GENETIC DIVERSITY AND *IN-VITRO* REGENERATION OF TARO (*Colocasia esculenta* (L.) Schott.) GERMPLASM IN KENYA

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Genetic Diversity and *In-vitro* Regeneration of Taro (*Colocasia esculenta* (L.) Schott.) Germplasm in Kenya

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DECLARATION

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

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This thesis has been submitted for examination with our approval as the University Supervisors.

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DEDICATION

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ACRONYMS AND ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
APE	Analysis of Phylogenetics and Evolution
DArT	Diversity Array Technology
EMBL	The European Molecular Biology Laboratory
FAO	Food and Agricultural Organization of the United Nations
GA	Genome Analyzer
GBS	Genotyping by Sequencing
GWAS	Genome Wide Association Studies
IPGRI	International Plant Genetic Resources Institute
PIC	Polymorphic Information Content
QTL	Qualitative Trait Loci
RAPD	Random Amplification of Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
SGS	Second Generation Sequencing
SIM	Shoot Induction Media
SOLiD	Sequencing by Oligo Ligation Detection
TaBV	Taro Bacilliform Virus

TAE	Tris-Acetate-EDTA
TDZ	Thidiazuron
TGS	Third Generation Sequencing
UV	Ultra Violet Light

ABSTRACT

Taro (*Colocasia esculenta*) is a clonally propagated aroid and is largely grown in humid tropical areas worldwide. This crop was first domesticated in South-east Asia, spreading throughout the world and is now an important crop in Asia, the Pacific, Africa and the Caribbean. Besides, it is the most important edible species of the monocotyledonous family Araceae. Almost all parts of the taro plant are utilized. The corms are baked, roasted, or boiled and are a good source of carbohydrates. Its leaves are frequently consumed as a vegetable representing an important source of vitamins, and even petioles and flowers are utilized in certain parts of the world. Taro production is majorly affected by low genetic diversity and lack of quality planting materials. This study aimed at assessing genetic diversity and developing an *In-vitro* regeneration protocol for Kenyan Taro germplasm. First, the genetic diversity of all 186 samples were classified into four major groups (A, B, C and D), using the statistical R software and a simple circular phylogenetic tree was generated, showing within population variation than among the population variation. Secondly, an efficient protocol for direct organogenesis was established for two Taro varieties (Dasheen and Purple wild), using sterilized upper part of the corm and the base of the petioles. These explants were evaluated for their potency for shoot induction on varied concentrations of 6- Benzylaminopurine (BAP) concentrations that included 0, 0.5, 1, 2 and 3 mg/l. The highest shoot induction was observed on media supplemented with 2 mg/l BAP for both Dasheen and Purple wild varieties while the lowest was on media with 0.5 mg/l for both varieties. The highest rooting response that also gave the shortest roots was observed in media supplemented with 0.5mg/l IBA (12.867) for Dasheen and 11.933 for Purple wild variety. Apical meristems excised under a microscope and cultured in callus induction medium showed swelling and formation of embryo-like structures within the first four weeks of culture. The first stages of callusing, including swelling of the embryo and colour change were seen after 8 weeks of culture. After subsequent subculturing into similar media for 4 weeks, callus became more distinguishable from swollen embryos. The callus texture was more visible after two weeks and friable and compact calli could be seen. Calli was best formed in media containing 10 µM 2,4-D and 2 µM TDZ for both Dasheen and Purple wild varieties. However, Dasheen had the highest formation of 77.8% and Purple wild had 71.1%. This regeneration protocol is very important for future Taro production to enhance quality and quantity planting materials.

CHAPTER ONE

INTRODUCTION

1.1 General Background Information

Taro (*Colocasia esculenta* (L.) Schott) is an important food crop of the Araceae family. It is a monocotyledonous, succulent, glabrous and perennial herb (Verma *et al.*, 2017) . Colocasia has two varieties, *C. esculenta* var *esculenta,* commonly called Dasheen and *C. esculenta* var *antiquorum* often called eddoe (Pe *et al.*, 2015). The Dasheen varieties have large central corms unlike the eddoes that have a small central corm and many smaller cormels (Lebot *et al*., 2010. Physiologically, Taro has large heart shaped sagittate leaves (Pe *et al.*, 2015). It originated in the tropical America and spread later to South East Asia, the Pacific islands and Africa (Doungous *et al.*, 2015).Worldwide Taro production is estimated at 11.8 million tons per annum (Sujina *et al.*, 2017) and its production is majorly tilted to west Africa compared to East Africa . The FAO reports have no statistics showing East Africa including the Kenyan Taro production levels (Pe *et al.*, 2015).

Despite the importance of Taro in food and nutrition security, incomes, and livelihood of rural people, the current global productivity of Taro is estimated around 5.39 t/ha, which is about 5% of its experimental yield (Lebot, 2009). Low productivity and limited cultivation of Taro are attributed to several factors, including a high incidence of pest and diseases; scarcity of quality planting materials; labor-intensive traditional production system; difficulties in post-harvest handling and marketing; and low investment in Taro research and extension (Onwueme, 1999). Taro is commonly propagated through vegetative means like side suckers, small corms, and corm pieces. These propagation materials are bulky in nature and their availability as planting materials is seasonal. Moreover, these vegetative planting materials often serve as vehicles for various pests and diseases from one Taro crop to the next crop and subsequently compromise the quality and quantity of Taro production. Therefore, the availability of quality planting material in sufficient quantities has been a major challenge among Taro producers. More importantly,

the Taro seeds system lacks an efficient and high throughput micropropagation system that helps in the mass propagation of quality planting materials.

Micropropagation provides a sustainable solution to the problems associated with conventional propagation by enabling rapid production of high-quality, disease-free, and uniform planting materials (IAEA, 2004). Since tissue culture is performed in a controlled laboratory environment, the multiplication of planting materials could be achieved all year-round. The tissue culture technique has been successfully applied on thousands of plant species (Fay, 1992; Villalobos and Engelmann, 1995; Jackson *et al*., 2001; Sarasan *et al*., 2006), but the use of this technique for *in-vitro* regeneration and mass production of Taro planting material has been limited in Kenya primarily due to the lack of efficient regeneration and mass multiplication protocols. Several protocols for Taro tissue culture are available (Yam *et al.,* 1990; Tuia, 1997; Minas, 2002; Hossain, 2012), including the one that uses locally available nutrients to substitute MS medium (Ngetich *et al.,* 2015). All these efforts to develop Taro micropropagation protocols could be due to variety-dependent response of Taro to micropropagation methods, variations in growth media and culture conditions, types of explants used in micropropagation, and to reduce the costs.

The direct and indirect shoot organogenesis are considered the best micropropagation method due to a low cost, minimal soma-clonal variations, and high throughput production system (Mukami *et al*., 2018; Burner & Grisham, 1995). Apical meristem culture eliminates viruses in many plant species, thus helps in the production of disease-free planting materials and achieving better yields than conventional planting materials (Wang and Valkonen, 2008, 2009).

1.2 Economic Importance of Taro.

Taro sustains food security in local markets and also brings import earnings. A lot of Taro is produced and consumed on a subsistence basis, whereas a considerable amount is utilized as a cash crop. However, surpluses from the subsistence production manage to find their way to the market, thereby playing a role in poverty alleviation. Besides, it is

considered a prestige crop, and the crop of choice for royalty, gift-giving, traditional feasting, and the fulfilment of social obligations. In Oceania and South-east Asia, Taro features prominently in the folklore and oral traditions of many cultures. Samoa and Tonga have a depiction of Taro as the main feature on one of their currency coins.

The socio-cultural attachment to Taro means that Taro itself has become a totem of cultural identification. People of Pacific Island origin continue to consume Taro wherever they may live in the world, not so much because there are no substitute food items, but mainly as a means of maintaining links with their culture. This cultural attachment to Taro has spawned a lucrative Taro export market to ethnic Pacific Islanders living in Australia, New Zealand, and western North America. Taro corms can be boiled, baked, roasted or even fried. The corms can also be used as a soup thickener. Taro is a good source of both amylase and amylopectin (Obidiegwu *et al.*, 2016) that are 98.8% digestible and this is very important for persons with digestive problems. The Taro leaves contain protein, carotene, Potassium, Calcium, Phosphorous, iron, riboflavin, thiamine, niacin, vitamin A, vitamin C and dietary fibre (Bradbury and Holloway *et al*., 1998). Taro leaf extracts are used as decongestants, anti-oxidants, anti-bacterial and expectorants (Brown *et al.*, 2005). In fact, in the Malay Peninsula, various parts of Taro are used in traditional medicinal practice. The Taro inflorescences are used as local food spice (Ukpong *et al*., 2014). In addition, Taro is a good source of vitamin B complex than whole milk (Rashmi *et al*., 2018), therefore it is a better option for the infant food formulae, for children who are allergic to milk. Furthermore, the Taro industry provides meaningful employment to a large number of people, mostly in rural areas. Where Taro exportation occurs, facilities for cleaning, sorting, packing, and shipping Taro provide additional avenues for poverty alleviation and employment generation in the rural areas.

1.3 Climatic Requirements of Taro

Taro gives optimum yields under rainfall between 1500-2000mm and cannot tolerate frosty conditions but average temperatures of 21 ^{0}C. Yields in high altitudes tend to be

poor compared to those in lowland areas due to its sensitivity to temperature. Taro is capable of tolerating heavy soils on which flooding and waterlogging can occur since it is able to transport oxygen from the aerial parts down to its roots (Omwueme *et al*., 1999). The optimal pH range for Taro production ranges between 5.5- 6.5. At this range Taro is able to form beneficial associations with vesicular arbuscular mycorrhizae, which facilitate nutrient absorption. In Egypt, Taro has been used in reclaiming saline soils and this is a possibility that Taro is able to exploit difficult ecologies where other crops cannot. (Kahane *et al*., 2013.

Partly because of their large transpiring surfaces, Taro plants have a high requirement for moisture for their production. Taro thrives best under very wet or flooded conditions. Dry conditions result in reduced corm yields. Corms produced under dry conditions also tend to have a dumb-bell shape; the constrictions reflect periods of reduced growth during drought. Partially due to its temperature sensitivity, Taro is essentially a lowland crop. Yields at high altitudes tend to be poor. In Papua New Guinea, for example, the maximum elevation for Taro cultivation is 2,700m. The highest yields for Taro are obtained under full sunlight intensity. However, they appear to be more shade-tolerant than most other crops. This means that reasonable yields can be obtained even in shade conditions where other crops might fail completely. This is a particularly important characteristic which enables Taro to fit into unique intercropping systems with tree crops. Daylight also affects the growth and development of Taro. The formation of corms/cormels is promoted by short-day conditions, while flowering is promoted by long-day conditions. Taro is able to tolerate heavy soils on which flooding and waterlogging can occur. Indeed, the Dasheen type of Taro does best when grown in such soils. It seems that under flooded or reducing soil conditions, Taro plants are able to transport oxygen (through their spongy petioles) from the aerial parts down to the roots. This enables the roots to respire and grow normally even if the surrounding soil is flooded and deficient in oxygen. In practice, however, flooded Taro fields must be aired periodically in order to avoid iron and manganese toxicity under the reducing soil conditions. Poor soils, such as the red soils in certain parts of Fiji, tend to give low yields of Taro (Palanivel et al., 2021).

1.4 Constrains of Taro production

Taro production in Kenya is extremely low in comparison to other tuber crops such as cassava, sweet potatoes and yams. This is mainly attributed to pests and diseases.

1.4.1 The Taro Beetle

The Taro beetle belongs to the genus Papuana. An adult beetle is black, shiny, and 15-20 mm in length. These pests feed on the Taro corm, leaving large holes that degrade the market quality of the corm. Additionally, these wounds promote attack by rot-causing organisms. The eggs are laid 5-15 cm beneath the soil close to the host plant (Rana *et al*., 2017). A wide range of plants, including Elephant grass have been found to be hosts for Taro beetle breeding (Joshi *et al*., 2020).

1.4.2 Taro Leaf Blight Disease

Taro leaf blight is caused by the fungus *Phytophthora colocasiae*. It was first reported in Java about a century ago, and has since spread to various parts of Asia and the Pacific. The list of countries where it has been reported include Indonesia, Papua New Guinea, Solomon Islands, Hawaii, Samoa, American Samoa, Thailand and the Philippines (Miyasaka *et al*., 2019).

The disease begins as purple-brown water-soaked lesions on the leaf. A clear yellow liquid oozes from the lesions. These lesions then enlarge, join together and eventually destroy the entire lamina in 10-20 days. Free water collecting on older leaves, as well as high temperature and high humidity are conducive to onset and spread of the disease and germination of the spores. The disease can be spread from plant to plant by wind and splashing rain. Spores survive in planting material for three or more weeks. Thus, infected planting material is one common means of spreading the disease over long distances and from season to season.

1.4.3 Bobone Virus Disease

The Alomae-Bobone virus disease complex is caused by a complex of two or more viruses acting together. The two viruses that are definitely involved are the Taro large bacilliform virus (TLBV) which is transmitted by the plant hopper Tarophagus proserpina, and the Taro small bacilliform virus (TSBV) which is transmitted by the mealybug Planococus citri (Praneetha *et al*., 2022).

Alomae first starts as a feathery mosaic on the leaves. Lamina and veins become thick. The young leaves are crinkly and do not unfold normally. The petiole is short and manifests irregular outgrowths on its surface. The entire plant is stunted and ultimately dies. The symptoms of Bobone are similar to those of Alomae, but the leaves are more stunted and the lamina is curled up and twisted. With Bobone, complete death of the entire plant does not usually occur. Severe cases of Alomae can result in total crop loss, while Bobone can cause up to 25% yield loss.

1.4.4 Dasheen Mosaic Virus

Dasheen Mosaic virus (DsMV) is the most common viral disease that attacks Taro. It is caused by a stylet-borne, flexuous, rod-shaped virus, which is spread by aphids. It is a yield depressing disease. (Pe *et al.*, 2015).

1.4.5 Other Diseases and Pests Associated with Taro

In several instances these diseases and pests have been considered minor yet they become quite severe in certain locations or at certain times during the cropping season. One very common disease is the corm and root rots caused by the fungi *Pythium spp* and *Phytophthora* (Radmer *et al*., 2017). Additionally, nematodes, Taro plant hopper, aphids, Taro horn worm, and armyworms are Taro pests that have been generally underrated (Altieri *et al*., 2018). The plant hopper, *Tarophagus Proserpina,* transmits virus diseases and could cause wilting and death of the plant after heavy infestation (Gosai R, 2016). The Taro horn worm defoliates the plant (Reddy P, 2015). Lastly, the armyworms are

cluster caterpillars which can also do extensive damage to the leaves (Maruthadurai *et al*., 2020).

1.5 Methods of Taro Propagation

Taro is a vegetative propagated plant through the use of side suckers (Antwi *et al*., 2017) produced as a result of lateral proliferation of the main plant in the previous crop (Manju *et al*., 2017), small corms that are unmarketable (Vidigal *et al*., 2016), pieces of apical corm with the bases of the petioles attached from harvested plants (huli) (Sagoe *et al*., 2018) or the use of large corms cut into smaller pieces (Kamarudin *et al*., 2018). Farmers mainly prefer the use of apical corms with the bases of petioles since they grow very fast and they do not utilize the edible corm part of the Taro plants (Tumuhimbise *et al*., 2009). However, these planting materials are perishable and this forces the farmers to plant them back to the soil immediately after harvesting the corms.

The use of huli is particularly advantageous because it does not entail the utilisation of much material that is otherwise edible (Buke & Gidago, 2016). Moreover, huli establish very quickly and result in vigorous plants (Glasse, 2018). However, huli are best adapted to situations where planting occurs shortly after harvesting, since protracted storage of huli is not advisable (Okoye & Oni, 2017). Where corm pieces are used, it is sometimes advisable to pre-sprout the pieces in a nursery before they are planted in the field (de Chavez *et al*., 2019). This enables sprouts to appear on the pieces before they are moved to the field. Side suckers and small corms may also be kept in nurseries to develop good sprouts, especially if there is a long time between the previous harvest and the next planting (Laxminarayana *et al*., 2016). The availability of planting material is a ubiquitous problem in the production of Taro. This is particularly so in places like Tonga, an island country in Polynesia, where occasional droughts reduce the quantity of available planting material for years after every drought (Davidson J.M., 2013).

Ngetich *et al.,* 2015, established a low cost alternative protocol for the micropropagation of Taro plantlets. This is an effective method but time consuming and vey cumbersome. Also, it is not easy to follow so as to generate clean tissue culture planting material.

1.6 Problem Statement

Taro is an underutilized crop that has the potential to ameliorate malnutrition and contribute to food security in East Africa. However, reports by the International Institute of Tropical Agriculture (IITA) postulate that the demand for Taro is always higher than the actual supply. This trend is projected to continue, particularly due to population increase (Asiedu & Sartie, 2010). Taro production has limited information on genetic diversity that constrains Biotechnologists and Breeders from developing varieties that are tolerant to biotic and abiotic stresses. However, this study aims at finding different varieties of the Kenyan germplasm. This germplasm characterization is an important future resource for Taro breeding programs and genomic studies in Kenya.

Besides, Kenyan Taro production lacks quality and quantity planting materials, which promotes both fungal and bacterial infections hence low yields for farmers. The various biotic and abiotic factors, include diseases and pests, low yield potential, inadequate planting material, and decreasing soil fertility (Korada *et al.,* 2010). Furthermore, there are approximately 30% tuber losses (Mignouna *et al.,* 2014). This direct organogenesis protocol is easy to follow and it can be used for production of large number of *in vitro* plantlets for commercial production.

This protocol is reproducible and research agencies should adopt it to mass regenerate Taro and conduct genetic engineering experiments that will improve biotic and abiotic adaptability of Taro to enhance food security and generate cash for the poor farmers.

Genetic engineering has consistently proven to supplement conventional breeding towards the improvement of many crops, including vegetative 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 Taro variety has been improved by the transgenic approach. This delayed progress is primarily due to the lack of Taro regeneration and transformation systems. Thus, immense efforts are needed to develop Taro regeneration systems.

1.7 Justification

Despite being an orphan crop, Taro has a wide range of economic importance. Its corms are primarily a good source of starch, providing a wide range of vitamins and amino acids (Rao *et al*., 2010). Additionally, its leaves are a good source of protein (Shekade *et al*., 2018). However, reports (FAOSTAT, 2019) have shown that the demand for Taro is always higher than the actual supply. This trend is projected to continue, particularly due to population increase (Matthews & Ghanem, 2021). The low productivity is due to various biotic and abiotic factors. These include diseases and pests, low yield potential, and inadequate planting material (Fufa *et al*., 2021). These challenges are further compounded by 60% losses during storage (Kaushal *et al*., 2015). Conventional methods have been employed towards the improvement of Taro to produce high-yielding, pest, and disease resistant varieties (Helmkampf *et al*., 2018). However, the lack of genetic characterization and clean planting material for Taro is a main challenge towards conventional Taro breeding.

