyer *et al.,* 2012). Before the 20th century, plant breeders depended on natural and spontaneous mutations to breed novel traits. In the twentieth century, it became clear that altering DNA/ gene sequences induces phenotypic variations. Researchers, therefore, supplemented breeding programs with chemical mutagenesis or high-dose irradiation, cleaving or modifying chromosomes in random locations and select the plant with phenotypic variations (Shu *et al.,* 2012). Mutated plants rely on their natural mechanisms for DNA

repair, which may incorporate chromosomal rearrangements and random mutations throughout the plant genome. Even though mutagenesis was random and there existed few methods of screening and selecting mutants, induced mutations yielded immense success with the generation of more than 1300 crop varieties from 1940 to 1990 (Meyer *et al.*, 2012). A notable example is Calrose 76, a semi-dwarf mutant japonica rice variety released in 1976. The semi-dwarfing gene in Calrose 76, sd-1 (Mackill and Rutger, 1979), was thereafter identified as the same "green revolution gene" found in indica rice that greatly increased rice production in the early 1960s (Spielmeyer *et al.,* 2002).

An enormous breakthrough in crop genetic engineering was achieved following the advent of recombinant DNA technology (rDNA). The technology allowed for the transfer of genes or gene elements without the species barrier and successful transgene integration/ expression in the host plant. Transgene transfer is most commonly done using organisms that can naturally escort genetic elements such as *Agrobacterium tumefaciens* (Shiboleth & Tzfira, 2012) or plant viral vehicles. DNA delivery is also done by protoplast transfection or using physical means like particle bombardment. Following transgene expression, the host plant exhibits the phenotype and effect corresponding to the transgene role. Compared to previous technologies, rDNA allows scientists to overcome the species barrier by enabling the transfer of desired traits across different species, families, or even kingdoms. With rDNA, there is also a probability to either increase or reduce the expression levels of a gene of interest. Using these tools, it is also possible to incorporate either single traits or quantitative traits (Georges & Ray, 2017).

Genetically modified crops were first introduced into the food supply chain in 1994 following a commercialization approval of the Flavr Savr tomato by the United States

Federal Food and Drug Administration (FDA). This was soon followed by other major GM crops such as corn, cotton, soybean, and canola, along with minor food crops such as squash. The number of commercialized GM crops has been growing ever since, with several other approved GM crops such as papaya, eggplant, rice, potato, sunflower, sugarbeet, and sugarcane in production around the world (Georges & Ray, 2017). The improved varieties possess a variety of desirable traits such as herbicide tolerance, insect resistance, virus-resistance, improved nutrition, or higher yield, among others (Chen & Lin, 2013).

Despite the success in the technology, major drawbacks continue to flair up the adoption and commercialization of the transgenesis as evidenced by minimal or no access to GM foods in certain agricultural markets such as the EU. Key among the drawbacks of rDNA is the randomness with which transgenes are inserted in the hostplant. The main concern regarding this approach is the possibility of the introgressed gene affecting or inactivating other important nearby genes (Bradford *et al.*, 2005). Additionally, restrictive government policies and a skeptical public perception of GM crops have thus far restricted the application of the technology for generating crops with desirable traits. Technologies that allow precise modification of crop genomes have therefore been long desired, and this necessity resulted in the birth of genome editing techniques. Unlike its predecessors, genome editing provides a rapid system of introducing precise changes at specific locations in the genome hence preventing cell toxicity and offering perfect reproducibility. Genome editing could be more acceptable to the public than GM crops with foreign DNA in their genomes (Komen *et al.*, 2020).

2.7.2 Homologous and non-homologous recombination

For the last three decades, the field of crop improvement has produced specific mutant crops via a variety of techniques that nonetheless depend upon the repair systems of homology-directed repair (HDR) or non-homologous end joining (NHEJ) (Puchta, 2005). This came following the discovery that bacteria and yeast respond to DNA double-stranded breaks (DSBs) by repair and recombination mechanisms and the realization that systems of generating specific DSBs could serve as an avenue for targeted genome modification. Several facile methods for generating precise DSBs to modify specific chromosomal loci in plant systems are now available (Songstad *et al.*, 2017).

The systems, which are designer nucleases, function by generating a DNA double-strand break in the target loci. The cell then harnesses its endogenous repair machinery to repair the break. If the break is repaired via the imprecise process of NHEJ, some nucleotides could be either inserted or deleted (indels), resulting in a functional knockout of the gene(s). Repair via NHEJ could also alter the open reading frame (ORF) of the target gene generating a premature stop codon or translation of a scrambled amino acid sequence (Malzahn *et al.*, 2017). When more than one DSBs are generated, the repair process can generate gene inversions, chromosomal deletions, and chromosomal translocations if the DSBs are on two separate chromosomes. Additionally, creating indels in promoters can alter key regulatory elements and disrupt the gene expression process (Bortesi & Fischer, 2015). When a homologous DNA template is made available, the damage in the DNA is corrected by HDR, resulting in gene insertion or precise gene modifications (Puchta, 2005) (Figure 1). The repair template can either be a sister chromatid, homologous chromosome, or user-supplied DNA homologous to the DSB. User-supplied DNA templates can harbor

several DNA sequence modifications, ranging from single base substitutions to a few hundred nucleotides or even entire genes (Puchta & Fauser, 2013).

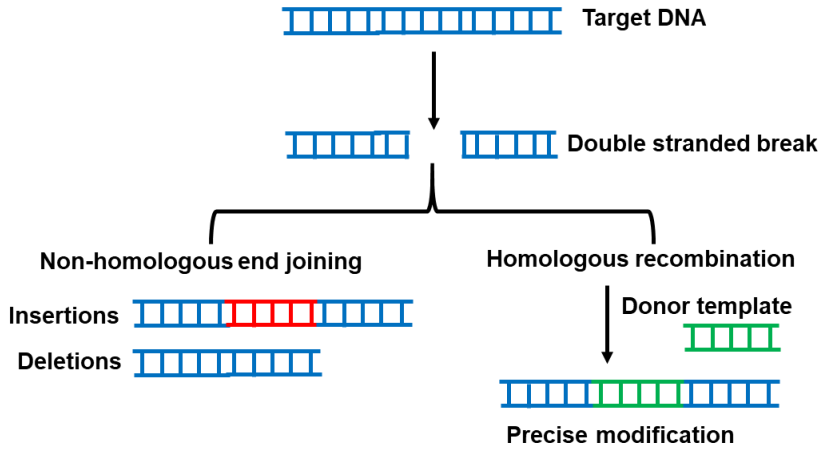

Figure 2.1: Repair pathways for double sranded breaks generated during genome editing with site-specific nucleases

2.7.3 Genome modification using engineered nucleases

Precise genome editing needs the action of a molecular machine consisting of two main components: a DNA or RNA-binding domain that facilitates sequence-specific DNA recognition and binding and an effector domain that mediates DNA cleavage or controls transcription near the binding site (Gaj *et al.*, 2016). This machinery can be customized to bind any nucleotide sequence of interest and generate a sequence-specific DSB, which are subsequently repaired hence effecting precise gene modification at the target loci Lowder *et al.*, 2015; Puchta, 2005). The nuclease-mediated genome editing toolbox has four major platforms: Meganucleases, or homing nucleases, Zinc-finger nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), and CRISPR/Cas systems.

2.7.4 Meganuclease-based engineering

Meganucleases (MNs), discovered in the late 1980s, are a group of naturally occurring endonucleases that recognize and cleave long nucleotide sequences (12 to 40 bp). The large recognition sequence of MNs may occur only once within a genome, making them perfect tools for site-specific genome editing (Gallagher *et al.,* 2014; Rosen *et al.,* 2006). Additionally, MNs are less toxic to cells than other systems, including ZFNs. One of the major drawbacks of MNs is that its naturally occurring number is inadequate and does not sufficiently cover all potential loci of interest (Burt & Koufopanou, 2004). To mitigate this challenge, various strategies such as molecular evolution, protein engineering, and structure-based design have been used to reengineer MNs to target novel sequences. Modifying MNs has, however, proven to be technically challenging and labor-intensive because each new target requires an initial protein engineering stage to produce a custom meganuclease. Additionally, the use of MNs is hindered by some patent disputes (Abdallah *et al.,* 2015; Smith *et al.,* 2006).

2.7.5 Zinc Finger Nuclease-based engineering

The zinc finger nucleases (ZFNs), synthesized in the early '90s, have so far been widely utilized for genome editing in both plant and animal systems (Govindan & Ramalingam, 2016). ZFNs exploit type IIs restriction enzyme FokI, which has separable recognition and non-specific cleavage domains. A zinc finger recognizes three nucleotides (nts) and is formed following repeated sets of cysteine and histidine amino acid residues. Zinc finger nucleases consist of proteins with multiple zinc finger monomers that can together recognize a sequence of 9 or 12 consecutive nucleotides in the genome of an organism (Malzahn *et al.,* 2017). The C-terminal end of the system has a nonspecific nuclease domain

from the FokI restriction enzyme that makes up the first half of the ZFN pair. The other half of the molecule has a similar structure, and it recognizes/binds to a nucleotide sequence approximately 6 nts from the first ZFN on the complementary DNA strand. This spacing gives an allowance for the two inactive FokI nuclease domains to dimerize, become active and cleave DNA at the region between the two ZFNs, creating a DSB (Kim *et al.,* 2009). DSB by either homologous or non-homologous recombination results in gene disruption (Vanamee *et al.,* 2001). More recent studies have increased the number of fingers to six per ZFN to enhance the specificity (Paschon *et al.,* 2019). The dimerization requirement for ZFNs cleavage coupled with the fact that FokI has weak native self-interaction greatly reduces potential off-target effects by ZFNs and contributes to its highly specific genome editing. Though ZFNs have been effectively used in several crop plants for generating targeted gene modifications**,** the complexity of its technical procedures has limited its extensive application. Additionally, ZFNs based on modular assembly are highly toxic to cells and have poor activity (Ramirez *et al.,* 2008). Generally, ZFNs are less preferred due to a low number of target sites, low target specificity, high off-targets effects, and labor-intensive nature (Jaganathan *et al.,* 2018).

2.7.6 TALENs - based engineering

TAL effectors are naturally occurring proteins secreted by the plant pathogenic bacteria *Xanthomonas* into host cells during plant infection. Once in the host cells, TAL effectors regulate host plant gene expression mechanisms to facilitate bacterial infection (Joung & Sander, 2013). Each TALE recognizes and binds to a specific DNA sequence in a target gene in the host plant. TALEs are composed of nearly identical repeat units of 34 amino acids, each with two hypervariable amino acid residues called RVDs (repeat variable

diresidues) at positions 12 and 13. The variable residues dictate the DNA binding specificity of the TALE (Morbitzer et al., 2010; Mussolino & Cathomen, 2012). Just like ZFNs, TALENs are chimeric proteins consisting of two sub-units, a *FokI* nuclease domain and a domain for DNA recognition and binding. The *FokI* nuclease domain can dimerize with the *FokI* domain of another TALEN bound to a DNA sequence on the complementary strand. This results in DSB between the two TALENS, activation of DNA repair, and subsequent gene modification (Joung & Sander, 2013).

TALENs are reengineered by altering the TALE domain repeats to target a gene of interest and fusing it with a FokI nuclease domain. Engineered TALENs typically recognize a stretch of 18–20 bp, and dimerization of a pair of adjacent TALENs, 14-20 bp apart is required for TALEN activation and cleavage (Ousterout & Gersbach, 2016). TALENS have been successfully employed by several research groups to modify endogenous genes in Arabidopsis, corn, rice, wheat, and Brachypodium, among others. TALENs can be used to control gene expression by coupling TALEs with gene activation or gene repressor proteins (L. Li et al., 2012; Scholze & Boch, 2011). TALENs are more precise than ZFNs in target binding, but the complexity of their design and assembly coupled with the requirement of a thymidine base at the starting position challenges their application for genome editing (Gaj *et al.*, 2013; Gupta and Musunuru, 2014).

2.7.7 CRISPR/Cas - based engineering

The Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated Cas genes (CRISPR/Cas) is a novel genome editing tool based on the acquired immune system of bacteria and archaea. Three types of CRISPR systems have been identified, but only the type II system from *Streptococcus pyogenes* has been well characterized and

modified for use in artificial gene targeting (Chylinski *et al.,* 2014). The CRISPR-Cas9 system adopted for genome editing has two components: a synthetic guide RNA (gRNA) and a Cas 9 endonuclease (Brooks & Gaj, 2018).

The main methods for delivery of genome editing reagents to target plant tissues involve either biolistic or *Agrobacterium*-mediated transformation. In *Agrobacterium*-based delivery, the Cas9 and gRNA expression cassettes are cloned onto the T-DNA (transfer DNA). Following T-DNA delivery onto plant tissues, the CRISPR reagents are expressed, resulting in DSB, repair, and subsequent mutation (Altpeter *et al.,* 2016). At present, the *Agrobacterium*-based approach is the most widely used because it is simple and able to transfer low copy numbers of the gene, thus less genome disruption. The biolistic transfer of CRISPR reagents is the second popular method for plant genome editing. Compared to *Agrobacterium*-based systems, tissue bombardment is not limited by the requirement of complex interactions with the host plant tissues (Banakar *et al.,* 2019). Besides, the system can deliver various types of organic molecules, including proteins, mRNA, DNA, and even oligonucleotides (Martin-Ortigosa & Wang, 2014). However, the force exerted on plant tissues during bombardment could shear the DNA, thus generating numerous genome disruptions. Besides, the biomolecules are randomly inserted into the genome *(Banakar et al.,* 2019; Zhang *et al.,* 2018). Recently, the delivery of CRISPR reagents as ribonucleoprotein complexes (RNPs) is gaining prominence due to the limited half-life of RNPs. Thus, the reagents are not integrated into the genome, and this offers the opportunity for DNA-free and selection-free genome editing (Svitashev *et al.,* 2016a). The potential for virus-mediated transfer of genome editing reagents has also been demonstrated (Mahas *et al.,* 2019).

2.7.8 Advantages of the CRISPR/Cas9 system

With the CRISPR/Cas9 system, it's possible to target a range of genomic sites since the only prerequisite for a potential CRISPR/Cas target is a 20-bp sequence preceding a protospacer adjacent motif (PAM). On the contrary, the available targets for ZFNs are limited due to the absence of fingers for all possible DNA triplets. Secondly, the delivery of CRISPR/Cas vectors to cells is easier due to the short length of the sgRNA as compared to the longer and highly repetitive ZFN/TALEN vectors (Belhaj *et al.,* 2015).

Unlike its predecessors, CRISPR/Cas9 vectors are easy to construct; only the 20 nt sequence of the gRNA needs to be changed to confer specificity to a different target (Razzaq *et al.,* 2019). On the contrary, targeting a single ZFN or TALEN sequence requires the engineering of two different proteins, with each consisting of repetitive ZF and TALE elements (Bortesi & Fischer, 2015). Co-transfection of multiple sgRNAs can be used for simultaneous editing of two or more loci, a cause that cannot be achieved with previous genome editing techniques (Lowder *et al.,* 2016).

Most importantly, the CRISPR research group has hastened the extensive application of the platform by providing an open access policy, in contrast, to the exclusiveness of the ZFN technology. The researchers host active discussion groups, provide access to plasmids and web-based tools for designing gRNA sequences (Bortesi & Fischer, 2015). In conclusion, the CRISPR/Cas9 system supersedes previous genome editing tools because of its simplicity, accessibility, cost-effectiveness, and adaptability (Malzahn *et al.,* 2017).

2.7.9 Limitations of CRISPR/Cas9 technology

Similar to every other technology, the CRISPR/Cas9 system also faces some obstacles that could hinder the fast realization of the associated benefits by the farmer and yam consumers. (i) The big size of the CRISPR/Cas9 system impedes its mutation efficiency and is not suited for delivery into viral vectors. This challenge has been solved by developing alternative smaller-sized nuclease systems, including cas9 orthologs (SaCas9 and CjCas9), CRISPR/Cas14a, and FnCpf1 (Manghwar *et al.*, 2019). (ii) PAM (protospacer adjacent motif) specificity – The SpCas9 has a stringent requirement for a 5′-NGG-3′ motif immediately adjacent to the target site, which limits its application in AT-rich genomes. However, this challenge has been mitigated by developing Cas9 variants with wider PAM compatibilities, including GAT, GAA, and NG (Hu *et al.*, 2018). (iii) The potential for off-target mutations. Although less frequent in plants, the DNA modifications by CRISPR/Cas can occur in unintended and nonspecific sites. Efforts to mitigate off-target mutations have been made by developing efficient approaches for detecting off-target sites and engineering high-fidelity CRISPR systems (Langner *et al.*, 2018). (iv) Dependence on *Agrobacterium*-mediated transformation. For most crops, including yam, CRISPR/Cas9 mutant events are generated by *Agrobacterium*-mediated transformation, which is time and resource consuming. Besides, the systems are dependent on tissue culture for the recovery of transformed cells, hence require optimized regeneration protocols (Manghwar *et al.*, 2019). Recently, however, the potential for *de novo* genome editing has been demonstrated (Maher *et al.*, 2020), which could circumvent the need for intricate tissue culture processes. (iv) Regulatory concerns of crops improved by the CRISPR/ Cas9 system. In some countries, the commercialization of CRISPR/Cas9 products generated by transgenic

technology is challenging due to the constraints and costs enforced by regulatory authorities for the commercial release of genetically modified organisms (Komen *et al.,* 2020).

2.7.10 Improvements to CRISPR/ Cas systems

2.7.10.1 Multiplex genome editing

Despite the achievement of innumerable success in precise genome edition using a single gRNA in CRISPR–Cas expression systems, the mono-guide approach is frequently associated with low targeting efficiencies. Therefore, current CRISPR/Cas systems achieve *high* throughput genome modification via the design of multiple sgRNA expression systems to simultaneously target multiple related or unrelated sequences in the same cell (Xing *et al.,* 2014).

Multiplex genome engineering allows for the simultaneous edition of multiple loci and therefore saves on time and other resources. The original efforts towards multiplexed genome engineering were based on the combined delivery of several individual gRNA expression cassettes. Multiple gRNAs can also be delivered as genome-integrated transcripts processed into mature gRNAs by endogenous or introduced nucleases. The earliest reports of multiplex genome editing, two gRNAs were used to achieve precise deletion of multiple sequences within the rice acetolactate synthase gene (*ALS1*) (Sun *et al.,* 2016).

At present, numerous toolkits for multiplex genome editing have been developed in both monocots and dicots, capable of the simultaneous edition of up to eight genes (Wang *et al.,* 2018; Zhang *et al.,* 2016). Multiplex genome editing has been used to modify various plants

to improved resistance to biotic and abiotic stresses. In banana, for instance, multiple gRNAs were used to produce plants resistant to banana streak virus (Tripathi *et al.,* 2019) and has also been used to produce grapevine plants resistant to the fungal pathogen *Botrytis cinerea* (Wang *et al.,* 2018). Since multiplex genome editing enables the development of crops with more than one improved trait, it can aid in the rapid production of yam events with several desired characteristics by simultaneous downregulation, activation, and editing of multiple target genes.

2.7.10.2 Base editing

Unlike the conventional CRISPR-based tools, base editors make single nucleotide substitutions in the genome without the creation of double stranded breaks. Besides, base editors do not require the availability of a homologous donor template and are not dependent on either HDR or NHEJ. Since its invention in 2016, several systems for creating single base mutations have been established in various plant, animal, and microbial cell events (Hess *et al.,* 2017; Molla & Yang, 2019). Notably, the mutation frequency of base editing is higher than that of HDR-mediated base-pair substitutions. CRISPR base editors were created to circumvent several of the challenges associated with the conventional CRISPR/ Cas systems, such as the toxicity of Cas9, random creation of indels, protospacer adjacent motif (PAM) compatibility, and genome instability caused by the DNA double-strand breaks (DSBs) (Li *et al.,* 2018; Safari *et al.,* 2019).

Base editing platforms are chimeric proteins composed of a DNA targeting module and a catalytically impaired nuclease domain capable of deaminating a cytidine or an adenine. Some of the base editing systems currently in use include the adenine base editor (ABE) that alters an A–T nucleotide pair into a G–C and cytosine base editor (CBE) that mutates

a C–G to a T–A. RNA base editors, such as ADAR2, which deaminates adenosine to inosine, have also been developed. The versatility of the base editors BE3, BE4 (Komor *et al.,* 2017), Targeted-AID (Safari *et al.,* 2019), and dCpf1-BE (Li *et al.,* 2020) has been confirmed in various organisms, including major crops.

2.7.10.3 Paired CRISPR-Cas9 nickase for improving editing specificity

One of the greatest shortcomings in using RNA-guided Cas9 nucleases for precise genome engineering is its predisposition to inducing off-target mutations. However, the frequency of on-target mutation can be increased by using a paired nickase strategy that combines a pair of mutant nickase Cas9 (Cas9n) with a pair of offset sgRNAs (Mali *et al.,* 2013). In the Cas9 nickase, one of the endonuclease domains is deactivated to produce a mutant version that generates a nick in only one of the strands. Paired mutant Cas9 nucleases produce DSBs by simultaneously nicking two regions that are complementary to the offset guide RNAs. By extending the target site from ~20 to ~40 bp, the paired nickase strategy improves the targeting specificity by up to 1500 folds (Ran *et al.*, 2013).

2.7.10.4 The Inactive dCas9 for RNA-guided transcriptional regulation

The Cas9/CRISPR system can be repurposed to effect genome regulation instead of the conventional genome editing functions of gene knock out, replacement, or insertion. To achieve this, the DNA cleavage activity of the Cas endonuclease is abolished by generating mutations on specific amino acids on both the RuvC and HNH domains, thereby creating nuclease-null mutants or ‘dead’ Cas9 (dCas9) (Sander *et al.,* 2014). The nuclease-null mutants are fused with effector domains, such as activation or repression domains to facilitate transcriptional regulation by CRISPR-mediated activation (CRISPRa) and inhibition (CRISPRi), respectively (Gilbert *et al.,* 2013; Zheng *et al.,* 2019).

Mechanistically, CRISPRa effector domains either recruit endogenous transcriptional activators or RNA polymerase. In contrast, CRISPRi achieves transcriptional repression by interfering with the activity of RNA polymerase or, by binding on the open reading frames, to inhibit transcriptional elongation. Notably, the catalytically inactive Cas9 can also be used to alter transcriptomes and for site-specific epigenetic regulation by fusion with chromatin-modifying enzymes such as inhibitors of DNA methyltransferases or histone deacetylases, respectively (Dong *et al.,* 2018; Hilton *et al.,* 2015).

2.8 Potential of genome editing for yam crop improvement

2.8.1 Genome Modification for Nutrition Improvement

Many crops, including yams, experience browning due to the presence of polyphenol oxidase, especially during storage. The enzymatic actions not only change the color but also affect the organoleptic features of fruits or vegetables, thus reducing the quality of products for the consumers. Polyphenol oxidase (PPO) changes the flavor, texture, and color of yams, thus reducing their commercial value (Jia *et al.,* 2015). Cas9 can generate heritable and stable mutations on targeted loci without affecting other attributes of the crop. The feasibility of applying this technology for nutrition improvement has been proven via knock-out of the *PPO* genes in potatoes, mushrooms, and apples (Halterman *et al.,* 2016; Nishitani *et al.,* 2016; Waltz, 2016) to create non-browning varieties. Thus, the browning in yams can be mitigated by knocking out the PPO gene. Various accessions of edible yam contain increased levels of anti-nutritional compounds like alkaloids and saponins, which could cause acute toxicity and reduce the bioavailability of minerals and proteins in these tubers (Adebowale *et al.,* 2018). Liang *et al.* (2014) generated low phytic acid maize via targeted mutation of three genes (*ZmIPK*, *ZmIPK1A*,

and *ZmMRP4*) involved in phytic acid synthesis, an anti-nutritional compound. Thus, this system can be employed to improve the palatability of the yam crop and reduce the toxicity by knocking out the genes that code for these anti-nutritional compounds.

According to Adepoju *et al.* (2018), raw yellow yam has significantly low levels of beta carotene, riboflavin and thiamine. This study proposes application of the CRISPR/ Cas9 system to improve the nutritional potential of this root tuber, as demonstrated in other crops. Recent studies have demonstrated that the beta-carotene contents of various crops can be enhanced via manipulation of the carotenoid biosynthetic pathway. For instance, Kaur *et al.* (2020) demonstrated up to 6-fold increase in the β-carotene contents of banana following CRISPR/Cas9 knock-out of the *lycopene epsilon-cyclase* gene.

2.8.2 Abiotic stress resistance via CRISPR/Cas9

Fresh yam tubers have a low shelf life due to post-harvest physiological deterioration caused by microbial and biochemical processes (Nyadanu *et al.*, 2014). Generally, abiotic stress factors, including temperature extremes, waterlogging, soil salinity, and drought, cause significant yield decline worldwide. Previous efforts by conventional breeding to improve abiotic stress tolerance have yielded undesired results, majorly due to a lack of precise knowledge of the key genes underlying the QTLs (Bhatnagar-Mathur *et al.*, 2008). Thus, more advanced technologies such as the CRISPR/ Cas9 system can be employed to generate crop varieties resilient to these stresses via precise modification of *cis*-regulatory elements and structural and/or regulatory genes. The levels of soil moisture grossly affect root development and therefore have a significant impact on the yield of yam tubers. Subsequently, sustainable yam production will require the development of drought-tolerant varieties for environments with reduced water levels. Yam breeders and technology

developers will, therefore, need to focus on providing accessions that can flourish in unsuitable weather conditions and in soils with reduced nutrient profiles.

In maize, drought-tolerant events were generated via the CRISPR/Cas9 by precise modification of the *AUXIN REGULATED GENE INVOLVED IN ORGAN SIZE8* (*AGROS8*) gene (Shi *et al.*, 2017). Knock-down of the rice annexin gene (*OsAnn3*) reduced the survival of T_1 mutant events under cold stress conditions (Shen *et al.*, 2017). Although minimal gene function analyses have been conducted in yam, various stress-related genes identified in other plants are highly conserved and can thus be targeted in less researched crops, such as yams. For instance, ethylene response factors (ERFs) are involved in several abiotic stress tolerance, including extreme temperatures, salinity, and drought, with highly-conserved DNA-binding domains in various crops (Debbarma *et al.*, 2019).

2.8.3 CRISPR/Cas9 for virus resistance

Eukaryotic translation initiation factors, including *eIF4E*, *eIF(iso)4E*, and *eIF4G*, are host factors with redundant functions in plants and aid in the replication of plant RNA viruses (Sanfaçon, 2015). Thus, mutations on the eIF locus could generate virus resistance. In cucumber, Chandrasekaran *et al.* (2016) generated potyvirus resistant events via targeted mutations in the *eIF4E* alleles. Further, rice plants with resistance to the tungro spherical virus (RTSV) have been generated by targeted editing of the host susceptibility gene *eIF4G* (Macovei *et al.*, 2018). Among the various genera that inflict the yam crop, viruses from the potyviridae family, particularly the yam mosaic virus, are the most widespread and economically significant within the yam belt of West Africa (Thouvenel & Fauquet, 1979).

Thus, this review proposes that targeted mutations in the *eIF4E* gene could contribute to virus resistance in yams, particularly to potyviruses.

2.8.4 CRISPR/Cas9 for resistance to fungal pathogens

Genome editing by the CRISPR/ Cas9 system has demonstrated significant potential in generating fungal resistance in various crops, particularly by loss of function mutations on host susceptibility (S) genes. For instance, powdery mildew resistance has been generated in many crops via knockout of genetic materials for Mildew resistance locus proteins (MLO). The *MLO* loci encode plasma membrane protein and are evolutionarily conserved in monocots and dicots (Acevedo-Garcia *et al.,* 2014). Nekrasov *et al.* (2017) generated powdery mildew resistance in tomatoes by mutating the MLO locus. In hexaploid wheat, resistance against the downy mildew pathogen was obtained by CRISPR/Cas9 targeted mutations in the *TaMLO-A1* allele (Wang *et al.,* 2014). In rice, the gene *OsERF922* was targeted to induce resistance against the fungal rice blast disease. Evaluation of transgenic events for blast lesions demonstrated reduced pathogenic infections (Abdelrahman *et al*., 2018).

2.8.5 CRISPR/Cas9 for the control bacterial diseases

The knock-out of host susceptibility genes by the CRISPR/ Cas9 system has been proven to generate durable plant resistance against bacterial diseases. In tomato, for instance, broad-spectrum resistance against bacterial pathogens, including *Xanthomonas* spp., *Phytophthora. capsici*, and *Pseudomonas. syringae*, was obtained by knocking out the tomato orthologue of *DMR6* (downy mildew resistance 6). The expression level of DMR6 is upregulated during pathogen infection, and its protein product is a negative regulator of plant defense responses (Sun *et al.,* 2016; Damme *et al.,* 2008). Knock out of

the *Citrus sinensis Lateral organ boundaries 1* (CsLOB1) susceptibility gene promoter in citrus conferred resistance to the citrus canker caused by *Xanthomonas citri* (Peng *et al.,* 2017). In another study, precise modification of *SWEET* susceptibility genes (*OsSWEET14* and *OsSWEET11)* or their promoters induced resistance to the bacterial pathogen *Xanthomonas oryzae pv. Oryzae* in rice (Oliva *et al.,* 2019; Xu *et al.,* 2019). These successes in generating crop resistance to the various bacterial pathogens suggest substantial promise in mitigating yam yield losses due to infestations by bacteria.

2.8.6 CRISPR/Cas9 for insect and pest management

The current biotechnological strategies for managing insect pests in crops involve genetic transformation to introgress novel genes or classical breeding for insect resistance. The most significant is the transgenic *Bt* technology, whose efficacy has been demonstrated in numerous crops, including corn, tobacco, potato, and cotton (Abbas, 2018). Although the utility of CRISPR/ Cas9 for pest resistance has been scarcely reported, modifying both the plant and the insect could offer innovative opportunities for pest management. For instance, the insect could be modified to stall its infesting capacity, or the plants could be edited to increase their competence to deter pests. Wang *et al.* (2016) successfully knocked down cadherin receptors genetically associated with Cry1Ac toxin resistance in *H. armigera*; this approach could be adopted to edit midgut receptors involved in developing resistance against insecticidal proteins. According to Tyagi *et al.* (2020), modifying the pest detoxification genes, such as the gossypol-inducing cytochrome P450 by CRISPR/ Cas9, could offer promising strategies for crop pest management.