This study has established genetic diversity and an *In-vitro* regeneration system to overcome several challenges breeders and biotechnologists face towards improving Taro. Genotyping by sequencing (GBS) is an appropriate technique that generates single nucleotide polymorphisms (SNP's) data for genome -wide analysis of genetic diversity. Besides, it is an approach that detects SNPs in a large segregating or mutant population and combines with scoring hence a rapid and direct study of its diversity is targeted towards the mapping of a trait or a mutation of interest (Deschamps, Llaca and May, 2012). There are outstanding advantages of this technique over other platforms in that it identifies abundant SNPs at low cost, does discovery and genotyping concurrently, reduces ascertainment biasness compared to array- based markers, and is relatively easy in automation according to Poland *et al.*, 2012 and Elshire *et al.*, 2011. In conclusion GBS

has successfully been used in Genome- wide association studies (GWAS), genomic diversity studies, genetic linkage analysis, molecular marker discoveries, and genomic selection under a large scale of plant breeding programs therefore it will help identify SNPS in this study.

Secondly, *In-vitro* regeneration of Taro will enhance the production of quality and high vigour plantlets, leading to mass plant production. Additionally, the whole tissue culture concept has several advantages over the traditional methods of cultivation. These include; the production of exact copies of plants that produce particularly good flowers, fruits, or have other desirable traits; quick production of mature plants; the production of multiples of plants in the absence of seeds or necessary pollinators to produce seeds; the regeneration of whole plants from plant cells that have been genetically modified, and the production of plants in sterile containers that allows them to be moved with greatly reduced chances of transmitting diseases, pests, and pathogens.

1.8 Objectives

1.8.1 General Objective

To assess genetic diversity and develop *In-vitro* regeneration protocols for Kenyan Taro (*Colocasia esculenta* (L.) Schott) germplasm.

1.8.2 Specific Objectives

1. To determine genetic diversity of Taro germplasm using single nucleotide polymorphisms (SNP's).
2. To regenerate Kenyan Taro germplasm through direct organogenesis.
3. To regenerate Kenyan Taro germplasm through indirect somatic embryogenesis.

1.9 Null hypothesis

1. There is no genetic diversity in Taro germplasm in Kenya.
2. It is not possible to regenerate Kenyan Taro germplasm through direct organogenesis.
3. It is not possible to regenerate Kenyan Taro germplasm through indirect somatic embryogenesis

CHAPTER TWO

LITERATURE REVIEW

2.1 **Overview** of Taro

2.1.1 **Origin**, Distribution, and Taxonomy

Taro belongs to the family Araceae in the genus *Colocasia* (Ahmed *et al*., 2020). There are two species of Taro (Yin *et al*., 2021). They include; *Esculenta* var *esculenta* commonly the Dasheen and *Esculenta* var *antiquorum* commonly the eddoe (Maretta *et al*., 2020). Taro probably originated in the Indo-Malaysian Peninsula over 50,000 years ago (White JP *et al*., 1982.) In addition, there is evidence of human use of the plants 28,000 years ago in the Solomon Islands (Loy TH *et al*., 1992). Its dispersal likely began in about 1600 to 1200 BC when long distance voyaging canoes were introduced. The crop was further taken eastwards into Fiji and western Polynesia and then into eastern Polynesia by migrating voyagers around 800 to 900 AD (Coates DJ *et al.,* 1988.). The geographic distribution of *Colocasia* species indicates that it is distributed from South Asia to South East Asia including China and Indonesia, in the lowland tropical areas as well as the Himalayan mountains (Ahmed I *et al*.,2014). Taro likely arrived the Madagascar Islands through the migrating Indonesians as early as 500 AD from where it spread across Africa to the Guinea coast (Yamaguchi M, *et al.,* 1983.). Currently Taro production is throughout the tropics, sub- tropics and warm temperate regions of Asia, Oceania, Africa and America (Prakash *et al.,* 2000.)

2.1.2 Cultivated Varieties in Kenya

Taro's morphology is quite variant (Palapala *et al.*, 2016)**.** Taro has two species that are mainly cultivated. They include the "Dasheen" and the "eddoes "varieties (Hidayatullah *et al*., 2020). In Kenya the purple and green wild types are also cultivated by farmers. According to IPGRI, 1999, the main differentiating characteristic between the Dasheen and the eddoe varieties is that the eddoe varieties have side tubers (cormels) that may be

5-20 in number and that they grow as big as the mother corm. In Kenya Taro cultivation is mainly in the central and western counties of Kenya, however they are also found in some parts of the Eastern Kenyan counties. This is due to the presence of water catchment areas in those counties. These areas also have fertile soils that are suitable for Taro farming.

2.2 Molecular markers.

Molecular markers are portions of DNA sequences dispersed along the genome used to identify a given organism (Shamim *et al*., 2017). They are useful in different areas such as genetic mapping, paternal tests, detecting mutant genes which are connected to hereditary diseases, cultivars identification, marker assisted breeding of crops and population history (Hasan *et al*., 2021). Today many plant breeders utilize molecular markers to proof and identify desirable traits of importance in different plants (Bhat *et al*., 2016). There are many advantages of molecular markers compared with morphological and biochemical markers which are laborious and time consuming (Kumar *et al*., 2018). A good molecular marker should be easily available, their assay should be rapid and easy, reproducible, highly polymorphic and selectively neutral to environmental conditions (Ramesh *et al*., 2020).

Different molecular markers are used to estimate DNA polymorphism and are classified as hybridization-based markers and polymerase chain reaction (PCR)-based markers (Amon T & Nongdam P, 2017). In hybridization based markers DNA profiles are visualized by hybridizing the restriction endonuclease digested DNA fragment, to a labelled probe, which is a DNA fragment of known sequence (Amiteye S, 2021). PCR based markers involve *in vitro* amplification of particular DNA sequences with the help of specifically or arbitrarily chosen oligonucleotide sequences (primers) and a thermostable DNA polymerase enzyme (Parveen *et al*., 2016). The amplified DNA fragments are separated by electrophoresis and banding patterns are detected by different methods like staining (using ethidium bromide dye) and autoradiography (Gomes-Pereira M & Monckton D, 2017). With the advent of thermostable DNA polymerase, the use of

PCR in research and clinical laboratories has increased tremendously. PCR is extremely sensitive and operates at a very high speed (Hedman J & Radstrom P, 2013). Its application for diverse purposes has opened up a multitude of new possibilities in the field of molecular biology.

2.2.1 Types of Molecular Markers

Molecular markers are grouped based on their different abilities of showing homozygosity (dominant marker) or heterozygosity (co-dominant marker) (Ramesh *et al*., 2020). The most commonly used dominant DNA marker for genetic diversity in plants are: Random Amplified Polymorphic DNA (RAPD) (Williams *et al*., 1990), DNA amplification fingerprinting (DAF) (Caetano-Anolles *et al*., 1991), Arbitrarily primed polymerase chain reaction (AP-PCR) (Welsh & McClelland 1990), Intersimple sequence repeat (ISSR) (Zietkiewicz *et al*., 1994 and Amplified Fragment Length Polymorphisms (AFLP) (Vos *et al*., 1995), whereas the most common used co-dominant markers are: Restriction Fragment Length Polymorphisms (RFLP) (Botstein *et al*., 1980), Microsatellites (SSR) (Akkaya *et al*., 1992); Sequence characterised amplified regions (SCAR) (Paran & Michelmore, 1993), Cleaved amplified polymorphic sequence (CAPS) (Konieczny & Ausubel, 1993), Expressed sequence tag (EST) (Adams *et al*., 1991) and Single Nucleotide Polymorphism (SNP) (Jordan & Humphries, 1994) and sequence tagged sites (STS) (Olsen *et al*., 1989). Both dominant and co-dominant markers can be used to detect DNA polymorphism, which is further used to assess the level of genetic variation in diverse populations and can indicate population history, patterns of migration, and breeding structure.

2.2.1.1 Dominant DNA Markers

2.2.1.1.1 Arbitrarily Sequence Markers

Random Amplified Polymorphic DNA (RAPD), Arbitrarily Primed PCR (AP-PCR), and DNA Amplification Fingerprinting (DAF) have been collectively termed Multiple

Arbitrary Amplicon Profiling (MAAP) (Caetano-Annolles, 1994). These three techniques were first used to amplify any species DNA fragments without prior sequences information (Semagn *et al*., 2006). The difference among MAAP techniques include modifications in amplification profiles by changing primer length, sequence and annealing temperature (Caetano-Anolles *et al*., 1992), the thermostable DNA polymerase (Bassam *et al*., 1992), the number of PCR cycles (Caetano-Anolles *et al*., 1991; Welsh and McClelland 1991; Micheli *et al*., 1993; Jain *et al*., 1994), enzymatic digestion of template DNA or amplification products (Caetano-Anolles *et al*., 1993) and alternative methods of fragment separation and staining. These three techniques produce markedly different amplification profiles, varying from quite simple (RAPD) to highly complex (DAF) patterns. These marker techniques are quick, easily generated by PCR and require no prior sequence information.

2.2.1.1.2 Inter-Simple Sequence Repeat (ISSR)

Inter-Simple Sequence Repeat (ISSR) involves amplification of DNA segment present at an amplifiable distance in between two identical microsatellite repeat regions oriented in opposite direction (Abate T, 2017). ISSR markers are highly polymorphic and are used on genetic diversity, gene tagging, phylogeny, evolutionary biology and genome mapping studies (Reddy *et al*. 2002). ISSR PCR is a technique, which overcomes the problems like high cost of AFLP, low reproducibility of RAPD, and the flanking sequences to develop species specific primers for SSR polymorphism (Amiteye S, 2021). ISSR is quick, simple, highly reproducible and the use of radioactivity is not essential (Jabbarzadeh *et al*., 2010). ISSR markers usually show high polymorphism (Kojima *et al*. 1998), and with the most important advantage that no prior information about genomic sequence is required (Bornet & Branchard 2001).

2.2.1.1.3 Amplified Fragment Length Polymorphsim (AFLP)

AFLP technique combines the power of RFLP with the flexibility of PCR-based technology by ligating primer recognition sequences (adaptors) to the restricted DNA

(Krawczyk *et al*., 2016). The key feature of AFLP is its capacity for "genome representation": the simultaneous screening of representative DNA regions distributed randomly throughout the genome (Caballero & Quesada, 2010). AFLP markers can be generated from DNA of any organism without initial investment in primer/probe development and sequence analysis (Tiwari *et al*., 2013). Both good quality and partially degraded DNA can be used for digestion but the DNA should be free of restriction enzyme and PCR inhibitors. AFLP analysis involves restriction digestion of genomic DNA with a combination of rare cutter (EcoRI or PstI) and frequent cutter (MseI or TaqI) restriction enzymes. Double stranded oligonucleotide adaptors are then designed in such a way that the initial restriction site is not restored after ligation. This technique has several advantages such as it is highly reproducible and reliable (Jones *et al*. 1997). Secondly, it does not require any DNA sequence information from the organism under study. Thirdly, it is information-rich due to its ability to analyze a large number of polymorphic loci simultaneously with a single primer combination on a single gel as compared to RFLPs and microsatellites (Russell *et al*. 1997). Besides, co-migrating AFLP amplification products are mostly homologous and locus specific with exceptions in polyploidy species. Lastly, both good quality and partially degraded DNA can be used for digestion but the DNA should be free of restriction enzyme and PCR inhibitors.

2.2.1.2 Co-Dominant Markers

2.2.1.2.1 Restriction Fragment Length Polymorphism (RFLP)

RFLPs are inherited naturally occurring Mendelian characters. They have their DNA rearrangements due to evolutionary processes, unequal crossing over, mutations within the fragments, and point mutations within the restriction enzyme recognition site (Schlotterer & Tautz, 1992). In RFLP analysis, a restriction enzyme digests genomic DNA and then resolved by gel electrophoresis and western blotting (Southern 1975). Specific banding patterns are then visualized by hybridization using a labeled probe. These probes are mostly species-specific of about 0.5–3.0 kb in size, obtained from a cDNA library or a genomic library. The genomic libraries are easy to construct but a large number of

scattered duplicates are found in inserts that makes complex patterns. This problem can be overcome by using methylation sensitive restriction enzyme PstI which facilitates DNA sequences of small sizes, preferred in RFLP analysis (Figdore *et al*., 1988). In contrast cDNA libraries are difficult to construct, however, they are more popular as actual genes are analyzed and they contain fewer repeat sequences (Miller & Tanksley 1990; Landry & Michelmore 1987).

This technique has several advantages including genetic map construction. Besides, they are codominant and reliable markers in linkage analysis and breeding. Also, they can be easily determined in homozygous or heterozygous state of an individual. Nevertheless, it has several disadvantages such as it requires a large amount of DNA for restriction digestion and Southern blotting. It is expensive, time-consuming and hazardous.

2.2.1.2.2 Microsatellites (SSR)

Microsatellites, also known as simple sequence repeats (SSR), variable number tandem repeats (VNTR) and short tandem repeats (STR) are tandem repeats motifs of 1-6 nucleotides found at high frequency in the nuclear genomes of most taxa (Beckmann & Weber, 1992). A microsatellite locus typically varies in length between 5 and 40 repeats, but longer strings of repeats are possible. Dinucleotide, trinucleotide and tetranucleotide repeats are the most common choices for molecular genetic studies. Dinucleotides are the dominant type of microsatellite repeats in most vertebrates characterized so far, although trinucleotide repeats are most abundant in plants (Beckmann & Weber, 1992; Kantety *et al*., 2002; Chen *et al*., 2006). With the abundance of PCR technology, primers that flank microsatellite loci are simple and quick to use, but the development of correctly functioning primers is often a tedious and costly process. However, once they are developed and characterized in an organism, microsatellites are powerful for a variety of applications because of their reproducibility, multiallelic nature, codominant inheritance, relative abundance and good genome coverage (Liu & Cordes, 2004).

These markers are used for plant breeding, conservation biology and population genetics (Coates & Byrne, 2005). In this technique, little amount of DNA is required, which does not have to be of high quality. Lastly, the results obtained from this technique are simple to interprete (de Vicente & Fulton, 2003). Despite having several advantages, they also have drawbacks. For instance, there is need for a known sequence to be amplified (Weising *et al*., 2005). Besides, developing new microsatellites are expensive and time consuming (Coates & Byrne 2005).

2.2.1.2.3 Sequence Characterized Amplified Regions for Amplification of Specific Band (SCAR)

Michelmore (1991) and Martin (1991) were the first to introduce this technique, in which RAPD marker termini are sequenced and longer primers of 22–24 nucleotide bases long are designed for specific amplification of a particular locus. It shows similarity with STS markers in construction and application. The presence or absence of the band represent variation in sequence.

When compared to RAPD markers, SCARs have the ability to detect only a single locus, their amplification is less sensitive to reaction conditions and they can potentially be converted into codominant markers (Paran & Michelmore, 1993). Subsequently, by comparing SCARs to arbitrary primers, SCARs exhibit several advantages in mapping studies (codominant SCARs are informative for genetic mapping than dominant RAPDs). Besides, they are map based cloning as they can be used to screen pooled genomic libraries by PCR. Thirdly, SCARs enhance locus specificity and physical mapping. Lastly, SCARs allow comparative mapping or homology studies among related species, thus making it an extremely adaptable concept in the near future (Tanaka *et al*., 2006; Michhelmore *et al*., 1991).

2.2.1.2.4 Cleaved Amplified Polymorphic Sequence (CAPS)

CAPS are a combination of the RFLP and PCR techniques and was originally named PCRRFLP (Maeda *et al*., 1990). The technique involves amplification of a target DNA through PCR, followed by digestion using restriction enzymes (Michaels & Amasino, 1998). Hence, CAPS markers rely on differences in restriction enzyme digestion patterns of PCR fragments caused by nucleotide polymorphism between samples. Therefore, the critical steps in the CAPS marker approach include DNA extraction, the number or distribution of polymorphic sites, and PCR conditions.

The CAPS markers are important since the analysis of restriction fragment length polymorphisms is based on PCR amplification. Therefore, it is much easier and less time-consuming than analyzing alternative types of markers that require southern hybridizations.

2.2.1.2.5 Expressed Sequence Tags (EST)

The production of ESTs starts with the construction of cDNA libraries. The identification of ESTs has proceeded rapidly, with over 6 million ESTs now available in computerized databases. ESTs were originally intended as a way to identify gene transcripts, but have since been instrumental in gene discovery, for obtaining data on gene expression and regulation, sequence determination, and for developing highly valuable molecular markers, such as EST-based RFLPs, SSRs, SNPs, and CAPS. ESTs have been used for designing probes for DNA microarrays that is used to determine gene expression. ESTs also allow the efficient development of single or low-copy RFLP markers. RFLP markers developed from ESTs (EST-RFLP) have been extensively used for the construction of high-density genetic linkage maps and physical maps (Kurata *et al*., 1997). Often EST-based RFLP 2556 markers allow comparative mapping across different species, because sequence conservation is high in the coding regions. ESTs also allow a computational approach to the development of SSR and SNP markers (Eujayl *et al*., 2001) for which previous development strategies have been expensive. Pattern-finding programs can be

employed to identify SSRs in ESTs. The available sequence information allows the design of primer pairs, which can be used to screen cultivars of interest for length polymorphisms.

2.2.1.2.6 Single Nucleotide Polymorphism (SNP)

Single nucleotide polymorphism (SNP) is a DNA sequence variation occurring when a single nucleotide (A, T, G or C) differs among members of a species. SNP is the most abundant marker system both in animal and plant genomes and has recently emerged as the new generation molecular markers for various applications. Being binary or in co-dominant status, they are able to efficiently discriminate between homozygous and heterozygous alleles. Moreover, unlike microsatellites their power comes not from the number of alleles but from the large number of loci that can be assessed (Foster *et al*., 2010). Once the rare SNPs are discovered in a low diversity species, the genetic population discrimination power can be equivalent to the same number of loci in a genetically diverse species. The more evolutionary conserved nature of SNPs makes them less subject to the problem of homoplasy (Brumfield *et al*., 2003). Most importantly, SNPs are amenable to high throughput automation, allowing rapid and efficient genotyping of large numbers of samples (Tsuchihashi & Dracopoli, 2002). In plants, SNP can be designed from ESTs (Coles *et al*., 2005), and single-stranded pyrosequencing (Miller *et al*., 2003). A high throughput genome analysis method called diversity array technology (DArT), based on microarray platform, has been developed for the analysis of plant DNA polymorphism (Jaccoud *et al*., 2001).

2.2.1.2.7 Sequence Tagged Site (STS)

STS was first developed by Olsen *et al*., 1989 as DNA landmarks in the physical mapping of the human genome, and later adopted in plants. STS is a short, unique sequence whose exact sequence is found nowhere else in the genome. Two or more clones containing the same STS must overlap and the overlap must include STS. Any clone that can be sequenced may be used as STS provided it contains a unique sequence. In plants, STS is characterized by a pair of PCR primers that are designed by sequencing either an RFLP

probe representing a mapped low copy number sequence (Blake *et al*., 1996) or AFLP fragments. STS markers are codominant, highly reproducible, suitable for high throughput and automation, and technically simple for use (Reamon-Buttner & Jung, 2000).