Targeting genes, such as olfactory receptors that could interrupt the identification of mating partners or chemical communication between pests could control pest populations. In the insect pest moth *Spodoptera litura,* CRISPR/Cas9-mediated mutation on an olfactory receptor coreceptor (*Orco*) gene interrupted the insect's ability to identify host plants and select mating partners (Koutroumpa *et al.,* 2016). It has also been suggested that pest developmental genes, such as the Abdominal-A (*abd-A*) gene, could be mutated by CRISPR/ Cas9, a concept that has been proven in various agricultural insects, including the diamondback moth, cotton leafworm (Sun *et al.,* 2017) and in the fall army worm (Wu *et al.,* 2018). The mutant insects demonstrated embryonic lethality, anomalous gonads, disarmed prolegs, and deformed body segments. The possibility of utilizing the super Mendelian inheritance of CRISPR/ Cas9 insect gene drives to manage insect populations has also been suggested, although this approach has the potential for causing ecological havoc (Sun *et al.,* 2017).

The CRISPR/ Cas9 mediated modification of plant volatiles could aid in pest management by deterring insects from host plants (Tyagi *et al.,* 2020). Another approach could be editing the crop pigmentation biosynthetic pathways to alter the plant's visual appearance. As such, pests cannot identify the plant because the visual appearance of a plant aids in its identification by pests. A proof of concept study modified the anthocyanin pathway in tobacco by the transgenic approach, which resulted in red-leaved transgenic events (Malone *et al.,* 2009). Such results can be obtained by CRISPR/ Cas9 targeting of the biosynthetic pathways of plant pigments. Although these suggested strategies for insect and pest management are intriguing, caution should be practiced in trait selection because of the potential for affecting the food chain or causing an ecological imbalance.

2.8.7 CRISPR System for yam metabolic engineering

The yam crop represents under-exploited biodiversity rich in various bioactive compounds, including terpenoids, steroids phenols, tannins, anthraquinones, alkaloids, polyphenols, tannins, and flavonoids. These phyto metabolites have immunomodulatory, hypoglycaemic, antioxidant, antimutagenic, anti-fungal, toxigenic, anti-dyslipidemic, and anti-microbial properties (Padhan & Panda, 2020; Price *et al.,* 2017). Thus, the yam crop offers a rich bio-resource and numerous biosynthetic pathways that can be engineered to generate products for applications in agriculture, medicine, industry, and bioterrorism. At present, the CRISPR/ Cas9 system has been applied for phyto-metabolic editing through a variety of ways, including transcriptional regulation, enzyme manipulation, enzyme inhibition, branch pathway blocking, switching path to alien phyto-metabolites, removing the limited availability to the precursor, manipulating protein modifications, and uORF-dependent regulation (Fu *et al.,* 2018). The enzymes involved in the biosynthesis of specific metabolites can be manipulated by the CRISPR/ Cas9 system to increase or decrease the metabolite levels, generate new products or novel biosynthetic pathways (Sabzehzari *et al.,* 2020).

2.8.8 Yam yield improvement by the CRISPR/ Cas9 system

Relative to other tuber crops such as sweet potato and cassava, the *Dioscorea* species is generally a low-yielding crop, and its cultivation is labor-intensive (FAOSTAT, 1990). The average yam yield is 8.8 t ha^{-1}, that of sweet potato is 12.2 t ha^{-1} (FAOSTAT, 2016), while that of cassava is 12.8 ha^{-1} (FAOSTAT, 2013). Besides, yams have a low multiplication ratio ranging from 1:4 to 1:8; thus, a large fraction of each harvest has to be preserved as subsequent planting material (Aighewi *et al.,* 2015).

The knock-out of negative yield regulators has demonstrated potential in increasing crop yields. In rice, for instance, the CRISPR/ Cas9-mediated knock-out of *GS3, DEP1, and Gn1a* genes generated mutant events with bigger grain sizes, compact, erect panicles, and higher grain numbers, respectively (Li *et al.*, 2016). In maize, the grain yield of mutants with precise modifications at the ARGOS8locus was increased under flowering stress conditions. However, most yield-related traits are complex quantitative traits controlled by multiple genes; the knock-out of individual factors may, therefore, not enhance the crop yield potential under field conditions. Like most other crops, the yield-related traits in yam have not been sufficiently characterized. Extensive efforts in gene identification are therefore needed to exploit the potential of this technology for the improvement of yam yield.

2.8.9 Gene function analysis

Advances in programmable sequence-specific nucleases, precisely the CRISPR/ Cas9 system, have revolutionized basic and applied genetics research in the postgenomic era. Apart from crop genetic improvement, targeted mutagenesis has found wide applications in the functional annotation of plant genomes, significantly augmenting traditional strategies of gene identification and characterization. Compared to the previous gene functional analysis by chemical and physical mutagenesis, TILLING (targeting induced local lesion in genomes), and RNAi, sequence-specific nucleases are more precise, faster, efficient, and reproducible (Liu *et al.*, 2019). Besides, some genes are controlled by quantitative traits, and the conventional QTL mapping and genome-wide association studies (GWAS) prove laborious in such situations. However, the CRISPR-Cas9 technology coupled with whole genome sequencing and pedigree analysis can achieve

these endeavors rapidly and efficiently (Huang *et al.,* 2018). The primary approach for the application of genome editing for gene functional annotation involves the creation of genetic mutants and then evaluating the subsequent loss-of-gene-function phenotype to elucidate the gene function (Syombua et al., 2021).

2.8.10 DNA-free genome editing in yam

The yam genome editing approach currently being used in our lab relies on *Agrobacterium* delivery of T-DNA that harbors both the sgRNA and Cas9. This conventional delivery approach results in T-DNA integration within the host genome and can thus be classified as a genetically modified organism (GMO). Usually, the integration is random and may lead to the production of undesired traits. The prolonged expression of editing reagents could also add to the undesirable effects (Jupe *et al.,* 2019). Generally, the delivery of CRISPR–Cas reagents by transgenic methods have significant drawbacks, including regulatory restrictions governing transgenesis (Voytas & Gao, 2014), prolonged breeding cycles for segregation of foreign DNA, and unanticipated genome damage or changes (Jupe *et al.,* 2019). As such, our lab is currently exploring the possibility of DNA-free genome editing of yam to mitigate these challenges associated with the transgenic approach. The protocol currently under optimization involves delivering preassembled ribonucleoproteins (RNPs) onto yam protoplasts, regeneration of the protoplasts, and molecular analysis of the putative mutants to select for plants with the desired mutations.

Protoplasts offer excellent targets for DNA-free Genome Editing as the RNPs can be easily delivered by polyethylene glycol (PEG) mediated fusion. Therefore, the RNP complex or mRNA is enclosed in PEG vesicles and fused with protoplasts. The editing efficiency of this system is approximately 10%, which is significantly lower than that of

DNA-based systems (Svitashev *et al.*, 2016). Besides, protoplast regeneration is technically challenging, thus complicating the feasibility of its widespread application in most crops. The other common approach for DNA-free genome editing involves the biolistic bombarded of CRISPR reagents into callus or immature embryos. More recently, Ma *et al.* (2020) have demonstrated the feasibility of using viral vectors for DNA- and selection-free genome editing. RNPs rapidly mutate the target sites soon after transfection and then are immediately degraded by endogenous cell proteases, reducing the possibility of off-target mutations and ensures no traces of foreign DNA (Tripathi *et al.*, 2019; Woo *et al.*, 2015).

Yam is vegetatively propagated and backcrossing for T-DNA segregation is challenging because the crop has a poor seed set and a lengthy breeding cycle. Thus, the ability to generate mutants without integrated foreign DNA is an attractive approach for generating yam plants with desirable traits.

CHAPTER THREE

Optimization of *in vitro* regeneration and *Agrobacterium* mediated transformation in the water yam (*D. alata*) by somatic embryogenesis and organogenesis

3.1 Introduction

Regeneration is the process, in which differentiated tissues/cells revert or convert their developmental fate and reconstruct a new tissue. It is a strategy exhibited by a wide variety of multicellular organisms across the animal and plant kingdoms so that individuals can recover from local damage or organ loss (Pulianmackal *et al.,* 2014; Sugimoto *et al.,* 2019). Plants, being immobile, have an enormous regeneration ability for survival, although the extent of this ability varies among species and tissue types (Bidabadi & Jain, 2020). In any form of regeneration, cells need to quit the normal development program and initiate the regeneration program. This releases the fate restriction of the original tissues, and allows the cells to acquire regenerative competence (Hill & Schaller, 2013; Pulianmackal et al., 2014).

Somatic embryogenesis refers to an orderly series of developmental processes during which non-zygotic cells develop into structures that resemble zygotic embryos without gamete fusion. Somatic embryos lack vascular connection with the parental tissue and are typically bipolar in structure (Méndez-Hernández *et al., 2019*; Nolan & Rose, 2010). Somatic embryogenesis enables the production of *in vitro,* true-to-type plants by clonal propagation, and regeneration of genetically modified plants by genetic transformation, somatic hybridisation and *in vitro* mutant induction and selection. Moreover, this technique

offers an indespensable tool for basic studies on plant morphogenesis and totipotency. Somatic embryogenesis also serves as a model for zygotic embryo development, as it provides unlimited source of biological material for biochemical, molecular, and cellular research (Bhatia & Bera, 2015; Pulianmackal *et al.*, 2014).

The wide applications of somatic embryogenesis in both basic and applied research have stimulated studies on the optimization of *in vitro* conditions for somatic embryo induction and subsequent whole-plant regeneration. Consequently, robust protocols on somatic embryo induction and plant regeneration are currently available for most crop species, including *Dioscorea rotundata* (Manoharan *et al.*, 2016). Beside *Dioscorea. rotundata*, *D. alata* L., commonly called the water yam, greater yam, or the winged yam, is the most widely distributed species of the *Dioscorea* genus in the humid and semi-humid tropics. Notably, *D. alata* does not exist in the wild and is not known to hybridize with other *Dioscorea* species. The species is supposedly a true cultivar formed from human selection from its wild forms, although there is no concrete evidence so support this claim (Price *et al.*, 2017). Compared to other cultivated yam species, *D. alata* is superior in tuber storability, competition with weeds (early vigor), ease of propagation and yield potential, especially in poor dilapidated soils (Sartie & Asiedu, 2014). According to (Barrau, 1965), the species was first domesticated in Indochina, where two of its ancestors, *D. persimilis* Prain & Burk. and *D. hamiltonii* J. D. Hook., exist in the wild. The major centre of *D. alata* cultivar diversity is the Solomon Islands and Papua New Guinea. It is a crucial food security crop in the Pacific Islands and the Caribbean, where it has substantial cultural and social importance. This species is also grown in sections of upland Asia (Coursey, 1967).

Somatic embryogenesis and plant regeneration in the water yam have been previously reported in two accessions, *'Kinampay'* and 'VU-2', from leaf, petiole, and nodal stem tissues (Belarmino & Gonzales, 2008). However, the study reported low callus response rates (approximately 50%), which necessitates more research to improve the embryogenic competence of this essential food security crop. In *D. rotundata,* Manoharan *et al.* (2016) demonstrated that the embryogenic competence of axillary bud is superior to leaf, petiole, and nodal stem tissues. Thus, the present study explored both nodal tissues and axillary buds. Herein, a very robust system for somatic embryogenesis and plant regeneration in two *D. alata* accessions, TDa 1304 and TDa 00/00600, was developed. This study reports the possibility of *D. alata* genetic improvement via *Agrobacterium* mediated transformation. Various factors that influence *Agrobacterium* infection efficiency, such as explant type, *Agrobacterium* suspension medium, *Agrobacterium* concentration, tissue wounding, and co-cultivation time were optimized.

3.2 Materials and methods

3.2.1 Plant material

Yam accessions TDa 00/00600 and TDa 1304 were obtained as *in-vitro* plantlets from the International Institute of Tropical Agriculture (IITA) – Ibadan, Nigeria. The plants were maintained and multiplied in yam basic media (YBM) at the IITA-Nairobi, according to the standard micropropagation method described by Nyaboga *et al.* (2014). The YBM medium consisted of MS basal salts with vitamins (Murashige & Skoog, 1962), 2 % sucrose, 0.05 mg/L benzylaminopurine (BAP), 0.02 mg/L α-naphthaleneacetic acid (NAA), 25 mg/L ascorbic acid, and 2.4 g/L gelrite. The medium pH was adjusted to 5.7 then autoclaved at 121 °C for 15 min. Cultures were kept in a growth room maintained at

25±2 °C and 16 h/ 8h light/ dark photoperiod (30 µmol m^{-2} s^{-1} provided by cool-white fluorescent lamps).

3.2.2 Explant culture and somatic embryogenesis

The embryogenic competence of *D. alata* was evaluated based on previous reports on yam regeneration from two explants, nodal stems (Belarmino and Gonzales, 2008) and auxiliary buds (Manoharan *et al.,* 2016). Nodal explants consisted of 5 mm-long stem segments cut from the nodal section of 8-week old *in vitro* plantlets. To obtain auxiliary buds, the nodal explants were cultured in shoot bud medium (SBM) consisting of MS salts with vitamins, 2 % sucrose, 0.5 mg/L copper II sulphate, 1 mg/L BAP, and 3 g/L gelrite, pH 5.8. The cultures were then incubated in the dark for three days, after which the enlarged auxiliary buds were excised using a sterile needle under a dissecting microscope.

To induce callus formation and somatic embryogenesis, the two explants were separately cultured in callus induction medium [CIM consisting of MS basal salts with vitamins (Murashige & Skoog, 1962), 2 % sucrose, 0.5 mg/L copper II sulphate, 600mg/L casein hydrolysate, 1 g/L proline, and 3 g/L gelrite] supplemented with either 2,4-dichlorophenoxy acetic acid (2,4-D) or picloram each at 0.5 mg/L or 1 mg/L. A total eight independent experiments were set up: (i) nodal tissue explant cultured at either 0.5 mg/L 2,4-D, 1 mg/L 2,4-D, 0.5 mg/L picloram, or 1 mg/L picloram (ii) auxiliary bud explants cultured at either 0.5 mg/l 2,4-D, 1 mg/L 2,4-D, 0.5 mg/L picloram, or 1 mg/L picloram. After 4-week of the culture, the calli were sub-cultured onto fresh CIM medium for further four weeks. Subsequently, the efficiency of callus induction, embryogenic competence, callus fresh weight, and the number of embryos per callus were evaluated. The embryogenic competence (%) was calculated by expressing the number of explants

forming embryogenic callus as a percentage of the total number of explants cultured. Each experiment consisted of 100 explants (10 Petri dishes with 10 explants each) and was set up in at least three biological replications.

3.2.3 Embryo maturation and plant regeneration

Embryo maturation and plant regeneration were achieved as described in a previous study (Manoharan *et al.,* 2016). Embryogenic callus was transferred onto a hormone-free MS basal medium containing 1% activated charcoal to induce secondary embryogenesis and embryo desiccation. These cultures were maintained in light for 45 days then transferred to maturation medium containing MS basal salts with vitamins (Murashige & Skoog, 1962), 2% sucrose, 0.4 mg/L BAP, 10 mg/L ascorbic acid, and 3 g/L gelrite. Embryos were sub-cultured onto the same fresh medium every four weeks.

After two weeks of culture in this medium, the number of cotyledonary stage embryos per callus was evaluated. The number of shoots per callus was evaluated after four weeks of culture in the same medium. The cotyledonary embryos with defined root and shoot apices were transferred to YBM for shoot elongation and root development. Embryo desiccation, maturation, and rooting/ shoot elongation were done at 25±2°C and 16 h/ 8h light/ dark photoperiod.

3.2.4 Histological study of the callus

Callus tissues at different stages of somatic embryogenesis were fixed using Carnoy's solution composed of 60% ethanol, 30% chloroform, and 10% glacial acetic acid. This was followed by sequential dehydration of tissues in an ascending ethanol series at 70%, 80%, 90%, and 100%, 30 min per step. All the other steps of histological analysis were done

using Technovit 3040 and 7100 kits (Kulzer, Germany), as described by (Filonova *et al.*, 2000) with slight modification.

The ethanol was gradually replaced with Technovit 7100 (Kulzer, Germany), at ratios of 2: 1, 1: 1, 1: 2 (v/v) ethanol: Technovit for 30 min per step (at 20ºc with vacuum infiltration) then with pure Technovit overnight. Tissues were stained with neutral red dye and embedded on Eppendorf tube lids using fresh Technovit. Technovit 3040 (Kulzer, Germany) was used to fix histoblocks onto wooden blocks, after which 5 µm serial sections were made using a rotary microtome (Leica RM 2155, USA) and mounted on cytological glass slides. The sections were then stained with 0.05 % toluidine blue for 4 min and photographed using a light microscope (Coslab, India) with an integrated digital camera system (optika vision lite 2.1).

3.2.5 Optimization of factors affecting *Agrobacterium*-mediated transformation in *D. alata*

The various factors that affect gene uptake during *Agrobacterium*-mediated plant transformation were evaluated, including the yam accession (TDa 1304 and TDa 00/00600), *Agrobacterium* cell density (OD_{600} of 0.3, 0.5, 0.7, and 1.0), explant type (nodal segment, auxiliary bud, callus), acetosyringone concentration (0, 100, 200, 300, and 400 µM), and tissue wounding (scalpel wounding, sonication, agro-infiltration). The effect of co-cultivation time (1, 2, 3, 4, 5, and 6 days) on the efficiency of transient transformation was also evaluated. Optimization was done based on the efficiency of transient expression of the reporter gene in the transformed explants assessed as blue staining after

histochemical GUS assay on the third day of co-cultivation. Figure 3.1 below gives a summary of the various parameters optimized.

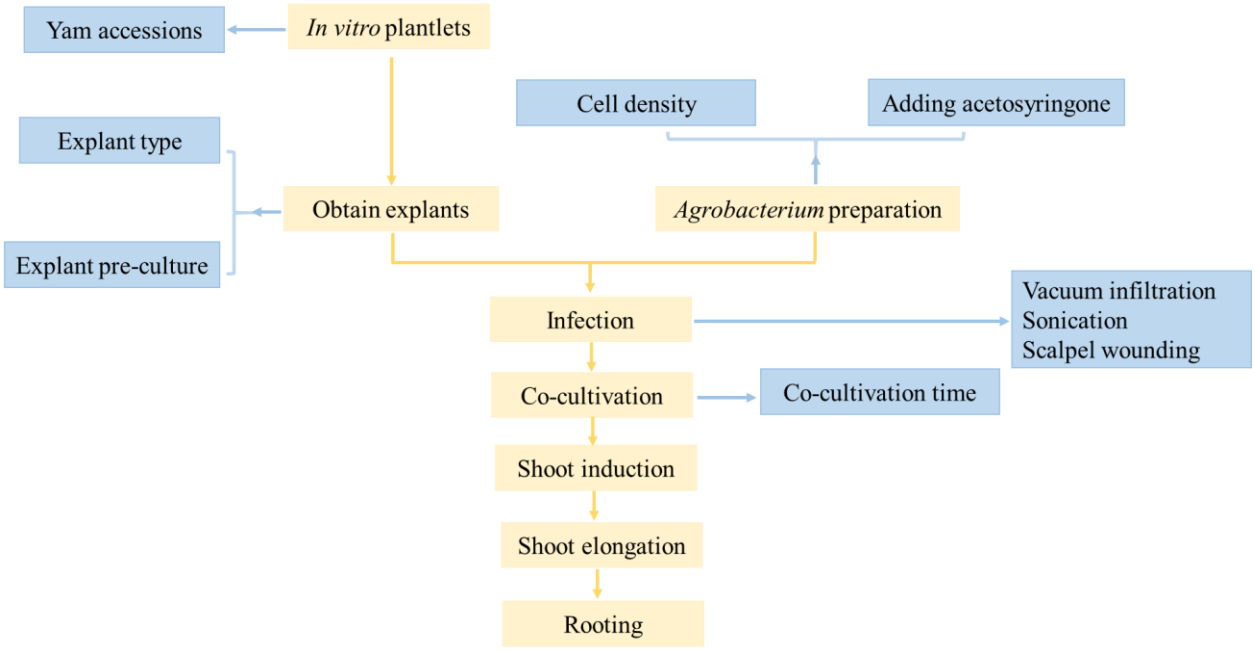

Figure 3.1: Experimental design adopted to optimize *Agrobacterium*-mediated transformation in *D. alata*. The protocol is shown in the yellow color, while the optimized parameters are shown in blue.

3.2.5.1 *Agrobacterium* Strain and Vector

The *A. tumefaciens* strain EHA105 (Hood *et al.*, 1986) and the binary plasmid PCAMBIA2301 (http://www.cambia.org) maintained in our laboratory were used in this study. The PCAMBIA 2301 vector contains a *gus*A gene (with intron) as the reporter gene, *npt*II gene as the plant selectable marker gene (conferring resistance to aminoglycoside antibiotics such as kanamycin, geneticin, and neomycin). The *gus*A and *npt*II genes were under the control of the cauliflower mosaic virus (CaMV35S) and NOS promoters, respectively. The bacteria were maintained on Luria-Bertani (LB) agar plates containing 50 mg/L rifampicin and 50 mg/L kanamycin.

3.2.5.2 *Agrobacterium* culture

To prepare the *Agrobacterium* infection medium, a single colony of *A. tumefaciens* EHA105 harbouring PCAMBIA 2301 was scooped from a solid LB plate and transferred into 25 mL liquid LB (Duchefa biochemicals) containing 50 mg/L kanamycin, 50 mg/L rifampicin, and cultured for 48 h at 28°C (200 rpm) to obtain the starter culture. Subsequently, 50 µL of the starter culture were transferred to 50 mL of liquid LB and cultured overnight in a shaker incubator at 28°C. The *Agrobacterium* culture was collected and centrifuged at 5000 rpm (22°C) for 15 minutes, the supernatant was discarded, and then the pellet was re-suspended in liquid infection medium SBM. The OD_{600} was adjusted to 0.7, the infection medium was shaken for 45 minutes (70 rpm, 22°C), and the OD_{600} was adjusted to four different densities: 0.3, 0.5, 0.7, or 1.0.

3.2.5.3 Explant preparation

Three types of explants, including nodal segments, auxiliary buds, and callus, were used in this study. 1) The nodal segments (approximately 5mm length) were excised from 8-week old *in-vitro* plantlets. 2) The auxiliary buds were excised (under the microscope) from nodal segments cultured in SBM for three days. 3) Embryogenic callus was obtained by culturing nodal segments on CIMmedium for four weeks.

3.2.54 *Agrobacterium* infection and co-cultivation

The explants were re-suspended in *Agrobacterium* infection medium, wounded (vacuum infiltration, sonication, or scalpel wounding), then shaken for 30 minutes to enhance contact between explants and the *Agrobacterium* cells. After infection, the excess suspension was drained off between filter papers then six explants were evenly placed on

solid co-cultivation medium in 90 mm by 15 mm Petri dishes. Co-cultivation was achieved by dark incubation at 23°C for 1, 2, 3, 4, 5, or 6 days.

3.2.5.5 Determination of *Agrobacterium* infection efficiency

Explants were washed thrice using SBM medium (nodal explants and auxiliary buds) or CIM (callus) supplimented with 500 mg/L carbenicillin and 50 mg/L cefotaxime to remove the excess *Agrobacterium* on the explant surface. After drying between pieces of sterile soft tissues, the explants were used for regeneration of transgenic events or histochemical GUS assay. The *Agrobacterium* infection efficiency was evaluated from the rate of transient GUS expression in the respective explants. The rate of transient GUS expression (%) = number of explants with blue coloration/ total number of explants stained × 100.

3.2.6 Explant culture to regenerate transgenic events

For plant regeneration, the calli were taken through the somatic embryogenesis route (protocol optimized in this paper), while auxiliary buds and nodal segments were regenerated through shoot organogenesis (Nyaboga *et al.,* 2014). The explants were transferred to resting medium containing 500 mg/L carbenicillin for seven days, and to selection media containing 100 mg/L Kanamycin and 500 mg/L carbenicillin for 14 days (CIM 0.5 for callus, or YBM for nodal segments and auxiliary buds). The calli were sub-cultured onto the same medium twice (14 days each), transferred to activated charcoal (MS basal salts with vitamins, 3 % sucrose, 10 % activated charcoal) for 15 days, then embryos were matured in 0.4 mg/L BAP medium containing 150 mg/L kanamycin and 250 mg/L carbenicillin. Embryo germination was done in YBM medium supplemented with 200 mg/L Kanamycin. In nodal segments and auxiliary buds, shoot regeneration was attained by successive sub-culture onto YBM medium with selection (200 mg/L Kanamycin) every

14 days and cultured in a 16 h/8 h (light/dark) photoperiod. Eventually, the plants were transferred to the greenhouse at 28/24°C with a photoperiod of 16 h/8 h (light/dark). The *in-vitro* plantlets were first transplanted in peat pellets (two weeks), small pots containing soil: manure mixture (1:1) for four weeks, then to big pots containing soil.

3.2.7 GUS Histochemical Staining

Explants and tissues from putative transgenic events were submerged in GUS staining buffer (Appendices, Table 2) and dark-incubated overnight at 37°C. The tissues were then rinsed twice in 70% ethanol to remove chlorophyll (Jefferson *et al.,* 1987) then examined for blue staining.

3.2.8 Genomic DNA isolation and PCR analysis

Kanamycin-resistant transgenic events were evaluated for transgene presenence by PCR using *npt*II and *gus*A gene-specific primers. Total genomic DNA was isolated from 100 mg of *in vitro* leaves using cetyl trimethylammonium bromide (CTAB, Appendices, Table 3) as described by Stewart and Via (1993). The plasmid DNA of pCAMBIA2301 was used as a positive control, and a non-transgenic plant DNA served as the negative control. The specific primers for amplifying a 780 bp region in the *npt*II gene were: forward 5′ GATGGATTGCACGCAGGTTCTC 3′ and reverse 5′ CAGAAGAACTCGTCAAGAAGGC 3′. The *gusA* gene primers were: forward 5’-CTGCGACGCTCACACCGATACC-3’ and reverse 5’-TCACCGAAGTTCATGCCAGTACAG-3’, and the expected band size was 500 bp. The PCR reactions were done in a 20 µL reaction mixture containing 50 ng of sample DNA and a Qiagen HotStart master mix. The amplification conditions for the *gus*A gene were

set as follows: one cycle at 95°C for 15 min, followed by 30 cycles of 94°C for 30 s (denaturation), 55°C for 30 s (annealing), 72°C for 1 min (extension), and a final 10 minute extension at 72°C. The annealing temperature for the *npt*II gene was 68°C for 30 s. The amplicons were visualized and photographed on the gel imaging system after electrophoresis on a 1% (w/v) agarose gel containing gel red. The transformation efficiency (%) was calculated as the number of positive transgenic plants/ the number of infected explants × 100%.

3.2.9 Experimental design and statistical data analysis

All our statistical experiments were conducted in a completely randomized design, and each experiment was replicated at least three times. Data on the percentage response, embryogenesis competence, number of shoots per explant, regeneration rate, and transformation percentages were analyzed by MINITAB 14 Macros, and significant differences between the means of multiple treatments were compared using Duncan's multiple range test at 5% significance level. Graphs were generated by Microsoft Excel, PowerPoint, and GraphPad Prism.

3.3 Results

3.3.1 Induction of embryogenic calli

Nodal explants (Figure 3.2A) cultured in SBM developed enlarged auxiliary buds (Figure 3.2B) within three days post-culture. The excised auxiliary buds cultured in callus induction medium showed swelling (Figure 3.2C) and formation of embryo-like structures (Figure 3.2D) within the first three weeks of culture. Within the next two weeks, extensive proliferation of the embryo-like structures was observed, resulting in clusters of globular

embryos (Figures 3.2E, F and G). However, some swollen masses developed into loose, watery, non-embryogenic callus. The non-embryogenic watery callus of cultivar TDa 00/00600 were purple in color, making them easier to distinguish from the translucent, gelatinous globular embryogenic callus. Meanwhile, cultivar TDa 1304 produced globular calli that were golden yellow in color and were also easily distinguishable from the loose white watery callus.