2.2.2 Genetic Diversity of Taro in Kenya

In Kenya, Palapala *et al.,* 2016 used SSR markers that showed existence of comparative significant genetic diversity differences between the Kenyan and Pacific Island Taro germplasm accessions. However, the high mutation rates of SSR markers also mean that microsatellites suffer from homoplasy problems (Schlotterer *et al*., 1998) and may also increase within-population component of variation.

Genetic analysis and crop improvement with the use of molecular markers has gained great importance (Dorado *et al.,* 2015) with genetic diversity assessments being of great priority in crop breeding (Nybom & Bartish, 2000). Additionally, it is important to understand the genetic diversity of a crop for its conservation and utilization of their germplasm in breeding programs.

In the recent past, molecular markers have been used to quantify genetic diversity of many wild and cultivated plants (Schulman AH, 2007). From RFLPs to simple sequence repeats (SSRs) and then to NGS of SNPs, different molecular markers have been used to characterize genetic diversity (Vinson *et al*., 2018). However, due to the large number of markers that can be generated at a reduced cost. SNPs are becoming the choice marker for genetic analysis and breeding. This is majorly because SNPs are also the most frequent source of variation in eukaryotic genomes and their bi-allelic nature offers accuracy in variant calling (Vignal *et al*., 2002). GBS techniques such as DArT have the ability to simultaneously sequence and discover SNPs from a targeted subset of the whole-genome. The more recent DArT sequencing (DArTseq), which sequences only the most informative representations of genomic DNA, improves the rate of genotype calling and ability to sequence more samples for less cost (Kilian *et al*., 2012). Furthermore, DArTseq produces dominant (SilicoDArT) and co-dominant (SNP) markers that have been widely

assessed for genetic analyses in several crops (Macko-Podgorni *et al*., 2014; Brinez *et al*., 2012) and allow the characterisation of population structure without prior knowledge of the genome or diversity (Elshire *et al*., 2011; Muktar *et al*., 2019).The identification of SNP has represented interesting approaches (Ipek *et al*., 2016; Akpinar *et al*., 2017) in different studies. Taro genetic diversity assessment through SSR markers have been previously employed. For instance, sixteen microsatellites have been developed for *C . esculenta* (Mace and Godwin, 2002) with seven of them having been used in SSR analysis for the Pacific Island Taro germplasm (Mace *et al*., 2006).

2.2.3 Genotyping by Sequencing (GBS)

GBS is a novel application of Next generation sequencing protocols for discovering and genotyping Single nucleotide polymorphisms (SNPs) in crop genomes and populations. This approach detects SNPs in a large segregating or mutant population and combines with scoring hence a rapid and direct study of its diversity is targeted towards the mapping of a trait or a mutation of interest (Deschamps, Llaca and May, 2012).This technique was used in the Buckler lab (Elshire *et al.*, 2011), for constructing reduced representation libraries for the Illumina Next Generation sequencing platform. According to Beissinger *et al*., 2013 this technique produces a large group of SNPs' that are used in genetic analysis and genotyping. It can be performed either through a reduced-representation or whole genome re-sequencing approach. This technique is important because it has the potential of detecting many unbiased loci in genome than genotyping arrays (Ganal *et al.*, 2012).In addition GBS when combined with genome-independent imputation provides an efficient and simple method for genetic map constructions in any pseudo-testcross progeny (Ward *et al*.,2013). GBS is important and will play a vital role in plant breeding and biotechnology since methylation sensitive restriction enzymes can be utilized in the targeting of euchromatic, gene rich regions and more so hundreds of samples can be processed together using different unique barcodes and the resulting libraries are taken through Polymerase chain reaction (PCR) amplification and Illumina sequencing (Kolb *et al.*, 2016). There are outstanding advantages of this technique over other platforms in that it identifies abundant SNPs at low cost, does discovery and genotyping concurrently,

reduces ascertainment biasness compared to array- based markers and is relatively easy in automation according to Poland *et al.,* 2012 and Elshire *et al.,* 2011. In conclusion GBS has successfully been used in Genome- wide association studies (GWAS), genomic diversity studies, genetic linkage analysis, molecular marker discoveries and genomic selection under a large scale of plant breeding programs therefore it will be of help in this study in the identification of SNPS.

2.3 Tissue Culture

Plant tissue culture is a method to culture the cells, tissue organs and other components of the plant following the aseptic in-vitro culture under a well-defined environment (Twaij *et al*., 2020). It is also a collection of techniques used to maintain or grow plant cells, tissues or organs under sterile conditions on a nutrient culture medium of known composition (Singh *et al*., 2017. In a simple terms, a part of the plant body is dissected into a small part which is called explant and grown into a complete plant (Tigrel *et al*., 2022). The explant exhibits a very high degree of plasticity *in-vitro*, thereby allow the explant to develop into another type and this way a whole new plant can be subsequently regenerated. Plant tissue culture is an alternative method of commercial propagation and is being used widely for the commercial propagation of a large number of plant species, including many medicinal plants (Hussain *et al*., 2012). It is widely used to produce clones of a plant in a method known as micro-propagation. This regeneration is done *In–vitro* so that the environment and growth medium can be manipulated to ensure a high frequency of regeneration. German Botanist Golliob Haberlandt is regarded as the father of plant tissue culture. He later continued work in the area and developed palisade tissue grew on knob's salt solution. Later, Hanning (1904) excised matured embryos and grew them *in-vitro* on a mineral salt sugar solution. This was a turning point when embryo culture was developed. In the 1950s, tissue culture was used on a large scale by the orchid industry. After many years in 1972, Carlson *et al*., 1972 created the first somatic hybrid of *Nicotiana gluca* and *N.langschorffii* by fusion of their protoplast. The main aim of tissue culture is to produce as easily and as quickly as possible, a large number of regenerative cells that are accessible to gene transfer. There are two main concepts of tissue culture in plants,

which is plasticity and totipotency. Plasticity is the ability of plants to endure extreme conditions. Totipotency on the other hand is the ability of part of a plant to regenerate into whole plants. This plasticity allows plants to alter their metabolism, growth and development to best suit their environment. Tissue culture has advantages over the traditional methods of cultivation which include; The production of exact copies of plants that produce particularly good flowers, fruits, or have other desirable traits; quick production of mature plants; the production of multiples of plants in the absence of seeds or necessary pollinators to produce seeds; the regeneration of whole plants from plant cells that have been genetically modified and the production of plants in sterile containers that allows them to be moved with greatly reduced chances of transmitting diseases, pests, and pathogens.

2.3.1 Plant Tissue Culture Process

The growth of explants rests on two fundamental properties of plant cells these are cell totipotency and cell plasticity (Bharat *et al*., 2020). These two properties of plant cells explore the capacity living cells possess to develop into a new genetically identical cell and after differentiation processes would be able to form tissues, organs, systems and complete individuals (Albersheim *et al*., 2010).

In a well maintained controlled environment using defined culture media, a whole plant is generated from small tiny explants and this is now called “tissue-culture raised plants” (Espinosa-Leal *et al*., 2018). The whole process is followed in aseptic conditions with raised plants that are disease-free, having healthier root systems and being more fibrous, and have a higher survival rate Ioannidis *et al*., 2022). While culturing most important is to have an appropriate medium together with auxin and cytokinin, which gives a good growth to explants into unorganized, growing mass of cells also called callus (Neumann *et al*., 2020). Callus has a variable appearance in texture, and shape (Talitha *et al*., 2023). Explants follow through the mechanisms which trigger their growth from a cell or a tissue section and the rate of growth depends on various factors varying according to the age, species, type of the tissue, the composition of the culture media and the environmental

conditions managing its growth empirically. Once the explants grow the required part of it is cut off and placed into an entirely fresh new media which will allow growth with altered morphology. All above an expert hand, skill together with the experience of the tissue culturist are other most important aspects required during a time when one needs to judge which pieces to culture and which to discard (Twaij *et al*., 2020). For example, if shoots emerge it may be cultured freshly with auxin to produce plantlets which, if plotted in potting soil can grow further as normal plants.

2.3.3 Media in Plant Tissue Culture Plant Tissue Culture Media

The culture media for *In –vitro* regeneration is composed of three basic components that include; essential elements, organic supplements and a source of carbon (Yuan *et al.*, 2013).The essential elements could be macro elements, microelements or an iron source. The macro elements include; Nitrogen, phosphorus, potassium, magnesium, calcium and Sulphur. Micro elements are required in trace amounts, they include; Manganese, iodine, copper, cobalt, boron, molybdenum, iron and zinc. Sucrose is the main carbon source used since it is cheap, easily available, readily assimilated and relatively stable. The gelling agents could be solid or in liquid form, depending on the type of culture being grown (Twaij *et al*., 2020). Agar and gelrite are the most commonly used gelling agents (Bharat *et al*., 2020).

2.3.4 Plant Growth Regulators

These are hormones that determine developmental pathway of plant cells (Talitha *et al*., 2023). To date, structurally diverse phytohormones have been characterized, such as auxin, cytokinin, abscisic acid, ethylene, gibberellin, brassinosteroid, salicylic acid, and jasmonate (Sauer *et al.*, 2013). The functions of plant hormones are diverse but all have profound effects on growth and development. They affect all phases of the plant life cycle (Ioannidis *et al*., 2022). Due to the fundamental roles of these hormones being integrators and regulators, their study and those of genes that control their synthesis, transport and

downstream effects have identified many new tools for agricultural improvement (Albersheim *et al*., 2010).

Auxins are a family of related compounds that were originally identified as promoters of growth (Ludwig-Muller, 2011). They promote cell division and growth. Auxins include Indole-3-acetic acid (IAA), Indole-3- butyric acid (IBA), 4-chloroindole-3-acetic acid (4-Cl-IAA) and Phenylacetic acid (PAA) (Damodaran S & Strader L, 2019). Indole-3-acetic acid (IAA) is a naturally occurring auxin that is most abundant and physiologically relevant. Exogenously applied IBA induces rooting more efficiently than IAA, hence widely used as a rooting agent in agricultural applications (Alizadeh & Dumanoglu, 2022). Besides, IBA is involved in other auxin-mediated developmental processes such as leaf epinasty, cell division, stem bending and root hair elongation (Mazzoni-Putman *et al*., 2021). 4-Cl-IAA is active at lower concentrations compared with IAA due to its greater chemical stability (Jayasinghege *et al*., 2019). It has however not been detected in the main model plant *Arabidopsis.* It stimulates pericarp growth in pea, maize coleoptile elongation and protoplast swelling. PAA plays a role in root interactions with soil microorganisms and is active at much higher concentrations.

Cytokinins (CKs) are adenine-derived, small-molecule plant growth regulators that control aspects of almost all plant growth and development processes (Emery R & Kisiala A, 2020). Cytokinins are a family of related compounds that are derived from Adenine. They include Zeatin, kinetin and 6-benzylaminopurine. Internally, CKs play significant roles in plant cell division, nutrient allocation, and photosynthetic performance (Jorge G *et al*., 2019). CK functions in plant metabolism include plant adaptations to various abiotic stresses as well as their regulatory role in plant interactions with biotic components of the environment (Khan *et al*., 2020). Lastly, it is the most widely used PGR in adventitious shoot induction and initiation of somatic embryogenesis in tissue culture (Long *et al*., 2022).

Gibberellins (GA) and abscisic acid (ABA) are plant hormones that play antagonistic roles in regulation of numerous developmental processes (Shu K *et al*, 2018). GA is associated

with promotion of germination, growth and flowering. However, ABA inhibits these processes. Additionally, the antagonistic relationship and the ratio between these two hormones regulates the transition from embryogenesis to seed germination. A different mechanism of interaction between GA and ABA in the regulation of root growth has been described (Saidi A & Hajibarat Z, 2012). For instance, in Arabidopsis GA promotes and ABA suppresses root growth and these two effects are mediated by DELLA proteins.

Jasmonic acid is a fatty acid derived plant hormone that is associated with pathogen defense pathways (Mendez-Bravo *et al*., 2011). The physical stimuli of certain insects can trigger the synthesis of jasmonic acid which increases the expression of genes involved in defending the plants. Moreover, this plant regulator is necessary during the engineering of transgenic plants that are disease resistant. Salicylic acid is a phenolic phytohormone that is found in plants. It is associated with plant growth and development, photosynthesis, transpiration, ion uptake and transport (Hayat S *et al*., 2013). Besides, it plays a role in the resistance pathogen pathway by inducing the production of pathogenesis related proteins. Brassinosteroids are sterols that are critical for normal plant growth and development. They play significant roles in stem elongation, leaf development, pollen tube growth, vascular differentiation and stress responses (Oh *et al*., 2020).

2.3.5 Sterilization Agents In Tissue Culture

There are a range of sterilizing agents in tissue culture (Sivanesan *et al*., 2021). One of the very first is water. This is mainly used to remove soil particles in the plants and also in rinsing out detergents. This water could be tap water, distilled water or autoclaved distilled water. Soap is also used as a cleaning agent and this is usually accompanied by an anti-septic such as Dettol or savlon. Ridomil is an anti-fungal used to clean the explants. Ethanol is a major sterilant that kills bacteria. Lastly, Sodium hypochlorite is one of the major sterilizing agents and tween 20 is usually added to it and it acts as a surfactant.

2.3.6 Plant Regeneration Techniques

There are two main methods of plant regeneration; somatic embryogenesis and organogenesis.

2.3.6.1 Somatic Embryogenesis

Somatic embryogenesis was first introduced by German and United states independent groups, whereby they regenerated plants from cultured carrot as the mother cell (Steward *et al*., 1958). This technique entails the formation of embryo-like structures called somatic embryos, which can develop into whole plants. These somatic embryos can be produced either directly or indirectly. Somatic embryogenesis occurs through a series of stages characteristic of zygotic embryogenesis. They can differentiate either directly from the explant without an intervening callus phase or indirectly after a callus phase (Zimmerman, 1993). Explants from which direct embryogenesis is most likely to occur include microspores (microsporogenesis), ovules, zygotic, somatic embryos and seedlings (Von Arnold *et al.*, 2002).This technique usually proceeds through two distinct stages. The initial stage is the embryo initiation where a high concentration of 2,4-D is used. The second stage is embryo production and this takes place in medium with minimal levels of 2,4-D. For successful establishment of a somatic embryogenesis system, a proper choice of plant material and the determination of physical and chemical factors that influences the switching on of the embryo genetic pathway of development is a requirement (Sivaram and Mukundan, 2003). This technique is of importance since it plays a vital role in biotechnological tools such as tissue culture, protoplast fusion and genetic transformation that to some extend helps overcome sexual reproduction related obstacles (Pan *et al.*, 2009).

2.3.6.1.1 Direct Somatic Embryogenesis

In this technique, the embryo is formed directly from a group of cells without the production of an intervening callus.

Leaf explants of *Phalaenopsis amabilis* var. formosa formed somatic embryo clusters within 20-30 days (Chen *et al*., 2006) directly from epidermal cells without an intervening callus. They were cultured on ½ strength modified Murashige and Skoog medium supplemented with 0.1, 1, and 3 mg TDZ. Here, plantlet conversion from embryos was successfully achieved on regulator-free growth medium.

Elsewhere, an effective, rapid, and efficient Arabidopsis regeneration system via direct somatic embryogenesis has been established (Gaj, 2001). Herein, immature zygotic embryos were used. Explants in different developmental stages were cultured on B5 agar medium containing 5 µM 2, 4-dichlorophenoxyacetic acid and the highest frequency (up to 90%) of somatic embryogenesis was observed in zygotic embryos with fully-developed cotyledons.

2.3.6.1.2 Indirect Somatic Embryogenesis

In this process the callus intervening stage is first formed from the explant. Embryos can then be produced from the callus tissue or from a cell suspension produced from the callus.

For instance, several plant growth regulators BA, TDZ, 2,4-DD, and Kinetin were tested alone or in combination for their capacity to induce indirect somatic embryogenesis from leaf and internode explants of *Paulownia elongata* (Ipekci & Gozukirmizi, 2004). Notably, calli were produced after 3 weeks and the initiation rate was 54.1%. Subsequently, a reproducible protocol for indirect somatic embryogenesis was established in a small aromatic tree, *Murraya koenigii* (Paul S. *et al*., 2011). Embryogenic callus was obtained from 90% zygotic embryonic axis (ZE) and 70% cotyledon (COT) explants in Murashige and Skoog (MS) basal medium supplemented with 8.88 µM 6-benzyladenine (BA) and 2.675 µM a-naphthaleneacetic acid (NAA). Globular somatic embryos were induced and further matured from such embryogenic callus by subsequent culture on the same basal media containing thidiazuron (TDZ) (2.27–9.08 µM). The highest frequency of somatic embryos (14.58 – 0.42 µM) was recovered from ZE-derived callus after 6 weeks.

2.3.6.2 Organogenesis

Organogenesis refers to the regenerative process that does not use a somatic embryo but rather the differentiation of the meristematic centre, reflecting the pluripotency of plant cells (Lardon & Geelen, 2020).This is a technique that relies on plant regeneration through a process analogous to zygotic embryo germination. It relies on the production of organs either directly from an explant or from a callus culture (Deo *et al.,* 2009).This technique relies on the plasticity of plant tissues and altering the medium components regulates it.

2.3.6.2.1 Direct Organogenesis

This technique relies on the production of organs directly from an explant.

A study aimed to develop an efficient micropropagation method for true-to-type date palm plants through direct organogenesis (Bekheet S., 2013). Here, nodular cultures were obtained from shoot tips on MS medium plus 2 mg/l 2ip and1 mg/l NAA. Among the combinations used, 5 mg/l 2ip alone gave the highest organogenesis frequency. For *in vitro* multiplication, culturemedium amended with 5 mg/l 2ip + 2 mg/l Kin gave the maximum shootbud proliferation and shoot bud length.

Consequently, Pereira *et al*., 2000, established a micropropagation protocol for *Pothomorphe umbellate* using leaf segments cultured on 1/4 strength Murashige and Skoog medium supplemented with 0.5 mg l−1 6-benzyladenine and 0.1 mg l−1 gibberelic acid added with 10 g l−1 sucrose. Rooting was achieved using MS medium devoid of growth regulators.

2.3.6.2.2 Indirect Organogenesis

This involves the formation of organs from a callus culture. An effective protocol has been developed for *in vitro* regeneration of the *Melothria maderaspatana* via indirect organogenesis in liquid and solid culture systems (Baskaran *et al*., 2009). Organogenesis was achieved from liquid culture calluses derived from leaf and petiole explants of mature

plants. Organogenic calluses (98.2±0.36 and 94.8±0.71%) were induced from both leaf and petiole explants on Murashige and Skoog (MS) liquid medium containing 6.0μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 μM thidiazuron (TDZ); and 6.0 μM 2,4-Dand 1.0 μM benzyladenine (BA) combinations, respectively.