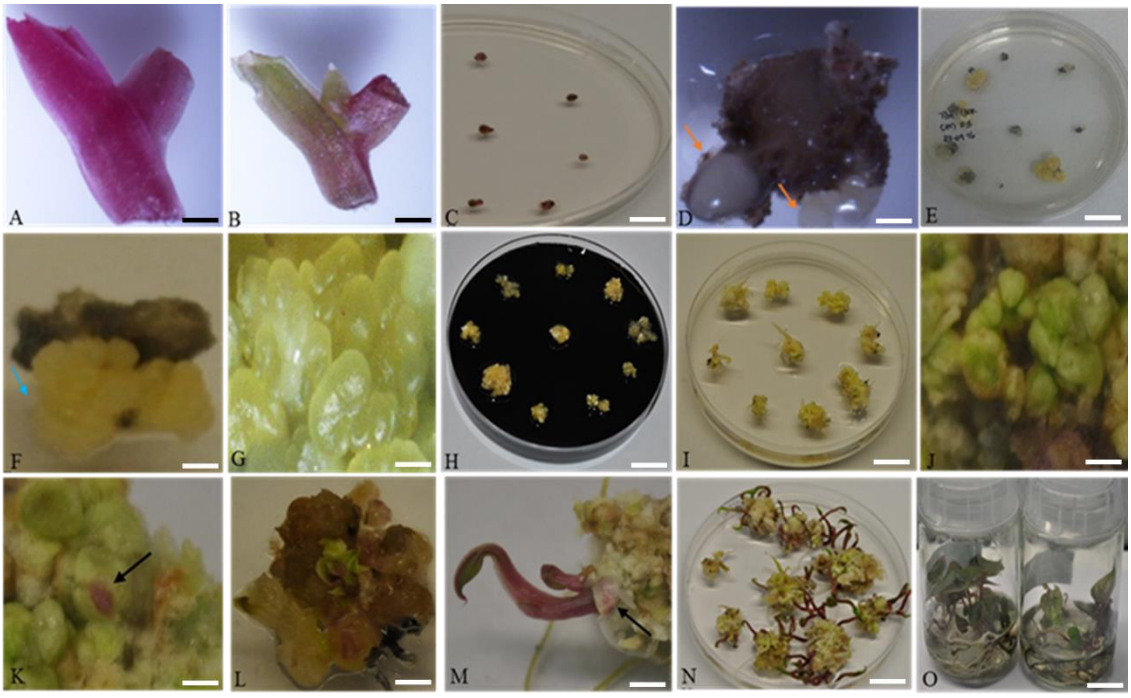

Figure 3.2: Somatic embryogenesis in *D. alata*. A: Nodal explants cultured in Axillary bud induction medium, B: Nodal explants in A after 4 days of culture, C: Swollen mass of callus formed three weeks after bud culture in CIM, D: Callus formed after four weeks of axillary bud culture in CIM, arrows indicate direct embryos, E: Callus formed after eight weeks culture of buds in CIM F: Embryogenic callus formed after eight weeks of axillary bud culture in CIM, the arrow indicates soft friable callus, G: A microscopic image the soft friable part of callus in F, H: Soft friable callus clusters cultured in MS medium supplemented with activated charcoal, I: Callus cultured in regeneration medium supplemented with BAP, J: Germination of a somatic embryo from cotyledonary collars, K: Callus producing shoots in regeneration medium, L: A shoot emerging from cotyledonary embryo, M: An emerged shoot showing cotyledonary collar, N: Callus cultured on 0.4 BAP medium O: Fully developed shoots in YBM medium. Scale bars; A, B, D, F, G, J, K, L, and M, 2 mm; C, E, H, I, N and O, 2 cm.

The callus formation frequencies in TDa 1304 and TDa 00/00600 were 90% and 85%, respectively. Cultivar TDa 1304 gave a better response (30%) to somatic embryogenesis than TDa 00/00600 (24%) (Table 3.1). The two cultivars also differed significantly in the number of embryos produced per explant; TDa 1304 produced an average of 13 embryos per callus while six embryos per callus developed in cultivar TDa 00/00600. Callus proliferation in TDa 00/00600 was, however, significantly higher than TDa 1304, with the average fresh weights of each callus being 150 and 101 mg, respectively (Table 3.1). Additional proliferation and formation of secondary embryos were observed when callus were transferred to in 1% activated charcoal medium (Figure 3.2H). When transferred to regeneration medium (Figure 3.2I), embryogenic callus successfully matured into cotyledonary embryos, as demonstrated by callus greening (Figure 3.2J). However, non-embryogenic callus transferred onto maturation medium formed rhizhogenic structures and underwent necrosis. The cotyledonary embryos transferred on to fresh maturation medium demonstrated a hooked type of germination (Figures 3.2K-M), with each callus generating two to six shoots (Figure 3.2N). The plantlets were successfully rooted in YBM (Figure 3.2O) and attained a height of upto 6 cm within three weeks. A regeneration frequency of 32% was attained in cultivar TDa 1304, while in cultivar TDa 00/00600, a regeneration frequency of 20% was achieved (Table 3.1). The two *D. alata* cultivars also differed with respect to the number of shoots produced per callus, with cultivar TDa 1304 producing an average of 8 shoots per callus and cultivar TDa 00/00600 producing an average of 4 shoots per callus (Table 3.1).

Table 3.1: Effect of auxin type/ concentration and explant type on callus formation and embryogenesis response in *D. alata*.

Cultivar	Explant	Auxin	% response	% Embryogenesis	Embryos/ callus	Wt/ callus (mg)
TDa 1304	Node	0.5D	44.3±2.2^{g}	13.8±1.9^{ef}	3±0.6^{fgh}	105.3±3.9^{fg}
	A. bud		74.8±1.5^{d}	35.3±2.9^{b}	7.8±0.6c	85.3±2.9^{i}
	Node	1.0D	37.3±2.1^{h}	9±3.5^{fgh}	2.3±0.6^{gh}	94.5±2.7^{hi}
	A. bud		79.3±2.1^{c}	31.7±1.7^{b}	6.3±0.6^{cd}	69±4.6^{j}
	Node	0.5P	51±1.8^{f}	19±1.5^{cd}	6.5±0.5^{cd}	166.3±3.3^{b}
	A. bud		91.8±1.3^{a}	46.8±1.3^{a}	15±0.5^{a}	113.3±4.1^{ef}
	Node	1.0P	46.8±0.9^{g}	12±1.3ef^{g}	4.8±0.5^{def}	147.3±5.6^{c}
	A. bud		88±1.97^{ab}	42±0.9^{a}	11±1.4^{b}	102±3.6^{gh}
TDa 00/0060	Node	0.5D	36.3±0.9^{h}	7.3±2.2^{ghi}	3.7±0.8^{efg}	129.5±2.4^{c}
	A. bud		73±1.4^{de}	11.5±1.8^{fgh}	9.8±0.5^{b}	108.8±1.7^{efg}
0	Node	1.0D	28±1.4^{i}	3.3±2.2^{i}	1.3±0.5^{h}	117.8±2.2^{e}
	A. bud		68.7±1.2^{e}	6.7±1.0^{hi}	4.8±0.8^{def}	102±3.6^{gh}
	Node	0.5P	51.5±1.7	16.8±1.3^{de}	2.3±0.5^{gh}	177±4.2^{a}
	A. bud		85±1.3^{b}	31.3±0.6^{b}	11±0.9^{b}	152.5±4.8^{c}
	Node	1.0P	44.8±1.7^{g}	11.8±2.4^{fgh}	1.3±1.0^{h}	164.5±3.4^{b}
	A. bud		83.3±1.5b^{c}	23.3±1.3^{c}	5.3±0.5^{de}	143.3±4.2^{c}

Values are means±standard error. Values followed by different superscripts in the same row are significantly different at P≤0.05 by Duncan's multiple range test. Letter D represents the hormone 2,4-D while P represents Picloram. % response denotes the number of explants that responded by forming either callus or direct embryos, expressed as a percentage of the total number of explants cultured. % embryogenesisi represents the number of embryogenic callus expressed as a percentage of the total explant number cultured.

3.3.2 Histological evaluation of embryogenic callus

No significant differences were observed in the histological appearance of callus tissues from the two *D. alata* cultivars. This study confirmed that callus induction in *D. alata* yields two different morphologies of callus: watery and globular. Sectioning of the watery callus showed that the cells were large with obscured nuclei (Figure 3.3A), an indication of non-embryogenic callus. The cells of the globular callus, on the other hand, had a high

nucleus/cytoplasm ratio, evidence of meristematic regions that could generate somatic embryos.

Most of the developmental stages of somatic embryos were observed. To begin with, pre-embryos with approximately six or more cells (Figure 3.3B) and surrounded by many starch grains (Figure 3.3C) were observed. Following cell division, globular embryos with an evident protoderm were formed (Figures 3.3 D, E). Some of the globular embryos were connected to the mother tissue by a suspensor structure (Figure 3.3D), others did not have a vascular connection to the mother tissue (Figure 3.3E), while the bulk of embryos were fused (Figure 3.3F). The embryos were characterized by a dense cytoplasm, prominent nucleus, and small vacuoles. The globular embryo differentiated further to form a bipolar heart-shaped embryo that had a depressed notch at the upper pole (Figure 3.3G). Further development of this bipolar structure generated a scutella node (terminal leaf node), which preceded the formation of the scutellum (Figure 3.3H). The scutellum was composed of irregularly shaped, richly cytoplasmic cells bordered by epithelial cells (Figure 3.3H).

Following successive division, the number of cell layers in the scutellum increased, and the coleoptile was evident (Figure 3.3I). Increased cell division was also observed in the scutella node, and the shoot apical meristem emerged (Figure 3.3J). The resultant embryo had a distinct shoot apical meristem and a root apical meristem (Figure 3.3K). When cultured further, the coleoptile and scutellum developed to form an evident leaf primordium and a shoot apex (Figure 3.3L). The shoot apex of mature somatic embryos had well-developed leaf primordia and a fully formed root apex (figure 3.3M).

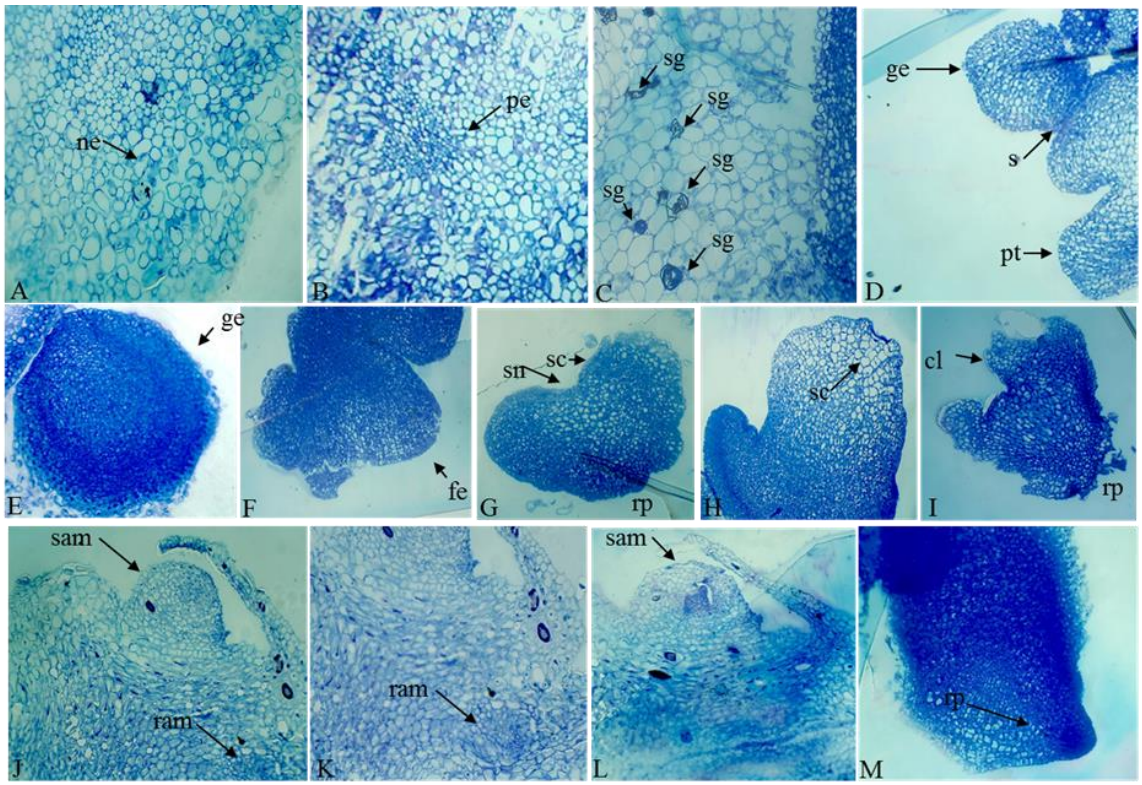

Figure 3.3: Histological evidence of direct somatic embryogenesis from axillary bud explants of *D. alata*. A: Non-embryogenic callus, B: pro-embryos, C: Callus tissue with many starch grains, D: Globular embryo with visible protoderm and a suspensor-like structure (arrow), E: Globular embryo without a vascular connection to the mother tissue F: Fused embryos, G: Heart-shaped embryo, H: Scutellum, I: Embryo with a coleoptile and a root pole, J: Embryo with a shoot apical meristem and a root apical meristem, K: Root apical meristem of the embryo in J, L: Shoot apex of a somatic embryo, M: Root pole of a somatic embryo. ne: non-embryogenic, pe: pro-embryo, sg: starch grain, ge: globular embryo, s: suspensor, pt: protoderm, fe: fused embryos, sn: scutella node: sc: scutellum, rp: root pole, cl: coleoptile, sam: shoot apical meristem, ram: root apical meristem.

3.3.3 Optimization of *Agrobacterium*-Mediated Transformation of *D. alata*

3.3.3.1 Explant type and yam accession

Three explant types, including nodal segments, auxiliary bud, and callus, were used to optimize the *D. alata* agro-mediated transformation system. The results demonstrated higher transient *gus*A expression in nodal explants (68.7% in TDa 1304 and 55.7% in TDa 00/00600) compared to the callus (52.7% in TDa 1304 and 40.3% in TDa 00/00600) and auxiliary bud (32.0% in TDa 1304 and 21.3% in TDa 00/00600) explants (Figure 3.4A). Following these results, nodal explants and callus tissues were chosen as the target explant

for subsequent transformation experiments. Due to the small size of auxiliary buds, most of them -turned brown due to the necrosis caused by the over-infection of *A. tumefaciens.* For all the explants tested, the accession TDa 1304 gave significantly higher infection rates compared to TDa 00/00600. Cultivar TDa 1304 was therefore chosen for further experimentation. Since auxiliary buds had very low survival rates caused by tissue necrosis from *A. tumefaciens* over-infection, only nodal segments and callus tissues were used for additional experimentation.

3.3.3.2 Explant pre-culture

Based on the GUS assay, the highest transformation efficiency (71.3%) was obtained when explants were agro-infected on the day they were excised (0 days of pre-culture) (Figure 3.4B). An increase in the pre-culture period significantly reduced the transformation efficiencies, with 3- and 4-days pre-culture recording the least rate of 45.3% and 37.3%, respectively.

3.3.3.3 Wounding

The various forms of explant wounding had different effects on the transient *gus*A expression of yam tissues (Figure 3.4C). Vacuum infiltration of tissues with the bacterial suspension significantly increased the transient expression rates, with callus tissues scoring 67.0% and nodal segments 80.7%. Due to some slight tissue necrosis, sonication marginally reduced the *Agrobacterium* infection rates as demonstrated in nodal segments (66.7%) and callus (58.3%). Although the frequencies did not differ significantly ($P < 0.05$) from the non-wounded control nodal explants (67.7%) and callus (58.3%). While, scalpel wounding caused substantial tissue wounding and significant ($P < 0.05$) reduction in the transient *gus*A expression, ranging from 40.7% for callus to 49.0% for nodal explants. It

was concluded that vacuum infiltration is the appropriate way of wounding the yam explants for increasing the transformation efficiency.

3.3.3.4 Bacterial density

The nodal explants were infected with *Agrobacterium* suspensions with different OD_{600}, ranging from 0.3 to 1.0 (Figure 3.4D). The transient transformation frequencies of the different bacterial suspension densities showed differences. The highest transient *gus*A expression were obtained at an OD_{600} of 0.7, followed by 0.5, while 1.0 gave the lowest efficiency. The corresponding infection rates were 72.0%, 58.3%, and 33.3%, respectively for nodal segments and 67.7%, 52.7%, and 25.7%, respectively for callus (Figure3.4D). Although the infection rates obtained at an OD_{600} of 0.3 (44.3% for nodal segments and 35.7% for callus tissues) were lower than that obtained at ODs of 0.5 and 0.7, they were higher than that obtained at an OD_{600} of 1.0. It was concluded that an OD_{600} of 0.7 is the optimal bacterial density for infection of yam explants; hence OD of 0.7 was used in further experiments. The higher *Agrobacterium* suspension density of 1.0 optical density allowed attachment of numerous bacterial cells on the plant tissues, which caused bacterial overgrowth by the third day of co-cultivation and subsequent tissue necrosis. Therefore, an OD_{600} of 1.0 is not suitable for *Agrobacterium* transformation of yam tissues.

3.3.3.5 Acetosyringone

Yam infection and co-cultivation medium were supplemented with five acetosyringone concentrations, including 0, 100, 200, 300, and 400 μM, to determine whether the phenolic compound could enhance the infection rates. The GUS assay revealed that infection of yam tissues could occur in medium with or without acetosyringone (Figure 3.4E). In acetosyringone-free medium, the infection rates of callus and nodal explants were 13.7% and 28.0%. Increasing the acetosyringone concentrations from 100 to 200 μM increased

the transient infection rates of yam callus from 34.0% to 56.3% and that of nodal segments from 49.0% to 56.3% (Figure 3.4E). Increasing the acetosyringone concentrations from 200 to 300 μM slightly reduced the transient expression of gusA gene in the agro-infected callus and nodal explants to 52.7% and 64.3%, respectively. However, a further increase to 400 μM significantly reduced the transformation efficiency to 41.7% in callus and 44.7% in nodal explants. Since acetosyringone 300 and 400 μM acetosyringone caused tissue browning and necrosis while 0 and 100 μM had low infection efficiency, 200 μM was considered the optimal concentration for further experimentation.

3.3.3.6 Co-cultivation

Yam explants were co-cultivated with *A. tumefaciens* for 1, 2, 3, 4, 5, and 6 days to determine the optimal co-cultivation period for maximal transient *GUS* transformation rates. The findings revealed that a 3-day co-cultivation period generated the highest bacterial infection rates (70.7% in nodal segments and 60.0% in callus tissues) (Figure 3.4F). The one-day co-cultivation generated the least transient infection rates, scoring 17.7% in nodal segments and 15.0% in callus tissues. Increasing the co-cultivation period beyond three days significantly reduced the transient GUS infection rates. The transient *GUS* transformation rates between days 2 and 4 did not differ significantly in both explant types. Further increase in co-cultivation time caused bacterial overgrowth, tissue necrosis and significantly reduced the transient *GUS* infection rates (Figure 3.4F).

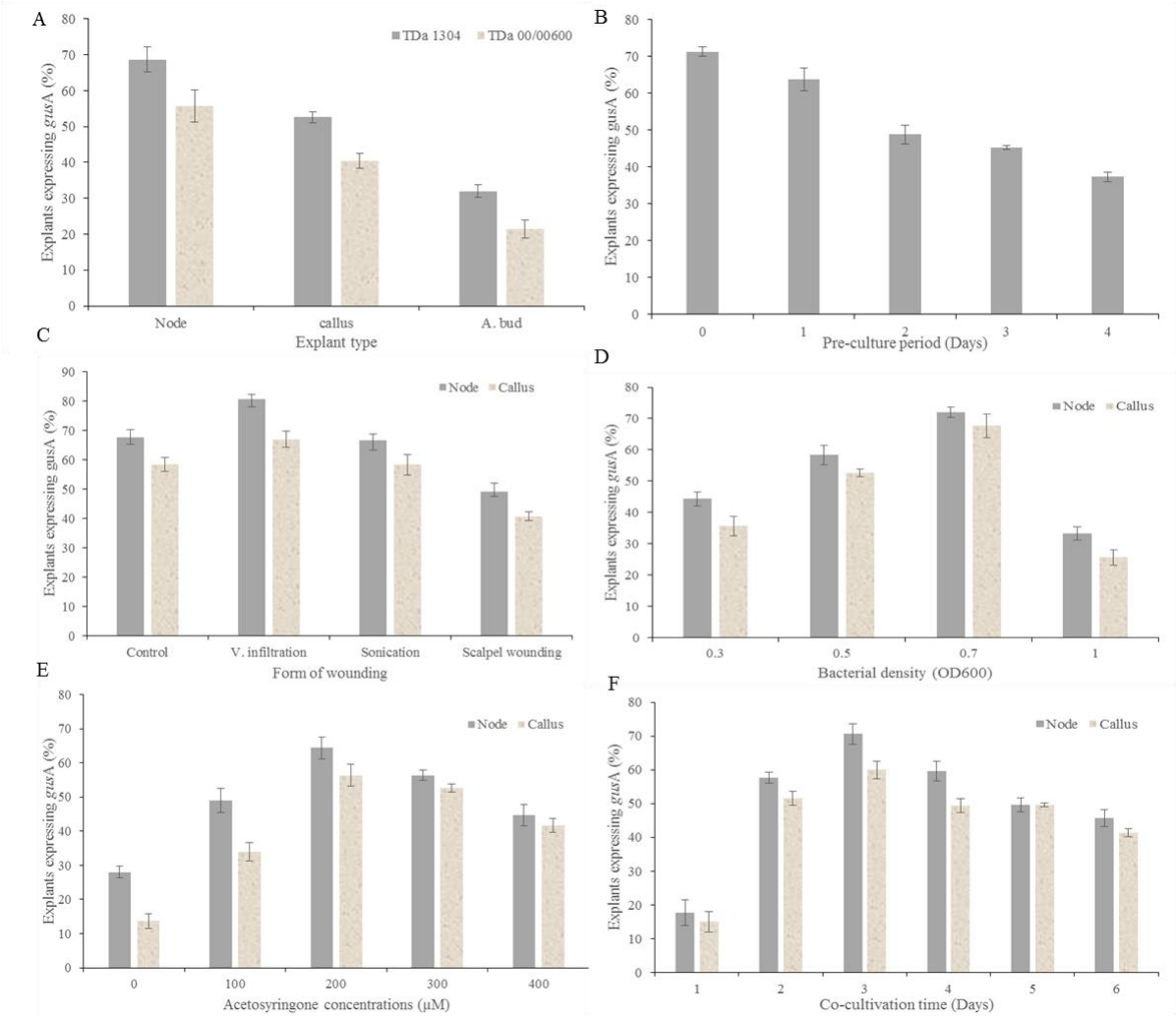

Figure 3.4: Optimization of the *Agrobacterium*-mediated transformation of *D. alata* based on transient *gusA* expression. A: explant type and yam accession. B: pre-culture period. C: tissue wounding. D: bacterial density. E: acetosyringone concentration; and F: co-cultivation period. The data from three replicates were analyzed by one-way ANOVA, and means were separated by Tukey's test.

3.3.4 Confirmation of transgenic plants by histochemical GUS assay

Transient GUS expression assay after three days of co-cultivation showed blue coloration, confirming transient expression of the reporter gene in the nodal explants and callus of both TDa 1304 and TDa 00/00600 (Figures 3.4 A-D). A total of six kanamycin-resistant plantlets were regenerated from nodal explants of TDa 1304. However, no plant was regenerated from callus tissues. All the six putative transgenic events gave a uniform blue

coloration after GUS staining assay (Figures 3.4 E & F), confirming stable expression of the *gusA* gene throughout the plant.

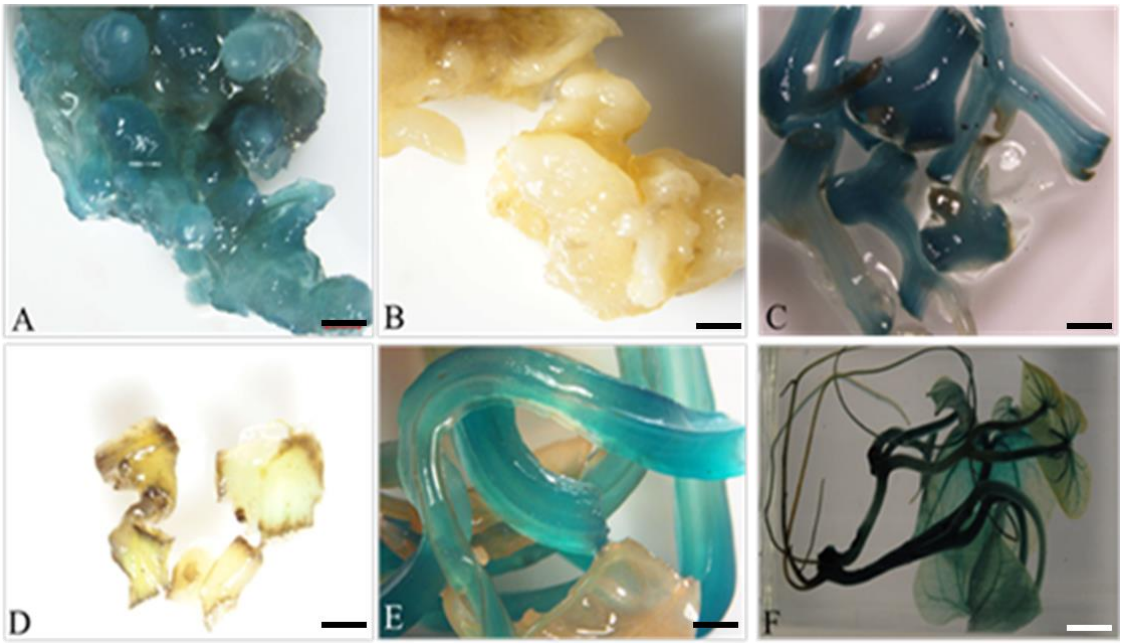

Figure 3.4: Transient and stable expression of GUS in *D. alata*. A: transient GUS expression in transformed yam callus. B: no GUS expression in non-transgenic control callus C transient GUS expression in transformed nodal explants. D: no GUS expression in non-transgenic control nodal explants. E: stable GUS expression in transgenic yam segments. F: stable GUS expression in yam complete plantlet. Scale bars; A, B, C, D, and E 2 mm, F 2 cm.

3.3.5 Transgene detection by PCR Analysis

A 500 bp amplicon corresponding to the *gus*A gene and 700bp corresponding to the *npt II* gene was amplified from the genomic DNA of transgenic plants (Figure 3.5), confirming transgene integration.

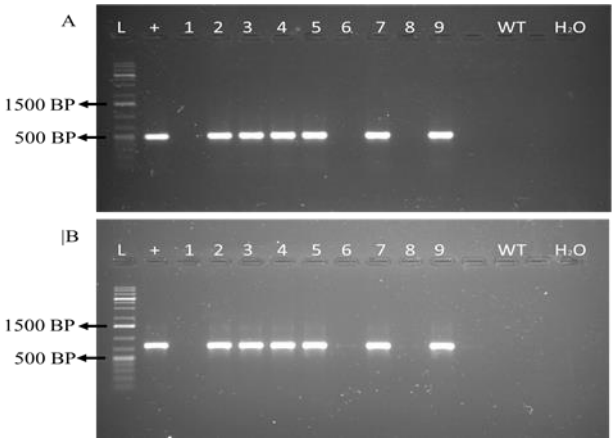

Figure 3.5: PCR analysis of transgenic yam events

A: *gus*A. B: *npt II*-specific primers. Lanes: L, 1 kb plus DNA ladder; +, PCambia2301 plasmid DNA used as a positive control; 1–9, mutant yam events; WT, non-transgenic plantlet DNA.

3.4 Discussion

The present study demonstrated that axillary buds have better callus response (91.8%), embryogenesis rates (46.8%), and the number of somatic embryos per callus (15) than nodal tissues (best rates of 51.0%, 19.0%, and seven embryos per callus). In cassava, numerous studies have also proven that axillary buds form the explant of choice for primary somatic embryogenesis, the formation of friable embryogenic callus, and plant regeneration (Nyaboga *et al.,* 2015; Rossin & Rey, 2011). These differences in responses by various explant types could also be because axillary buds are younger than nodal stem tissues and, therefore, at a lower differentiation state.

Further, this study observed a genotype-dependent response to somatic embryogenesis and plant regeneration. Lower callus responses, somatic embryogenesis rates, number of somatic embryos per callus, and regeneration efficiency were found in TDa 00/00600 in all treatments, while TDa 1304 gave significantly better responses in all the above parameters. Nyaboga *et al.* (2015), Syombua *et al.* (2019), and Narváez *et al.* (2019) also reported genotype-dependent responses to somatic embryogenesis and plant regeneration.

Somatic embryogenesis and subsequent plant regeneration are generally initiated and manipulated via the addition of phytohormones in the culture medium for synchronized production, maturation, and conversion of embryos into plants. The phytohormones promote tissue dedifferentiation, chromatin remodeling, and gene expression reprogramming of somatic cells to generate tissues akin to zygotic embryos (Kumar *et al.,* 2015). The present study used 2,4-D and picloram to induce tissue dedifferentiation in the water yam. Similar to the findings of Belarmino and Gonzales (2008) and Manoharan *et al.* (2016), picloram induced a significantly better embryogenic response in *D. alata*, relative to 2,4-D. We also observed that lower auxin concentration promoted a better embryogenic competence than higher concentrations. This phenomenon has been reported in other monocotyledonous plants, including wheat (Adero *et al.,* 2019) and sugarcane (R. Kaur & Kapoor, 2016). Generally, elevated auxin levels hinder pro-embryo development and convert embryogenic callus into non-embryogenic ones (Taylor *et al.,* 1992).

Analysis of the histo-differentiation process of *D. alata* callus confirmed that this study regenerated yam plantlets via somatic embryogenesis. Most of the somatic-embryo developmental stages were observed. The histological sections of *D. alata* had a morphology similar to that reported in other monocotyledonous species, including sugarcane (Alcantara *et al.,* 2014) and wheat (Delporte *et al.,* 2014). In *D. rotundata*, the formation of somatic embryos from meristematic cells that had many starch grains was also reported (Manoharan *et al.,* 2016). The somatic embryos obtained exhibited the typical bipolar orientation, which has been reported in *S. tuberosum* (Sharma & Millam, 2004), capsicum (Avilés-Viñas *et al.,* 2013), sugarcane (Alcantara *et al.,* 2014), and wheat (Delporte *et al.,* 2014) among others. Additionally, the embryos did not have a vascular

connection to the mother tissue. The scutellum was composed of irregularly shaped, richly cytoplasmic cells bordered by epithelial cells, similar to the findings of Alcantara *et al.* (2014).

The efficiency of *Agrobacterium*-mediated transformation depends on the successful induction of bacterial virulence, chemotaxis, tissue attachment, T-DNA transfer, and gene integration into plant cells. Notably, these processes can be enhanced by modifying the physiological and physicochemical parameters in the infection procedures or the media (Nyaboga *et al.,* 2015). For instance, tissue wounding, including normal wounds generated during explant preparation, needle or scalpel wounding, sonication, and particle gun wounding, could enhance the bacterium's ability to infect target tissues (Trick & Finer, 1997). Wounding increases the production of phenolic substances from the wounded tissue, which attracts more bacterial cells to the site and enhances the transformation efficiency (Matheka *et al.,* 2019). In the present study, wounding by sonication increased the *Agrobacterium* infection efficiency, although not significantly different from the control. Mariashibu *et al.* (2013) reported that although sonication enhances *Agrobacterium* infection, it may damage most cells, restricting the ability to recover transformed events. In this study, scalpel wounding caused tissue necrosis and significantly reduced the transformation frequency. According to Gnasekaran *et al.* (2014), scalpel wounding may generate severe injuries on the epidermis and penetrate deeper within the wounded tissue. Subsequently, excess *Agrobacterium* accumulates within the cells, causing over-infection and subsequent tissue necrosis (Uddain *et al.,* 2015).