Elsewhere, Arellano *et al*., 2009 have developed an *in vitro* regeneration system for *Phaseolus vulgaris* cv. Negro Jamapa via indirect organogenesis. The explants used were apical meristems and cotyledonary nodes dissected from the embryonic axes of germinating seeds. Here, several auxin/cytokinin combinations were tested for callus induction. The best callusproduction was obtained with medium containing 1.5 μM 2,4-dichlorophenoxyacetic acid. After 2 weeks of growth calli were transferred to shooting medium containing 22.2 μM 6-benzylaminopurine. Shoots regenerated with a frequency of approximately 0.5 shoots per callus, and upon transfer to rooting medium these shoots produced roots with 100% efficiency. Histological analyses of the regeneration process confirmed the indirect organogenesis pattern.

2.3.7 Tissue Culture of Taro

According to Ngetich *et al.*, 2015 micro propagation using low cost media for the production of Taro plantlets is possible. However, this did not really show the use of common Murashige and skoog media, therefore this study aims to test whether these plants can really grow on MS media just like the low cost media. In addition, according to Verma *et al*., 2017, a combination of both 2, 4-D and TDZ, played a decisive role in the induction of direct somatic embryogenesis in Taro. Therefore, by using the above hormones I will get embryos that I will mature, shoot and root them for mass production of Taro plantlets.

For this research callus induction will be done, the embryos that develop will be matured, shooting and rooting will be induced and later acclimatization of the plantlets done. This technique is more preferable since at the end of this research there will be an established protocol for Taro transformation and this aims at improving Taro varieties in Kenya.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area

Field studies were conducted from a wide range of ecological conditions of soil, temperature and water availability from nine Kenyan counties (Kiambu, Murang'a, Meru, Nyeri, Siaya, Busia, Kakamega, Kisii and Machakos) (Figure 3.1). These counties were mainly chosen because some of their regions mostly experience high rainfall throughout the year.

Figure 3.1: Map of Kenya Showing Study Areas

3.2 Plant Material and Study Site

A total of 270 young Taro plantlets were collected and used as plant materials to determine genetic diversity. These plantlets were preserved in zip-locked bags and transported to the Biosciences Eastern and Central Africa (BecA) greenhouse in the International Livestock Research Institute (ILRI). The plantlets were planted on pots containing well mixed heated and cooled soil with forest soil and manure in the ratio of 2:1 and a handful size of gravel placed at the base of each pot that enhanced proper drainage of excess water to prevent rotting. These plants were watered using a two-litre container thrice a week. Weeding was performed regularly and the plants were maintained for four months before they were used for subsequent experiments. Additionally, during sampling, one leaf was plucked from each of the 270 plantlets, divided into three sections and preserved in three falcon tubes containing silica gel. Upon transfer to ILRI, these leaf samples were transferred to clean falcon tubes with no silica gel and placed on -80 ^{0}C freezer, awaiting DNA extraction. The grown plantlets were maintained for use in genetic diversity analysis; in the case where the leaf sample was not enough for DNA extraction. Subsequently, the maintained plantlets were also used for tissue culture.

3.3 Sample size

Purposive sampling technique (Benard, 2002) was applied during sample collection, whereby only farmers in the wet regions in these counties were visited, since Taro only grows in wet regions. To determine genetic diversity, a total of three samples were collected from each farm and a total of ten farms were visited in each county. Therefore, a total of 270 samples were collected. All the collected samples were well labelled with the county name, sub county, ward, village, farmer name and phone number, and Global positioning system (GPS) Co-ordinates (appendix 1).

3.4 Genetic Diversity

3.4.1 DNA Extraction and Quality Check

A total of 173 freeze dried leaf tissue samples out of the 270 sampled for genetic diversity analysis and a total of 15 samples representing the samples from the greenhouse (GH) were used here. Therefore, a total of 188 samples were used for genetic diversity assessment. They were finely crushed with the aid of liquid nitrogen and their total genomic DNA extracted according to the QIAGEN Plant DNEasy mini kit protocol. It is important to note that GBS is a very expensive technique and only two plates were budgeted in this study. Usually, one plate has 96 wells. However, two blanks were needed on each plate to cater for the enzyme and water as control. This is the reason why some counties had to give 19 samples and others 20 samples (Table 3.1). The distinguishing existing phenotypic characteristics in literature was used to try and classify the different genotypes in the greenhouse. Notably, these samples were labelled against the specific varieties (Table 3.2). This was to try and assess whether the characteristics given in literature could help identify the different Kenyan Taro cultivars. The genomic DNA was quantified using a Nanodrop 2000c (Thermo Fisher scientific) machine (appendix 2).

Table 3.1: County Name and the Total Number of Samples Used in Sequencing

County Name	Total samples
Murang'a	19 (1-19)
Siaya	19 (1-19)
Nyeri	19 (1-19)
Kiambu	19 (1-19)
Busia	19 (1-19)
Meru	19 (1-19)
Kisii	20 (1-20)
Machakos	20 (1-20)
Kakamega	19 (1-19)
GH (Green house)	15 (1-15)

Table 3.2: The Green House (GH) Varieties Based on Phenotypic Classification

Sample Number	Variety matching phenotypic characteristic
1 and 2	Dasheen
3 and 4	Eddoe
5,6,and 7	Green wild Taro
8 and 9	Purple wild Taro
10 and 11	Green tania
12 and 13	Purple tania
14 and 15	Home flower

3.4.2 Genotyping by Sequencing

Genotyping by sequencing of 188 Taro accessions was performed by using a whole genome profiling service for SNP and DArTseq markers. First, 100µl of 50ng/µl was sent to Integrated Genotyping by Sequencing and Support unit in BecA for SNP and DArTseq analysis following the protocol described by (Akbari *et al.*, 2006). A similar DArTseq method is described by (Akpinar et al., 2017) which entailed complexity reduction, cloning of polymorphic fragments to create a library, amplification of the generated library by polymerase chain reaction (PCR), cleaning and evaluation of amplicons by capillary electrophoresis sizing, sequencing of fragments, creation of FASTQ files with generated sequencing reads, performance of an internal alignment using the reads from the library, filtration of SNP and Silico DArT markers using algorithms and lastly the resulting SNP and DArT data scored as presence /absence (1 / 0 respectively) matrices. Here, DNA was processed using the DArTseq™ platform using a protocol optimized for Taro. All DNA samples were processed in digestion/ligation reactions using a combination of PstI and HpaII restriction enzymes (RE) (Kilian *et al*., 2012) with modifications, where a single PstI-compatible adaptor was substituted with two different adaptors corresponding to two different RE overhangs. Subsequently, the PstI- compatible adapter was designed to include Illumina flow cell attachment sequence, sequencing primer sequence and a "staggered" varying length barcode region (Elshire et al., 2011). On the other hand, a reverse adapter contained a flow cell attachment region and HpaII-compatible overhang sequence.

Only “mixed fragments” (PstI-HpaII) were effectively amplified in 30 rounds of polymerase chain reaction (PCR) using the following reaction conditions: 94℃ for 1 min, 30 cycles of; 94℃ for 20 sec, 58℃ for 30 sec, 72℃ for 45 sec, followed by a final hold of 72℃ for 7 min. Consequently, equimolar amounts of the amplified products from each sample of the 96-well microtiter plate were bulked and applied to c-Bot (Illumina) bridge PCR followed by sequencing on Illumina Hiseq2500. The sequencing (single read) was run for 77 cycles. Then, proprietary DArT analytical pipelines were used to generate sequences from each lane and poor-quality sequences were filtered away.

Single nucleotide polymorphisms (SNP) were identified by aligning reads to create clusters across all individuals sequenced. SNP markers were aligned to the reference genomes to identify chromosome positions. The BLASTN algorithm with an e-value ≤ 5e-7 and percentage identity of 90% was used. SilicoDArTs and SNPs were scored as "dominant" markers, with "1" = Presence and "0" = Absence of a restriction fragment with the marker sequence in genomic representation of the sample. SNPs were scored as codominant markers with 0 for the homozygous allele aa, 1 for the heterozygous allele Aa and 2 for the homozygous allele AA. Finally, identical sequences were collapsed into “fastqcoll files” which were “groomed” using DArT PL’s proprietary algorithm which corrects low quality base from singleton tags into a correct base using collapsed tags with multiple members as a template. The “groomed” fastqcoll files were used in the DArTs proprietary SNP and presence/absence variation (SilicoDArT) calling pipeline,

DArTsoft14. For SNP calling all tags from all libraries included in the DArTsoft14 analysis were clustered using DArT PL’s C++ algorithm at the threshold sequence distance of three base pairs, followed by parsing of the clusters into separate SNP loci using a range of technical parameters, especially the balance of read counts for the allelic pairs. In addition, multiple samples were processed as technical replicates (from DNA to allelic calls) and scoring consistency was used as the main selection criteria for high quality/low error rate markers.

3.4.3 Quality Analysis of Marker Data

The markers were tested for reproducibility (%), call rate (%), polymorphism information content (PIC) and one ratio. Reproducibility is the proportion of technical replicate assay pairs for which the marker score exhibited consistency. Call rate is the success of reading the marker sequence across the sample. PIC is the degree and usefulness of marker diversity in the population for linkage analysis. One ratio is the proportion of the samples for which genotype scores equalled '1'. Lastly, the Spear-man correlation between the Euclidean distances of the matrices of DArTseq and SNP markers was determined using the Mantel test in R.

3.4.4 Data Filtering Process

Using the R statistical programme, data was filtered using the dartR v 1.9.9.1 package (Gruber *et al*., 2018) to remove all SNPs and silicoDArT markers that had > 5% missing data and individuals with > 10% missing data. Markers with a reproducibility score (RepAvg) < 100% were also removed as well as those that originated from the same fragment. Non-informative monomorphic markers were also removed. SNPs with a minor allele frequency (MAF) of < 1% were also discarded. MAF filtration was not done for presence/absence of silicoDArT. The markers were further filtered based on the one ratio value, where markers with an extremely low one ratio (<0.05) were not included in the analysis.

3.4.5 Genetic Diversity Analyses

Here, selected markers were used and all genetic diversity indices were estimated using the R statistical package "ADEGENET" (Jombart, 2008). This package uses principal components discriminant analysis to allow for data dimensionality reduction in large genomic datasets. Furthermore, it computed diversity indices to illustrate the overall genetic divergence among the sub-populations. These included; observed (Ho) and expected heterozygosity (He), total gene diversity (Ht), genetic differentiation (Fst) and

population inbreeding coefficient (Fis), and fixation index (Fst). Subsequently, marker allele frequency which is the frequency at which the second most common allele occurs in a given population (Tabangin *et al*., 2009), was also computed. Finally, an analysis of molecular variance was performed using the hierfstat package in R (Goudet, 2005).

3.4.6 Sequence Similarity Search

SNPs sequences were randomly selected at different nodes and their similarity against published sequences searched in the NCBI database using the BLASTN algorithm. A minimum e-value of $1e^{-5}$, >80% identity, query coverage and total score were considered. Lastly, a circular phylogenetic tree of Taro was generated using MEGA X (Kumar *et al*., 2018) and interactive tree of life (iTOL).

3.5 Direct Organogenesis

3.5.1 Media

Full strength Murashige and Skoog (MS) (1962) media was prepared using 4.4g/l of MS media with basal salts, 30 g/L (w/v) of sucrose dissolved in distilled water, plant growth regulators (PGRs) depending on the stage of growth, pH adjusted to 5.8 using 0.1N HCL and/or 0.1N NaOH, and 3g/L of gelrite added to one-litre glass bottles. Notably, media with 4.4 g MS basal salts, 30g sucrose, 3 g gelrite and distilled water with no plant growth regulators was used as control. This media, glassware and metallic equipment used in all experiments were autoclaved at 121 ^{o}C at 15 1bs pressure for 20 minutes. All media were kept at room temperature after being dispensed in the laminar flow on clean autoclaved glassware jam jars for three days before culture and kept for three days before culture. The jam jars with media are kept for three days awaiting culture to ensure that no contamination develops in them. Besides, the jam jars were half full with media. Lastly, only the jam jars that were free of contamination were used for explant initiation.

All chemicals used in this experiment were either Duchefa or Sigma analytical grades and all experiments were performed under aseptic conditions in the laminar flow. All the

scalpels were clean and were opened in the laminar flow and were sterilized in a bead sterilizer before use. Frequent spraying of the laminar flow working space was done frequently using 70% ethanol (analytical grade). All cultures were maintained at 24± 2 ^{0}C growth rooms.

3.5.2 Explant and Its Preparation towards the Establishment of a Sterilization Protocol

It is easy to distinguish the Dasheen and Purple wild varieties phenotypically. This is because the Dasheen has green leaves while the Purple wild variety has purple leaves. The Dasheen variety was observed to be more popular among farmers unlike the eddoe variety. Also, despite the fact that tania and the home flower are wild types, some had several cormlets, therefore this could bring a confusion between them and the eddoe variety. Therefore, we opted to select the Dasheen and the Purple wild variety. This is because based on the farmers opinion the Purple wild variety was also not easily affected by diseases and needed no much water to grow and mature. Here, three experiments for the Dasheen and Purple wild varieties were performed with each having three replications. Each replication had 10 explants and the experimental sample size was 30 for each variety.

Explants comprising of the corm top attached to the base of the petioles were harvested from the greenhouse using a panga. These explants were trimmed to a length of 5 cm (figure 3.2) and taken to the laboratory where they were thoroughly washed with running tap water for 30 minutes then immersed in a one litre glass beaker containing 500 ml tap water, soap and antiseptic (Dettol) and two drops of 100 µl tween 20 and swirled gently for 30 minutes. The explants were rinsed thoroughly with autoclaved double distilled water, then placed in another beaker containing 3g/L (w/v) Redomil (antifungal) and 100µl/l tween 20 for an hour with gentle swirling at intervals, and then rinsed thoroughly with autoclaved double distilled water. These explants were then placed on a beaker containing autoclaved distilled water. Under a laminar flow chamber, the explants were sterilized with 70% ethanol for 1 min and then rinsed thoroughly using autoclaved distilled

water. Subsequently, the explants were sterilized using 20%, 40%, and 60% (w/v) Sodium hypochlorite (NaOCl) (containing 100µl/l tween 20) solution for 0, 10, 20 and 30 minutes

Finally, the explants were rinsed 5 times to remove the NaOCl traces and left on the autoclaved distilled water awaiting further trimming to about 1cm size using sterilized forceps and blades. Survival rate was the calculated using the following equation:

% Survival (Explants) = (Number of survived explants/ Total no. of explants cultured) x 100.

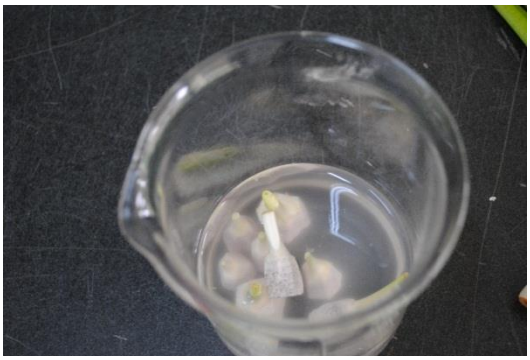

Figure 3.2: Trimmed Corm Top Attached to the Base of the Petioles Ready for Sterilization

3.5.3 Shoot Induction and Elongation

The trimmed explants were cultured on shoot induction medium (SIM) that contained MS basal medium supplemented with 30g/l sucrose and different concentrations of 6-benzylaminopurine (BAP) hormone of 0, 0.5, 1.0, 2.0, 3.0 in mg/l. The culture bottles were then placed in the growth room at 24 ± 2^0C for 30 days and the formed shoots were transferred to fresh SIM and were grown further for 15 days at a similar growth room to enhance shoot elongation and multiplication. Data on contamination and survival rates, for one month daily, shoot induction time, length, number and colour was recorded on fortnight bases.

3.5.4 Root induction

The elongated shoots of about 7 cm long were transferred to rooting media that comprised of MS basal salt supplemented with 30mg/l sucrose and different concentrations of IBA (0, 0.25, 0.50, 0.75, 1.00 mg/l). The plantlets were then placed in the growth room for incubation at 24 ± 2^0C to induce rooting. Data on the total number of roots initiated per shoot and their length in cm were recorded after every fortnight for one month of culture.

3.5.5 Assessment of the Tissue Culture Plantlets

3.5.5.1 Hardening and Acclimatization of the Plantlets

The fully developed tissue cultured plantlets which were at least 5cm with three leaves and 5cm roots were de-flasked and rinsed with autoclaved double distilled before transferring them to plastic cups containing sterilized potting media. Two different substrates were tested including sterilized soil: sand: manure in the ration of 3:1:1(A) and vermiculate (B). Both media were sterilized in the autoclave at 121ºC for 15 minutes. The plantlets were acclimatized at 28±2ºC and relative humidity ranging from 70 - 80%. Besides, foliar spray was done once after every 20 days for 3 months. Watering of plantlets (0.5litre per plantlet in a potting jar), was made after every 2 days for those in soil: sand: manure media and every day for those in vermiculate media because it drains water easily. Observations were made for a period of 10 weeks and plant survival recorded after every week.

3.6 Somatic Embryogenesis

3.6.1 Effect of Plant Growth Regulators on Callus Induction

Media preparation was done as in section 3.4.1.Three sets of plant growth regulators (PGR's) as shown in table 3.3 were used for callus induction of two Taro (Kenyan) varieties (Dasheen and Purple wild).

Table 3.3: Three Sets of PGRs Used for Callus Induction

2,4-D (µM)	TDZ (µM)	Labelling in the experiment
5	0	1A
5	1	1B
5	2	1C
5	3	1D
10	0	2A
10	1	2B
10	2	2C
10	3	2D
15	0	3A
15	1	3B
15	2	3C
15	3	3D

3.6.2 Effect of Plant Growth Regulators on Somatic Embryo Development

The same PGRs used in section 3.6.1 above were used to test the rate of somatic embryos formation from callus of dasheen and purple wild varieties. Sub culturing after every 4 weeks was carried out. Somatic embryos formation per treatment was evaluated for 2 months after every 2 weeks. The morphology of the embryos; colour and texture was also determined using eye and microscopic observation.

3.6.3 Effect of Plant Growth Regulators on Shoot and Root Induction

MS fortified with 10 µM 2,4-D and 2g/L BAP was used in shoot formation, multiplication and elongation of dasheen and purple wild varieties. This was done for 2 months to evaluate the regeneration rate. Induction of micro shoots and their length (cm) was evaluated at an interval of 2 weeks after sub culturing.

Subsequently, MS media containing 3% (w/v) sucrose and 0.3% (w/v) gelrite were used to induce rooting in both dasheen and purple wild varieties. This was done for 2 months to evaluate the regeneration rate. Induction of roots and their length was evaluated at an interval of 2 weeks after sub culturing.

3.6.4 Hardening and Acclimatization

Hardening of the plantlets was done as described in section 3.5.5 above.

3.7 Data Analysis

Using the R statistical programme, all SNPs and silico-DArT markers that had > 5% missing data and individuals with > 10% missing data, respectively, were removed using the dartR v 1.9.9.1 package (Gruber *et al*., 2018). DArTsoft v.7.4.7 (DArT P/L Australia) was used to analyze the images from DArTseq and SNP platforms and their markers scored using DArTsoft as 1or 0,indicating the presence or absence of the marker in the genomic representation of each sample according to Akbari *et al.*, 2006.This DArT software computes parameters of quality such as call rate, polymorphic information content (PIC), and reproducibility of SNP and DArTseq markers.

Using statistical software R, in reference to adegenet, tidyr and dplyr packages, scripts were developed to manipulate the whole data set routinely done in R package. After the calculation of relationship matrix among all samples, the APE (Analysis of phylogenetic and evolution) package according to Paradis *et al*., 2004 was used to infer Neighbor-joining tree on the developed matrix according to Saitou and Nei *et al.*,1987. Finally, a circular phylogenetic tree of Taro was generated using MEGA X (Kumar *et al*., 2018) and interactive tree of life (iTOL).

In the direct organogenesis and callus induction experiments, a completely randomized block design was used. Three experiments were performed with each having three replications. Each replication had 10 explants and the experimental sample size was 30. Data on shoots and roots development was recorded after 15 days. The number of induced roots and their length after culturing them for 15 days on the rooting medium was recorded. The percentage survival of the plantlets transferred for acclimatization was recorded after 15 days. Data were subjected to analysis of variance (ANOVA) and treatment means were compared using Duncan Multiple Range Test (DMRT) using the

XLSTAT 2020 software (Addinsoft 2021), and the graphs were plotted using R statistical software (Wickham, 2016).

CHAPTER FOUR

RESULTS

4.1 Genetic Diversity

4.1.1 Taro Silico DArT and SNP Detection

A total of 33,422 SNPs and 80,222 silicoDArT markers were generated from 186 individuals of *C. esculenta*. The sequencing of two samples (GH14 and Siaya 16) was unsuccessful (Table 4.1). The SNP data frame had 207 columns, while the silico data frame had 200 columns. The first sample was on column 15 in the silico, while it was on position 22 in the SNP file. Therefore, 200 - 14 = 186 samples and 207 - 21 = 186 samples.

The silicoDArT markers had a call rate that varied between 81–100%, with an average of 98% (Figure 4.1). Missing values ranged from 6 to 10% for individual trees, and 0 to 35% for the markers. Reproducibility of the silicoDArT markers ranged from 95% - 100% with an average of 99%. For SNPs, missing values ranged from 0 to 50% for individual trees, and 0 to 44% for the markers. Their call rate had an average of 92% ranging from 58 to 100%. The reproducibility of markers had an average of 96% with a range from 90% to 100%. In the silicoDArT markers there was a normal distribution of the call rates at around 0.88 which is closer to 100%, whereas the SNPs have a range of approximately 50-100%. Therefore, the silico DArT markers have shown consistent marker score and near 100% reproducibility.

Table 4.1: A summary of the Sampled Samples Based on SNP and Silico DArT Data

County Name	Total samples	Successfully sequenced samples	Missing sample sequence
Murang'a	19 (1-19)	19	0
Siaya	19 (1-19)	18	1 (Sample 16)
Nyeri	19 (1-19)	19	0
Kiambu	19 (1-19)	19	0
Busia	19 (1-19)	19	0
Meru	19 (1-19)	19	0
Kisii	20 (1-20)	20	0
Machakos	20 (1-20)	20	0
Kakamega	19 (1-19)	19	0
GH (Green house)	15 (1-15)	14	1 (GH 14)

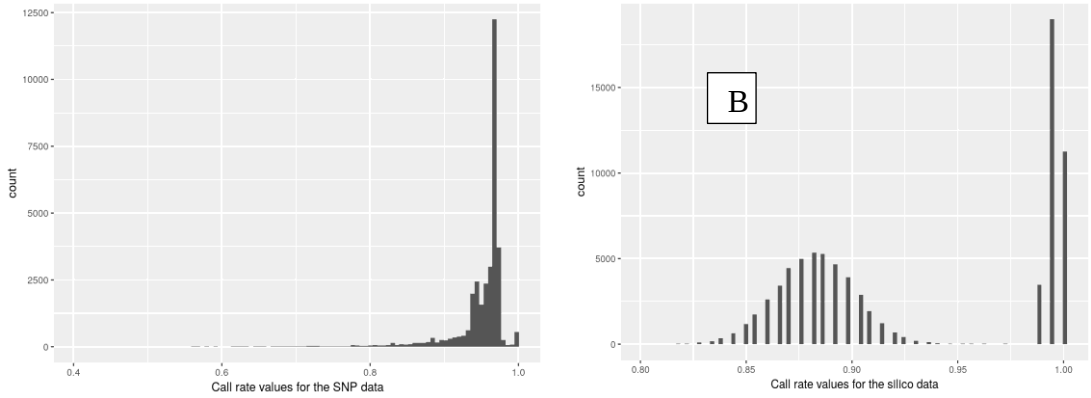

Figure 4.1: The Call Rate Values for the (A) SNP Markers and (B) Silico DArT Markers

4.1.2 Polymorphism Information Content (PIC)

Overall, the PIC values of the SNPs ranged from 0.01-0.12 (average=0.03) (Figure 4.2 A). On the other hand, the PIC values for the silico DArT markers ranged from 0.01-0.5 (average=0.06) (Figure 4.2 B). These PIC values are extremely low, indicating low

diversity among the samples. The silico DArT markers had a higher range than the SNP markers and their frequency distribution are shown in Fig. 4.2.

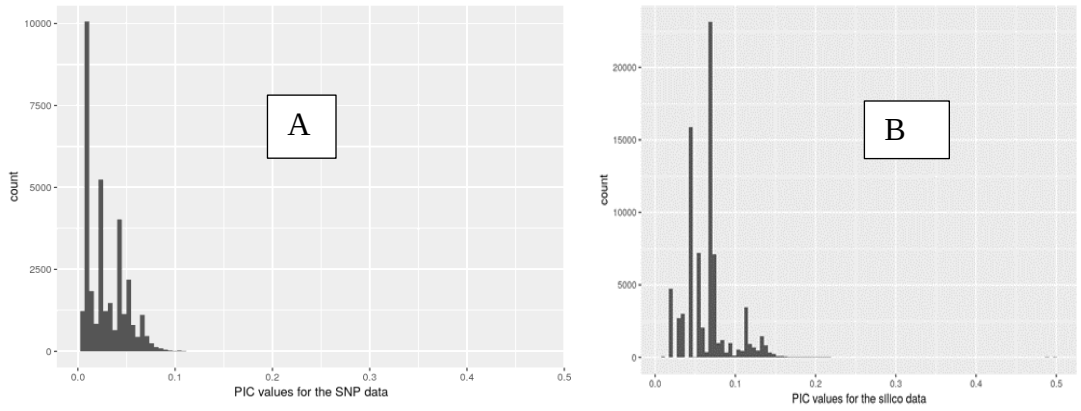

Figure 4.2: The PIC Values of (A) SNP Markers and (B) Silico DArT Markers

4.1.3 Sequence Similarity

The DArTseq marker data set was used to calculate the Jaccard genetic distance index values among the 186 Taro samples. The average genetic distance among all landraces was 0.28 and the highest genetic distance (0.41) was calculated between all the samples against the GH varieties. Subsequently, a dendrogram on both the SNP and silicoDArT marker data sets was constructed for all the varieties based on the Jaccard genetic distance. The SNP data set showed minimal differentiation (Figure 4.3). Here three clusters were obtained. Cluster A had 174 varieties and cluster B had 2 varieties (Machakos 5 and GH 6). Based on the phenotypic characteristics, GH 6 was classified as green wild Taro. It was to fall in the same group as GH 5 and 7, which were also green wild Taro, but this was not the case. Cluster C had 10 varieties in total. In cluster C, two sub-groups were obtained, C1 and C2. Sub-group C1, had GH 15, 11, 13, 12, 10 and Kisii 5, whereas C2 had GH 5, 7, 8 and 9. The GH 5 and 7 fell under group C (figure 4.3). Despite not classified under the green wild Taro, their leaves were green and the corm resembled both the green wild Taro and Purple wild Taro. Based on the farmers' opinion, this type of corm took too much time to cook but is resistant to pest and diseases. These green leaves resembles that

of eddoe and Dasheen. The only difference is that the corm takes long to cook. The GH 5 and GH 7, were classified in the same group with GH 8 and GH 9, which were Purple wild Taro. The Purple wild Taro has purple leaves. This proves that the green wild Taro is a close relative of the Purple wild Taro. However, there is a 'wild' type that is neither Purple wild Taro nor green wild Taro. Therefore the wild varieties that the farmers are using to control pest and diseases in the Taro fields are three.

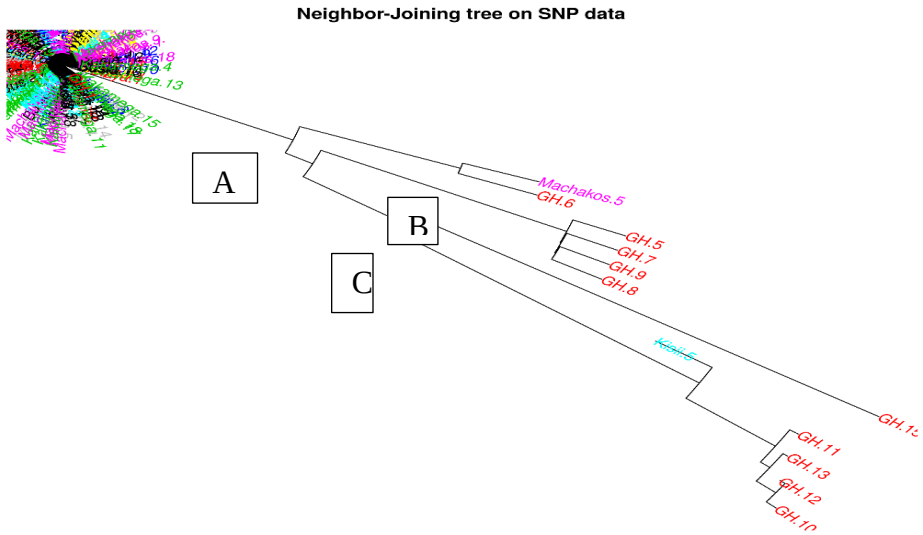

Figure 4.3: A Neighbor Joining Tree Obtained From SNP Data

On the other hand, a dendogram was also drawn using the silico DArT marker data sets (Figure 4.4). Here, four clusters were obtained (A, B, C and D) as shown in table 4.1. Besides, its evident that the groups had samples drawn from different geographical locations.

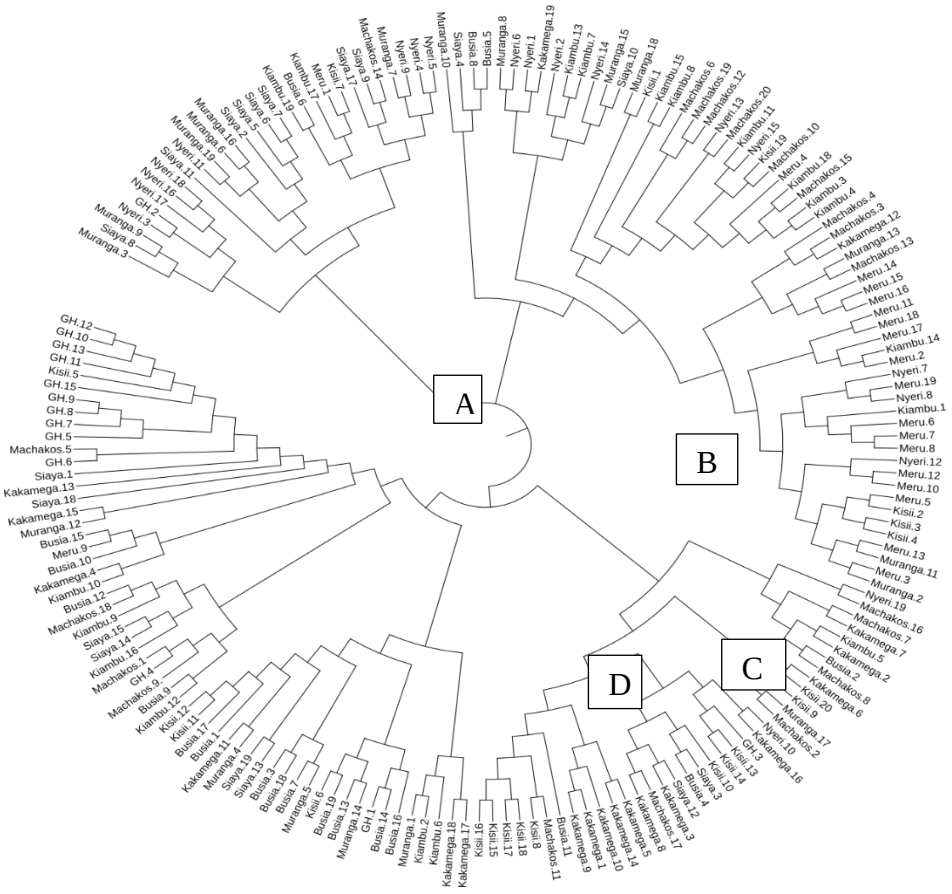

Figure 4.4: Results Showing a Circular Phylogenetic Tree Obtained from DArT Sequencing

In group A, all the counties were represented except Kakamega County. Notably, the GH2 variety was classified here. However, these samples were only 29 in total. The GH 2 sample was phenotypically selected as Dasheen. Therefore, the Dasheen variety lacks in Kakamega County. In group B, all the counties were represented but no GH sample was classified here. Therefore, based on the phenotypic characteristics known to differentiate Taro genotypes, there is one genotype that is difficult to differentiate from both eddoe and Dasheen. Meru County had 17 samples represented here from a total of 19 samples. Therefore, this 'unknown' genotype has is most popular in this County. This group had a total of 62 samples.

Table 4.2: Four Clusters Representing a Summary of the Silico DArT Marker Data

CLUSTER	SUB-GROUP 1	SUB-GROUP 2
A	Murang'a 3,9	Siaya 11,2,5,6,7,17,9,
	Siaya 8	Nyeri 11,9,4,5
	Nyeri 3, 16,17,18	Murang'a 19, 6,16,7
	GH 2	Kiambu 17, 19
		Busia 6
		Meru 1
		Kisii 7
		Machakos 14
B	Murang'a 10	Murang'a 8,13,15,18,11,2
	Siaya 4	Nyeri 6,1,8,2, 14,13,15,7,12
	Busia 8, 5	Kakamega 19,12
		Kiambu 13, 7,15,8, 11, 18,3,4,14,1
		Siaya 10
		Kisii 1, 19,2,3,4
		Machakos 6,12,19,20,10,4,3,15,13
		Meru 4,11,14,15,16,18,17,2,19,6,7,8,12,10,5,13,3
C	Nyeri 19	Kakamega 6,16,3,8,5,14,10,1,9
	Machakos 16, 7,8	Kisii 20,9,13,14,10,8,18,17,15,16
	Kakamega 7,2	Murang'a 17
	Kiambu 5	Machakos 2,11,17
	Busia 2	Nyeri 10
		GH 3
		Siaya 3,12
		Busia 4,11
D	Kakamega 11,17,18	GH 4,5,6,7,8,9,11,15,13,10,12
	Kiambu 6,2	Kiambu 12,16,9,10
	Murang'a 1,4,5,14	Busia 9,12,10,15
	Busia 16,14,13,19,7,18,3,1,17	Machakos 9,1,18,5
	GH 1	Siaya 14,15,18,1
	Kisii 6,11,12	Kakamega 4,15,13
	Siaya 13,19	Meru 9
		Murang'a 12
		Kisii 5

In group C, all 8 counties were represented except Meru County. Notably, the GH3 variety was classified here and it represented the Eddoe variety. A total of 37 samples were grouped in this category. Lastly, in group D, a total of 56 samples were classified here. The majority of the GH family including GH1, 4, 5, 7, 8, 9, 10, 11, 12, 13, and 15 were categorized here. This shows that it is difficult to differentiate the green wild Taro and Dasheen because GH1 was selected to be Dasheen. The silico DArT technique was able to group all the wild types of Taro together.This group represented all counties with Meru, Murang'a and Kisii having one sample each. Generally, only sample GH14 and Siaya 16 were unsuccessfully sequenced.

To have more insight into the varieties grouping and the pattern of variation, principal coordinate analysis (PCA) was used to assess variation. This showed 61.1% and 77.9% (figure 4.5 and 4.6, respectively) of the total variation in the samples based on silicoDArT and SNP markers, respectively. Besides, it is in agreement with the Neighbor Joining analysis for the SNP data and the phylogenetic circular dendogram for the silicoDart markers which showed that the varieties were not classified according to their geographical provenance but based on the different existing genotypes. The GH 1 was classified under group D, sub-group 1, while GH 4, 5,6,7,8,9,10,11,12,13, and 15 were classified under sub-group 2. Phenotypically the GH1 variety was classified as Dasheen. This GH1, was selected because it had characteristics similar to Dasheen but its corm was white with no red, purple or yellow colouration. Therefore, it is confirmed to be a wild type.

The GH 4 variety that was phenotypically classified as eddoe was grouped in group D sub-group 2, showing that it is a close relative of the wild varieties. It had several cormlets together which resembled that of eddoe, but the leaves and leaf venation resembled that of the home flower. Therefore, there are wild types that have corms similar to those of eddoe.

GH 2 was classified in group A, sub-group 1. Here, 28 other samples were classified here. This was the white variety that farmers grew as Taro, but it had yellow or light purple venation on the corm. This group had samples from all the counties except Kakamega county.

Phenotypically, GH 3 was classified as eddoe but after sequencing it was grouped under group C sub-group 2. In group C, no samples from Meru County were classified here. The eddoe variety has a lot of the purple coloration.

Notably, the GH 4,5,6,7,8,9,10,11,12,13, and 15 were classified together in group D, sub-group 2, but GH 1 was also in this group but in sub-group 1. Therefore, the ‘Dasheen’ variety whose leaves phenotypically resemble the Dasheen characteristics is a wild type

that farmers have grown and utilize as Taro. This variety completely resembles the Dasheen variety but the corm is completely white with no purple, red or yellow venation on the corm.

Lastly, there is group B, where no cluster of the GH was classified here. All counties were represented in this group and a total of 63 samples were clustered here. This group had the majority of the samples compared to other groups. Here, 17 samples were from Meru County. Besides, Machakos, Kiambu, Nyeri, and Murang'a also had a majority of their samples here. This could be due to the fact that these counties are close to each other and therefore farmers borrow planting materials from each other. This is a genotype that is unique to Kenya. During sampling, the Meru samples resembled the eddoe variety, but from our GH classification, GH 3 was classified separately and no Meru sample appeared here. Therefore, there is a genotype that resembles the eddoe variety, but is not eddoe.