Vacuum infiltration has increasingly been reported as an effective method for promoting plant transformation, especially in recalcitrant cultivars (Oliveira *et al.,* 2008; Gupta *et al.,* 2006; Shrawat *et al.,* 2007). In the present study, vacuum infiltration significantly increased the *Agrobacterium* infection of *D. alata* tissues. The decreased pressure and subsequent re-pressurization during vacuum infiltration bring the bacterial and plant cells into closer contact, facilitating T-DNA transfer into plant tissues (Mariashibu *et al.,* 2013).

One-day explant pre-culture was found to significantly increase transformation rates. Besides, pre-culture enhanced the explant viability, as demonstrated by a higher number of germinating explants following pre-culture. Similar findings have been reported in *Brassica rapa*, *Primula Vulgaris,* and tomato (Baskar *et al.,* 2016; Hayta *et al.,* 2018; Rai *et al.,* 2012), where short term pre-culture enhanced transformation efficiencies. Conversely, extended pre-culture reduced the transformation efficiencies. Explant pre-culture enhances the tissue vitality to overcome *Agrobacterium* infection stress, and therefore increasing the explant regenerative capacity (Rai *et al.,* 2012). Hayta *et al.* (2018) reported that explant pre-culture enhances *Agrobacterium* adhesion on plant tissues during co-cultivation, hence the increased infection rates. The increase in *Agrobacterium* infection after one-day pre-culture could also be because actively dividing cells, particularly in the S-phase of the cell division cycle, have a higher competence for *A. tumefaciens* uptake (Rai *et al.,* 2012).

During explant infection with bacterial suspension, a high *Agrobacterium* concentration could cause bacterial overgrowth, *Agrobacterium* contamination, tissue necrosis due to over-infection, and inability to recover transformed events. Conversely, a low bacterial density results in weak infection ability, thus low T-DNA integration into tissues

(Sreeramanan *et al.,* 2008). Therefore, the optimal bacterial suspension that would cause high infection rates with little or no tissue necrosis should be determined. In this study, an OD_{600} of 0.7 was found optimum for yam tissue infection; lower bacterial densities had significantly low infection rates while higher densities caused *Agrobacterium* overgrowth and tissue necrosis. Similar findings on the effects of bacterial densities on transformation efficiencies were reported in safflower (Orlikowska *et al.,* 1995) and *Dendrobium Savin* (Subramaniam *et al.,* 2009).

The presence of optimal acetosyringone concentration (200 μM) in the infection medium significantly enhanced GUS expression rates. However, an increase beyond the optimal concentration caused tissue browning, indicative of tissue necrosis due to *Agrobacterium* over-infection. These findings suggest that the presence of optimal concentrations of acetosyringone is crucial for success in *Agrobacterium*-mediated transformation of *D. alata*. In *Dioscorea zingiberensis*, the optimal concentration of acetosyringone was also 200 μM, but an increase to 300 or 400 μM had no apparent toxic effects on the callus. Further, these findings concur with reports in *Brassica napa* and broccoli, where 200 μM was found necessary for maximal *Agrobacterium* infection (Baskar *et al.,* 2016; Henzi *et al.,* 2000). Acetosyringone induces the transcription of virulence genes in *Agrobacterium tumefaciens*, which increases the transformation rate (Opabode, 2006).

Another important factor affecting transformation efficiency is the co-cultivation duration. In the present study, different co-cultivation periods (1, 2, 3, 4, 5, and 6 days) generated significantly variable transformation frequencies, with a maximum rate at 3-day co-cultivation. The transformation frequencies reduced with time increase from the third day, and the tissues turned brown due to *Agrobacterium* overgrowth. Similar findings have been

reported following extended co-cultivation of *Pinus pinea* (Humara *et al.,* 1999), *Lycium barbarum* (Hu *et al.,* 2006), and delight orchid (Gnasekaran *et al.,* 2014). For most protocols, 2-3 days of co-cultivation is standard for maximal infection, although some crops may require longer cultivation. Therefore, it is vital to optimizing the co-cultivation period for each species of interest to ensure minimal tissue necrosis and maximum infection rates (Gnasekaran *et al.,* 2014).

This study achieved significantly higher transformation frequencies of nodal tissues (68.7% in TDa 1304 and 55.7% in TDa 00/00600) relative to axillary buds (32.0% in TDa 1304 and 21.3% in TDa 00/00600) and callus explants (52.7% in TDa 1304 and 40.3% in TDa 00/00600). These results reveal genotype and tissue-dependent response to *Agrobacterium*-mediated transformation of *D. alata*. In stevia (Gnasekaran *et al.,* 2014), *Withania somnifera* (Udayakumar *et al.,* 2014), and *D. rotundata* (Nyaboga *et al.,* 2014), nodal tissues are also the explant of choice for genetic transformation. In this study, stably transformed TDa 1304 events were recovered (1.1% transformation efficiency), while no transgenic line was obtained in TDa 00/00600. In the white yam (*D. rotundata*), Nyaboga *et al.* (2014) reported 9.4 to 18.2% transformation efficiency, suggesting the rates obtained in the present study are considerably less than that of the white yam.

Another remarkable difference between the protocol optimized herein and that of *D. rotundata* is the time taken to regenerate transgenic events. It took three to four months from infection to the regeneration of complete *D. rotundata* transgenic plants (Nyaboga *et al.,* 2014); however, the present study took up to nine months to regenerate whole transgenic events of *D. alata*. Genotype-dependent response to genetic transformation is

a prevalent phenomenon, with some maize (Masters *et al.,* 2020), orchid (Gnasekaran *et al.,* 2014), cassava (Nyaboga *et al.,* 2015) being regarded as recalcitrant. In this study, all the transgenic events were recovered by organogenesis, and no plant was obtained by regeneration from embryogenic callus. In the future, further optimization of this protocol is needed to establish a transformation system based on embryogenic cultures, increase the transformation efficiency, and shorten the time for regeneration transformed events.

3.5 Conclusion

The lack of optimized protocols for *D. alata* regeneration and *Agrobacterium* mediated gene transfer have impeded the genetic improvement of this essential food security crop. The protocols developed in this study will open avenues for genetic transformation and genome editing of *D. alata* to solve the various challenges encountered during its production and consumption.

CHAPTER FOUR

Production and regeneration of friable embryogenic calli (FEC) in *D. rotundata*

4.1 Introduction

Guinea yam (*Dioscorea cayenensis* - *Dioscorea rotundata* complex) is one of the most important root and tuber crops contributing to food security and poverty alleviation in tropical and subtropical regions of the world (FAOSTAT 2013). Of the 600 species of the genus *Dioscorea*, the most important edible species are *D. rotundata* (white yam), *D. alata* (water yam), *D. cayenensis* (yellow yam), *D. dumetorum* (bitter yam), *D. esculenta* (Chinese yam), and *D. bulbifera* (aerial yam). Among these, *D. rotundata* is the most preferred and cultivated, accounting for a large proportion of yam production in West Africa. Notably, the yam belt of West Africa accounts for 93% of the world's yam production *(Darkwa et al.,* 2020).

The availability of a morphogenic culture system amenable to gene transfer techniques is an indispensable prerequisite for the development of a genetic transformation protocol in plants. In a recent study, Manoharan *et al.* (2016) reported a robust system for inducing somatic embryogenesis in yam and subsequent regeneration from somatic embryos. However, the histological analysis reported a mixed form of regeneration, consisting of both somatic embryogenesis and organogenesis. For plant genetic transformation, somatic embryogenesis may be more suitable than organogenesis because, in most cases, somatic embryos are of single-cell origin, and chimeric trangenic plants are less likely to develop (Bespalhok & Hattori, 1998). Therefore, application of the system for gene transfer in *D. rotundata* will require substantial refinement of the heterogeneous mixture of the cell

morphotypes generated by Manoharan *et al.* (2016) to obtain a homogenous assortment of tissues at the same stage of development.

At present, friable embryogenic callus (FECs) form the tissues of choice for regeneration and transformation in various crops, including the ornamental Astromelia (Lin *et al.*, 2000) and cassava (Syombua *et al.*, 2021). Friable callus consists of numerous embryogenic units that disperse easily and can proliferate rapidly in liquid medium to produce high-quality embryogenic suspension cultures in which the large majority of the cells are totipotent (Taylor *et al.*, 1996). Thus, FECs are an ideal target tissue for direct gene transfer systems because they maximize the probability of the insertion and integration of the genetic material into large numbers of morphogenically competent cells. Besides, FECs provide versatile systems for crop genetic manipulations at the cellular level (Bull *et al.*, 2009) and are an excellent source for isolating totipotent protoplasts (Wen *et al.*, 2020).

Following stringent selection during subculture, the present study reports a system through which the embryogenic tissues developed by Manoharan *et al.* (2016) can be selected, enriched, maintained, and taken through a series of sub-cultures to generate friable embryogenic callus (FECs). To optimize a protocol for generating yam FECs, this study evaluated various factors, including the basal salt composition, effects of callus wounding, washing wounded callus, adding ascorbic acid on washing medium, and addition of tyrosine to the culture medium.

4.2 Materials and methods

4.2.1 Plant material and growth conditions

In vitro yam plantlets of the accession number TDr 2436 were obtained from the germplasm collection of the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. The plantlets were maintained by sub-culturing on yam basic medium, consisting of Murashige and Skoog basal salt (MS) with 30 g/L sucrose, 2 mg/L glycine, 100 mg/L Myo-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine·HCI and 0.1 mg/L vitamin B1, and 2.4 g/L gelrite at 4–8 weeks interval. The medium pH was adjusted to pH 5.8 using 1M NaOH then autoclaved at 121°C for 15 min (Nyaboga *et al.*, 2014). The cultures were maintained at 25 ± 2°C under a 16h light/8h dark photoperiod (30 µmol m^{-2} s^{-1}).

4.2.2 Production of primary somatic embryos

Somatic embryo formation in yam was induced from axillary buds using the method used in our lab for the cassava cultivar TME 14 (Nyaboga *et al.*, 2015). Briefly, single nodal stem sections of size 10–20 mm were prepared from 4-8 week old *in vitro* plantlets. The nodal sections were placed horizontally in bud induction medium SBM (Murashige and Skoog basal salts (MS basal salts) with 2 µM $CuSO_4$, 1 mg/L of 6-benzyl amino purine (BAP), 2% sucrose, 3 g/L gelrite, 2 mg/L glycine, 100 mg/L myo-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine·HCI and 0.1 mg/L vitamin B1) for three days, at 28°C in the dark. Subsequently, the emerging axillary buds were excised using sterile syringes under a dissecting microscope and cultured in callus induction medium, CIM (MS basal salts with 0.5 mg/L picloram, 2% sucrose, 3 g/L gelrite, 2 mg/L glycine, 100 mg/L myo-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine·HCI and 0.1 mg/L vitamin B1) for

four weeks at 25 ± 2°C in the dark to induce somatic embryo formation. To determine the effect of amino acids on the callus proliferation and formation of embryogenic tissues, the callus induction medium was supplemented with different quantities of proline and casein hydrolysate. All subsequent experiments were supplemented with the optimized quantities of amino acids.

Three basal medium formulations were used to optimize the system, including MS salts, half MS, and FEC Induction Medium (FIM) (Appendix 4). The FIM consisted of MS salts with reduced contents of nitrogen, phosphate and potassium, and extra thiamine (10 mg/L). The comparative potential of the various basal medium to induce primary embryogenesis was evaluated based on the percentage of explants that formed embryogenic callus, the callus weight, and the number of embryos per callus. The explants producing direct somatic embryo-like structure (DSLS) and the number of DSLS/explant were recorded after four weeks of culture. The percentage of DSLS was calculated as the number of explants showing DSLS induction/total number of explants cultured × 100.

4.2.3 Friable callus formation from compact embryogenic callus

The formation of FECs was induced from somatic embryos by successive culture in medium supplemented with 0.5 mg/l picloram. Four basal salt formulations were used to optimize FEC formation, including Full strength MS salts, half-strength MS salts, FIM and Greshoff and doy (GD) salts (Gresshoff & Doy, 1974). The somatic embryos were maintained in this medium for six months at 26 ± 2°C in the dark, at a 4-week sub-culture schedule. Different forms of wounding were induced, including crushing through a stainless steel mesh (pore size 1–2 mm) as described by Nyaboga *et al.* (2015) or separating the compact calli into single units (sizes of approximately 2-5 cm diameter) with a pair of

forceps without damaging (Lin *et al.,* 2000). The control consisted of compact callus transferred without any form of wounding. All treatments were performed in triplicates, and each experiment consisted of 10 Petri plates of 10 units each. For the meshed callus, each Petri plate set up consisted of 1.5g of meshed compact callus. The different media were evaluated based on the ability to induce FEC formation, four months after culture.

4.2.4 Effect of callus washing, ascorbic acid, and tyrosine on callus browning

The compact callus that developed on callus induction media were crushed through a stainless-steel wire mesh with 1-2 mm pore size. The fine callus pieces were collected onto a sterile 50 ml falcon tube and washed thrice with either of four solutions; (i) double distilled water, (ii) liquid MS medium, (iii) liquid MS medium containing 10 mg/L ascorbic acid, and (iv) liquid MS medium containing 20 mg/L ascorbic acid. The washed calli were therafter transferred to a nylon filter mesh (100 μm pore size) and the excess liquid drained off on sterile soft tissues. The nylon filter mesh containing the meshed callus was then transferred onto GD medium containing 0.5 mg/L picloram. The control experiment consisted of meshed calli that were not washed. To determine whether L-tyrosine could improve callus proliferation rates and FEC formation, some meshed calli were cultured in GD medium containing 0.5 mg/L picloram and supplemented with 12 mg/L L-tyrosine. Variations in culture browning were evaluated visually two weeks after incubation at 26 ± 2 °C in the dark.

4.2.5 FEC germination and regenerant acclimatization

The FECs were desiccated in a hormone-free MS medium containing 1% activated charcoal for 45 days. The cultures were maintained at 25 ± 2°C and a 16h/ 8h photoperiod provided by a cool-white fluorescent lamp. Subsequently, the cultures were transferred to

MS containing 0.4 mg/l BAP and 10 mg/l ascorbic acid for embryo maturation. The cultured were sub-cultured onto the same medium every four weeks until the formation of cotyledonary embryos with distinct root and shoot. Germinated cotyledonary embryos were rooted in YBM medium then successively acclimatized in the screen house. Briefly, well-rooted plantlets were carefully pulled out from the culture vessels, and the roots were washed to remove all traces of medium. The plants were then planted in pellets (soaked overnight), kept in a plastic weaning cup, and covered with a transparent plastic bag. After 14 days, pores were progressively made in the plastic bag for a period of 7 days, during which the plastic bag was completely removed. The plants were maintained in the peat pellet for an additional seven days, then moved to a small bucket containing peat: soil mixture (1:1).

4.2.6 Statistical analysis

A completely randomized design was used for all treatments. All the experiments were repeated twice with three replicates for each treatment of 100 explants. Data were statistically analyzed using analysis of variance (ANOVA) and presented as the mean ± standard error (SE). The means were separated using Duncan's multiple range tests, and significance was determined at a 5 % level using the Minitab software version 17.0.

4.3 Results

4.3.1 Effect of amino acids and thiamine on embryogenesis and callus weight

Nodal explants (Figure 4.1A) cultured in SBM medium began to form axillary buds by the second day of culture. Two weeks after culturing the axillary bud explants in callus induction medium (CIM), direct somatic embryos began forming on the surface of the

explants. Some of the explants, however, formed non – embryogenic white hard structures or compact hard structures. It was evident that the addition of proline, thiamine, and casein hydrolysate was necessary for enhanced callus proliferation and high embryogenesis rates (Table 4.1). Medium containing casein hydrolysate, proline, and thiamine significantly increased the callus fresh weight (498.4 mg), percentage of embryogenic callus 76.2%), and the number of embryos per callus (19.3). On the contrary, the corresponding parameters in medium not augmented with these components were 221.3 mg fresh weight, 41.9% embryogenesis, and 4.5 embryos per callus. Thus, all medium used for callus induction in subsequent experiments were supplemented with these components.

Table 4.1: Effect of amino acids and thiamine on callus embryogenesis and proliferation

Amino acids (g/l) & Thiamine (mg/l)	**DSLS induction after 4 weeks of culture (%)**	**No. of DSLS/explant after 4 weeks of culture**	**Callus fresh weight (mg) /explant after 8 weeks of culture**
CH 0 + Pro 0 + Tmn 0	41.9 ± 4.6^{e}	4.5 ± 1.8^{e}	221.3 ± 24.6^{d}
CH 600 + Pro 0 + Tmn 0	52.4 ± 5.3^{d}	7.3 ± 3.1^{de}	314.8 ± 9.6^{c}
CH 0 + Pro 1000 + Tmn 0	53.1 ± 3.1^{d}	8.7 ± 2.5^{d}	307.6 ± 14.6^{c}
CH 600 + Pro 1000 + Tmn 0	64.2 ± 2.8^{c}	12.3 ± 3.2^{c}	368.7 ± 22.7^{b}
CH 600 + Pro 0 + Tmn 10	65.4 ± 5.7^{c}	14.8 ± 2.4^{bc}	385.6 ± 19.5^{b}
CH 0 + Pro 1000 + Tmn 10	68.6 ± 2.6^{bc}	15.2 ± 1.6^{b}	376.8 ± 28.8^{b}
CH 600 + Pro 1000 + Tmn 10	76.2 ± 3.5^{a}	19.3 ± 1.5^{a}	498.4 ± 17.3^{a}

Note: DSLS – direct somatic embryo-like structure; CH - casein hydrolysate; Pro - proline; Tmn - thiamine.

4.3.2 Effect of the basal salt mixture on primary embryogenesis

The composition of the basal salt mixture in embryo induction were compared in terms of explants producing somatic embryos and number of somatic embryos per explant (Table 4.2). When assessed four weeks after explant culture, both the percentage of explants producing somatic embryos (77.3%) and the number of somatic embryos per explant (19.2) were highest on FIM medium. The percentage of explants producing somatic embryos and the number of somatic embryos per explant in normal MS medium were 76.5% and 18.6,

respectively. In medium containing half-strength MS, 48.6% of the explants formed embryos, with an average of 4.7 embryos per explant. Callus proliferation also varied depending on the basal salt mixture used. Maximum proliferation was obtained when explants were cultured in either FIM medium (504.8 mg per callus) or normal MS medium (486.7 mg per callus), while half-strength had the least callus proliferation (236.9 mg). The DSLS numbers per callus and the percentages of callus forming DSLS did not differ significantly between FIM and normal MS medium. Following these results, normal MS medium was used for embryo induction in subsequent experiments because of the ease of medium preparation.

Table 2.2: Effect of basal salt composition on callus embryogenesis and proliferation

Basal salt composition	**DSLS induction after 4 weeks of culture (%)**	**No. of DSLS/explant after 4 weeks of culture**	**Callus fresh weight (mg) /explant after 8 week of culture**
FIM	77.3±1.6[a]	19.2±2.4[a]	504.8±24.6[a]
MS	76.5±2.4[a]	18.6±1.7[a]	486.7±13.5[a]
Half strength MS	48.6±3.9[b]	4.7±3.5[b]	236.9±35.4[b]

4.3.3 Effect of callus wounding on FEC formation

The three callus morphotypes were carefully distinguished at the end of the 8-week culture in CIM 1 (first round of culture in CIM media), and the non-embryogenic ones were discarded (Figure 4.1B). Crushing yam callus through the mesh (Figure 4.1C) effectively reduced the unit sizes to approximately 2 mm diameter. Callus washing and blotting on sterile soft tissues (Figure 4.1D) were found crucial to obtaining clean callus (Figure 4.1E), free of the slimy liquid produced during callus meshing. Within the first two weeks after culture in CIM 2 medium (second round of culture in CIM media), substantial tissue browning and necrosis (Figure 4.1F) were observed at the wounded callus edges.

Compared to unwashed callus, the washed and blotted callus had significantly reduced and tissue necrosis. As from the fourth week, small callus began forming, and the browning slowly cleared off (Figure 4.1G).

4.3.4 Effect of washing wounded callus on callus browning and tissue necrosis

Washing also reduced the recovery time (Table 4.3), which was assessed according to the time taken for the callus to begin proliferating. Compared to callus washed in distilled water (21.3-day recovery time and browning degree 4), liquid CIM significantly reduced both the browning degree (3) and the recovery time (16.7 days). A further reduction in recovery time was observed when liquid CIM washing medium was supplemented with ascorbic acid. Increasing the concentration of ascorbic acid in the washing medium also reduced the degree of tissue browning and recovery time, with an optimum at 10 mg/L (12.8 recovery days and browning scale 1). However, increasing the ascorbic acid concentration to 20 mg/L significantly increasing tissue browning (scale 2) and the recovery time (17.6 days). Addition of L-tyrosine (12 mg/l) to either the liquid washing medium or the solid culture medium did not demonstrate any effect on the browning level or the recovery time.

Table 4.3: Recovery time and browning level of meshed callus

Washing medium	Browning degree	Recovery time (Days)
Not washed	5	25.4 ± 2.3^{d}
Distilled water	4	21.3 ± 1.5^{c}
Liquid CIM	3	16.7 ± 1.8^{b}
Liquid CIM + 5 mg/l Ascorbic acid	1	13.5 ± 1.4^{a}
Liquid CIM + 10 mg/l Ascorbic acid	1	12.8 ± 2.1^{a}
Liquid CIM + 20 mg/l Ascorbic acid	2	17.6 ± 0.7^{b}
Liquid CIM + 12 mg/l L-tyrosine	3	16.3 ± 0.2^{b}

Note: The browning degree was assessed on a scale of 0 to 5, where 0 represented no browning while 5 was maximum browning.

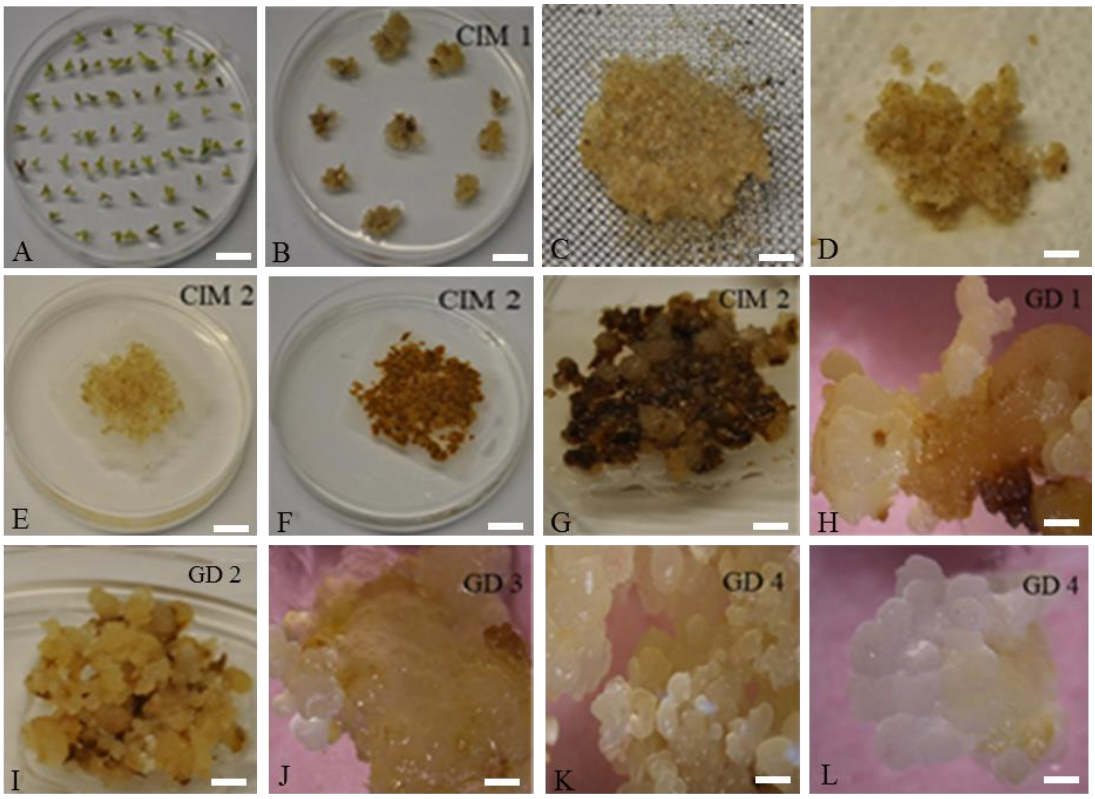

Figure 4.1: Development of friable embryogenic callus (FECs) in yam accession TDr 2436. A: Nodes cultured in Axillary bud induction medium. B: Yam callus derived from auxiliary bud culture in CIM 1. C: Meshing of callus through 1–2 mm size metal wire mesh. D: Blotting of meshed callus in soft tissues. E: Meshed callus cultured in CIM 2. F: Meshed callus in E after one-week culture in CIM 2, G: Meshed callus in F after eight weeks culture in CIM 2. H: Meshed callus cultured in GD 1. I: Meshed callus in GD 3, J: Callus in GD 3 beginning to form embryos. K: Callus in GD3 with embryo clusters. L: Embryo clusters in N cultured in GD 4. Scale bars; A, B, E and F 5 mm; C, D, G, H, I, J, K, and L 1 cm.

4.3.5 Effect of basal salt mixtures on FEC formation

Continued sub-culturing of meshed callus through a series of GD medium (Figure 4.1H and I) promoted callus proliferation. FEC formation was observed as from GD 3 (Figure 4.1J). Sub-culturing the FECs onto GD 4 promoted massive proliferation (Figure 4.1 K and L). Compared to meshed callus, callus wounded by removing the non – embryogenic culture in CIM 2, and successive sub-culture on GD did not form FECs. On the contrary, a lot of scarring and hardening was observed in the detached callus.

The various basal salt mixtures demonstrated significant differences in the ability to promote FEC formation. FIM mediu[a] had significantly higher ability (11.78%) to promote FEC formation than GD (4.1%). No FECs were formed from either half strength or full-strength MS (Figure 4.2).

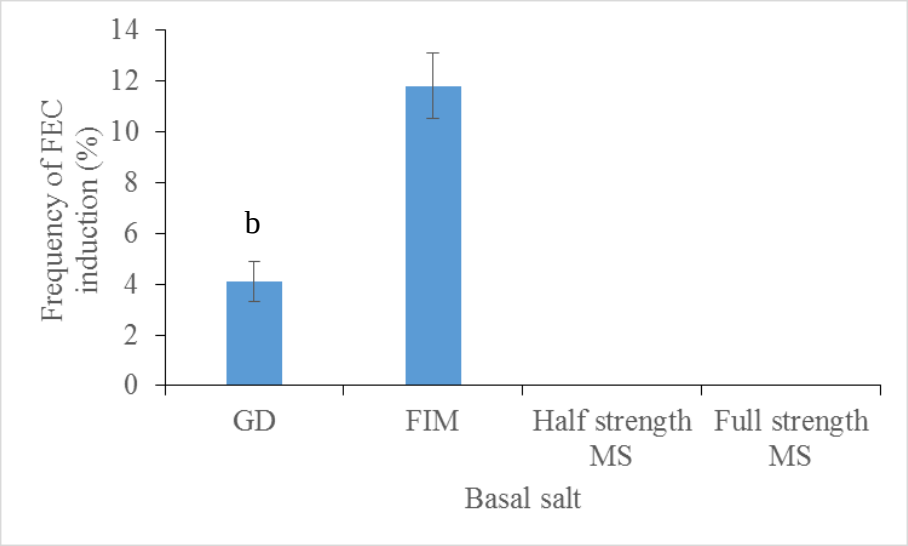

Figure 4.2: Effect of basal salt composition on FEC production. The frequency of FEC formation was estimated by expressing the number of Petri dishes initiated at Fig 1E that developed an FEC cluster by GD 4, as a percentage of the total number of cultures initiated. The friable appearance, as shown in Figure 4.1K, was counted as FEC. Values labeled with different letters are significantly different by Duncan's multiple range test at $P<0.05$.

4.3.6 FEC proliferation, maturation, and regeneration

FEC clusters sub-cultured on to GD 5 (Figure 4.3A) only proliferated for the first two weeks, after which they began to turn purple (Figure 4.3B), a sign of embryo maturation. After transferring the FECs to activated charcoal medium for 45 days, they all turned purple (Figure 4.3C). while on this medum, the embryos differentiated to form a distinct root apical meristem and shoot apical meristem (Figure 4.3D), after which the embryos turned green in maturation medium supplemented with BAP (Figure 4.3 E and F) to form either single or fused cotyledons (Figures 4.3G and H). The cotyledonary embryos successfully

rooted and elongated in YBM medium (Figure 4.3I). In soil, the regenerants exhibited normal growth, and no somaclonal variations were evidenced (Figure 4.3J).