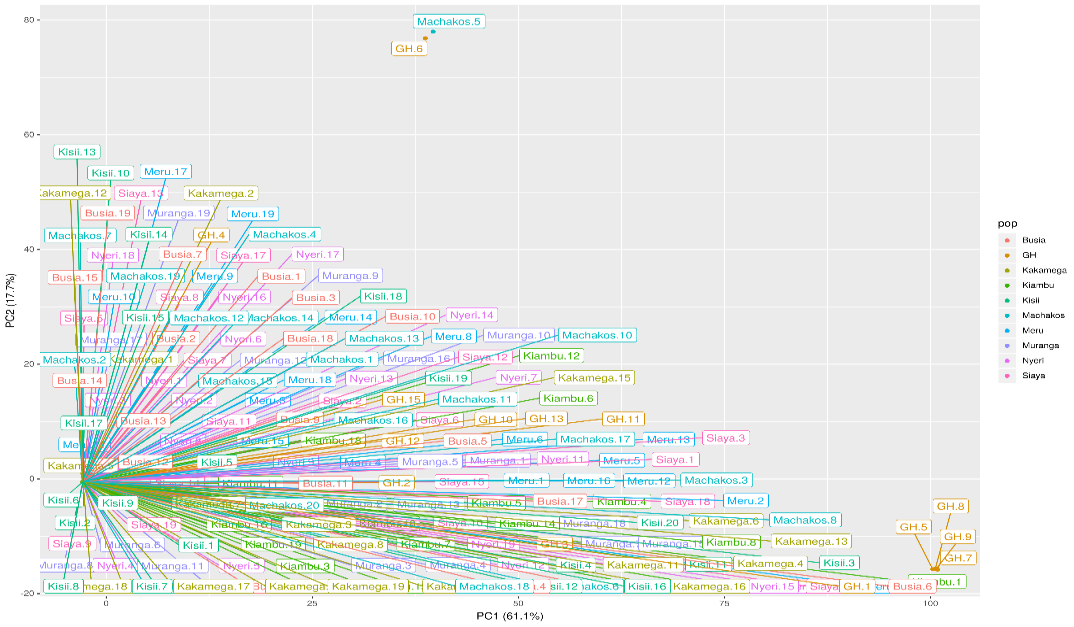

Figure 4.5: Principal Coordinate Analysis on SNP Data

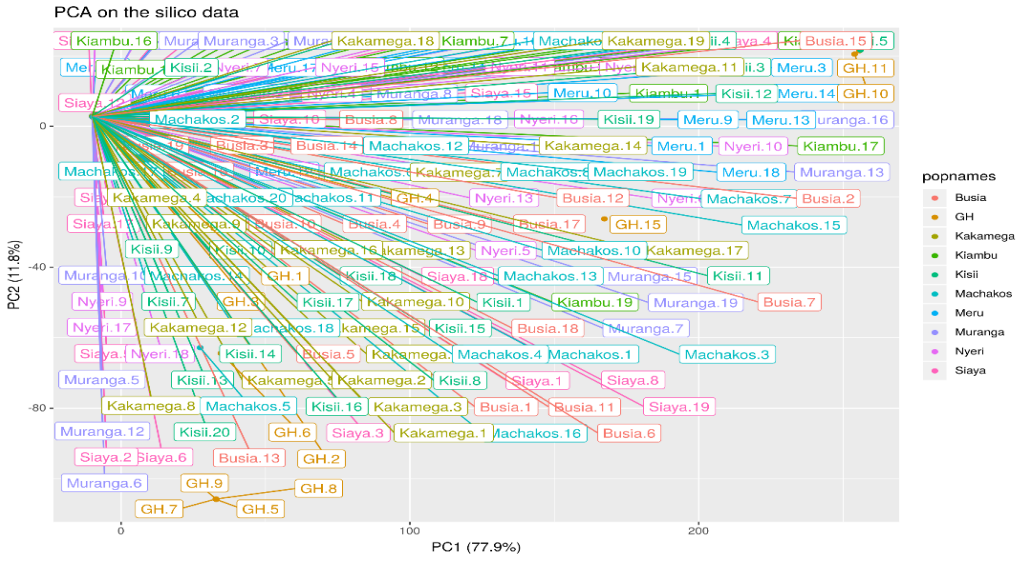

Figure 4.6: Principal Coordinate Analysis on Silico DArT Marker Data

4.2 Direct Organogenesis.

The shoots of the Dasheen and Purple wild varieties were successfully induced in 15 days from the time of initiation. Their multiplication and rooting was done in 15 days each. Lastly, their survival rate after hardening in the green house was done after 15 days. Therefore, this is a short protocol of only 60 days.

4.2.1 Effect of Different Concentrations of Sodium Hypochlorite (NaOCl) and Exposure Time on Explant Survival

NaOCl and exposure time significantly affected the survival of the Taro explant. The greatest proportion of survival was observed when the explants were exposed to 40% NaOCl for 20 minutes (90%) followed by 40% NaOCl for 30 minutes (80.33%) (Table 4.3). Increasing NaOCl concentration significantly reduced contamination. However, at a concentration of 60% NaOCl, the survival rate decreased. Here, an exposure time of 30 minutes had the least contamination (20%) but with minimal survival rate (6.66%).

Table 4.3: Effect of Different Concentrations of Sodium Hypochlorite (NaOCl) with Varying Time Exposure on Contamination and Survival on Explants after Two Weeks of Culture on MS Medium

Concentration of NaOCl (w/v)	Exposure time (min)	Contamination (%)	Survival (%)
20%	0	100.00^{j}	0.00^{i}
	10	83.33^{i}	6.66^{h}
	20	61.66^{g}	18.00^{g}
	30	47.33^{e}	41.00^{c}
40%	0	100.00^{j}	0.00^{i}
	10	62.33^{h}	21.00^{e}
	20	6.66^{b}	90.00^{a}
	30	3.33^{a}	80.33^{b}
60%	0	100.00^{j}	0.00^{i}
	10	60.33^{f}	20.00^{f}
	20	40.00^{d}	31.33^{d}
	30	33.33^{c}	6.66^{h}

Means were compared using Duncan multiple range test and bars with different letters in a figure are significantly different ($p \leq 0.001$).

4.2.2 Effect of BAP Concentrations on Shooting.

The effect of different BAP concentrations on shoot induction and multiplication in two Taro varieties is shown in Table 4.4. The BAP concentrations evaluated in this study were significantly different for shoot induction and multiplication in two Taro varieties ($P<0.001$). At 15 days, the number of shoot induced at 2.0 mg/L BAP concentration was 3.256±0.126 for Dasheen and 3.033±0.126 for Purple wild varieties. These were the highest number of shoots induced. Subsequently, for shoot multiplication, the shoot of the Dasheen variety was best at 7.611±0.205) in 2.0 mg/L BAP. Similarly, the Purple wild variety had the best multiplication of 7.444±0.205 at the same concentration of 2.0 mg/L BAP. When the BAP concentration was 3.0 mg/L, shoot induction for Dasheen and Purple wild reduced to 1.867±0.126 and 1.878±0.126, respectively. Notably, a significant effect of media was observed between lower BAP concentration (0.5 mg/l) and varieties. For instance, the Dasheen variety had a shoot induction of 0.911±0.126 and a multiplication of 3.189±0.205. This was lower compared to the shoot induction (1.811±0.126) and multiplication (4.800±0.205) of the Purple wild variety. The regeneration of Taro through organogenesis is presented in Figure 4.7.

Table 4.4: Responses of Taro Varieties (Dasheen and Purple Wild) To Four Different Concentrations of Benzyl-Aminopurine (BAP) for Shoot Induction (at 15 days) and Shoot Multiplication (30 Days)

Genotypes	BAP concentration (mg/L)	Shoot induction (15 days)	Shoot multiplication (30 days)
Dasheen	0.0	0.033± 0.126^{d}	0.500± 0.205^{f}
	0.5	0.911± 0.126^{c}	3.189± 0.205^{d}
	1.0	1.856± 0.126^{b}	5.211± 0.205^{bc}
	2.0	3.256± 0.126^{a}	7.611± 0.205^{a}
	3.0	1.867± 0.126^{b}	4.911± 0.205^{c}
Purple wild	0.0	0.189± 0.126^{d}	1.311± 0.205^{e}
	0.5	1.811± 0.126^{b}	4.800± 0.205^{c}
	1.0	2.178± 0.126^{b}	5.544± 0.205^{b}
	2.0	3.033± 0.126^{a}	7.444± 0.205^{a}
	3.0	1.878± 0.126^{b}	4.722± 0.205^{c}
Mean	-	**1.701**	**4.524**
Pr > F	-	< 0.001	< 0.001

C

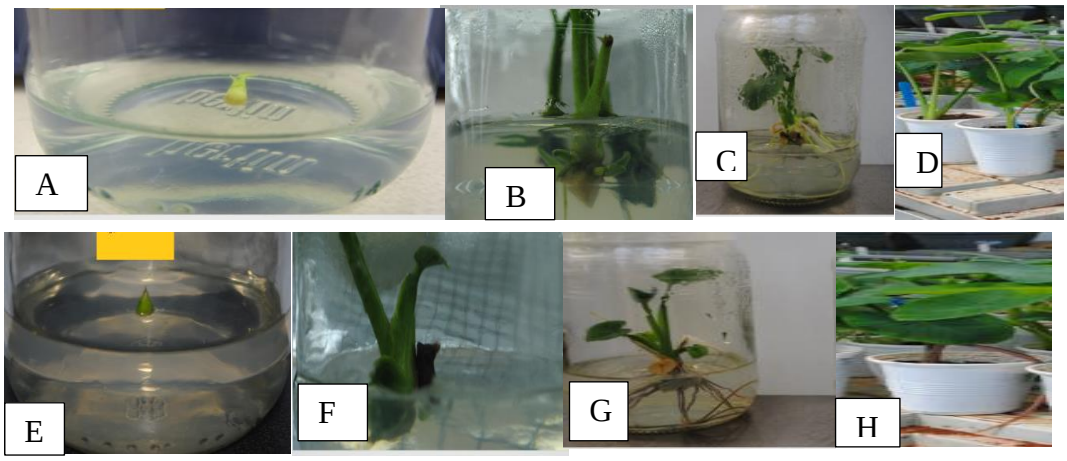

Figure 4.7: Direct Organogenesis

Figure 4.7: Direct organogenesis (A) 15-day old Dasheen apical meristem initiated on MS medium containing 2 mg/l BAP hormone; (B) 30-day old initiated Dasheen apical meristem shoots on MS medium containing 2mg/l BAP hormone; (C) Root development of the Dasheen variety in MS medium containing IBA hormone; (D) Hardened Dasheen plantlets in plastic cups in the glasshouse; (E) 15-day old Purple wild apical meristem initiated on MS medium containing 2 mg/l BAP hormone; (F) 30-day old initiated Purple wild apical meristem shoots on MS medium containing 2mg/l BAP hormone; (G) Root development of the Purple wild variety in MS medium containing IBA hormone; (H) Hardened Purple wild plantlets in plastic cups in the glasshouse.

4.2.3 Effect of IBA Concentration on Rooting.

The effect of different IBA concentrations on number of roots and root length in two Taro varieties are presented in Figure 4.8. The different IBA concentrations evaluated in this study differed significantly in number of root production and root length ($P<0.001$). The IBA concentration of 0.5 mg/l induced the highest mean number of roots of 12.867±0.521 for Dasheen and 11.933±0.481 for Purple wild varieties. We observed a gradual decline in number of root inductions in both Taro varieties when IBA concentration was increased to 0.75 mg/l (7.267 ± 0.176 and 5.400 ± 0.945, for Dasheen and Purple wild, respectively)

and 1 mg/l (5.067 ± 0.467 and 3.600 ± 0.702 for Dasheen and Purple wild, respectively). Dasheen performed better than Purple wild for root induction in all levels of IBA concentrations and control treatment, indicating significant effect among treatments evaluated in this study for root induction (*P<0.001*). The effect of IBA concentration on root length has been summarized in figure 4.8. The media concentration with no IBA hormone (0 mg/L) had the longest roots at 7.967±0.519 and 6.713±0.491 for Dasheen and Purple wild varieties, respectively. The shortest roots were observed on 0.5 mg/L BAP media that induced the most roots. This was observed to be 1.460±0.170 and 1.220±0.139 for Dasheen and Purple wild varieties, respectively. Data clearly showed that IBA treatment had negative effect on the root length though magnitude of effect was variable in different concentrations. Irrespective of IBA level, Dasheen mostly outperformed Purple wild in root length.

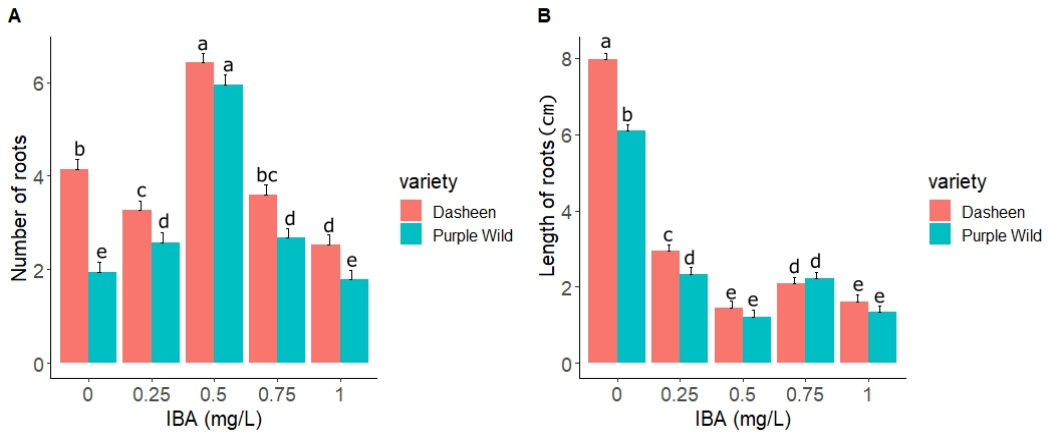

Figure 4.8: The Effect of Different IBA Concentrations on the Number of Roots

Figure 4.8: The effect of different IBA concentrations on the number of roots induced in Dasheen and Purple wild Taro varieties at 25 days of culture (A), and the effect of different IBA concentrations on the root length (cm) induced in Dasheen and Purple wild Taro varieties (B). Means were compared using Duncan multiple range test and bars with different letters in a figure are significantly different ($p \leq 0.001$).

Table 4.5: Effect of Different Concentrations of IBA Hormone on the Number and Length of Roots Induced.

Variety	IBA (mg/l)	Number of roots induced.	Length of roots
Dasheen	0	8.267 ± 0.176^{b}	7.967 ± 0.519^{a}
	0.25	6.533 ± 0.371^{c}	2.940 ± 0.156^{b}
	0.5	12.867 ± 0.521^{a}	1.460 ± 0.170^{c}
	0.75	7.267 ± 0.176^{bc}	2.093 ± 0.069^{bc}
	1	5.067 ± 0.467^{d}	1.613 ± 0.211^{c}
Purple wild	0	3.867 ± 0.751^{b}	6.713 ± 0.491^{a}
	0.25	5.133 ± 0.786^{b}	2.340 ± 0.142^{b}
	0.5	11.933 ± 0.481^{a}	1.220 ± 0.139^{d}
	0.75	5.400 ± 0.945^{b}	2.213 ± 0.323^{bc}
	1	3.600 ± 0.702^{b}	1.333 ± 0.227^{cd}

Means (± SE) followed by different alphabets in each column are significantly different ($p \leq 0.001$) using Duncan Multiple Range Test (DMRT) in the XLSTAT 2020 software (Addinsoft 2021).

4.3 Callus Induction

Apical meristems showed swelling and formation of embryo-like structures within the first four weeks of culture. The first stages of callusing, including swelling of the embryo and colour change were seen after 8 weeks of culture. After subsequent subculturing into similar media for 4 weeks, callus became more distinguishable from swollen embryos. The callus texture was more visible after two weeks and friable and compact calli could be seen.

4.3.1 Effect of Explants Type, Light and Dark Treatments on Callus Initiation

Preliminary experiments were performed to assess the effect of light and dark treatments on callus initiation using apical meristems, root tips, and leaf discs of Dasheen and Purple wild Taro varieties. MS medium with 2,4-D (5, 10, and 15µM) and TDZ (1, 2, and 3 µM) concentrations were determined and explants incubated both in light and dark conditions. Three experiments were performed using each explant and each treatment had 10 explants.

These experiments were replicated thrice. After two weeks, callus was only formed on apical meristem explants maintained in the dark (Table 4.6). However, no callus was observed forming on any of the explants placed in light conditions. Callus was best formed on MS media supplemented with 10µM 2, 4-D and 2µM TDZ hormones in dark conditions. Therefore, based on these observations, embryogenic callus initiation experiments were performed using apical meristems as the explants and dark treatment conditions.

Results in Table 4.6 are derived from three experiments for each 2,4-D and TDZ hormone combinations which had three replicates each and ten explants per replicate. No callus formation (-), 1-2 explants forming callus (+), more than two explants forming callus (++).1A (5µM 2,4-D, 0 µMTDZ),1B (5 µM 2,4-D, 1 µMTDZ),1C (5 µM 2,4-D, 2 µMTDZ),1D (5µM 2,4-D, 3 µMTDZ); 2A (10µM 2,4-D, 0 µMTDZ), 2B (10 µM 2,4-D, 1 µMTDZ), 2C (10 µM 2,4-D, 2 µMTDZ),2D (10µM 2,4-D, 3 µMTDZ); 3A (15µM 2,4-D, 0 µMTDZ), 3B (15 µM 2,4-D, 1 µMTDZ), 3C (15 µM 2,4-D, 2 µMTDZ), 3D (15µM 2,4-D, 3 µMTDZ).

Table 4.6: The effect of Light and Dark Treatment on Callus Initiation from Apical Meristems, Root Tips and Leaf Discs of Dasheen and Purple Wild Taro

Explant type	2,4-D and TDZ combined concentrations (µM)	Light conditions effect	Dark conditions effect
	1A	-	-
	1B	-	-
	1C	-	-
	1D	-	-
	2A	-	+
	2B	-	+
	2C	-	++
	2D	-	+
	3A	-	-
	3B	-	-
	3C	-	-
	3D	-	-
Root tip	1A	-	-
	1B	-	-
	1C	-	-
	1D	-	-
	2A	-	-
	2B	-	-
	2C	-	-
	2D	-	-
	3A	-	-
	3B	-	-
	3C	-	-
	3D	-	-
Leaf disc	1A	-	-
	1B	-	-
	1C	-	-
	1D	-	-
	2A	-	-
	2B	-	-
	2C	-	-
	2D	-	-
	3A	-	-
	3B	-	-
	3C	-	-
	3D	-	-

4.3.2: Effect of 2, 4-D and TDZ Combined Concentrations on Callus Induction

Apical meristems excised under a microscope and cultured in callus induction medium showed swelling (Figure 4.9A) and formation of embryo-like structures (Figure 4.9B) within the first four weeks of culture. The first stages of callusing, including swelling of the embryo and colour change were seen after 8 weeks of culture. After subsequent subculturing into similar media for 4 weeks, callus became more distinguishable from swollen embryos. The callus texture was more visible after two weeks and friable and compact calli could be seen (Figure 4.9 C).

Calli was best formed in media containing 10 µM 2,4-D and 2 µM TDZ for both Dasheen and Purple wild varieties. However, Dasheen had the highest formation of 77.8% and Purple wild had 71.1%. After four weeks, the formed calli were sub cultured into embryo formation media which had each original TDZ and 2,4-D combinations and 2mg/L BAP. Therefore, all 2,4-D and TDZ combinations were used with 2 mg/L BAP hormone to induce embryos. The Dasheen variety gave 42.7% while Purple wild gave 39.1%. Notably, the embryogenic calli that were induced after 4 weeks in the embryo formation media were observable under a microscope and different stages of an embryogenic calli were seen, including globular, that was round with shiny protrusions. Besides, the torpedo and heart shape were also seen (Figure 4.9 D).

Subsequently, continuous subculturing onto freshly prepared media containing 2mg/L BAP alone resulted in both the compact and friable embryogenic calli maturation and after 4 weeks they developed shoots. The shoots were at first white cream in colour since they were in dark conditions (Figure 4.9 E). After further sub culturing, in light conditions, multiple shoots were induced and their colour changed from cream white to pale green (Figure 4.9 G). Continuous sub culturing resulted in well germinated shoots with an average height of 5 cm. Consequently, these well-developed plantlets were rooted in hormone free MS media (Figure 4.9 H) and acclimatized (Figure 4.9 I) in the glasshouse.

After 2 weeks 100% survival with no alteration in height, leaf shape and number of leaves was observed.

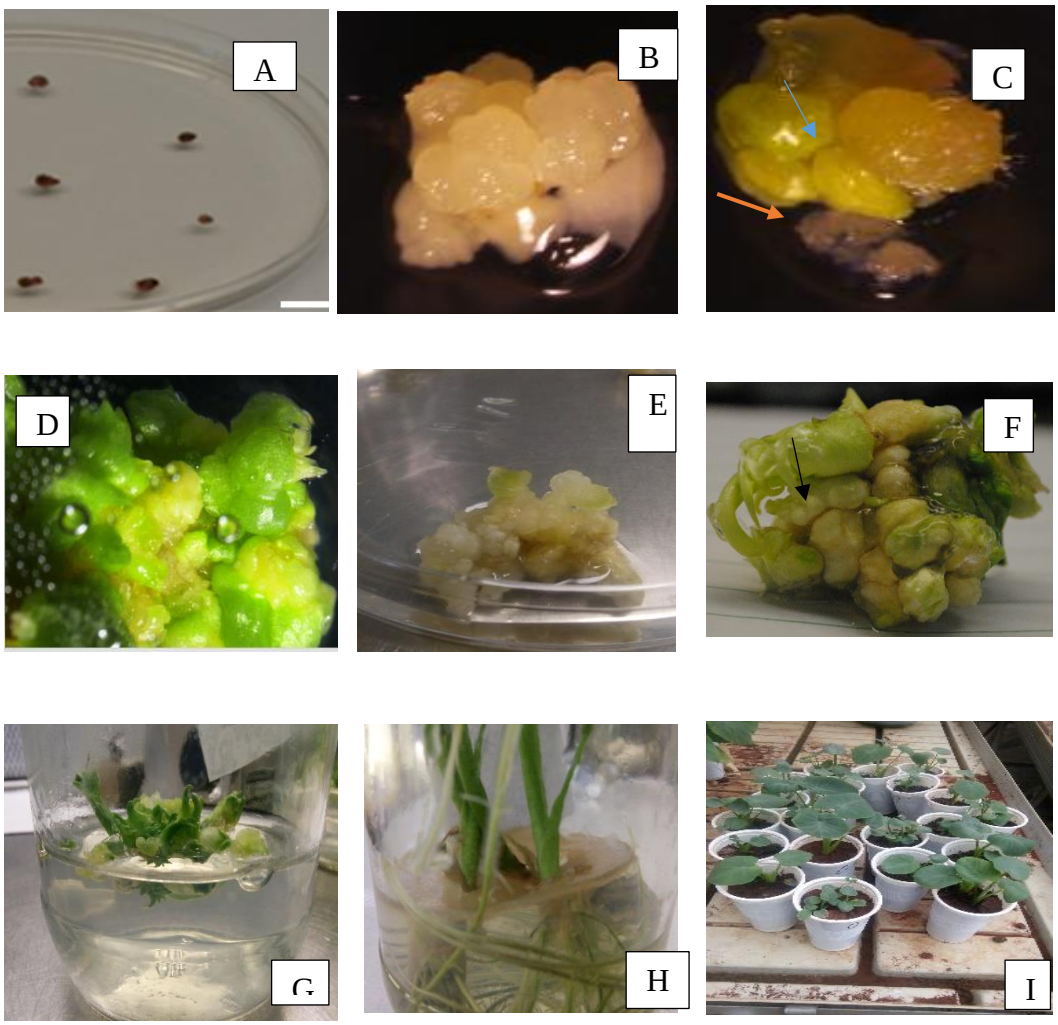

Figure 4.9: Somatic Embryogenesis in C. Esculenta

Figure 4.9: Somatic embryogenesis in *C. esculenta* A: Swollen mass of callus formed three weeks after apical meristem culture in CIM, B: A microscopic image showing primary callus formed after four weeks of apical meristem culture in CIM, C: Compact (blue arrow) and friable (orange arrow) embryogenic calli formed after eight weeks in embryo formation media , D: A microscopic image showing embryogenic calli with all

stages of embryogenic callus development including globular, heart shape, and torpedo stages, E: Shoot induction from the compact embryogenic calli after eight weeks in maturation media containing 2mg/L BAP**,** F: Multiple shoot induction from friable embryogenic calli in media containing BAP, arrow shows a shoot emerging from cotyledonary embryo, G: Fully developed and elongated shoots from green embryogenic calli, H: Rooted shoots after 8 weeks in root induction media, I: Acclimatization of plantlets in the C, B and D.

Table 4.7: Effect of Different Combined Concentrations of 2, 4-D and TDZ on Callus Formation and Combined 2,4-D and TDZ Concentration and 2mg/L BAP on Embryogenesis Using Apical Meristems

Variety	Concentration	% callus formation	% Embryogenesis
Dasheen	1A	0.0 ±0.0^{d}	0.0±0.0^{d}
	1B	11.1 ±1.4^{cd}	4.4±3.5^{cd}
	1C	35.6 ±2.0^{b}	9.7±1.3^{b}
	1D	13.3 ±1.9^{cd}	7.1±1.9^{c}
	2A	0.0 ± 0.0^{d}	0.0±0.0^{d}
	2B	17.8 ±2.2^{c}	8.4±1.5^{c}
	2C	77.8 ±0.4^{a}	42.7±0.9^{a}
	2D	13.3 ±2.3^{cd}	6.3±2.9^{cd}
	3A	0.0 ±0.0^{d}	0.0±0.0^{d}
	3B	13.3 ±1.9^{cd}	6.6±1.6^{cd}
	3C	35.6 ±2.7^{b}	8.3±1.2^{c}
	3D	13.3 ±2.9^{cd}	7.6±1.3^{c}
Purple wild	1A	0.0 ±0.0^{d}	0.0±0.0^{d}
	1B	11.1 ±1.9^{cd}	2.1±2.6^{d}
	1C	33.3 ±2.7^{b}	7.3±1.3^{c}
	1D	6.7 ±1.9^{cd}	4.7±1.8^{cd}
	2A	0.0 ±0.0^{d}	0.0±0.0^{d}
	2B	13.3 ±0.9^{cd}	6.3±1.4^{cd}
	2C	71.1 ±0.2^{a}	39.1±0.8^{a}
	2D	15.6 ±0.8^{c}	6.4±0.4^{cd}
	3A	0.0 ±0.0^{d}	0.0±0.0^{d}
	3B	15.6 ±1.4^{c}	4.2±1.3^{cd}
	3C	35.6 ±2.2^{b}	7.1±1.4^{b}
	3D	13.3 ±0.7^{cd}	5.3±1.1^{cd}

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. 1A (5µM 2,4-D, 0 µMTDZ),1B (5 µM 2,4-D, 1 µMTDZ),1C (5 µM 2,4-D, 2 µMTDZ),1D (5µM 2,4-D, 3 µMTDZ); 2A (10µM 2,4-D, 0 µMTDZ), 2B (10 µM 2,4-D, 1 µMTDZ), 2C (10 µM 2,4-D, 2 µMTDZ), 2D (10µM 2,4-D, 3 µMTDZ); 3A (15µM 2,4-D, 0 µMTDZ), 3B (15 µM 2,4-D, 1 µMTDZ), 3C (15 µM 2,4-D, 2 µMTDZ), 3D (15µM 2,4-D, 3 µMTDZ).

4.3.3 Effect of Different Media on Acclimatization of Taro Plantlets

Thirty tissue cultured plantlets from both direct organogenesis and indirect somatic embryogenesis which were between at 7cm to 12 cm in height, with 7 roots and at least 3 leaves were selected for green house acclimatization. Here, 10 plantlets were placed in each of the three media selected. After 1 week of acclimatization in the greenhouse, there was 100% survival of all the plantlets. However, there was no change in leaf number and height (cm). The average height of plantlets transplanted on soil: sand: manure media (3:1:1 ratio) was 8.95cm while on vermiculate was 7.35cm and finally on a control media (soil only) was 7.01cm after 2 week of transplanting in the greenhouse. Notably, by the end of 10weeks, the average height of plantlets on soil: sand: manure media was 10.28cm while those on vermiculate media were 8.7cm. All the plantlets transplanted on the control withered and died. The highest mortality rate was due to the rotting of the plantlets from the leaves and full acclimatization was actually observed from the 6^{th} week. Though plantlets continued to grow increasing height, no new leaves formed. The survival rate in both soil: sand: manure and vermiculate media was 60% and 40% respectively.

Table 4.8: Effect of Media on Greenhouse Acclimatization

Weeks	Data recorded after every 2 weeks on different acclimatization media					
	Control (soil only)		Soils: manure		vermiculate	
	Mean height	Survival rate	Mean height	Survival rate	Mean height	Survival rate
0	7.01±0.87[b]	100	8.95±1.41[c]	100	7.35±2.37[c]	100
2	7.01±0.78[b]	50	9.01±1.42[b]	90	7.82±0.75[b]	70
4	8.2±0.83[a]	20	9.03±2.08[b]	80	7.51±1.21[b]	60
6	0	0	9.05±3.08[b]	70	7.89±2.61[b]	50
8	0	0	10.01±3.41[b]	60	8.13±2.61[b]	40
10	0	0	12.28±3.84[a]	60	8.41±1.96[a]	40

Means were compared using Duncan multiple range test and bars with different letters in a figure are significantly different ($p \leq 0.001$).

CHAPTER FIVE

DISCUSSION

5.1 The genetic diversity of Taro germplasm

Researchers, (Palapala and Akwee, 2016), have estimated the level and distribution of genetic variation within the East African Taro population. Here, they found out that there are similarities and dissimilarities within and among Taro accessions in Kenya. However, a higher within population variation was revealed than among populations and only three regions were mentioned (Nyanza, western and Rift valley), which does not represent the Eastern and central regions of Kenya where farmers also cultivate Taro. They further described the contribution of Taro to food security and to the profile of national research as low and underrated. Despite the fact that microsatellites such as SSR markers are very high per locus, they cause biasness in diversity estimates as a result of heterozygosity levels (Palapala and Akwee, 2016). In addition these microsatellites also suffer homoplasy problems (Schlotterer *et al.,* 1998) hence increasing chances of within population variation. Therefore, there is need to explore other platforms that are cost effective and more efficient to assess the genetic diversity of Taro.

This study explores the DArT platform, which has been previously exploited to genomically dissect different cultivated species (Brinez *et al*., 2012; Akbari *et al*., 2006). This platform gives advantage to the less developed countries because the average cost per data point of silicoDArT is less than that of SNP markers (Kilian *et al*., 2003). Besides, this system detects two types of markers; SNP and Silico DArT, which shows high call rates and reproducibility. This has been previously observed (Alam *et al*., 2018; Hassani *et al*., 2020) proving the dependability of DArT technology to genotype several plant species.

Additionally, other obtained results have indicated that DarTseq is a cost effective method of performing genotyping-by-sequencing technique to analyze genetic diversity of huge

and also organisms with no reference genome complex (Egea *et al.*, 2017). Consequently, with developing advances in molecular biotechnology, it is essential to focus on each and every crop so as to alleviate poverty and make the world a food secure place. Furthermore, molecular markers, manage and protect genetic diversity of crops (Egea *et al.*, 2017). In fact recent advances such as DArTseq have facilitated the reduction of complexity hence resolving complex genomic samples (Jaccoud et al., 2001). Therefore, the DArTseq technique has been used in the present work to evaluate the genetic diversity of 188 Taro samples. Data has been analyzed using statistical R software, with routine data manipulation in reference to packages such as adegenet, tidyr and dplyr. Relationship matrices with the aid of APE packages have been calculated and used to infer neighbor joining tree and lastly a phylogenetic tree obtained from the iTOL web tool.

A total of 33,422 SNPs and 80,222 silicoDArT markers were generated from 186 individuals of *C. esculenta*. The silico DArT markers have shown consistent marker score and near 100% reproducibility. Besides, statistical analysis of DArT data sets have shown high consistency with the results based on SNPs highlighting the aptness of DArT platforms.The SNP data set has shown minimal differentiation where three clusters have been obtained. One cluster had 174 varieties, and the other two clusters had 6 varieties each. Subsequently, there is evidence that the GH varieties that some farmers argued as edible is a distant relative of the Dasheen, eddoe and Purple wild varieties, but with no much genetic variation. They also differed phenotypically, where the GH family had large dark green leaves with an apparent venation. On the other hand, a dendogram was also drawn using the silico DArT marker data sets (Figure 4.4). Here, four clusters have been obtained (A, B, C and D) and it is evident that the grouping was not based on the geographical locations.

In group A, all the counties were represented except Kakamega County. Notably, the GH2 variety was classified here. However, these samples were only 29 in total. In group B, all the counties were represented but no GH variety was classified here. Meru County had 17 samples represented here. This group had a total of 62 samples. In group C, all 8 counties were represented except Meru County. Notably, the GH3 variety was classified here. A

total of 37 samples were grouped in this category. Lastly, in group D, a total of 56 samples were classified here. The majority of the GH family including GH1, 4, 5, 7, 8, 9, 10, 11, 12, 13, and 15 were categorized here. This group represented all counties with Meru, Murang'a and Kisii having one sample each. Generally, only sample GH14 and Siaya 16 were unsuccessfully sequenced.

To have more insight into the varieties grouping and the pattern of variation, principal coordinate analysis (PCA) was used to assess variation. This showed 77.9% (Figure 4.6) and 61.1% (Figure 4.5) of the total variation in the samples based on silicoDArT and SNP markers, respectively. Besides, it is in agreement with the Neighbor Joining analysis for the SNP data and the phylogenetic circular dendogram for the silico Dart markers which showed that the varieties were not classified according to their geographical provenance.

Indeed, this is not surprising due to the fact that farmers borrow planting materials from each other. In the near future it is expected that TGS techniques will advance (Dorado et al., 2015b), and more analysis such as the possibility of analyzing whole genomes will be a dream come true putting in mind the low cost reduction brought about by this techniques. Through this technique, it is possible to maintain and even manage biodiversity (Egea *et al.*, 2017), and this is a milestone for the Taro crop. In addition to biodiversity management, results obtained by DArTseq help in enhancing more analyses in germplasm collection even for asexual reproductive systems, that are mainly said to hinder diversity analysis (Gebhardt et al., 2013).

In conclusion, the Taro genotype in Kenya are four; the Dasheen, eddoe, wild and 'unknown'. Of note, some wild types are edible. The commonly grown varieties by the farmers for consumption include; the Dasheen, eddoe, 'unknown', and Purple wild. Phenotypically, the Dasheen, eddoe and 'unknown' can be easily be distinguished from the Purple wild, that is also consumed by some farmers, by just looking at the leaf colour, which is dark purple for the Purple wild and green for the Dasheen, eddoe and 'unknown.

5.2 In Vitro Regeneration of Kenyan Taro Germplasm through Direct Organogenesis

Micropropagation is the *in-vitro* clonal propagation of plants (Bhojwani and Dantu, 2013). Since food insecurity is still a serious global challenge, it is imperative to develop a direct shoot organogenesis protocol for crops of economic importance as these protocols enable us to produce large numbers of new high-quality plantlets in a relatively short time and space. Micropropagation is a low-cost technology, and plantlet derived from micropropagation helps in plants germplasm conservation (Gupta et al., 2020). In this study, micropropagation method was developed for two common varieties of Taro in Kenya, that is, Dasheen and Purple wild. Benzylaminopurine (BAP), an adenine derivative, is an important cytokinin used in shoot induction (Chand et al., 1999). BAP hormone is mainly preferred for *in-vitro* regeneration of monocots (Ramakrishnan et al., 2014). Results generated from shoot proliferation media showed a significant difference in the number of shoots induction between the two Taro varieties. Reports by Toledo et al. (1998) state that various varieties of potatoes respond to shoot induction differently because of different genetic background. In this study, shoots and leaves were best produced on the two varieties when cultured on a BAP level of 2 mg/L. Here, we noted that this regime gave the best shoot induction in both Dasheen and Purple wild varieties. BAP has previously enhanced growth and development of axillary buds in C. esculenta var. esculenta, and this has been documented in a previous study (Chung and Goh, 1994). Seetohul et al., 2008 has reported the highest *in-vitro* multiplication rates of Taro shoot tips in MS medium supplemented with 2 mg/L of BAP, which is agreeing with our findings. As observed in the current study, a higher concentration of BAP (6 mg/L) produced a fewer shoot compared to a lower concentration (El-Sayed et al., 2016). This could be attributed to the toxicity caused by high cytokinin concentrations, which causes a delay in shoot formation (Manju et al., 2017). During the multiplication stage, the requirement of cytokinin differs depending on the type of crop, explant, developmental phase, growth regulator concentration, and the interaction between growth regulators and the environment (Yokoya et al., 1999). Both Taro varieties used in this study successfully

formed root when grown in MS media supplemented with different concentrations of IBA as well as in the absence of IBA. Roots are essential to plants as they supply water and nutrients to plants (Schiefelbein et al., 1997). An earlier study reports better rooting in Taro shoots in half strength MS medium supplemented with 1.5 or 2.0 mg/L of naphthaleneacetic acid (NAA) than in half-strength MS medium supplemented with 1.5 or 2.0 mg/L IBA at 15 days of culture (Behera and Sahoo, 2008). This present study has shown that MS (4.4 g/L) premix supplemented with 0.5 mg/L of IBA was the best concentration for rooting in both Taro varieties evaluated. The application of auxins to micro-propagated shoots could intensify the production of the root by increasing the endogenous contents of enzymes (Asghar et al., 2011). Another study reports auxins induced complication in lateral root formation through repetitive cell division (Liu et al., 2002). In the present study, the roots were of a shorter length in IBA supplemented medium than those induced in media with no IBA, possibly due to inhibition of shoot bud formation at elevated IBA concentration and arrest of root production as the auxin in the root primordial is shifted from the apex shoot (Ozel et al., 2006). A previous study established that plants could be transplanted when they have rooted, but good results are achieved if the plants do not have too many long roots but have a greater number of roots (Singh et al., 2012). Despite initial difference in root length all the plants developed well during the acclimatization. Therefore, this present study has demonstrated that supplementation of MS media (4.4 g/L) with 2.0 mg/L BAP and 0.5 mg/L IBA enhance better shoot and root induction, respectively.