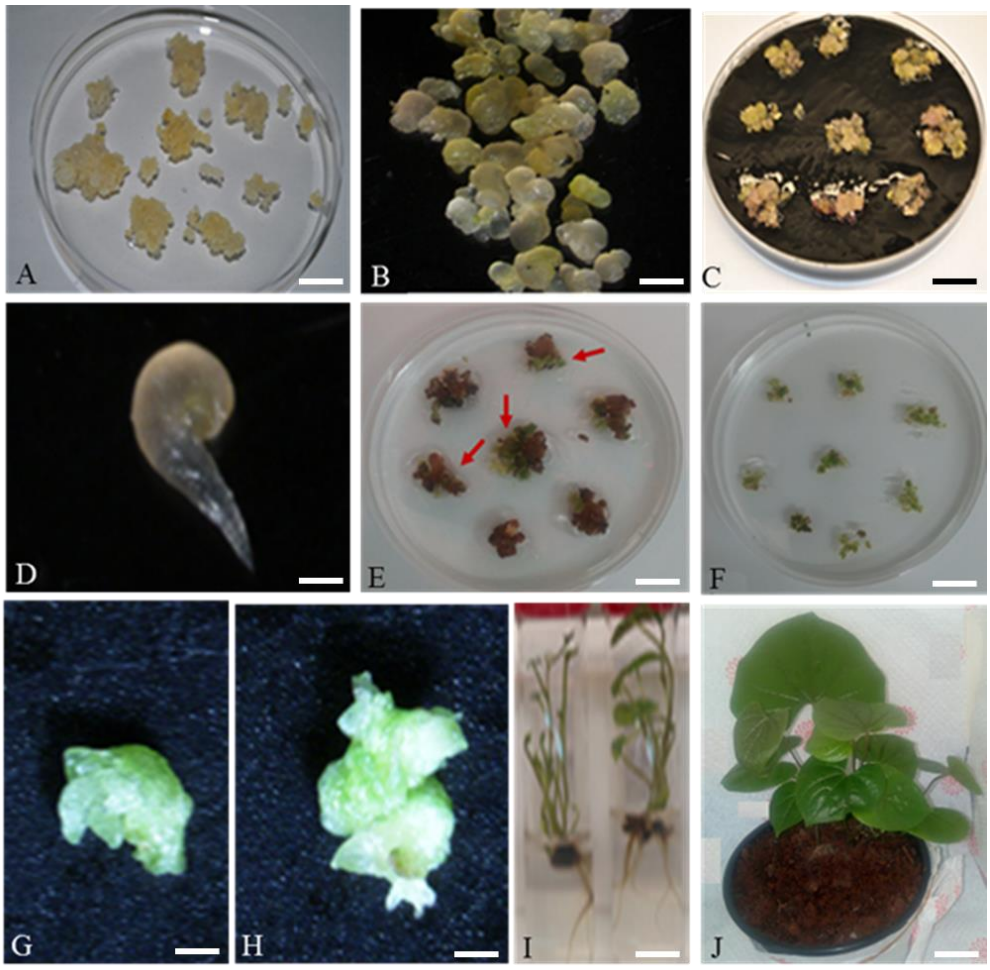

Figure 4.3: FEC proliferation, maturation, and regeneration. A: FECs proliferating in GD5 medium; B: Microscopic observation of FECs beginning to mature. C: FECs in activated charcoal medium. D: Microscopic observation of a single embryo with a distinct root apical meristem (RAM) and shoot apical meristem (SAM). E: FECs maturation in BAP medium, arrows show green cotyledonary embryos. F: Green cotyledonary embryos sub-cultured onto maturation medium 2. G: Single cotyledonary embryo. H: Fused cotyledonary embryo; I: Cotyledonary embryos well-rooted in YBM medium. J: Plantlets regenerated from FECs well established in the soil. Scale bars; A, B, C, E, F and J 5 mm; D, G, H 50 µm, I 1cm.

4.4 Discussion

This chapter reports, for the first time, the production of friable embryogenic callus (FECs) in the *Dioscorea* species. The FECs consisted of soft, friable, mucilaginous, and highly embryogenic structures that appeared on the surface of embryogenic callus cultures, typically from organized embryogenic callus. Notably, the FEC morphologies reported in this study are similar to that reported previously in cassava (Taylor *et al.,* 1996a; Nyaboga *et al.*, 2014). Compared to the compact, nodular, and organized embryogenic callus reported by (Manoharan *et al.* 2016), the callus reported in this study were smaller in size, more friable, mucilaginous in structure, and were capable of rapid proliferation in culture.

Similar to previous studies in sandalwood (Rugkhla & Jones, 1998), cassava (Syombua *et al.,* 2019; Utsumi *et al.,* 2017), switchgrass (Ogawa, 2015), and maize (Vasil & Vasil, 1986), this study found that medium supplementation with L-proline, casein hydrolysate, and L-thiamine is necessary for high embryogenic competence of yam callus. It is inadvertently accepted that somatic embryogenesis is a developmental switch in plant cell fate, typically induced under stressful conditions (Méndez-Hernández *et al.,* 2019). Exposure to various forms of biotic and abiotic stress inhibits plant cell growth and causes retardation. However, the application of exogenous amino acids scavenges reactive oxygen species, thus offering osmoprotection. Therefore, the exogenous application of amino acids such as proline enhances cell growth and development (Hayat *et al.,* 2012).

Several studies have reported that the addition of proline in the culture medium of immature maize embryos enhances somatic embryogenesis (Armstrong & Green, 1985; Claparols et al., 1993; Duncan & Widholm, 1987). Similarly, the application of exogenous proline on a groundnut culture medium alleviated oxidative damage to the cell lipid

membranes and reduced salinity-induced decline in fresh weight (Jain *et al.,* 2001). Thiamine is a cofactor involved in the catalysis of pyruvate to acetyl-COA for energy production (Dhillon *et al.,* 2011). According to Utsumi *et al.* (2017), the inclusion of additional thiamine in somatic embryogenesis cultures could facilitate energy metabolism from glycolysis to the TCA cycle, thus increasing the FEC formation rate. These reports concur with the findings of the present study. In a previous study by Nyaboga *et al.* (2015), the inclusion of the amino acid L-tyrosine in the culture medium was shown to promote FEC formation in cassava. However, this study did not find any beneficial effects of adding L-tyrosine on yam FEC production medium. These contrasting differences could be attributed to species-specific differences in tissue culture responses. This result could also be attributed to the fact that yam is rich in tyrosinase, a copper-containing enzyme ubiquitous enzyme that catalyzes the oxidation of tyrosine (Ilesanmi *et al.,* 2014; Ilesanmi & Adewale, 2020; Mulla *et al.,* 2018). The enzyme could, therefore, breakdown the amino acid, reducing its bioavailability and potential beneficial effects on the yam callus cells.

Callus formation and somatic embryogenesis involve cellular dedifferentiation and could be induced via tissue wounding to promote cell reprogramming and proliferation (Ikeuchi *et al.,* 2017). Previous studies have also reported that callus wounding can encourage FEC formation (Nyaboga *et al.,* 2015; Taylor *et al.,* 2012). In this study, detaching the embryogenic callus sections and culturing them through either GD or FIM resulted in callus hardening and browning, and no FECs were formed through this route. This response could be attributed to the induction of an oversensitive response in the cells, resulting in cell necrosis. It is possible that this form of wounding could not induce cellular reprogramming to initiate FEC formation. These contrasting findings suggest that it is crucial to optimize

various forms of tissue wounding to obtain the desired effects. Various studies also reported negative effects of tissue wounding on plant regeneration and transformation. In *Vitis vinifera,* Zhang *et al.* (2009) reported that tissue wounding could induce an oxidative outburst characterized by excessive production of reactive oxygen species. These events lead to necrosis, which manifests as tissue browning and subsequent cell death.

The present study results indicated that callus washing in CIM liquid and blotting on soft tissues is essential in reducing tissue browning and necrosis. Further reduction in tissue browning was observed when the washing medium was supplemented with ascorbic acid, a water-soluble vitamin commonly used in plant tissue culture medium as an antioxidant (Ko *et al.,* 2009). The *Dioscorea* species is rich in phenolics and secondary metabolites such as saponins, gracillin, diosgenin, dioscin, and catechins (Lebot *et al.,* 2018). The release of these metabolites to the tissue culture medium can darken the medium, inhibit the activity of plant growth regulators, and inhibit somatic embryogenesis and plant growth. Thus, several measures could be undertaken, such as supplementing the medium with activated charcoal, polyvinylpyrrolidone, cation exchange resins, or anion exchange resins to absorb the phenol-like substances (Ndakidemi *et al.,* 2014).

This study demonstrated that augmenting the liquid washing medium with ascorbic acid could effectively alleviate tissue browning after meshing, reduce the callus recovery time, and thus promote FEC formation. The beneficial effects of ascorbic acid in reducing the oxidative browning of tissues after cell injury have also been reported in the Faba bean (Abdelwahd *et al.,* 2008), the *Musa* species (Titov *et al.,* 2006), orchids (Chugh *et al.,* 2009), and Jatropha (He *et al.,* 2009). Besides its role as an antioxidant, ascorbic acid could enhance *in vitro* plant growth by promoting cell division and elongation (Titov *et al.,* 2006).

The use of FIM medium significantly increased the frequency of FEC formation compared to the GD medium. However, no FECs were formed on either half or full-strength MS medium. From this study, it can be inferred that the concentration of nitrogen, phosphorus, and potassium in the culture medium plays a crucial role in promoting somatic embryo formation in yam. Considering that both GD (Gresshoff & Doy, 1974) and FIM (Utsumi *et al.,* 2017) have significantly higher vitamin and amino acid contents than MS, the present findings suggest that FEC formation requires high concentrations of vitamins and amino acids. Similarly, the sources and levels of phosphorus and nitrogen in culture medium were shown to affect morphogenesis and somatic embryo formation in sorghum (Elkonin & Pakhomova, 2000). Therefore, manipulating the mineral composition of culture medium could hold the key to regenerating recalcitrant crops.

4.5 Conclusion

This is the first report of friable embryogenic callus (FEC) development and regeneration in the *Dioscorea* species. In this study, a protocol for *in vitro* regeneration of yam FECs was developed in *D. rotundata*. The study outlines the successive steps involved in the induction of primary organized embryogenic structures, induction of FEC formation, embryo maturation, germination, plantlet rooting, and acclimatization. Supplementing the primary embryogenesis medium with casein hydrolysate, L-proline, and thiamine significantly improved the embryogenic competence of the axillary bud explants. Callus meshing induced tissue reprogramming to initiate FEC development. Washing the meshed callus with liquid medium containing the antioxidant ascorbic acid substantially alleviated callus browning and tissue necrosis. Compared to the conventional GD medium, FEC induction, which has reduced quantities of nitrate, phosphate, and potassium, showed a

higher capability to trigger FEC formation. The plantlets regenerated from FECs showed no somaclonal variations when hardened in soil. The protocol optimized herein offers a system and material for yam protoplast isolation and genetic improvement by transgenesis or genome editing.

CHAPTER FIVE

Establish a protocol for protoplast isolation and culture in *D. rotundata*.

5.1 Introduction

Plant protoplasts are wall-less, fragile, but totipotent plant cells that can regenerate into various organs and even whole plants. They are generated via mechanical disruption or enzymatic treatments that leave the cell contents bound by an intact plasma membrane. Since plant protoplasts lack the typical polysaccharide wall, they provide versatile tools for the rapid introduction of foreign material into plant cells, including proteins, viral particles, DNA, RNA, chromosomes, and organelles (Pasternak *et al.,* 2020). Therefore, plant protoplasts have attracted immense interest as experimental units in various fields of plant biotechnology, including genome editing, functional gene characterization, protoplast transient gene expression, and genetic manipulation (Davey *et al.*, 2005). Gregory and Cocking (1965) first reported successful protoplast isolation from various plants and tissue types. Since then, numerous researches have reported on optimized protoplast isolation and regeneration systems in various crops, including rice (Jabnoune *et al.,* 2015), corn and wheat (H *et al.*, 2013), petunia (Kang *et al.,* 2020), cassava (Wen *et al.*, 2020), sweet potato (Guo *et al.*, 2006), tobacco and Arabidopsis (Shen *et al.,* 2014), apple (Malnoy *et al.*, 2016), grapevine (Bertini *et al.,* 2019), lettuce (Sasamoto & Ashihara, 2014), tomato (Horváth, 2009), banana (Wu *et al.,* 2020) and soybean (Wu & Hanzawa, 2018).

Despite the success achieved in CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated 9) mediated genome editing, most protocols are based on *Agrobacterium*-mediated transformation. The expression cassettes contain

selectable markers (herbicide and/ or resistance markers), transcription terminators, inducible or constitutive promoters (El-Mounadi *et al.*, 2020; Shimatani *et al.*, 2017). The vectors harboring the guide RNA and Cas9 protein are then transfected into a binary vector, mostly *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*. The positive colonies are further transformed into plants, and the first generation of transgenic plants are identified by antibiotic or herbicide selection (Veillet *et al.,* 2019). Reporter genes, such as the green fluorescent protein (GFP), are also incorporated into the cassette to distinguish mutant cells, tissues, or organs from the wild type (Doench *et al.,* 2014). Therefore, the mutated events are categorized as genetically modified (GM) organisms and thus subjected to the rigorous testing and evaluation systems of GM foods (Callaway, 2018; Eckerstorfer *et al.,* 2019). This fact suggests that transgene-free procedures for generating GMOs are desirable because they can circumvent regulatory obstacles (Globus & Qimron, 2018).

For sexually propagated crops, the transgene can be diluted out by Mendelian segregation, which eliminates the transgene in the third or subsequent generations, to generate transgene-free genome-edited events (Zhang *et al.,* 2019). These crops are considered conventional; the mutations resemble those generated by natural means or chemical mutagenesis, allowing evasion from the stringent biosafety regulations of transgenic plants (Yubing He & Zhao, 2020). Such back-crossing is, however, not feasible for vegetatively propagated crops, such as yam. Breeding in yam is constrained by long crop cycles, heterozygous genetic background, vegetative propagation, polyploidy, and poor flowering (Mignouna *et al.,* 2008). Therefore, novel approaches for production of transgene-free genome-edited yam are needed.

Several reports have indicated the feasibility of transfecting RNA-Cas9 ribonucleoprotein complexes (RNPs) into protoplasts to generate transgene-free rice, wheat, apple, rice, and corn. The protoplast-based genome editing could also significantly reduce the potential for off-target mutations relative to the *Agrobacterium*-based approach (Liang *et al.,* 2017; Svitashev *et al.,* 2016). However, the availability of a robust system for protoplast isolation, culture, and regeneration is a pre-requisite for RNP-based genome editing. Thus, the present study sought to develop a system for protoplast isolation, purification, culture, and regeneration in yam.

The success of protoplast-based plant regeneration systems relies on the consistent production of high yields of uniform and highly viable protoplasts. Thus, improvements in the various steps can be made to enhance the yield, viability, and regeneration competence, including the enzyme types, combinations, and concentration, enzyme digestion time, source tissue type and age, purification method, culture density and method, and phytohormone combinations (Davey *et al.*, 2005). By optimizing the above parameters, the present study developed a reproducible system for yam protoplast isolation, purification, and callus regeneration. However, whole-plant regeneration was not attained, which may need further optimization in the future. This optimized protocol provides a versatile tool for gene function analysis, macromolecule and gene transfection, cybridisation, and somatic hybridization for yam improvement.

5.2 Materials and methods

5.2.1 Plant Material and callus induction

The donor plants, *Dioscorea rotundata* accession TDr 2436, were obtained as *in vitro* plantlets from the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. The plants were multiplied and maintained on Yam basic medium as described by Nyaboga *et al*. (2014). For callus induction, nodal explants from 4-week old *in vitro* plantlets were cultured in shoot bud medium (Murashige and Skoog (MS) medium (Murashige & Skoog, 1962) containing 0.1 mg/L BAP, 0.2mM $CuSO_4$, 20 g/L sucrose, 3 g/L gelrite, and pH 5.7 for three days. Subsequently, the emerging axillary buds were excised under a dissecting microscope and cultured in callus induction medium (MS medium, 20 g/L sucrose, 0.2mM $CuSO_4$, 0.5 mg/L picloram, 600 mg/L casein hydrolysate, 1 g/L proline, 3 g/L gelrite, and pH 5.7).

5.2.2 Explant preparation and plasmolysis

For preparation of callus tissues, 2 mm long nodal explants were excised from 4-week-old *in-vitro* plantlets and cultured in shoot induction medium [SBM; MS salts and vitamins, 20 g/L sucrose, 1mg/L BAP, 0.2 µM $CuSO_4$, pH 5.7] for three days. The emerging auxiliary buds were then excised under a microscope and cultured in callus induction medium [(Murashige and Skoog salts and vitamins (Murashige & Skoog, 1962), 2 % sucrose, 0.5 mg/L copper II sulphate, 600mg/L casein hydrolysate, 1g/L proline, and 3 g/L gelrite]. To obtain mesophyll protoplasts, fully expanded leaves (1 g) were excised from yam *in vitro* plantlets of different ages (4, 6, 8, and 10 weeks of subculture) and finely chopped into 0.5-1 mm strips, using a sharp scalpel without tissue crushing at the cut site. The strips were then transferred to Petri dishes containing 10 ml of plasmolysis solution [0.8 M mannitol,

0.05 M $CaCl_2$, and 0.1% 2-(N-morpholino) ethanesulfonic acid (MES)] for 2 hours. Callus tissues (1 g) of ages 4, 6, 8, and 10 weeks after culture in CIM medium were also plasmolyzed for 2 hours.

5.2.3 Protoplast isolation and purification

Plasmolysed tissues were transferred to an enzyme digestion solution for cell-wall digestion. The enzyme solution contained different concentrations of the cellulase Onozuka R-10 (1%, 2%, and 4% in combination with either Macerozyme R-10 (0.2%, 0.4%, 0.8%, and 1.6%), or pectinase (0.2%, 0.4%, 0.8%, and 1.6%). All enzyme solutions were dissolved in plasmolysis solution, adjusted to 5.8 using KOH and HCl, then filter-sterilized using a 0.20 μm syringe filter (GVS, USA). To evaluate the effect of vacuum infiltration on protoplast yield and viability, half of each tissue type in the enzyme solution was vacuum infiltrated for 10, 20, or 30 mins. The cultures were dark-incubated at 25 ± 2 ^{o}C, in a gyratory shaker at 65 rpm, at different time intervals (4, 8, 12, 16, 20, and 24 hours). All enzyme solutions were sourced from Duchefa, The Netherlands.

Following incubation, protoplasts were purified through a series of filtration, washing, and centrifugation. The enzyme-protoplast-debris mixture was filtered through a 180 μm stainless steel mesh followed by an 80 μm mesh into a centrifuge tube then centrifuged at 900 rpm for 7 min. The supernatant was carefully discarded, the pellet re-suspended in plasmolysis solution, centrifuged again, and the pellet re-suspended in 7.5 ml of a 20% sucrose solution containing protoplast washing solution (PWS). This solution was then over-layered with 2.5 ml PWS containing 13% mannitol, pH 5.6, and centrifuged at 900 rpm for 5 min. Intact protoplasts were collected as an interface between the two layers and re-suspended in 2 ml of 9% Mannitol + PWS.

5.2.4 Protoplast Viability and yield

The protoplast yield was determined using a hemocytometer with a chamber depth of 0.2 mm, as described by (Widholm, 1972). The hemocytometer ruling consisted of 16 large squares of 1 mm each. Each large square was divided into 16 subsquares with a side of 0.25 mm and an area of 0.0625 mm. A 50 µl protoplast solution was stained with 1 µl of fluorescein diacetate solution (FDA, 5 mg/ml in acetone) and propidium iodide (PI, 1 mg/ml in PBS). Protoplasts were then observed under a fluorescence microscope (Leica DM2000 LED, Leica, Germany; excitation filter BP 450–490 nm, dichromatic mirror: 510 nm, and emission filter: LP 515 nm) equipped with a digital camera (Leica DFC420, Leica, Germany). Protoplasts exhibiting green color were regarded viable while red-stained were considered dead. The percentage viability was expressed as: Percentage viability = Number of viable protoplasts/the Total number of protoplasts (Viable and dead) × 100. The yield was expressed as the number of protoplasts per gram of fresh weight (gfw) and calculated by the formula: Yield = Average number of cells in one large square/ weight of leaves material used × 10^4. The protoplast viability and yield were determined based on three independent isolations with three replicates per treatment.

5.2.5 Protoplast culture

Effect of protoplast culture density on the viability of protoplast-derived micro-colonies

Protoplasts were adjusted to various densities, including $5x10^4$, $7.5x10^4$, $1x10^5$, $5x10^5$, $7.5x10^5$, and $1x10^6$, to investigate the effect of protoplast culture density on the viability of protoplast-derived microcolonies. Three culture medium and two culture methods were used to evaluate their effects on cell wall regeneration and protoplast survival rates. The

two culture methods included liquid and solid (agar) culture methods. The basal composition of all the three medium consisted of Kao and Michayluk (Kao & Michayluk, 1975) basal salt mixture, Gamborg B5 vitamins (Gamborg *et al.,* 1968), 0.7 M mannitol, 0.1% MES, and 125 g/L sucrose. Medium 1 (KM 1) consisted of the phytohormones 1 mg/L 1-naphthaleneacetic acid (NAA), 0.5 mg/L 2,4-D, and 1 mg/L kinetin, medium 2 (KM 2) consisted of 1.0 mg/L of 6-benzyl amino purine (BAP), 1.0 mg/L of NAA, while medium 3 (KM 3) consisted of picloram. The cultures were maintained in the dark for seven days and then transferred to 25˚C ± 2˚C with 16 h light regime of 30 µmol m^{-2} s^{-1} light radiation. Replacement medium (KP 1, KP 2, and KP 3) was prepared consisting of Kao and Michayluk basal salt mixture, Gamborg B5 vitamin, 0 mannitol, 0.1% MES, and 20 g/L sucrose. Progressive replacement of the culture medium with medium consisting of a mixture of KM and KP after 7 (3:1), 14 (2:1), 21 (1:1), and 28 (0:1) days. Cultures were inspected regularly under a fluorescent microscope to check for cell wall formation by calcofluor staining 7-8 hours after protoplast culture. Cell cultures were examined regularly under a microscope (Leica DM2000 LED, Leica, Germany) for cell division and microcolony formation. After the 28-days culture, microcalli with a diameter of 1–2 mm were transferred onto 5 cm Petri dishes containing solid medium KP.

5.2.6 Statistical analysis

Data were statistically analyzed using analysis of variance (ANOVA). Significance was determined using Duncan's multiple range test, least significant difference test, and t-test at $p < 0.05$.

5.3 Results

The steps for protoplast isolation, purification, and callus formation optimized in this study are outlined in Figure. 5.1. The protocol provides a fast and effective way for achieving up to $5.34*10^6$ protoplasts/ mL yield from 1gfw of yam tissues, with a 90% viability within 16 hours of enzymolysis. Incubating tissues on plasmolysis solution for one hour successfully plasmolyzed the cells, as demonstrated by separation of the protoplast from the cell and a shriveled appearance. The 16-hour digestion in the enzyme solution generated a mixture consisting of viable protoplasts, dead cells/ protoplasts, the enzyme mixture, and broken cells. Following purification, the mesophyll and callus protoplasts were collected as a green (Figure 5.2A) or brown band (Figure 5.2B) at the interface of sucrose and mannitol layers. Three protoplast morphologies were obtained; dense with chloroplasts distributed throughout the cytoplasm (Figure 5.2C), vacuolated with chloroplasts concentrated on one side of the cell (Figure 5.2D), and small protoplast without chloroplasts (Figure 5.2E).

Generally, the protoplasts were spherical (Figure 5.2F), with a diameter between 10 and 70 μm. When Evans blue stain was added to the protoplast solution, dead protoplasts stained blue, while viable protoplasts did not take up the stain (Figure 5.2G). On FDA, viable protoplasts fluoresced green under ultraviolet light (Figure 5.2H). Cell wall regeneration began within 7-8 hours after protoplast culture, as demonstrated by blue fluorescence (Figure 5.2I) of calcofluor white-stained cultures. Cell division occurred within three days after protoplast culture, as was evident under a light microscope (Figure 5.2J) and following FDA staining (Figure 5.2K). Almost all protoplast-derived cells that underwent first divisions developed into micro-colonies with a 2–5 mm, approximately 8–

12 weeks after culture. Microcolonies in the three, four, five, seven, ten, and sixteen cell stages were evident (Figures 5.2L-O). When transferred to solid medium, the microcolonies underwent additional cell division to form micro callus (Figure 5.2P) and big callus (Figure 5.2Q). Further callus proliferation was observed on medium supplemented with 1% activated charcoal (Figure 5.2R). The calli were transferred to regeneration medium supplemented with BAP (Figure 5.2S), but no plant regeneration was achieved.

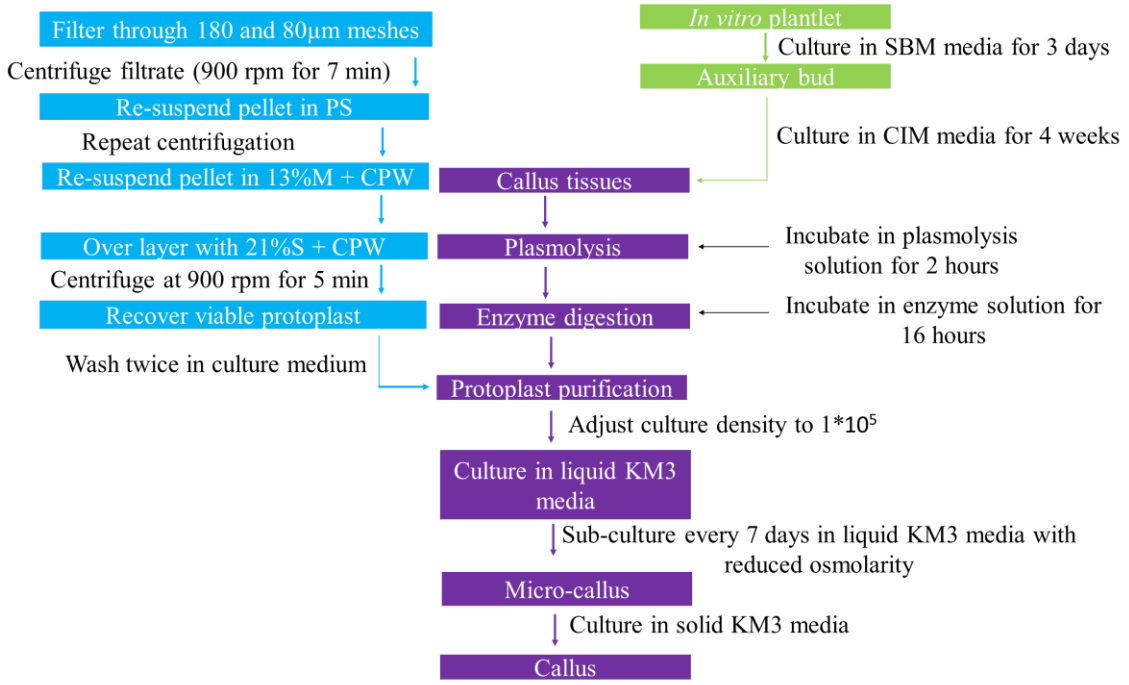

Figure 5.1: Flow chart illustrating the optimized steps for protoplast isolation in *D. rotundata*.

Note: The protocol marked in green (left) illustrates the protocol for preparing callus explants for protoplast isolation, the blue-marked steps (right) provide the procedure for protoplast purification while the purle-marked (centre) boxes illustrates the summarized protocol for protoplast isolation, purification and culture.

5.3.1 Effect of source explant on protoplast yield, viability and cell division

The results demonstrated that explant-dependent variations inthe protoplast yield, viability and callus formation (Table 5.1). Generally, callus tissues had a lower yield ($4x10^5$) and

viability (85%) than mesophyll tissues ($4.85x10^6$ yield and a 90% viability). However, the source explant had no effect on cell wall regeneration since protoplasts from both tissue types generated cell walls within 7 to 8 hours. However, the source tissue significantly affected the first division time, with the callus showing a faster response than mesophyll (three days in callus and four days in mesophyll). Besides, the division frequency of callus tissues (17%) was significantly higher than mesophyll (6%). The source tissue also affected colony formation; callus tissues had a significantly higher colony formation rate (0.3%) than mesophyll tissues (0.05%).