5.3 In Vitro Regeneration of Kenyan Taro Germplasm through Indirect Somatic Embryogenesis

Somatic embryogenesis is a developmental regenerative pathway that somatic cells develop into structures resembling zygotic embryos following orderly embryological stages without fusion of gametes (Mendez-Hernandez *et al*., 2019; Nolan &Rose, 2010). It is a regeneration technique that is mainly triggered by both auxins and cytokinins (Jia X X *et al*, 2008) with the later having the potential to enhance cell division of pre-embryonically cells (Kintzios S. *et al*., 2002). This technique is a powerful tool for genetic

improvement that enhances large production of plants throughout the year (Bhansali *et al*., 1991). Previously, a two-step protocol was developed to initiate embryogenic callus from Taro corm explants (Deo *et al*., 2009). This protocol was quite cumbersome and time-consuming. Therefore, this study has established a one step-protocol from apical meristem explants in Taro to speed up the embryogenic process of this food security crop.

After two weeks of culture, callus was only formed on apical meristem explants maintained in the dark conditions. However, no callus was observed forming on any of the explants placed in light conditions. This callus was best formed on MS media supplemented with 10µM 2,4-D and 2µM TDZ hormones. Despite the fact that TDZ, (cytokinin) has been shown in several monocots and dicots to induce somatic embryogenesis (Chen J T *et al*., 2004; Deo PC *et al*., 2009; Khan H *et al*., 2006; Lin CS *et al*., 2004; Panaia M *et al*., 2004 and Sheibani M *et al*., 2007), this was difficult in root tips and leaf discs. Therefore, based on these observations, embryogenic callus initiation experiments were performed using apical meristems as the explants and dark treatment conditions, proving that meristematic tissues are the most suitable explants for somatic embryogenesis (Lakshmanan and Taji, 2000). Besides, the suitability of dark treatment conditions have also been affirmed by previous studies on wheat (Mahalakshmi *et al*., 2003), and barley (Sharma *et al*., 2005).

Additionally, results have shown that the MS media concentration with 10µM 2, 4-D and 2µM TDZ gave better callus response (77.8% and 71% for Dasheen and Purple wild, respectively). The embryogenesis rates of this media concentration were highest (42.7% and 39.1% for Dasheen and Purple wild varieties, respectively). Furthermore, this study observed a genotype-dependent response. The Dasheen variety gave better callus response and embryogenic rate than the Purple wild variety. This is confirmed by previous studies (Nyaboga *et al*., 2015, Narváez *et al*., 2019, and Syombua *et al*., 2019) that also showed genotype-dependent responses to somatic embryogenesis.

Generally, somatic embryogenesis is initiated and manipulated by adding plant hormones in the culture medium to enhance synchronized production, maturation, and conversion of

embryos into whole plants (Syombua *et al*., 2021). These phytohormones generate tissues that resemble zygotic embryos by promoting tissue dedifferentiation, chromatin remodeling, and gene expression reprogramming of somatic cells (Kumar *et al*., 2015). The present study has used TDZ and 2, 4-D to induce dedifferentiation in Taro. Similar to the findings by Deo *et al*., 2019, 2,4-D and TDZ enhanced embryogenesis response in Taro. Besides, cytokines promote somatic embryo maturation (Lema-Ramnska J. *et al*., 2013). However, this protocol took long to induce callus. Therefore, this study uses lower concentrations of 2,4-D and TDZ to induce somatic embryogenesis since Taylor *et al*., 1992 showed that elevated auxin levels converted embryogenic calli into non-embryogenic status. This was achieved and was also reported in other monocotyledonous plants such as wheat (Adero *et al*., 2019) and in sugarcane (R. Kaur & Kapoor, 2016). Additionally, Verma *et al*., 2017, showed that direct somatic embryos could be induced using 2,4-D and TDZ media regimes but using axillary meristems. Therefore, depending on the target explant, it is possible to regenerate Taro, either through callus or by directly forming somatic embryos.

5.4 Acclimatization of Taro Tissue Cultured Plantlets

Tissue cultured plantlets acclimatized in soil: sand: manure ratio (3:1:1) and vermiculate after 12 weeks have shown a survival rate of 60% and 40%, respectively. Acclimatization is a key challenge in most of the tissue cultured plants due to the shock and stress experienced upon transfer from the *in vitro* environment to the *ex vitro* conditions in the greenhouse. However, a high rate of plantlet loss upon acclimatization hinders mass production (Fernando *et al*., 2004) hence availability of clean planting materials becomes a challenge.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

First, this study proves that there exists genetic diversity in Taro germplasm in Kenya, hence classifying the genotypes into four clusters; the Dasheen, eddoe, wild and 'unknown'. Subsequently, this study has shown that there exists an 'unknown' classification of Taro in Kenya. Notably, this variety is more powdery when cooked and dried and sweeter than other varieties. This was majorly found in the central and eastern region of Kenya.

Secondly, this study has affirmed that it is possible to *in-vitro* regenerate Kenyan Taro germplasm through direct organogenesis. Here, the greatest proportion of survival was observed when the explants were exposed to 40% NaOCl for 20 minutes (90%) followed by 40% NaOCl for 30 minutes (80.33%). Then, a concentration of 2mg/L BAP hormone was established to be the best for shoot induction and multiplication for Taro using apical meristems. Subsequently, a concentration of 0.5mg/L IBA hormone induced the best roots for these shoots. Lastly, acclimatization was done and 100% survival of the plantlets was observed and they phenotypically resembled the mother plants.

Thirdly, it is possible to induce callus and regenerate Taro germplasm. This is because this study has established a one step-protocol using apical meristems as explants. Here, callus was formed on apical meristems maintained in the dark and was best formed on MS media supplemented with 10µM 2,4-D and 2µM TDZ hormones.

6.2 Recommendations

- Breeders should utilize this data during parental selection for new breeding techniques and genome-wide association studies (GWAS).

- This direct organogenesis protocol is easy to follow and it can be used for production of large number of *in vitro* plantlets for commercial production.
- This protocol is reproducible and research agencies should adopt it to mass regenerate Taro and conduct genetic engineering experiments that will improve biotic and abiotic adaptability of Taro to enhance food security and generate cash for the poor farmers.

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APPENDICES

Appendix I; County name, germplasm number, location and GPS coordinates of Taro accessions studied.

County name	Germplasm Number per County	Location(Village, Ward or Sub-County)	Global positioning system(GPS) Co-ordinates
Kiambu	1,2	Ngewa Ward, Kikuyu sub-County	E 36° 51'57.5",S -1° 5'54.9"1636m asl
	3,4	Kikuyu ward, Kikuyu sub-County	E 36° 39'18.3",S-1° 14'47.2" 1986m asl
	5,6	Kikuyu ward, Kikuyu sub-County	E 36° 40'58.5",S -1° 16'47.3",1865m asl
	7,8	Karai ward, Kikuyu sub-County	E 36° 39'47.5",S -1° 18'38.1",1847m asl

	9,10	Mutarara village, kikuyu ward	E 36° 39'47.5",S -1° 18'38.1",1847m asl
	11,12	Gatumumu, Gitaru ward	E 36° 42'08.2",S -1° 13'32.5",1882m asl
	13,14	Gitaru ward	E 36° 41'33.9",S -1° 13'05.5",
	15,16	Kabete ward, Muguga area	E 36° 43'42.2",S -1° 14'01.3",1777m asl
	17,18	Dongoro village, Kabete ward	E 36° 42'57.4"S -1° 14'04.1",1841m asl
	19,20	Kikuni village, Nyathona ward	E 36° 41'51.4",S-1° 11'28.4",1925m asl
Muranga	1,2	Ngenda ward	E 37° 7’ 48.6’’S 0° 46’15.3’’,1310m asl
	3,4	Ngenda ward	E 37° 37'42.3",S 0° 46'15.5",1314m asl
	5,6	Ngenda ward	E 37° 7’ 52.6’’,S 0° 46’ 13.9’’,1309m asl
	7,8	Ngenda ward	E 37° 7’ 44.6’’,S 0° 45’ 58.6’’,1291m asl
	9,10	Itito village, Ngenda ward.	E 37° 7’36.1’’,S 0° 46’28.0’’,1348m asl
	11,12	Marumi village, Kigumo	E 36° 58’44.3’’,S 0° 48’39.7’’,1690m asl

		ward, Kigumo sub County	
	13,14	Marumi village	E 36° 58’ 55.0’’,S 0° 48’ 34.9’’,1682m asl
	15,16	Nguku village, Kigumo ward, kigumo sub County.	E 36° 59’ 24.3’’,S 0° 49’ 10.2’’,1684m asl
	17,18	Nguku village, Kigumo ward	E 36° 59’17.1’’,S 0° 49’15.5’’,1686m asl
	19,20	Kirere village	E 37° 0’33.5’’,S 0° 48’37.0’’,1612m asl
Meru	1,2	Kinwe village, Mikinduri ward, Tigania East sub County.	E 37° 48’ 39.7’’ N 0° 6’ 41.5’’
	3,4	Kagaine village, Nkomo ward, Tigania	E 37° 46’29.4’’,N 0° 5’14.8’’,1179m asl

		west sub County,	
5,6	Kioru	village, Nyaki ward, North Imenti sub County	E 37°44’ 32.9’’,N 0° 2’ 17.0’’,1139m asl
7,8	Kioru	village, Nyaki ward, Imenti north sub County	E 37° 44’31.0’’,N 0° 02’ 25.7’’,1148m asl
9,10	Konju	village, Mikindu ri ward, Tigania East sub County	E 37° 51’46.7’’,N 0° 5’10.0’’,1125m asl
11,12	Mwethe	village, Kigucwa ward, Tigania East sub County.	E 37° 51’ 23.3’’,N 0° 09’09’’,1453m asl
13,14	Imenti south sub County		E 37° 44’52.5’’,S 0° 6’ 01.3’’,1152m asl

	15,16	Nyagene ward	E 37°45’10.5’’,S 0° 6’32.8’’,1061m asl
	17,18	Gachero village, Mitunguu ward, South imenti sub County	E 37° 45’ 16.7’’,S 0° 6’ 41.4’’ 1049m asl
	19,20	Kithino village, Maraa ward, South Imenti constituency	E 37° 45'34.5",S 0° 7' 30.1",1009m asl
Nyeri	1,2	Sagana village, Ruguru ward, Mathira west sub County.	E 37° 5’ 57.5’’ , S 0° 20’ 26.5’’ ,1937M asl
	3,4	Iganjo Village, Ruguru Ward, Mathire west sub County	E 37° 5’58.7’’,S 0° 20' 0.8"

5,6	Karia village, Ruguru ward, Mathira west sub County	E 37° 5’ 42.9’’ S 0° 20’31.5’’
7,8	Karia village, Ruguru ward, Mathira west sub County.	E 37° 5’ 32.7’’ ,S 0° 20’ 40.9’’ 1903M asl
9,10	Wamunyoro village, Ruguru ward, Mathira West sub County.	E 37° 05’ 45.5’’ S 0° 20’51.5’’ 1915m asl
11,12	Karandi village, Ruguru ward, Mathira west sub County	E 37° 05’ 17.9’’ S 0° 20’ 53.4’’ 1873m asl
13,14	Jambo village, Karatina ward, Mathira	E 37° 8’ 8.8’’ S 0° 28’ 59.5’’ 1744m asl

		East sub County		
	15,16	Kiamwangi village, Iriaine ward, Mathira East sub County	E 37° 8’32.4’’	S 0° 28’ 31.4’’ 1737m asl
	17,18	Iriaine ward, Mathira East	E 37° 6’ 2.3’’	S 0° 28’ 42.1’’ 1718m asl
	19,20	Iriaine ward, Mathira east sub County	E 37° 5’ 34.3’’	S 0° 26’ 28.2’’ 1767m asl
Siaya	1,2	Gendro village, Central Alego ward, Alego Usonga sub County.	E 34° 8’ 51.8’’,N 0° 2’20.1’’,1143m asl	
	3,4	Gendro village, Central Alego ward, Alego	E 34° 9’ 15.8’,N 0° 2’31.6’’,1142m asl	

	Usonga sub County	
5,6	Gendro village, Central Alego ward, Alego Usonga sub County	E 34° 09’46.6’’,N 0° 2’19.7’’,1145m asl
7,8	Kanyango village, Central Alego ward, Alego Usonga sub County	E 34° 9’ 55.5’’,N 0° 2’ 10.2’’,1138m asl
9,10	Ulumbi village, Yala township ward, Gem sub County.	E 34° 33’0.4’’,N 0° 6’43’’,1397m asl
11,12	Ulumbi village, Yala township ward,	E 340 32’57.6’’,N 00 6’41.2’’,1395m asl

		Gem sub County	
	13,14	Anyiko Koncher a village, Yala township ward, Gem sub County.	E 34° 32’52.1’’,N 0° 7’8.5’’,1402m asl
	15,16	Tatro village, Yala township ward, Gem sub County	E 34° 32’42.5’’,N 0° 7’50.7’’,1407m asl
	17,18	Ochuor village, Yala township ward	E 34° 32’45.1’’,N 0° 8’13.9’’,1408m asl
	19,20	Ochuor village, Yala township ward	E 34° 32’41.3’’,N0° 8’20.7’’,1408m asl
Busia	1,2	Bubamba village, Bunyala central ward,	E 34° 0’53’’,N 0° 3’47.4’’,1134m asl

	Budalangi sub County	
3,4	Bubamba village	E 34° 0’54.3’’,N 0° 3’52.7’’1137m asl
5,6	Siangwede village	E 34° 0’59.4’’,N 0° 3’41.8’’,1140m asl
7,8	Siangwede village	E 34° 1'0.7",N 0° 3'40.7",1138m asl
9,10	Hunamwakwe village	E 34° 1’10.4’’,N 0° 3’37.7’’,1138m asl
11,12	Iseme village, Bunyala central ward	E 34° 01’26.7’’,N 0° 04’20.4’’,1132m asl
13,14	Bwaluanga village	E 34°01’28.2’’,N0° 0.5’40.2’’,1138m asl
15,16	Makhoma village, Hajula ward, Budalangi sub County	
17,18	Erukalabudala village, Hajula ward.	E 33°59’31.4’’,N 0° 3’55.4’,1136m asl

	19,20	Runyu village, Hajula ward	E 33° 59’31.4’’,N 0° 3’55.4’,1136m asl
Kakamega	1,2	Shitaho village, Mahiakalo ward, Lurambi Sub County.	E 34° 46’5.8’’, N 0° 17’18.7’’,1507m asl
	3,4	Ebihule village, Butsotso East ward, Rurambi sub County	E 34°C 43’36.2’’,N 0° 19’44.3’’,1462m asl
	5,6	Shitirira village, Butsotso east ward	E 34° 44’4.5’’N0° 18’57.7’’1493m asl
	7,8	Shikoti village, Butsotso East ward	E 34°43’58.2’’N 0°19’6.8’’1490m asl
	9,10	Shikoti village ,Butsotso ward	E 34° 46'32.7', N 0° 17'27.5", 1548m asl
	11,12	Mukwapa village, Ingotse	E 34° 44’ 4.3’’,N 0° 20’ 56.3’’,1467m asl

		matia ward, Navakholo sub County.	
	13,14	Mushiachi village, Ingotsematia ward	E 34° 44’32.5’’N 0° 21’42.6’’,1484m asl
	15,16	Viande village, Ingotsematia ward, Navakholo sub County.	E 34° 45’16.1’’N 0° 21’5.8’’1487m asl
	17,18	Msamba village, Ingotsematia ward	E 34° 41’8.9’’,N 0° 21’52.3’’,1423m asl
	19,20	Ingungu village, Ingotsematia ward.	E 34° 41’34.2’’N 0° 21’13.1’’,1463m asl
Kisii	1,2	Gitenga village, Obarachoward, Kitutu	E 34° 47’27.6’’ S 0° 40’41.8’’ ,1731m asl

		chache south sub County	
3,4	Kisii	prisons, Obarach o ward, Kitutu Chache south sub County	E 34° 46’26.5’’,S 0° 40’19.6’’,1637m asl
5,6	Mwobuba	village, Nyakoe ward, Kitutu chache south sub County.	E 34°46’41.8’’,S 0°36’34.0’’,1607m asl
7,8	Siara	village, Nyakoe ward.	E 34° 43’54.4’’,S 0° 37'17.4",1500m asl
9,10	Siara	village, Nyakoe ward.	E 34° 44’11.1’’,S 0° 37’21.4’’,1514m asl
11,12	Siara	village, Nyakoe ward.	E 34° 43’56.0’’,S 0° 37’13.7’’,1500m asl

	13,14	Siara village, Nyakoe ward	E 34° 43’57.4’’,S 0° 37’9.0’’,1496m asl
	15,16	Siara village.	E 34° 33’54.1’’,S 0° 37’18.7’’,1497m asl
	17,18	Siara village	E 34° 43’52.2’’,S 0° 37’20.2’’,1495m asl
	19,20	Siara village, Nyakoe ward, Kitutu chache south.	E 34° 43’49.5’’,S 0° 38’5.4’’,1545m asl
Machakos	1,2	Chumba village, Kathiani ward, Kathiani sub County.	E 37° 17’58.3’’S 1° 30’2.1’’,1958m asl
	3,4	Chumba village, Kathiani ward, Kathiani sub County.	E 37° 18’2’’,S 1° 30’4.3’’,1922m asl
	5,6	Chumba village, Kathiani ward, Kathiani	E 37° 18’2.8’’,S 1° 30’3.4’’,1919m asl

	sub County.	
7,8	Chumba village, Kathiani ward, Kathiani sub County.	E 37° 18’6’’,S 1° 30’3.9’’,1913m asl
9,10	Kinyumo village, Kathiani ward, Kathiani sub County.	E 37° 17.5’59.0’’,S 1° 30’22.4’’,1885m asl
11,12	Kinyumo village, Kathiani ward.	E 37° 17’57.3’’,S 1° 30’26.8’’,1876m asl
13,14	Kinyumo village, Kathiani ward.	E 37° 17’56.7’’,S 1° 30’28.4’’,1875m asl
15,16	Kinyumo village	E 37° 17’52.2’’,S 1° 30’22.2’’,1890m asl
17,18	Kinyumo village.	E 37° 17’54.0’’,S 1° 30’23.9’’,1884m asl
19,20	Kinyumo village.	E 37° 17’55.5’’,S 1° 30’26.9’’,1882m asl

Appendix II: Nanodrop machine results.

#	Nucleic Acid Conc.	A260	A280	260/280
1	297.7	5.953	3.254	1.83
2	83.5	1.671	0.918	1.82
3	193	3.86	2.135	1.81
4	160.4	3.207	1.776	1.81
5	45.7	0.914	0.545	1.68
6	105.7	2.114	1.192	1.77
7	188.4	3.767	2.088	1.8
8	64.4	1.289	0.723	1.78
9	105.7	2.113	1.183	1.79
10	103.1	2.062	1.163	1.77