Table 5.1: Effect of explant type on the protoplast yield and viability, cell wall formation, cell division, and microcolony formation

Source tissue	**Y – 0 D**	**V – 0 D**	**V – 14 D**	**CWR (Hrs)**	**FD (Days)**	**DF – 4 D**	**CF (%) – 4 wks**
Callus	0.4±0.05	85±6	48±3.4	7 – 8	3	17±3.74	0.3±0.1
Mesophyll	4.85±0.58	90±6.3	46±3.2	7 – 8	4	6±1.32	0.05±0.03

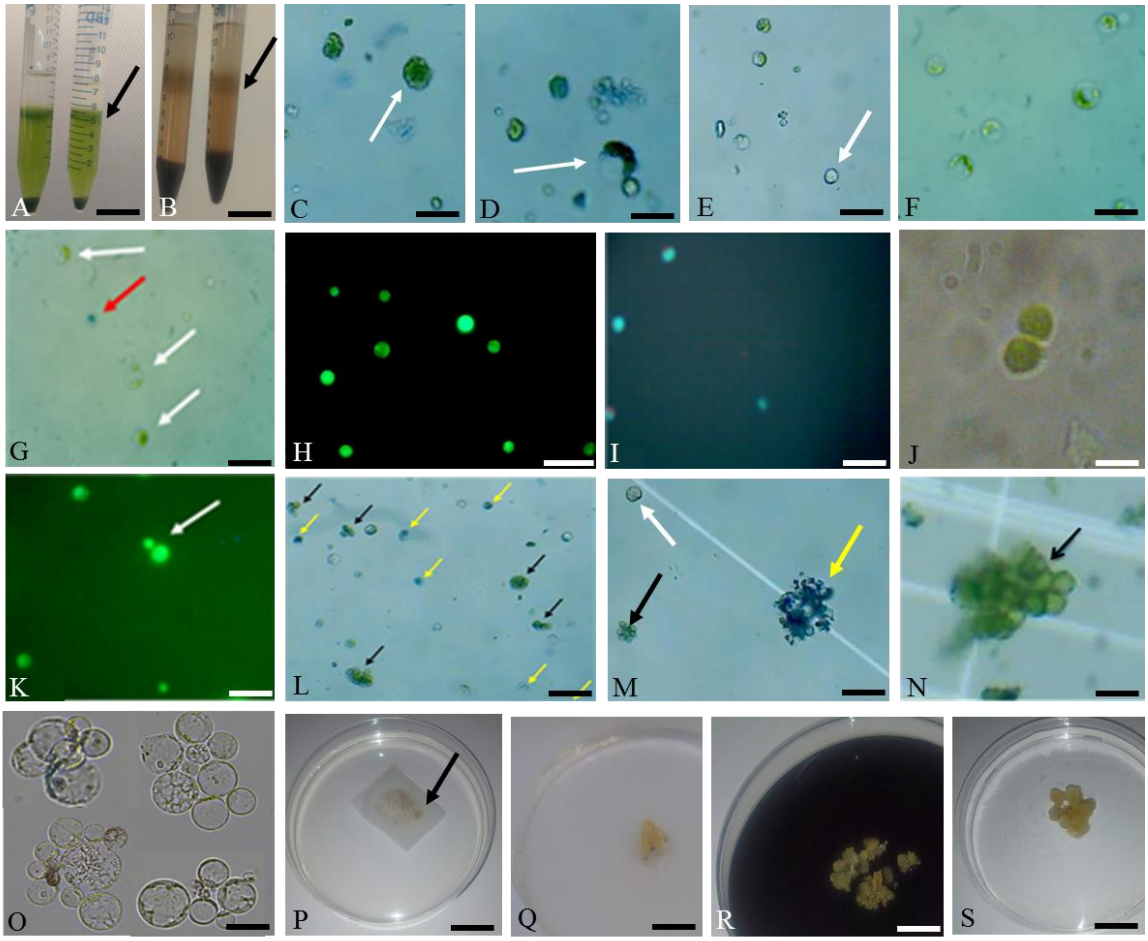

Figure 5.2: Protoplast isolation and culture in *D. rotundata.*

A: Purified mesophyll protoplasts forming a band at the interface of sucrose and mannitol layers. B: Purified callus protoplasts forming a band at the interface of sucrose and mannitol layers. C: Dense protoplast with chloroplasts distributed throughout the cytoplasm. D: Vacuolated protoplast with chloroplasts concentrated on one side of the cell, E: Small protoplast without chloroplasts. F: Appearance of yam protoplasts under a light microscope before staining. G: Appearance of yam protoplasts under a light microscope, after staining with Evans blue (white arrow indicates viable protoplasts, the red arrow indicates non –viable protoplast). H: Fluorescence microscopy of yam protoplasts stained with FDA (fluorescing green). I: Microscopic observation of cell wall regeneration 8 hours after culture (Staining done with calcofluor white). J: 3 days old protoplasts undergoing division. K: Protoplasts staining with FDA 3 days after culture (arrow indicates protoplasts undergoing division). L: 14 days old protoplast culture stained with Evans blue to assess the viability of microcolonies (Black arrow indicates viable microcolony, yellow arrows indicate non –viable protoplasts/ microcolonies). M: 28-day-old cultures showing dead microcolony (yellow arrow), viable microcolony (black arrow), and a protoplast that has not undergone cell division (white arrow). N: An enlarged view of a 28-day old microcolony from mesophyll protoplasts. O: 28-year old microcolonies from callus protoplasts, P: Microcallus cultured in solid medium (arrow shows a developing callus). Q: Callus cultured in solid medium to promote callus proliferation, R: Proliferated callus cultured in activated charcoal medium for plant regeneration. S: Callus cultured in BAP medium for plant regeneration; scale bars; A, B 1 cm; C-N 50 µm, O 100 µm, P-S 2 mm.

5.3.2 Effect of enzyme (cellulose and macerozyme) incubation periods on the protoplast yield and viability

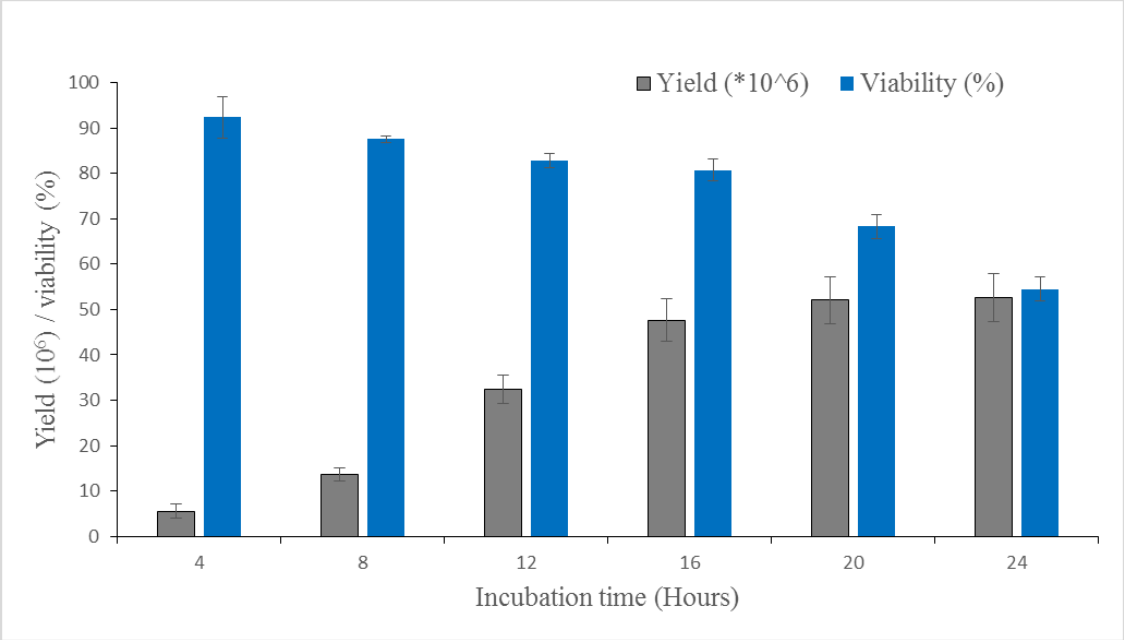

Figure 5.3: Effect of different enzyme (cellulose and macerozyme) incubation periods on protoplast yields and viability.
Note: Y – the number of protoplasts obtained from each gram of callus or mesophyll tissues, as evaluated under a microscope using a hemacytometer; V – the number of living protoplasts expressed as a percentage of the total number of protoplasts.

The results indicated that a shorter enzyme incubation period on the yam protoplast resulted in higher viability but a lower yield. After 4 hours of incubation, the protoplast yield was $5.5x10^5$, and the viability was 92.4 percent. Conversely, a prolonged enzyme incubation period increased the yield of yam protoplasts while reducing the viability. This was demonstrated by the high yield of yam protoplast ($5.27x10^6$) at the 24^{th} hour and a 54.5% variability during the same time. This result generally shows an increasing trend in yield with an increased incubation period but a corresponding decrease in viability. Thus, 16 hours was chosen as the optimal incubation period, as it yielded relatively high yield and substantial viability shown in the twelfth hour of incubation (yield $47.76x10^6$, viability 80.7%).

5.3.3 Effect of cellulase, macerozyme and pectinase concentrations on protoplast yield and viability

The results show that enzyme concentration had significant effects on the yield and variability of protoplasts from both leaf and callus tissues. The lowest yields were obtained at the lowest enzyme concentration (1C+0.2M), which were $2.1x10^6$ for leaf explants and $1.8x10^5$ for callus tissues (Table 5.2). At this low enzyme concentration, the viability was high, which were 82% and 90% for leaf and callus tissues, respectively. Increasing the enzyme concentrations resulted in a corresponding yield increase and reduced viability. In the combination with macerozyme, the highest yield ($5.01x10^6$ for the leaf and $5.6x10^5$ for the callus) was obtained at the highest cellulose and macerozyme concentrations (4C + 1.6M). However, the corresponding viabilities were low, 73% for the leaf and 77% for the callus tissues.

Generally, pectinase generated a better yield than macerozyme in all the tested concentrations. However, the corresponding viabilities did not differ significantly between macerozyme experiments and pectinase. For pectinase, the highest yield was obtained at the maximum enzyme concentration tested (4C+1.6P), which were $5.34x10^6$ for the leaf and $6.1x10^5$ for the callus. Increasing the enzyme concentration from 1C + 0.2P to 4C + 0.8P reduced the viability from 78% to 63% in mesophyll tissues and from 83% to 67% in callus tissues. An enzyme concentration of 2C+0.8M was chosen as the optimal because it generated relatively high yields ($3.43x10^6$ in mesophyll tissues and $4.7x10^5$ in callus) and viability (77% in mesophyll tissues and 81% in callus).

Table 5.2: Effect of enzyme types and concentrations on the protoplast yield and viability

	Leaf		Callus	
	Y (*10^6)	V (%)	Y (*10^6)	V (%)
1C + 0.2M	2.1±0.44^{d}	81.97±4.1^{ab}	0.18±0.04^{d}	90±6.26^{a}
1C + 0.4M	3.24±0.32^{cd}	76±3.82^{abc}	0.42±0.06^{abc}	82±5.74^{abc}
2C + 0.4M	2.43±0.34^{d}	83±4.14^{a}	0.23±0.04^{cd}	88.03±6.16^{ab}
2C + 0.8M	3.43±0.35^{b}	76.97±3.86^{abc}	0.47±0.07^{ab}	81±5.67^{abc}
4C + 0.8M	4.62±0.46^{ab}	79±3.97^{ab}	0.45±0.07^{ab}	83±5.83^{abc}
4C + 1.6M	5.01±0.5^{a}	73±3.67^{abcd}	0.56±0.09^{ab}	77±5.41^{abc}
1C + 0.2P	4.08±0.41^{abc}	77.97±3.9^{abc}	0.51±0.08^{ab}	83±5.79^{abc}
1C + 0.4P	5.12±0.51^{a}	65±3.25^{d}	0.58±0.09^{a}	70.03±4.9^{c}
2C + 0.4P	4.88±0.49^{a}	72±3.58^{bcd}	0.48±0.07^{ab}	78±5.44^{abc}
2C + 0.8P	5.21±0.52^{a}	65±3.22^{d}	0.62±0.09^{a}	68±4.76^{c}
4C + 0.8P	4.92±0.49^{a}	68±3.4^{cd}	0.36±0.05^{bcd}	72±5.03^{bc}
4C + 1.6P	5.34±0.54^{a}	63.03±3.15^{d}	0.61±0.09^{a}	67.03±4.71^{c}
Average	4.19	73.41	0.45	78.26

Note: Y – the number of protoplasts obtained from each gram of callus or mesophyll tissues, as evaluated under a microscope using a hemacytometer; V – the number of living protoplasts expressed as a percentage of the total number of protoplasts. C – cellulose; M – macerozyme; P - pectolyase. The numeric values 1, 2, 4, 0.2, 0.4, 0.8 0r 1.6 before the letters C, M or P represent the concentrations of enzymes used in each treatment.

5.3.4 Effect of explant age on protoplast yield and viability

Young explants of both tissue types generally resulted in higher protoplast yield and viability. At four weeks, mesophyll tissues had a yield of $5.25x10^{6}$ and 80% viability, which reduced to $6.6x10^{5}$ protoplasts per mL and 73% viability by the 10^{th} week (Table 5.3). Meanwhile, callus tissues had a yield of $4.7x10^{5}$ and 86% viability in the fourth week, which reduced to $2x10^{5}$ protoplasts per mL and 76% viability by the 10^{th} week. Six-week-old explants were chosen as the optimal explant age for protoplast isolation, as they resulted in high yields ($4.43x10^{6}$ in mesophyll tissues and $4.8x10^{5}$ in the callus tissues), with moderate viability (81% and 83% in the callus tissues) (Table 5.3).

Table 5.3: Effect of explant age on the first division time, division frequency, and colony formation

	Leaf	Callus

Explant age (Weeks)	Yield (*10^6)	Viability (%)	Yield (*10^6)	Viability (%)
4	5.25±0.53[a]	80±4.03[a]	0.47±0.07[a]	85.97±6.01[a]
6	4.43±0.44[a]	81±4.07[a]	0.48±0.08[a]	83±5.8[a]
8	2.78±0.28[b]	76±3.8[a]	0.13±0.02[b]	76±5.33[a]
10	0.66±0.07[c]	73±3.63[a]	0.02±0.01[b]	76±5.3[a]

Note: Y – the number of protoplasts obtained from each gram of callus or mesophyll tissues, as evaluated under a microscope using a hemacytometer; V – the number of living protoplasts expressed as a percentage of the total number of protoplasts.

5.3.5 Effect of vacuum infiltration on protoplast yield and viability

A 10 min vacuum infiltration significantly enhanced protoplast yield and viability. When vacuum infiltration was done for 10 min, the protoplast yield was relatively higher ($5.23x10^6$) compared to $4.61x10^6$ when callus tissues were not infiltrated (Table 5.4). Similarly, the viability of vacuum infiltrated tissues was higher (82.1%) than without infiltration (81.7%). However, increasing the vacuum infiltration period beyond 10 min significantly reduced the protoplast yield and viability.

Table 5.4: Effect of vacuum infiltration on protoplast yield and viability

Infiltration period (min)	Y (*10^6)	V (%)
0	4.61±1.48[b]	81.7±4.32[a]
10	5.23±0.46[a]	82.1±5.7a
20	3.76±1.82[c]	73.4±3.8[b]
30	0.85±1.67[d]	67.8±4.4[c]

Note: Y – the number of protoplasts obtained from each gram of callus or mesophyll tissues, as evaluated under a microscope using a hemacytometer; V – the number of living protoplasts expressed as a percentage of the total number of protoplasts.

5.3.6 Effect of culture density on cell division and colony formation

Protoplast culture density influenced cell division and colony formation, with the optimal densities being $1x10^5$ and $5x10^5$. At these two concentrations, 30% of the cultured protoplasts underwent cell division, compared to 10 attained at $5*10^4$ and $1*10^6$. Meanwhile, both $7.5*10^4$ and $7.5*10^5$ resulted in a 20% cell division (Table 5.5). A $1*10^5$ plating density resulted in 15% colony formation, while $7.5*10^4$ and $5*10^5$ gave 10% colony formation. No colonies were formed at either $5*10^4$ or $1*10^6$.

Table 5.5: Effect of culture density on the cell division and colony formation rates

Protoplast culture density	Cell division (%)	Colony formation (%)
$5*10^4$	10	-
$7.5*10^4$	20	10
$1*10^5$	30	15
$5*10^5$	30	10
$7.5*10^5$	20	-
$1*10^6$	10	-

Note: The cell division frequency denotes the number of protoplasts that had undergone cell division by the fourth day after culture; the colony formation was evaluated by expressing the number of colonies observed by the fourth week after culture as a percentage of the total number of protoplasts cultured.

5.3.7 Effect of culture method on protoplast isolation and regeneration

The culture method had substantial effects on the various steps of protoplast regeneration, including first division time, division frequency, and colony formation rate (Table 5.6). In the liquid, the first cell division occurred three days post-culture, while the solid (agar) culture never showed any cell division even at ten days post-culture. Similarly, no protoplast division was observed in the solid (agar) media culture method by the fourth day (0%), compared to 6 % in the liquid culture. Evaluation of colony formation four weeks post-culture showed no colony in the solid (agar) culture (0%), relative to 0.4% colony formation in the liquid culture technique.

Table 5.6: Effect of culture method on the protoplast first division time, division frequency, and colony formation

Culture method	First division (Days)	Division frequency (%) – 4 days	Colony formation (%) – 4 weeks
Liquid	3	6	0.4
Solid (Agar)	0	0	0

Note: First division refers to the time post-cuture initiation when division occurred; The cell division frequency denotes the number of protoplasts that had undergone cell division by the fourth day after culture; the colony formation was evaluated by expressing the number of colonies observed by the fourth week after culture as a percentage of the total number of protoplasts cultured.

5.3.8 Effects of plant growth regulators (PGRs) on shoot regeneration

Cell wall regeneration, cell division, and microcolony formation were attained in the three tested phytohormone combinations, albeit at different efficiencies. However, callus regeneration was only attained in KM2, which contained 1 mg/L 1-naphthaleneacetic acid (NAA), 0.5 mg/L 2,4-D, and 1 mg/L kinetin.

5.4 Discussion

Protoplast systems are indispensable tools in modern plant biology, with immense applications in gene function analysis, subcellular protein localization, and the study of protein-protein interactions. Most importantly, protoplast-based systems have been efficiently used for crop improvement by somatic hybridization, cybridization (Ephrussi, 2015), and genome editing (Woo *et al.*, 2015). Somatic hybridization by protoplast fusion overcomes pre- and post-zygotic barriers among incompatible species, enabling the production of crops with novel genetic combinations from intraspecific, interspecific, or even intergeneric plants (Kumari *et al.*, 2020). Conventional genome editing by *Agrobacterium* mediated transformation results in mutants with foreign genetic elements within their genome, thus regarded as GM and subjected to the rigorous biosafety regulations of transgenic crops (Friedrichs *et al.*, 2019). On the contrary, genome editing by RNP transfection onto protoplasts does not result in foreign gene integration; the resultant crops are thus considered conventional and may not be subjected to the regulations typical of GM (Schmidt *et al.*, 2020).

The practical application of genome editing and somatic hybridization for crop improvement requires the establishment of reproducible protoplast-to-plant systems. At present, reliable systems for the isolation of these osmotically fragile cells are available in

model plants and most crops of agronomic importance, including both monocots and dicots (H *et al.*, 2013; Kang e*t al.*, 2020; Guo *et al.*, 2006; Shen *et al.*, 2014; Malnoy *et al.*, 2016; Bertini *et al.*, 2019; Wu *et al.*, 2020; Wu and Hanzawa, 2018). However, successful preparation of abundant viable protoplasts and subsequent plant regeneration is an underexplored area in the *Dioscorea* species. This chapter reports the efficient isolation and callus regeneration from yam mesophyll and callus protoplasts. The study achieved yields of up to $5.25x10^6$ protoplasts/ml of fgw and 90% viability, which exceeds that previously reported by Tor *et al.* (1993).

Core to regenerating whole plants from protoplasts is the ability to isolate large quantities of viable protoplasts. However, various parameters, including the tissue type and age, enzyme type, concentrations, and combinations, environmental factors, and culture medium, affect the totipotency and plasticity of protoplasts and protoplast-derived cells (Davey *et al.*, 2005). It is, therefore, necessary to determine the optimal conditions that facilitate the generation of large populations of uniform protoplasts with high viability, including environmental and plant intrinsic factors (Sinha *et al.*, 2003). Previous studies have reported the necessity of tissue pre-plasmolysis prior to enzyme digestion (H *et al.*, 2013 Guo *et al.*, 2006; Malnoy *et al.*, 2016). In the present study, 2-hour culture of source tissues in a plasmolysis solution containing 13% mannitol and 7mM $CaCl_2$ was effective at plasmolyzing the cells, as demonstrated by loss of the intimate contact between the plasma membrane and the cell wall. Generally, protoplasts burst in hypotonic solution and collapse in a hypertonic solution. Thus, tissue plasmolysis in either salts and/or sugar alcohol solutions, such as mannitol or sorbitol, is necessary to reduce cytoplasmic damage and spontaneous protoplast fusion (Chamani *et al.*, 2012; Maćkowska *et al.*, 2014).

Primary plant cells are bound by the cell membrane and cellulosic walls with a pectin-rich matrix, which forms the middle lamella joining adjacent cells. Other components of the primary cell wall are the polysaccharides cellulose and hemicelluloses. However, in hypertonic solutions, the cell undergoes plasmolysis, during which the plasma membranes contract from their walls (Alberts *et al.,* 2002). Subsequent removal of the latter structures releases large populations of spherical, osmotically fragile protoplasts, where the plasma membrane is the only barrier between the cytoplasm and its immediate external environment. The easy availability of commercial, purified enzymes such as cellulase, cellulysin, pectinase, macerozyme, driselase, rhozyme, and hemicellulase has now increased the yield and viability of protoplasts and their subsequent response in the culture medium. Commonly, a combination of pectinase and cellulase is used to digest the cell walls and also liberate protoplasts in a single cell (Cocking, 2000).

The concentration and combination of digestion enzymes depend on the donor tissue age, genotype, and stage of differentiation. In the present study, all tested concentrations of pectolyase Y23 generated significantly higher protoplast yields than the corresponding concentrations of macerozyme. However, tissues digested by the two enzymes had comparable viabilities. The findings reported herein concur with Pongchawee *et al.* (2006), who reported that pectolyase Y-23 has higher protoplast isolation efficacy than macerozyme R-10 in releasing *Anubias nana* protoplasts. The pectic enzymes pectolyase Y-23 have very high pectin lyase and polygalacturonase activity, resulting in high maceration efficiencies. Notably, pectolyase Y-23 has 50X higher endo-polygalacturonase activity than macerozyme (Nagata & Ishii, 2011). The enzyme pectolyase Y-23 has successfully been used to isolate protoplasts of various species, including soybean (Wu &

Hanzawa, 2018), magnolia (Huo et al., 2017), *Cannabis sativa* (Beard *et al.*, 2021), *Stevia rebaudiana* (Lopez-Arellano et al., 2015), sugarcane (Wu *et al.*, 2021), coriander (Ali e*t al.*, 2018), banana (Khalid & Tan, 2016), and oil palm (Masani *et al.*, 2013), among other species.

Similar to the current findings, Babaoğlu (2000) reported that using isolation enzymes in combination, such as both cellulose R10 and pectolyase, increases the protoplast yield than when a single enzyme is used. In the present study, protoplasts generally had higher viability at low enzyme concentration, which could be explained by the fact that protoplasts produced remain spherical in shape, and the chloroplasts in the cells are left visible. At higher concentrations, the protoplast is released in good shape and in a well-separated form. However, the protoplasts tend to aggregate, which may rupture these fragile wall-less structures (Nanjareddy *et al.*, 2016).

The present study demonstrated that the period within which the plant tissues are exposed to the digesting enzymes significantly affects the protoplast yield and viability. Generally, shorter enzyme incubation periods resulted in higher viability but lower yields of yam protoplasts. These findings are consistent with those reported in petunia, where low enzyme concentrations generated low yield but higher viabilities. Similarly, increasing the enzyme concentrations increased the petunia protoplast yields but reduced the viability (Meyer *et al.*, 2009). In this study, the protoplast yield decreased with increase in incubation time. An initial increase in the tissue incubation time resulted in higher yields, up to a maximum at 16 hours. However, any further increase significantly reduced the protoplast yields and viability. According to Nanjareddy *et al.* (2016), prolonging the incubation period induces water stress on the cells, thus inducing protoplast rupture and

subsequently reducing yields. Park *et al.* (2016) also reported no benefits from extending the incubation period beyond the optimum and that increased incubation time compromises the protoplast viability.

Though protoplasts can be isolated from a variety of tissues, young *in vitro*-grown plants (He et al., 2016), tissues and explants such as root tips (Akashi *et al.,* 2000), hypocotyl (Sakamoto *et al.,* 2020), cotyledons (Huang *et al.,* 2013) and shoots (Lindberg *et al.,* 2007) and leaves from old or mature plants (Duquenne *et al.*, 2007; Grzebelus *et al.*, 2012; Lindberg *et al.*, 2007) generally form the tissues of choice. In the present study, leaf-sourced protoplasts had higher yields and viabilities than callus protoplasts. The two tissue types also demonstrated differences in the capacity to undergo cell division, with callus-sourced protoplasts giving higher cell division rates and higher ability to form microcolonies. Younger tissues generally favored higher yields and viabilities. These results are similar to Babaoğlu (2000) and Wiszniewska and Pindel (2013), who reported that the explant developmental stage influences plant regeneration. This result could be explained by the fact that younger cells generally have thinner cell walls and less lignification than older cells. Therefore, the thin cell walls are easily removed by the digestion enzymes (Pongchawee *et al.,* 2006). Younger cells are also more preferred because they have better viability and regeneration capacity (Sinha *et al.,* 2003).

A 10 min vacuum infiltration slightly increased the protoplast yield. However, any increase beyond 10 min reduced the protoplast yields. A short-term vacuum infiltration allows the enzymes to penetrate through the cellular spaces, facilitating faster and more efficient digestion of the cellulose, hemicellulose, and other cellular components (Nanjareddy *et al.,*

2016). However, a prolonged infiltration ruptures the protoplasts that had been released into the enzyme medium during the initial infiltration. Park *et al.* (2016) reported similar findings in Arabidopsis; short-term vacuum infiltration (up to 150 min) increased the yield, beyond which the protoplast yields reduced significantly. However, these findings contradict that of Sinha *et al.* (2003), who demonstrated that vacuum infiltration reduces protoplast yields in White Lupin.

The density at which protoplasts are plated during culture is critical since protoplasts must be above a minimum inoculum density to sustain new wall synthesis, mitotic division, and cell colony formation. Generally, the optimum plating density is $5 \times 10^2 - 1.0 \times 10^6$ protoplasts per mL, depending on the species/ cultivar (Davey *et al.,* 2005). This study demonstrated that the plating density is a crucial part of the yam protoplast culture protocol, with higher culture densities promoting better cell division and colony formation rates. These findings are similar to Kang *et al.* (2020), who reported higher plating efficiencies at higher culture densities.

Following cell wall regeneration and cell division, daughter cells develop into tissues, from which plants may be regenerated by somatic embryogenesis and/or organogenesis (Shen *et al.,* 2014). Several procedures have been described to culture isolated protoplasts, with incubation in liquid medium being the most simple to establish. Protoplasts can also be cultured in medium overlaying supports of nylon mesh and filter papers, semi-solid medium, or in hanging droplets (Eeckhaut *et al.,* 2013). The present study demonstrated that the choice of the protoplast culture method is important for the success of yam cell division, colony formation, and callus regeneration. The liquid culture method was more efficient at promoting protoplast regeneration (expressed first division within the first few

days) and percentage colony formation. Meanwhile, no division nor colony formation was observed in solid cultures. These results are similar to Clarke and Daniell (2011), who reported that liquid culture facilitates cell division and regeneration of protoplasts under osmotic protection. According to Clarke and Daniell (2011), isolated protoplasts grown in liquid culture commence cell wall regeneration within minutes after culture. The high ability to form microcolonies in liquid culture can be attributed to the ease of regulating the osmotic pressure; when the liquid osmotic pressure rises, it can be lowered by diluting the culture medium using a similar medium of lower osmotic potential. The change subsequently sustains mitotic division, facilitating callus regeneration. On the contrary, the osmotic potential of solid medium is not easily manipulated (Giles, 2013). The liquid medium also allows other subsequent procedures, such as changing the bath medium to promote protoplast growth. This removes other downstream processes like dissecting the medium layer into sectors or bathing the sectors to allow protoplast growth in the solid medium (Giles, 2013).

The differentiation or induction medium composition also influenced the protoplast regenerative capacity. Based on our results, it can be concluded that Kinetin is more efficient in inducing cell division and colony formation in yam protoplasts than medium supplemented with either NAA or Picloram. After one month on the induction medium, further transfer to regeneration medium F and MS (without growth regulators) showed no profound effect on regeneration frequency. Callus regeneration was also affected by medium phytohormone composition. Similarly, Kinetin was superior to NAA and Picloram in inducing callus regeneration. Lopez-Arellano *et al.* (2015) reported similar findings in

stevia, where a phytohormone combination of 2,4-D, NAA, and Zeatin was found necessary to induce cell wall formation, cell division, and colony formation.

5.5 Conclusion

Optimization of protoplast isolation conditions is essential for the efficient release of protoplasts. The present study demonstrates the effectiveness of our simplified protocol for protoplast isolation from yam mesophyll and callus tissues, and subsequent callus regeneration. From the results, a shorter incubation period of 16 hours was found optimal for generating high yields of viable protoplasts. Also, enzyme concentration, the age of the source explant, vacuum infiltration, and the culture density affected the yield, viability and regenerative capacity of protoplasts. The best and most cost-effective treatment for yam protoplast isolation (1.37×10^5 protoplasts per gFW) was 16-hour digestion of mesophyll (3.43×10^6 protoplasts per gFW, 77% viability) or callus tissues (4.7×10^5 protoplasts per gFW, 81% viability) with 0.8% macerozyme and 2% cellulase. The results revealed that cell wall and colony formation are more effivient in the liquid culture medium, compared to the semi-solid medium. The highest plating density, microcolony, colony, and callus regeneration were achieved in medium supplemented with 2,4-D, kinetin, and BAP. This protocol provides a versatile model and starting material for transient gene expression analysis, somatic hybridization, gene transfer, cybridisation, and CRISPR/ Cas9 genome editing, among other applications.

CHAPTER SIX

Generation of yam CRISPR/Cas9 mutants with a knock-out on the endogenous Phytoene desaturase (*PDS*) gene.

6.1 Introduction

The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein9 (Cas9)-mediated gene editing is a promising strategy for gene function analysis and improvement of economically important crops (Langner *et al.*, 2018). In comparison to other genome editing techniques such as the zinc finger nucleases (ZFNs) and TAL effector nucleases (TALENs), the CRISPR/ Cas9 system has obtained a broader adoption and application due to its simplicity, versatility, high efficacy, and low cost (Malzahn *et al.,* 2017). Genome editing by the CRISPR/Cas9 system has three requirements: (1) production of a 17–23-bp gRNAs with sequence homology to the target site, (2) expression of the nuclear-localized gRNA: (3) a protospacer adjacent motif (PAM) site (5′-NGG or 5′-NAG for *S. pyogenes* Cas9) located 3 bp upstream of the target sequence (Curtin et al., 2011; Joung & Sander, 2013; Sonoda et al., 2006). Edition occurs via the generation of DNA double-stranded break (DSB) at the target sequence. Subsequently, the DSB is repaired by either the nonhomologous end-joining (NHEJ) or homology-directed repair (HDR), both of which can be used to introduce genomic modifications (Doudna & Charpentier, 2014). For instance, NHEJ is error-prone and often introduces indels (small deletions or insertions) and nucleotide substitutions at the target site, hence generating gene knockouts and loss of function mutations. In the presence of a homologous template, the DSB is repaired via HDR, which results in gene replacement (Johnson & Jasin, 2000; Puchta, 2005).

Currently, the CRISPR/Cas9 system has been used to manipulate various genes in both monocots and dicots, and some agronomically important traits have been rapidly generated. This system has been successfully applied not only in seed-producing plant species such as maize (Svitashev *et al.,* 2016b), S*olanum lycopersicum* (Čermák *et al.*, 2015), wheat (Wang *et al.,* 2014), soybean (Li *et al.*, 2015), *Brassica napus* (Yang *et al.,* 2017), barley (Lawrenson *et al.,* 2015), *Oryza sativa* (Zhou *et al.,* 2014), and sorghum (Jiang *et al.,* 2013), but also in clonally propagated crops such as banana (Ntui *et al.,* 2020b; Tripathi *et al.*, 2019), cassava (Odipio *et al.,* 2017a), potato (Wang *et al.,* 2019), apple (Nishitani *et al.,* 2016), and grapes (Nakajima *et al.*, 2017).

Yam (*Dioscorea* spp.) is a multi-species tuber crop that serves as a staple food and an income source for approximately 300 million people worldwide, particularly in sub-Saharan Africa (Price *et al.,* 2017). Besides, yam produces various secondary metabolites, including alkaloids, diterpenoids, and steroidal saponins, which are important precursors of pharmaceutical excipients. In terms of global production of tuber crops, yam ranks fourth after potato, cassava, and sweet potato. With an annual production of 47.9 million tonnes, Nigeria is currently the largest producer of yam globally (Ita *et al.,* 2020) (FAOSTAT, 2017). Several attributes of the yam plant, such as diversity of maturity periods and the potential for long-term storage, make these tubers vital for food security in developing countries. Additionally, yam tubers are a rich source of vitamin C, essential minerals, dietary fiber, and starch (Chandrasekara & Josheph Kumar, 2016). Despite these positive attributes, yam production is severely limited by several biotic and abiotic constraints such as pests and diseases, especially viruses, anthracnose, and nematodes, low

yield potential of local landraces, inadequate planting materials, and poor soil fertility (Darkwa *et al.*, 2020).

Genetic improvement of the yam crop via classical breeding has not achieved substantial progress mainly due to the dioecious nature of the plant, polyploidy, poor seed set, non-synchronous flowering of elite genotypes, heterozygosity, and a prolonged breeding cycle (Mignouna *et al.*, 2008). Therefore, improvement of the yam germplasm necessitates the application of CRISPR/Cas9 tools that will allow scientists to directly manipulate the genome. As such, precise genome engineering systems can be exploited to complement transgenic approaches and conventional breeding strategies for gene function analysis and crop improvement. Application of the CRISPR/Cas technology in yam will provide an avenue for rapid gene function analysis to elucidate the genetics of this economically important tuber crop. When coupled with the current advancement in yam tissue culture techniques (Manoharan *et al.*, 2016), *Agrobacterium*-mediated transformation (Nyaboga *et al.*, 2014), and sequencing of the yam genome (Siadjeu *et al.*, 2020; Tamiru *et al.*, 2017), CRISPR/Cas9 can be used to introduce desirable changes in the yam genome directly, hence facilitating crop improvement for food security and income generation.

This chapter reports a simple protocol for CRISPR/ Cas9 editing of the yam genome, including gRNA design and molecular validation, construct design, genetic transformation of yam tissues, and molecular characterization of mutant events. By optimizing a leaf agro-infiltration system, a cheap and quick method for *in vivo* validation of gRNA activity was developed.

6.2 Materials and methods

6.2.1 Plant material and growth conditions

Plantlets of the yam (*D. rotundata*) accession Amola were obtained from the *in-vitro* germplasm collection at the International Institute of Tropical Agriculture (IITA)-Ibadan, Nigeria. The accession was maintained as *in-vitro* cultures on yam basic medium [YBM; Murashige and Skoog (MS) salts and vitamins, 25 mg/L ascorbic acid, 0.02 mg/L Naphthaleneacetic acid (NAA), 0.05 mg/L 6-Benzylaminopurine (BAP), 30 g/L sucrose, 2.4 g/L gelrite, pH 5.8]. The *in-vitro* cultures were maintained through routine sub-culturing on YBM at 25 ± 2°C and 16/8 h light/dark cycle.

6.2.2. CRISPR/Cas9 plasmid constructs

The plasmids used in this study were developed by Prof. Bing Yang (Missouri state University) and Dr. Zhengzhi Zhang (Iowa State University), Dr. Valentine Ntui and myself as described by Syombua et al. (2020). These included a total of Seven CRISPR plasmid constructs, each containing different gRNA sequences. The first and second plasmids (pCRISPR- *DrPDS1*-GFP and pCRISPR- *DrPDS2*-GFP) had a GFP tag and *nptII* selection marker (Figure 6.1). All the other plasmids had *hpt* selection marker that confers resistance to the antibiotic hygromycin.

The CRISPR/Cas9 construct 7 with two guide RNAs targeting the yam phytoene desaturase (*DrPDS*) gene was designed as described by Ntui et al. (2019). Briefly, phosphorylated and annealed oligos were ligated onto the gRNA expression vectors pYPQ131 (for gRNA1) and pYPQ132 (for gRNA2). The ligated products were transformed to DH5α *E. coli* cells, and colonies bearing the correct insert were verified by

Sanger sequencing. The clones were assembled into the Golden Gate recipient and Gateway vector pYPQ142 and transformed to DH5α *E. coli* cells. Plasmids from the Golden Gate assembly above together with the Cas9 entry vector pYPQ150 were cloned into the Gateway binary vector pMDC32 LR clonase™ (Invitrogen, New Zealand) recombination reaction. The Cas9 in vector pYPQ150 was of high GC content. The clones were mobilized to *Agrobacterium* strain EHA105 by electroporation and confirmed by PCR using Cas9 primers. The sequences of all gRNAs used in the present study are shown in Appendix 5.

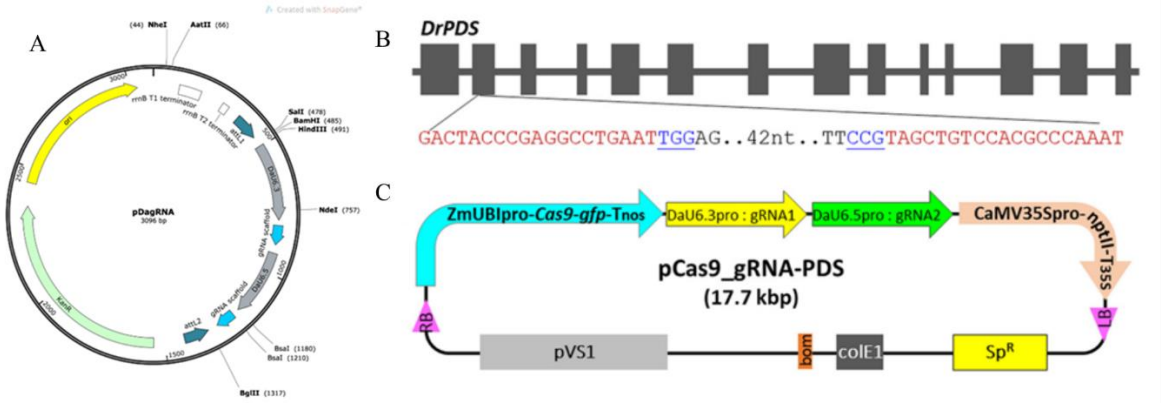

Figure 6.1: Schematic presentation of pCas9_gRNA-PDS used to generate genome-edited events

6.2. Transient *Agrobacterium*-mediated gene expression system in yam leaves

A system for transient gene expression in yam leaves was optimized using pCRISPR-Dr*PDS1*-GFP and pCRISPR-Dr*PDS*7, and the yam accession Amola. The optimized protocol was validated using the other five CRISPR constructs on the accession Amola and TDr 2579.

6.2.1 Preparation of *Agrobacterium* cultures for *Agro*-infiltration

Plasmids harboring the *Cas9* gene and gRNAs were mobilized into *A. tumefaciens* strains LBA 4404 (Ooms *et al.,* 1981) and EHA 105 (Hood *et al.,* 1993) via electroporation. A starter culture was prepared by inoculating a single colony of the recombinant bacteria into 20 ml LB broth containing 50 mg/L rifampicin and 100 mg/L kanamycin and incubated overnight in a rotary shaker (200 rpm) for 48 h at 28°C. Next, 50 uL of the starter culture was inoculated onto 50 ml liquid LB medium in an Erlenmeyer flask and grown at 28°C in a shaker incubator (200 rpm) until the OD_{600} reached 1.0. The bacterial cells were harvested by 10 min centrifugation of the culture at 4000 rpm (22°C), then re-suspended in liquid MMA infiltration buffer (10 mM MES (2-(*N*-morpholino) ethanesulfonic acid), 10 mM $MgCl_2$, pH 5.6, 200 μM acetosyringone). Cultures were then incubated for 2–4 hours at room temperature with gentle rocking.

6.2.2 *Agro*-infiltration procedure

Well rooted yam plants were transferred to peat pellets, covered with a transparent polythene bag, and placed in a glasshouse maintained at 28ºC with a photoperiod of 16 h/8 h (light/dark) for four weeks. Elongated plantlets were then transplanted in pots containing manure: soil mixture (1:1).

*Agrobacteri*um cultures containing the plasmid construct were injected into the abaxial side of the leaves of two- to three-month-old plants using the blunt tip of a plastic syringe by applying gentle pressure. Three leaves of each plant were Agro-infiltrated, and the experiment was repeated thrice. The negative control consisted of leaves infiltrated with MMA buffer alone.

6.2.3 Effect of infiltration buffer on leaf necrosis

Yam leaves were *Agro*-infiltrated with three different media devoid of any bacterium to optimize the infiltration buffer. The three buffers included yam bud induction medium [SBM; MS salts and vitamins, 20 g/L sucrose, 1mg/L BAP, 0.2 µM $CuSO_4$, pH 5.7, and 200 µM acetosyringone), MMA (10 mM MES, 10 mM $MgCl_2$, pH 5.7 and 200 µM acetosyringone), and phosphate buffer PB (17.851 g/L Na_2HPO_4-$7H_2O$, 4.61 g/L $NaH_2PO_4H_2O$, pH 7.2 and 200 µM acetosyringone). Macroscopic responses were scored three- and seven-days post-infiltration when the changes in leaf morphology were clear. Briefly, macroscopic scoring of cell death was done according to the percentages of cell death on the infiltrated area, on a scale from no symptoms (0) to confluent cell death (75% to 100%). Intermediate responses ranged from minimal necrosis (25%), such as chlorosis, to increasing levels (50%) of cell death.

6.2.4 Effect of chemical additives and heat shock treatment

Bacterial pellets (*A. tumefaciens* strain EHA 105) harboring the pDrg1g2-GFP construct were re-suspended to OD_{600} = 1.0 in SBM. The SBM/bacteria mix was supplemented with various chemicals, including acetosyringone (0 - 600µm), ascorbic acid (0 – 200 mM; Duchefa), polyvinyl pyrrolidine (PVP, 0 – 1g/L; Duchefa), and pluronic F-68 (0 – 0.2%; Sigma), to determine the effect of medium chemical supplementation on the infiltration efficiency. The medium was then filter sterilized and immediately used for infiltration. The ability of each additive to enhance *Agro*-infiltration was evaluated by assessing the intensity of fluorescence emitted from each of the infiltrated patches and analyzed by the Image J software. Chemical additives that were empirically determined to improve transient expression levels were combined to form the optimized medium SBM-AI (SBM

containing 400 µM acetosyringone and 0.002% Pluronic F-68). The effect of heat on transient GFP expression was assessed by placing whole plants in a 37°C incubator for 30 min, 0–4 days post-infiltration.

6.2.5 GFP imaging

Differences in protein expression levels following the various treatments were evaluated by assessing fluorescence emitted from infiltrated leaf patches in a microscope under UV light. Differences in the fluorescence intensities of the various patches were analyzed by the Image J software. The system allowed us to quantitatively monitor GFP intensity spatially over the surface of plant leaves and effectively show mutation induction. Yam leaf samples were collected between 0- and 10-days post-infiltration and visualized for fluorescence under ultraviolet light.

6.2.6 Detection of mutations in the Agro-infected leaves of yam

Infiltrated sections were sampled using a cork borer (8.5 mm), and total DNA was extracted as previously described by Stewart and Via (1993). The DNA was used for sequence analysis using *PDS* gene-specific primers: DrPDS-F (5′- GTTGCCGCTTGAGAGTTC -3′) and DrPDS-R1 (5′- AGG CTGTTTTACCTGCACCA -3′). Amplification was performed in a 20 µl reaction volume containing 1 µl genomic DNA (100 ng/µl), 10 µl of HotStarTaq master mix, 1 µl of 10 µM of each primer, and 7 µl nuclease-free water. The PCR amplification conditions were as follows: an initial denaturation step at 95 °C for 15 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s, extension at 72°C for 1 min, and final extension at 72°C for 10 min. Subsequently, 5 µl of the PCR product was resolved in a 1 % agarose gel stained with gel red to confirm the amplicon size. The remaining 15 µl of PCR product were purified using a QIAquick PCR purification

Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and used for Sanger sequencing.

The sequencing reaction mixture was composed of 4 µl sample of the purified PCR product, 4 µl of 5X sequencing buffer, 1 µl of Big Dye Terminator, 1 µl of 100 ng/µl of either PDS_F or PDS_R primer, and 10 µl of nuclease-free water. The thermocycler conditions were set according to the manufacturer's instruction (BigDye^{TM}Terminator, Applied Biosystems, Foster City, CA). Amplification products were purified by sequential addition of 50 µl of 100% ethanol, 2 µl of 3 M Sodium acetate, and 2 µl of 125 mM EDTA followed by a 15 min incubation at room temperature. The mixture was then pelleted by centrifugation at 13,000 rpm for 25 min, the pellet washed with 70% ethanol, and air-dried for 15 min on the laminar hood. The dried pellets were resuspended in 10 µl of HiDi formamide (Life Technologies) and incubated in a thermal cycler at 65°C for 5 min, 95 °C for 2 min, and cooled to 4 ºC. The samples were sequenced by the Sanger method using ABI 3130 DNA sequencer (Applied Biosystems, California, USA) and aligned with the yam reference genome using the SnapGene software (SnapGene v4.3.4, GSL Biotech LLC).

6.2.7 Generation of yam events with a mutated *PDS* gene

Nodal explants from *in-vitro* grown plantlets were transfected with *Agrobacterium* EHA 105 harboring pCRISPR1-Dr*PDS*1-GFP using the protocol described by Nyaboga *et al.* (2014). The Agro-infected nodal explants were regenerated on selective medium containing kanamycin (50–200 mg/l) to obtain transgenic shoots. Mutant plantlets with altered *PDS* activities were visually discriminated from control plants via the characteristic albino phenotype and observation of green fluorescence using a fluorescent

stereomicroscope (SMZ 1500, Nikon Corporation, Tokyo, Japan) at an excitation B filter for a wavelength range between 460 and 490 nm.

Molecular analysis of gene-edited plants

6.2.8 Detection of CRISPR/Cas9 induced mutations by PCR analysis

Total genomic DNA was isolated from 100 mg of freshly ground leaf samples of putative mutant events and wild-type plants using a modified cetyltrimethylammonium bromide (CTAB) protocol Stewart and Via (1993). The quality and concentration of DNA were assessed using a nanodrop spectrophotometer. Approximately 100 ng of genomic DNA was used as the template for PCR analysis using HotStarTaq master mix (Qiagen) and *Cas9* gene-specific primers complementary to a 500 bp amplicon. The primer sequences were: Cas9-F (5′-TTGCGCCTCATCTATTTGGC-3′) and Cas9-R (5′-TCGATGTACCCAGCATACCC-3′). The following amplification conditions were used: 95 °C for 15 min; 32 cycles of 95 °C for 30 s, 57 °C for 45 s, and 72 °C for 30 s followed by a final extension at 72 °C for 10 min. The PCR products were resolved in 1 % agarose gel stained with gel red. Gel pictures were taken by a gel documentation system.

6.2.9 Mutant characterization by sequence analysis

Genomic DNA from putative mutant events was used as template to amplify the endogenous *PDS* fragment by PCR. Sanger sequencing of PCR products was done as previously described to identify the presence of desired mutations. Sequence results of putative mutant events were aligned to that of the endogenous *DrPDS* gene of wild-type plants by the SnapGene software (SnapGene v4.3.4, GSL Biotech LLC). The editing

efficiencies were calculated by expressing the number of events showing mutation as a percentage of the total number of transgenic events sequenced.

6.2.10 Statistical analysis

All statistical analyses were done using the Minitab software. The experiments were performed thrice and subjected to analysis of variance (ANOVA) followed by mean separation by Duncan's multiple range test (DMRT). Fluorescence intensities were quantified using the Image J software and data from three separate experiments pooled and statistically analyzed. Data were expressed as mean ± the standard error of the mean (SEM), and a *P*-value < 0.05 was considered statistically significant.

6.3 Results

6.3.1 Transient assay of Cas9 activity and U6 promoter efficiency

pCas9, pDagRNA-gGFP, and pGFP+1 were mixed equally and used for protoplast transfection. pUbi-GFP and pGFP+1 served as positive and negative controls, respectively, during protoplast transfection. Forty hours after transfection, protoplasts transfected with pUbi-GFP showed strong GFP fluorescence, while fluorescence could not be observed in protoplasts transfected with pGFP+1 alone, demonstrating the complete non-function of GFP resulted from the 1nt-insertion. All protoplast samples transfected with pCas9, including pDagRNA-gGFP and pGFP+1, showed GFP fluorescence, suggesting that the GFP+1 was correctly inserted in the wild-type allele (Figure 6.2). However, the number of protoplasts with GFP fluorescence and their intensity varied among different U6 promoters and between the two Cas9 versions (Figure 6.3). Based on scale-scoring of the fluorescence, the rice-optimized Cas9 (OsCas9) showed lower activity than wheat-

optimized Cas9 (TaCas9). Promoter U6.1 showed the lowest efficiency both with OsCas9 and TaCas9, while U6.5 had the best activity either with OsCas9 or TaCas9. U6.2 showed high activity with TaCas9 but low activity with OsCa9. On the contrary, U6.3 demonstrated good activity only secondary to U6.5. The editing efficiency of U6.4 was intermediary. When the GFP guide RNA was driven by both U6.1 and U6.2, the fluorescence level was similar to U6.2 alone, for either OsCas9 or TaCas9. This finding further verified that the activity of U6.1 was negligible. Based on the transient assay results, promoters U6.3 and U6.5 were used for driving the guide RNA expression cassettes used for stable transformation.

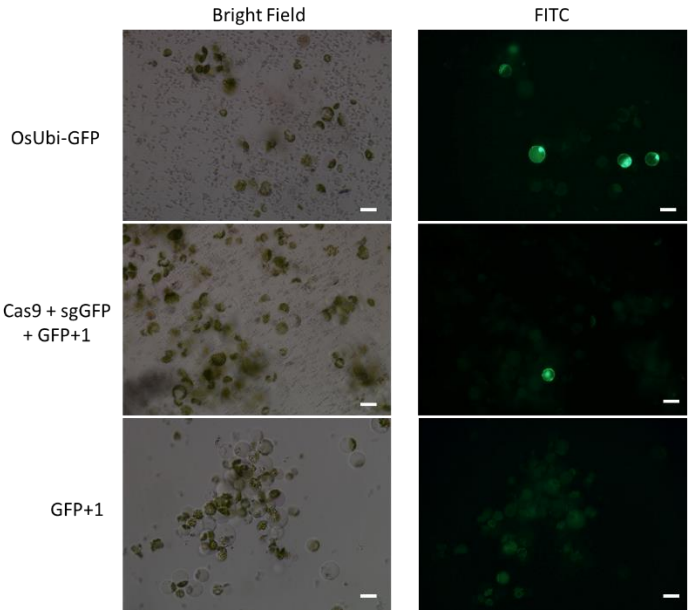

Figure 6.2: Expression of GFP in yam protoplasts transfected with Cas9, sgGFP, and non-functional GFP (GFP+1). BF: Bright field; FITC: Fluorescein isothiocyanate. Scale bars represent 25 µm.

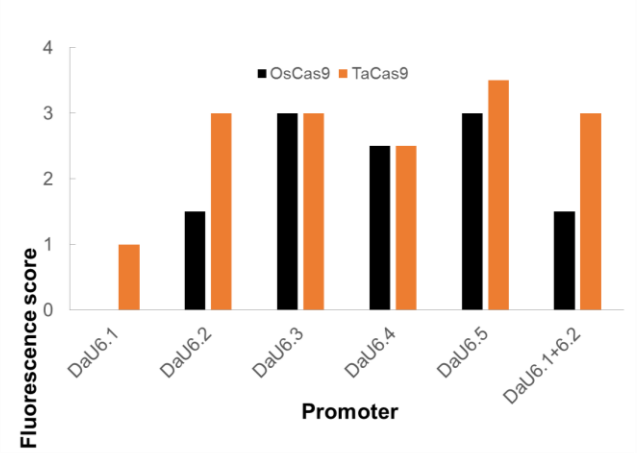

Figure 6.3: Editing efficiency of Cas9 and U6 promoters in yam protoplasts. Fluorescence was scored in a scale from 1 to 10, with no tracked fluorescence as in pGFP+1 scored as 0, while fluorescence in pUbi-GFP scored as 10.

6.3.2 *In vivo* validation of the CRISPR constructs by Agro-infiltration

The *in vivo* functionality of designed Cas9-gRNAs was tested via *Agro*-infiltration of the respective vectors onto the leaves of glasshouse yam plantlets. Phenotypically, Cas9 activity was assessed by observation of bleached patches on *Agro*-infiltrated sections and the emission of a green fluorescence under UV light (Figure 6.4). The ease of buffer penetration was influenced by the age of leaves; a poor penetration ability was observed in stage 1 leaves (young, unopened leaves), while maximal infiltration was achieved in stage 3 leaves (mature, firm, and fully expanded leaves). Besides, the gentle pressure exerted during infiltration caused necrosis on the infiltrated sections of stage 1 leaves. Meanwhile, intermediate ease of buffer penetration was observed in stage 2 leaves (fully expanded young leaves), along with the efficient formation of the characteristic white patches on infiltrated sections. However, the formation of characteristic white patches and fluorescence was not observed in stage 3 leaves, suggesting that mutation was not achieved. Therefore, only stage 2 leaves were infiltrated in subsequent experiments.

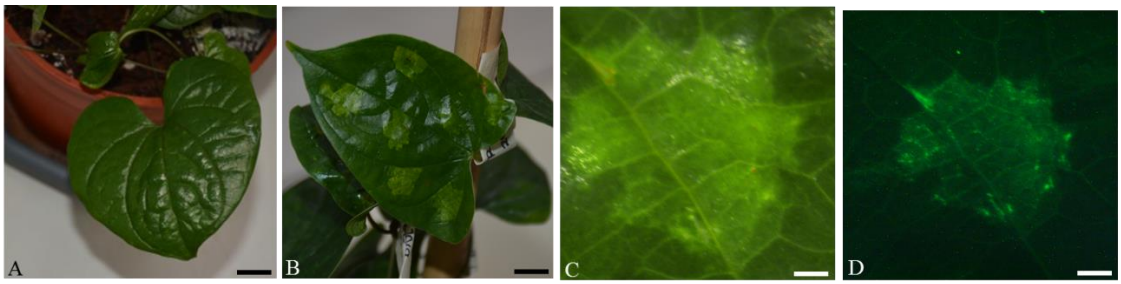

Figure 6.4: Phenotypic analysis of Agroinfiltrated leaves**.** A: Control leaf inoculated with yam infection medium without bacterial suspension. B: Leaf inoculated with yam infection medium containing bacterial suspension. C: Microscopic examination of an infiltrated leaf section. D: Fluorescent micrograph of the infiltrated section in C. Scale: A and B: 0.25 cm, C and D: 0.25 mm.

6.3.3 Optimization of *Agro*-infiltration conditions

6.3.3.1 Effect of infiltration media on leaf morphology

Three different buffer formulations, MMA, SBM, and PB, were tested for use in yam leaf Agro-infiltration. Buffer PB caused confluent cell death and tissue necrosis ranging from 75 to 100%, MMA caused weak cell death characterized by chlorosis of Agro-infiltrated sections, while SBM did not cause any apparent effect on leaf tissues (Figure 6.5). The buffer SBM was, therefore, selected for use in all subsequent infiltration experiments.

Figure 6.5: Quantification of Agro-infiltration responses. The photograph shows representative scoring scales for cell death, ranging from 0 % (no symptoms) to 100 % (confluent cell death). Intermediate responses range from weak responses such as chlorosis to increasing levels of cell death. Scale: 0.25 mm.

6.3.3.2 *Agrobacterium* strain and cell density

For both EHA 105 and LBA 4404 harboring pCRISPR1- *DrPDS1*-GFP, negligible levels of GFP expression were observed on day 0, suggesting the absence of endogenous plant-derived or bacteria-derived GFP activity. The highest fluorescence intensities were obtained 4 days post infiltration (dpi) for the two strains (Figure 6.4A). Notably, GFP expression rates decreased significantly from the 6^{th} to 10^{th} dpi. The highest fluorescence intensities were attained in the strain EHA 105 at 4 dpi, which was approximately 1.3 times higher than those obtained in the strain LBA4404 at 4 dpi.

The *Agrobacterium* strain EHA 105 harboring pCRISPR1-GFP was administered on to the underside of yam leaves at an OD_{600} ranging from 0.05 to 2.0 to examine the effect of A*grobacterium* concentration on transgene expression. Subsequently, leaves from each bacterial density were sampled at 4 dpi, data on GFP expression from three separate experiments pooled, statistically analyzed, and graphed (Fig. 6.6B). Relative to $OD_{600} = 1.0$, the intensities of GFP expression were significantly lower at $OD_{600} = 0.05$, 0.1, 0.25, 0.50. 1.5, and 2.0. The fluorescence intensities at $OD_{600} = 0.75$ and 1.0 were not significantly different. Thus, the strain EHA105 and a bacterial density of $OD_{600} = 0.75$ were used in all subsequent infiltrations.

Fig 6a

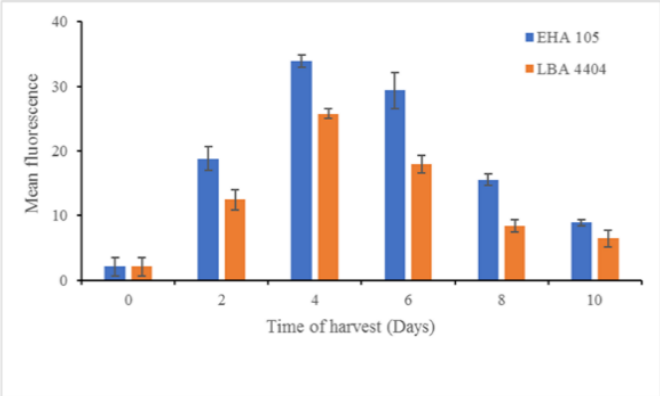

Fig 6b

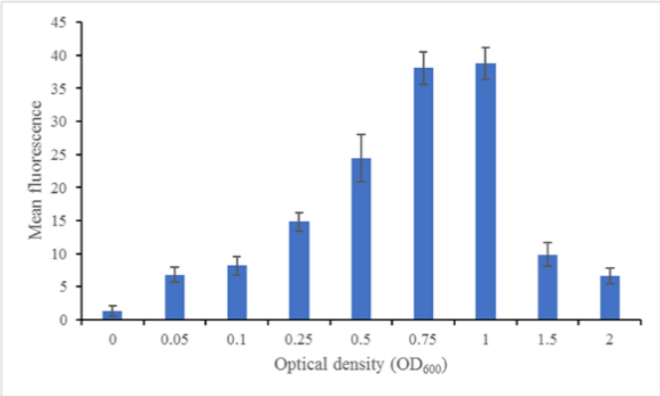

Figure 6.1: Effects of Agro-bacterial strains and cell culture density on GFP fluorescence intensity via Agro-infiltration. A: *Agrobacterium* strains EHA 105 and LBA4404 harboring pCRISPR1-GFP were infiltrated into yam leaves. Leaves were sampled at 0, 2, 4, 6, 8- and 10-days post infiltration (dpi). B: *Agrobacterium* strain EHA 105 harboring pCRISPR1 -GFP was infiltrated into yam leaves at increasing concentrations OD_{600} = 0.05, 0.1, 0.5, 0.75, 1.0, 1.5 and 2.0.

6.3.3.3 Supplementation of infection buffer with chemical additives

Further optimization was done by supplementing the infection with various chemical additives, including acetosyringone, ascorbate, pluronic F-68, and polyvinylpolypyrrolidone (PVPP). An increase in acetosyringone concentrations significantly increased fluorescence intensities, with the highest intensity obtained at a concentration of 400 µM. At this concentration, fluorescence intensities were about three-fold higher than those attained when the infiltration buffer is used alone (Fig. 6.7A). Tissue

browning is a prevalent challenge during *Agrobacterium*-mediated transformation. Therefore, antioxidants such as polyvinylpolypyrrolidone (PVPP), L-cysteine (L-cys), glutathione, dithiothreitol (DTT), α-tocopherol, α-lipoic acid, and ascorbic acid are incorporated in the infection medium to reduce tissue necrosis and thereby increase infection rates. In the present study, the addition of low levels of ascorbic acid (5 mM) increased fluorescence intensities, although this increase was not statistically significant. A further increase in the amounts of the antioxidant caused cell death of infiltrated leaf sections and thereby reduced the intensity of GFP expression (Figure 6.7B). The addition of PVP, even at the lowest concentrations, caused significant cell death on infiltrated sections (Figure 6.7C). The addition of low levels (5 and 10 μM) of Pluronic F-68 correlated with an increase in GFP expression levels by about twofold and fourfold, respectively. However, concentrations above this level did not elicit any stimulatory effect (Figure 6.7D).

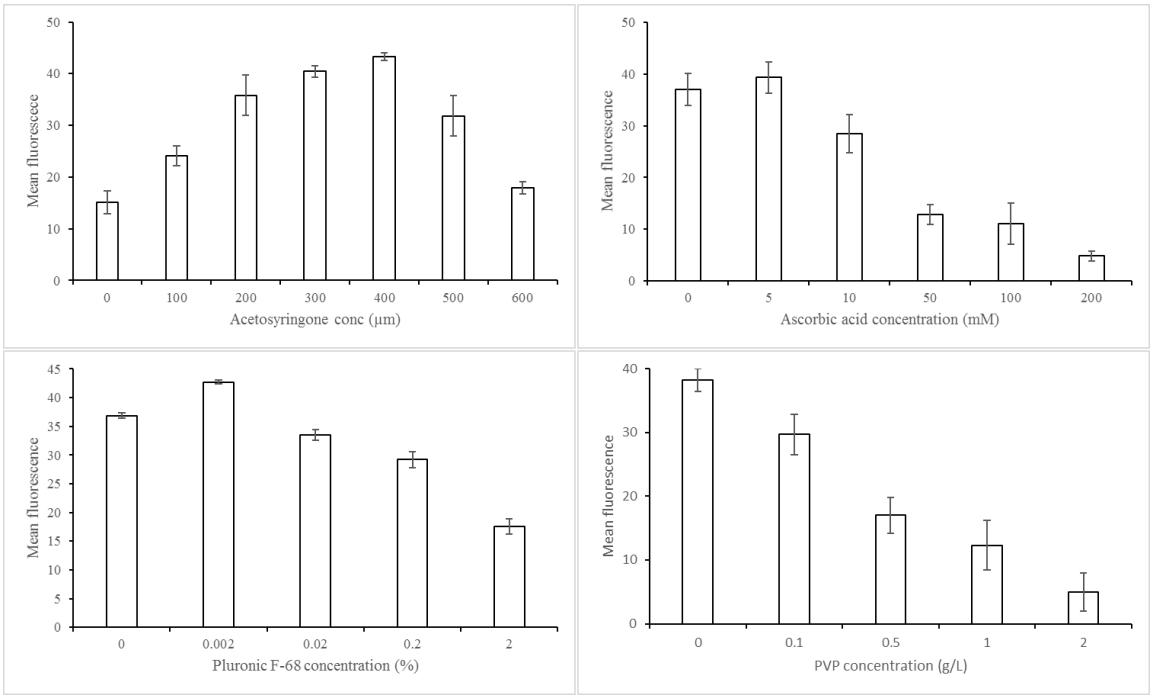

Figure 6.2: Effects of chemical additives on transient GFP expression. *Agrobacteria* strain EHA 105 harboring pDrPDSg1g2 were resuspended in medium containing different concentrations of chemical additives. A: Acetosyringone. B: Ascorbic acid. C: Pluronic F-68. D: PVP, into yam leaves.

6.3.3.4 Heat shock to whole plants

The effect of temperature on T-DNA delivery was assessed by exposing whole plants to 30 min heat treatment, at 37°C and at different periods post-infiltration (0, 1, 2, 3, and 4 DPI). The results showed that a 30 min exposure of whole plants to heat treatment on 2 and 3DPI significantly increased GFP expression levels (Figure 6.8). However, no significant changes in fluorescence intensities were observed when plants were heat-shocked 0, 1, and 4 DPI.

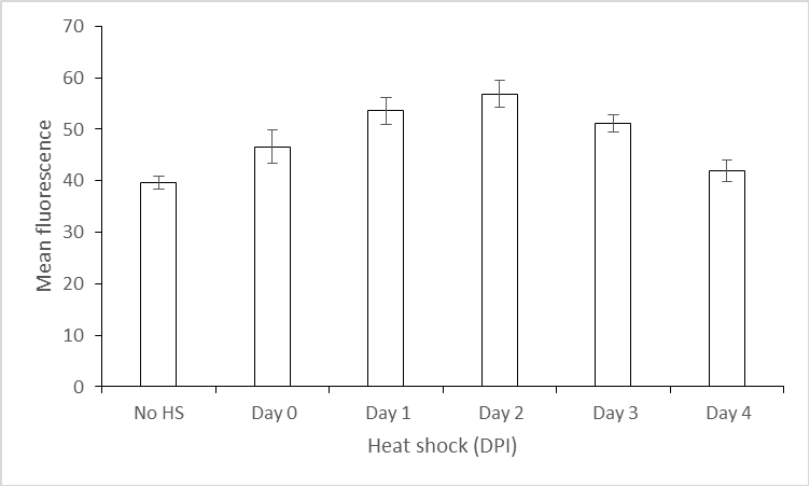

Figure 6.3: Effects of whole plant heat shock on transient GFP expression.

6.3.4 Detection of targeted mutations in *Agro*-infiltrated leaves

To evaluate the nature of mutations acquired, PCR amplification and targeted Sanger sequencing were conducted on the DNA of infiltrated patches using *PDS* primers. According to the number of mutations detected, the target efficiency of pCRISPR7-*DrPDS7* in yam leaves was 75% for gRNA1 and 0% for gRNA2. Out of the total of 28 DNA samples sequenced, 21 samples had mutations in gRNA 1 while no mutation was obtained on gRNA2. Moreover, the mutations detected were a mixture of substitutions, insertions, and deletions (Figure 6.9). These results suggest that the vector was viable for the generation of mutated events.

Figure 6.4: Sequence-based detection of mutations induced by DrPDS-7Cas9 following Agro-infiltration of yam leaves. Deletions are denoted by black dashes, insertions by blue letters, and substitutions by purple letters.

6.3.5 Generation and validation of transgenic yam with edited PDS gene

Agrobacterium-mediated transformation of yam nodal explants with the gene-editing construct DrPDS Cas9 g1/g2 resulted in the regeneration of eight transgenic events. Variations in the expression patterns of the yam *PDS* gene mainly manifested as either total albino or green plants with chimeric albinism (Figure 6.10A and B). While most mutated plants developed as secondary shoots from the base of originally green shoots, some mutants were directly generated from the nodal explants (Figure 6.10C). The regenerated plants were maintained on yam micropropagation medium YBM supplemented with 150 mg/l kanamycin. Complete albino plants exhibited a dwarf and bushy phenotype (Figure 6.10D) characterized by retarded growth, reduced leaf area, short petioles and internodes, and poor response to *in vitro* propagation compared to the control plants. However, mutated plants with a mosaic pattern of albinism exhibited vibrant growth that was comparable to

the control plants. Similar to wild-type plants, mutant plants exhibited a vibrant root system (Figure 6.10A). Phenotypic analysis of mutant plants was further confirmed by observation of green fluorescence under UV light. While wild-type plants did not emit any fluorescence, mutated plants emitted a bright green fluorescence (Figure 6.10 E and F).

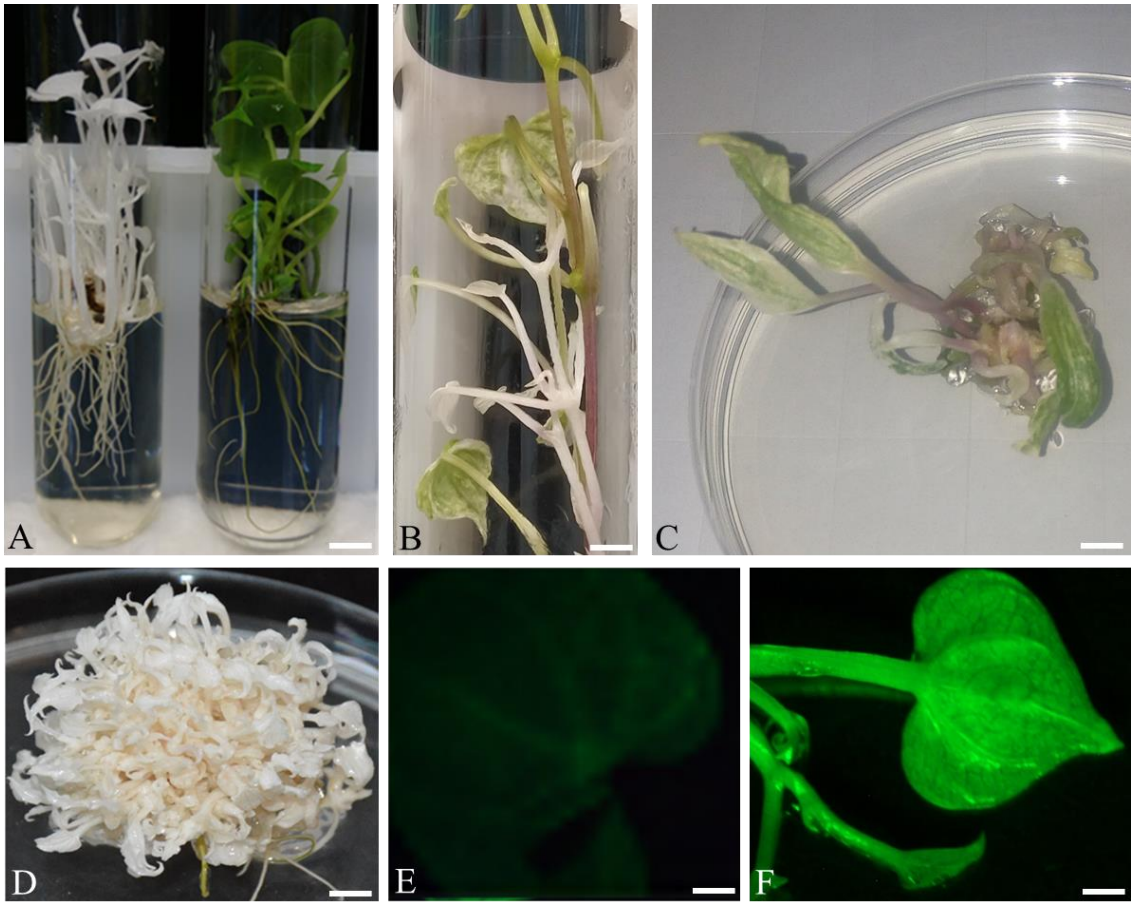

Figure 6.5: Phenotype of PDS gene mutated yam shoots. A: Left, well-rooted albino shoot. Right, wild-type plant. B: Variegated albino plant. C: Mutant emerging from the nodal explant. D: Clear albino shoot with a shrub phenotype and stunted growth. E: The leaf of a wild-type plant as observed under UV light. F: The leaf of a mutant plant as observed under UV light

6.3.6 Molecular analysis of edited plants

Results on PCR analysis of putative mutant plants using *Cas9* gene-specific primers confirmed the presence of *Cas9* gene in kanamycin-resistant events, including the green line and all events showing the characteristic albino phenotype (Fig 6.11A).

PCR analysis using *PDS* gene-specific primers revealed band shift in events 5 and 6, suggesting that the mutations consisted of substantial insertions and deletions (Fig 6.11B).

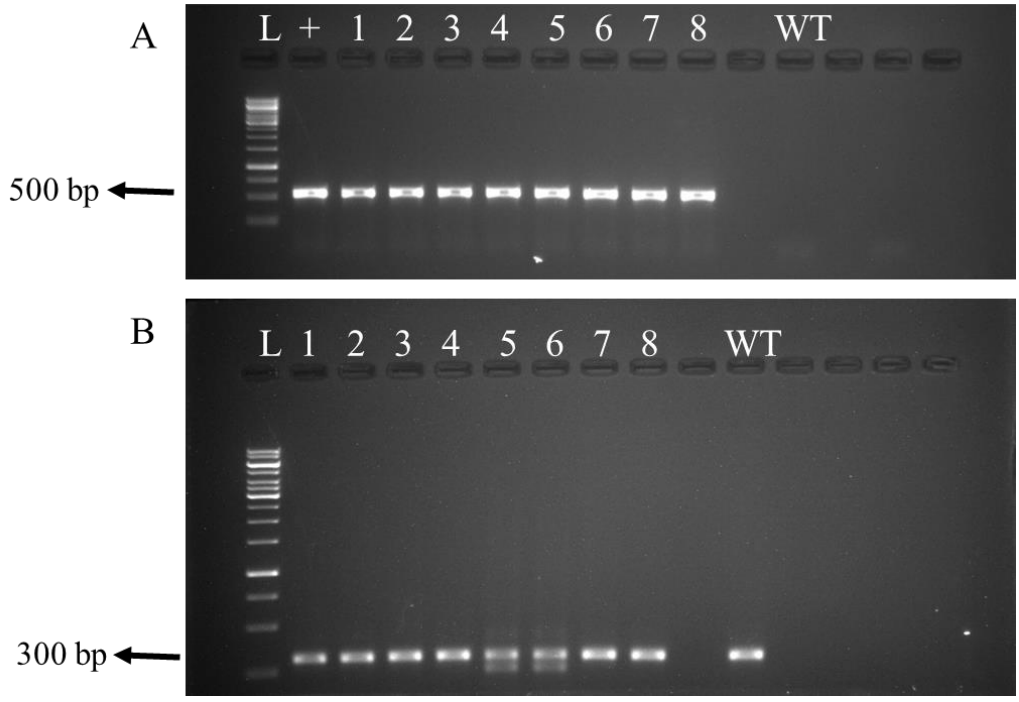

Figure 6.6: PCR analysis of mutant yam events. A: *Cas* 9. B: *PDS*-specific primers. Lanes: L, 1 kb plus DNA ladder; +, plasmid DNA used as a positive control; 1–8, mutant yam events; WT, non-transgenic plant DNA.

Sanger sequencing of the eight events revealed a mixture of deletions, insertions, and substitutions in the seven events showing either total or partial albinism, while no mutation was observed in the one green plantlet. Combined statistical analysis of our results indicated that deletions were the most prevalent form of mutation (70.01%), ranging from a single nucleotide to six base-pair deletions. Meanwhile, insertions accounted for 28.38%,

ranging from one to eight base-pair insertions. Among the total of fourteen mutations analyzed, we detected only one case of nucleotide substitution. The most frequent target site was four bases upstream of the PAM site, where we detected both single nucleotide insertions and deletions resulting in frameshifts (Figure 6.12).

Figure 6.7: Sequence-based detection of mutations induced by DrPDS-1Cas9 in yam accession Amola. Sequence-based detection of mutations induced by *DrPDS-1Cas9* in yam accession Amola. Deletions are denoted by black dashes.

6.4 Discussion

Mutagenesis offers a robust system for functional genomics research and trait improvement in crop plants. The present study is the first report of precise modification of the yam *PDS* gene via the CRISPR-Cas9 system. The *PDS* gene encodes a key enzyme in carotenoid biosynthesis (Pd *et al.*, 1994) and whose knock-out results in the generation of albino plants. As such, visual detection of mutated plants is feasible hence providing a rapid system for assessing the potency of the technology in generating gene edits. Besides, the

PDS gene is involved in many other metabolic pathways, including gibberellin and chlorophyll biosynthesis. Therefore, chromosomal aberrations in the *PDS* gene also cause dwarfism (Qin *et al.,* 2007). The versatility of the *PDS* gene in assessing the efficiency of the CRISPR/Cas9 system in generating gene edits has been demonstrated in *Nicotiana benthamiana* (Nekrasov *et al.,* 2017), Arabidopsis (Li *et al.,* 2013), rice (H. Zhang et al., 2014), apple (Nishitani *et al.,* 2016), sweet orange (Jia & Wang, 2014), populus (Fan *et al.,* 2015), petunia (Zhang *et al.,* 2016), tomato (Pan *et al.,* 2016), cassava (Odipio *et al.,* 2017), pear (Charrier *et al.,* 2019), banana (Ntui *et al.,* 2020b) and walnut (Walawage *et al.,* 2014). In this study, the construct was designed with two gRNAs targeting different exons of the *PDS* gene in order to increase the probability of creating the desired mutations. Loss of function of the *DrPDS* gene resulted in the generation of complete albino or total albino plants, as has been demonstrated in other species (Shan *et al.,* 2013). Sequence analysis showed that the mutations generated were predominantly deletions and insertions, which concurs with sequence analysis of modified events in other crops (Fan *et al.,* 2015; Nekrasov *et al.,* 2017; Sun *et al.,* 2016; Zhou *et al.,* 2014). While only single nucleotide insertions were obtained on gRNA2, several different insertions and deletion lengths were generated in gRNA1. These differences could be attributed to discrepancies in DSB repair mechanisms between the two loci.

The available yam transformation methods are technically demanding and time-consuming, taking up to 12 months to regenerate putative events. With these challenges, the development of a quick, efficient, and cheap system of validating the efficiency of *sgRNAs* is of great significance. Transient gene expression by syringe agroinfiltration offers a simple and efficient technique for different transgenic applications. As such, this

study optimized a syringe infiltration system for gene expression and rapid validation of sgRNA activity *in vivo*. Buffer PB caused cell death of up to 100 %, while MMA caused up to 25 % cell death. Yam bacterial infection media SBM did not, however, cause any cell death and was, therefore, selected for subsequent agroinfiltration experiments. Optimal GFP expression was achieved when young leaves were infiltrated with *A. tumefaciens* strain EHA105 at an optical density (OD_{600}) of 1.0. Acetosyringone (400 μM) and 0.002 % of the surfactant Pluronic F-68 significantly enhanced GFP expression. The effect of ascorbic acid on GFP expression was not significant, and supplementation with PVP significantly reduced GFP expression because of cell death. A 30 min 37 °C heat shock to plants, one-day post- infiltration, significantly increased GFP expression levels. All the optimal features were combined to produce an effective delivery system composed of: The *Agrobacterium* strain EHA105, bacterial culture density (OD_{600}) of 1.0, 37 °C heat shock one-day post infiltration, resuspension buffer composed of SBM medium supplemented with 400 μM Acetosyringone and 0.002 % Pluronic F-68. The efficiency of the combined conditions was validated in seven different CRISPR constructs targeting the PDS gene. Using this method, we were able to validate up to six different gRNAs within a week. Although bioinformatics-based design and selection of *sgRNA* are of importance, it only assesses the theoretical efficiency and specificity of the target loci *(*Fan *et al.,* 2015*;* Ma *et al.,* 2015; Sun *et al.,* 2016). The present study, therefore, offers a fast and efficient strategy of experimental validation of candidate *sgRNAs.* Unlike gRNA validation by PEG mediated transfection, this strategy is not technically demanding and does not require the use of expensive cell wall digesting enzymes.

6.5 Conclusion

Since the regeneration of mutated whole plants takes a long time, it is important to conduct an *in vivo* assessment of gRNA efficiency. Most gRNA validation systems in plants employ protoplast-based systems, which are costly and could therefore hinder technology transfer to poor, underprivileged laboratories. It is, therefore, of significance to provide cost-efficient strategies that offer a similar output within a short time duration. As such, the present study provides a short, cheap, efficient, and easy strategy for gRNA validation in the yam crop.

Since the advent of crop improvement via conventional breeding, *Agrobacterium*-mediated transformation, and genome editing, minimal progress has been achieved in the improvement of the yam crop. This slow progress can majorly be attributed to the inherent properties of the crop, including its high heterozygous nature and polyploidy. This study is the first report of successful editing of the yam *PDS* gene and the first precise modification of the most economically significant yam species, *D. rotundata*. The findings of the present study, therefore, unlock this orphan crop for more research towards its improvement for better productivity and reduced susceptibilities to biotic and abiotic stresses.

CHAPTER SEVEN

GENERAL DISCUSSION, CONCLUSION, AND RECOMMENDATIONS

7.1 GENERAL DISCUSSION

Yams are a crucial food security crop and a source of income for a significant human population in the tropical and sub-tropical world, particularly in the yam belt of West Africa. Compared to other root and tuber crops such as cassava, yam is more suited for combating food insecurity because it can be stored for up to 4-6 months at ambient temperatures. However, yam has high production costs, compounded by low yield per hectare, pest and disease infestations, and high labor costs (Kenyon *et al.*, 2008; Price *et al.*, 2017a). Yam improvement by classical breeding has been slowed by various intrinsic attributes of the crop, including polyploidy, poor seed set, long breeding cycle, and heterozygosity. Thus, the application of modern tools that allow direct modification of the genome could overcome some of the challenges encountered in conventional breeding (Syombua *et al.*, 2020).

The primary objective of this study developed a CRISPR-Cas9-based toolkit for yam genome editing using multiple gRNAs targeting the phytoene desaturase (*PDS*) gene. The genome-editing reagents were delivered into the nodal segments of a farmer preferred yam cultivar, Amola, by *Agrobacterium*-mediated transformation. The protocol developed herein presents avenues for yam improvement and gene function analysis. Further, the study demonstrates the potential for multiplex editing of two loci, which could enable simultaneous targeting of several traits, thus rapid yam improvement. Further, this study provides an optimized system for rapid gRNA validation by Agro-infiltration. The system was established through the infiltration of young leaves of two months old potted plants

with *Agrobacterium* harboring gRNAs targeting the *PDS* gene. Compared to gRNA validation by protoplast transfection, Agro-infiltration is cheaper and simpler, does not require the use of expensive cell wall digesting enzymes. Besides, the protocol is short, taking a maximum of 10 days.

The third chapter of this study demonstrates the capacity for gene integration in *D. alata*, the most widely cultivated *Dioscorea* species globally. The study first optimized plantlet regeneration from somatic embryos and conducted a histological evaluation to examine the sequence of events involved in obtaining a whole plant from the axillary bud explants. The evaluation describes the pro-embryoid origin and cytological transition from globular through heart, torpedo, and cotyledonary stages, aspects of starch occurrence, and tissue dedifferentiation. To optimize the various aspects that affect the potential for plant cell gene uptake and integration, the study delivered constructs harboring the *β-glucuronidase* reporter gene by *Agrobacterium* mediated transformation. The optimized parameters included explant type, explant pre-culture period, form of tissue wounding, bacterial density, acetosyringone concentration, and co-cultivation time. Positive *β-glucuronidase* assay and PCR analysis exhibit the feasibility of applying the system for *D. alata* genetic improvement.

Compared to compact embryogenic callus, friable embryogenic callus (FECs) can generate embryogenic cell suspensions more easily, have greater uniformity, occur in higher numbers, and are capable of continued proliferation in media (Taylor *et al.*, 1996). Thus, FECs are better targets for protoplast isolation, mutant selection, and cellular engineering experiments. However, protocols for FEC development and regeneration lack in most crops, which delays crop genetic improvement. The fourth chapter of this study is the first

report on the development of yam FECs. Using the model used in our lab to develop cassava FECs (Nyaboga *et al.,* 2015), this study reports the factors that contribute to FEC formation in yam, including the basal salt mixtures in media, amino acids, vitamins, and callus wounding.

The availability of robust and reproducible protoplast-to-plant systems is a necessity for the successful application of somatic hybridization, protoplast direct gene transfer for plant genetic manipulations. These systems are also essential for high-throughput analysis and functional gene characterization using protoplasts (Eeckhaut *et al.,* 2013). All protoplast-based techniques are depended on the availability of large populations of viable protoplasts; thus, the existence of robust and reproducible protoplast isolation methods is a pre-requisite for applying these techniques. The possibility for direct gene transfer to protoplasts has been immensely applied to generate DNA-free genome-edited events (Liang *et al.,* 2017; Murovec *et al.,* 2018). Thus, the system developed herein will be of great significance in developing yam plants without an integrated foreign gene.

7.2 CONCLUSIONS

- *D. alata* is amenable to *in vitro* regeneration via somatic embryogenesis and gene uptake by *Agrobacterium* mediated transformation.
- The development of regenerable friable embryogenic callus (FECs) in *D. rotundata* is feasible.
- Under culture, yam protoplasts are capable of cell wall formation, colony, and callus regeneration.
- The yam genome is amenable to targeted mutagenesis by the CRISPR/Cas9 system
- Leaf Agro-infiltration can enable quick and accurate *in vivo* validation of gRNAs.

7.3 RECOMMENDATIONS

The following are recommended for future studies in yam genome editing by the CRISPR/ Cas9 system:

1. Guide RNA (gRNA) multiplexing is recommended to increase the chances of obtaining desired edits when editing the yam genome by CRISPR/ Cas9.
2. The gRNA activity and specificity should be validated by either protoplast transfection or leaf Agroinfiltration prior to genetic transformation.
3. The CRISPR/ Cas9 system developed herein should be used to target genes of agronomic importance for yam crop improvement.

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9 APPENDICES

Appendix 1: YBM medium

	Component	Quantity/ L
1	MS basal salts with vitamins	Full strength
2	Sucrose	2%
3	BAP	0.05 mg
4	NAA	0.02 mg
5	Ascorbic acid	25 mg
	Gelrite	2.4 g

Appendix 2: GUS assay stock solutions and buffer Components

	Component	Quantity	Final Volume
1	Tris (10 µM), NaCl (50 mM) (pH 7.2)	1.21 g Tris, 2.92 g NaCl	1 L
2	X-Gluc (10 mg/ml)*	100 mg	10 ml
3	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

Appendix 3: Cetyltrimethylethyl ammonium (CTAB) extraction buffer

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

Appendix 4: Medium components used to optimize FEC regeneration

Reagents	Molecular weight	Full strength MS	Half strength MS	GD + Vitamins	FIM + Vitamins
		mg/L	mg/L	mg/L	mg/L
Ammonium Nitrate (NH4NO3)	80.04	1650	825.00	1000	165
Ammonium Phosphate, Monobasic	115.03				
Ammonium Sulfate	132.14				
Boric Acid (H3BO3)	61.83	6.2	3.10	0.3	6.2
Calcium Chloride, Anhydrous (CaCl2)	110.99				
Calcium Chloride (CaCl2, 2H2O)	146.99	440	220.00		440
Calcium Nitrate (Ca(NO3)2)	164.09				
Calcium Nitrate dihydrate Ca(NO3)2.2H2O	236.15			208.81	
Cobalt Chloride·6H2O (CoCl2.6H2O)	237.93	0.025	0.01	0.025	0.025
Cupric Sulfate·5H2O (CuSO4.5H2O)	249.69	0.025	0.01	0.025	0.025
C10H12N2O8FeNa = 367.0 (Fe.Na.EDTA)	367			36.7	
Na2 EDTA·2H2O	372.24	37.3	18.65		37.3
Ferrous Sulfate·7H2O	278.02	27.8	13.90		27.8
Ferrous Sulfate·7H2O	278.01				
Magnesium Sulfate, 7H2O (MgSO4,7H2O)	246	370	185.00		370
Magnesium Sulfate, Anhydrous (MgSO4)	120.38			17.1	
Manganese sulfate·4H2O (MnSO4.4H2O)	223.06	22.3	11.15		22.3
Manganese Sulfate·H2O (MnSO4.4H2O)	169.02			1	
Sodium Molybdate(VI)·2H2O (Na2MoO4,2H2O)	241.95	0.25	0.13	0.025	0.25
Nickel Sulfate·6H2O	262.85				
Potassium Chloride (KCl)	74.55			65	
Potassium Iodide (KI)	166	0.83	0.42	0.8	0.83
Potassium Nitrate (KNO3)	101.01	1900	950.00	1000	190
Potassium Phosphate, Monobasic, Anhydrous (KH2PO4)	136.09	170	85.00	300	17
Potassium sulfate (K2SO4)	174.26		0.00		
Sodium Nitrate	142.14		0.00		
Sodium Phosphate Monobasic·H2O	137.99		0.00		
Zinc Nitrate·6H2O	297.49		0.00		
Zinc Sulfate·7H2O	287.58	8.6	4.30	0.3	8.6
Vitamins					
		mg/L	mg/L	mg/L	mg/L
Glycine		2	2	4	2
myo -Inositol		100	100	100	100
Nicotinic Acid		0.5	0.5	1	0.5
Pyridoxine·HCI		0.5	0.5	1	0.5
Thiamine·HCI		0.1	0.1	10	10
Other components					
Proline		1000	1000	1000	1000
Casein hydrolysate		600	600	600	600
Sucrose (mg/L)		20000	20000	20000	20000
Picloram (mg/L)		0.5	0.5	0.5	0.5
pH		5.7	5.7	5.7	5.7

Appendix 5: Composition of protoplast washing solution (PWS)

S. No.	Component	mg/L
1	Calcium chloride	148
2	Cupric sulfate	0.025
3	Magnesium sulfate	246
4	2-(N-morpholino)ethanesulfonic acid (MES)	976
5	Potassium nitrate	101
6	Potassium iodide	0.160
7	Potassium phosphate monobasic	27.2
8	Mannitol	130,000

Appendix 6: Details of CRISPR/Cas9 plasmid constructs used in this study.

Construct	**Cas 9**	**Binary vector backbone**	**Plant selection marker**	**gRNA**
CRISPR 1 (p2300-Cas9-DrPDSg1g2-GFP)	P35S-Cas9	pCAMBIA2300	*npt*II	gRNA1: GACTACCCGAGGCCTGAAT gRNA2: TAGCTGTCCACGCCCAAAT
CRISPR 2 (p2300-Cas9-DrPDSg3g4-GFP)	P35S-Cas9	pCAMBIA2300	*npt*II	gRNA1: GACTACCCGAGGCCTGAAT gRNA2: TAGCTGTCCACGCCCAAAT
CRISPR 3 (p1300-Cas9-gDrPDSg3g4)	P35S-Cas9	pCAMBIA1300	*Hpt*	gRNA3: CTTGAGAGTTCAATCATCAT gRNA4: AGGCCTGAATTGGAGAACAC
CRISPR 4 (p1300-Cas9-gDrPDSg5g6)	P35S-Cas9	pCAMBIA1300	*Hpt*	gRNA5: GGACTTTTGCCAGCCATGGT gRNA6: TAAGACGATTGAGCTCAACT
CRISPR 5 (p1300–P19–Cas9-gDrPDSg3g4)	P35S-P19-Cas9	pCAMBIA1300	*hpt*II	gRNA3: CTTGAGAGTTCAATCATCAT gRNA4: AGGCCTGAATTGGAGAACAC
CRISPR 6 (p1300–P19–Cas9-gDrPDSg5g6)	P35S-P19-Cas9	pCAMBIA1300	*Hpt*	gRNA5: GGACTTTTGCCAGCCATGGT gRNA6: TAAGACGATTGAGCTCAACT
CRISPR 7 (pMDC32-Cas9-DrPDCg7g8)	P35S-Cas9	pMDC32	*Hpt*	gRNA7: GAACTCTCAAGTTAGTAGT gRNA8: TAACGAGTATATACCACGT