Nutritional quality of amadumbe (*Colocasia esculenta* L. Schott) and development of an efficient tissue culture propagation protocol

By

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Declaration

I declare that the dissertation herewith submitted to the Department of Biotechnology and Food Science, Durban University of Technology for the award of a master's degree in Food Science and Technology is my work and has not been previously submitted for a degree at any other University or any Higher Education Institution.

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Date

Dedication

This research work is dedicated to my beloved late grandmother Rose Beato and father Sipho Dunford Mntambo who have meant and continue to mean something to me. Although they are no longer of this world their memories continue to shape my life.

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List of abbreviations

GLVs – Green leafy vegetables
DW – Dry weight
FW – Fresh weight
NDF – Neutral detergent fibre
ADF – Acid detergent fibre
FAO – Food Agriculture Organisation
WHO – World Health Organisation
HDPE – High density polyethylene
FAOSTAT – Food Agriculture organisation statistics
CEC – Cation exchange capacity
SOM – Soil organic matter
EC – Electrical conductivity
USDA-NRCS – US Department of Agriculture- Natural Resources Conservation Service
GA – Gibberellic acid
BAP - Benzylaminopurine
PGR – Plant Growth Regulator
IBA – Indole-3-butyric acid
NAA - 1-Naphthaleneacetic acid
2.4D - 2, 4-Dichlorophenoxyacetic acid
PAA - Phenylacetic acid
TDZ - Thidiazuron
m*T* – meta-Topolin
ZEA - Zeatin
KIN - Kinetin
LED – Light emitting diode
PAR – Photosynthetically active radiation
RH – Relative humidity
AT – Air temperature
MS – Murashige and Skoog
NaDCC – Sodium dichloroisocyanurate
PPM^{TM} – Plant Preservative Mixture
SGS – Standard Global Services
SASRI – South African Sugar Research Institute
AB-DTPA - Ammonium bicarbonate-diethylene triamine pentaacetic acetic acid.
ICP-OES - Inductively coupled plasma - optical emission spectrometry
ANOVA – Analysis of Variance
RDA – Recommended Daily Allowance
TLB – Amadumbe leaf blight
ACS – Aseptic culture
BC – Bacterial contamination
FC – Fungal contamination
SD – Shoot development
PCT – Plant Cell Technologies

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ABSTRACT

Colocasia esculenta (L.) Schott commonly known as amadumbe in South Africa is a conventional underutilised crop. Nutritionally, amadumbe leaves and corms contain nutritionally rich micro- and macro minerals. Amadumbe also contains high fibre, carbohydrates, and protein reserves. Amadumbe can be used as a dual-purpose crop to satisfy undernourished individuals and to alleviate global food insecurity. This research aimed to investigate the quality of amadumbe for use as a green leafy vegetable and to optimize a decontamination procedure to eliminate surface and endogenous contaminants in explants for plant tissue culture. Two sites Umbumbulu (South) and Snembe (North) of KwaZulu-Natal, South Africa were sampled and studied. The two sites are located on opposite ends of the province experiencing varied weather patterns *viz.* temperature and precipitation. Umbumbulu experiences temperatures of 26.2±20.6°C with annual precipitation of 573 mm compared to Snembe with temperatures of 26.5±20.2°C and annual precipitation of 597 mm. Specifically, the (young and mature) leaves and corms were harvested and processed for the quantification of the nutritional (micro and macro nutrients), proximate composition (organic molecules), antinutritional (oxalate concentrations) and optimisation of plant tissue culture decontamination procedure. Furthermore, soil samples were collected from both sites for determination of the soil mineral composition. Potassium was the dominant macro element ranging from (2.0 – 5.1 g/100 g), calcium (0.08 – 1.5 g/100 g), magnesium (0.14 – 0.48 g/100 g), and phosphorus (0.14 – 0.43 g/100 g) in plant tissues. Furthermore, higher levels of micro nutrients were observed with iron (13.4 -88 mg/100 g) and manganese (2.2 – 64 mg/100 g) dominating. Amadumbe leaves also showed to be abundant in moisture, protein, ash, NDF, and ADF. The soil mineral concentrations were significantly different ($p<0.05$) between locations with soil:plant organ interaction. The effect of [Control, PPM^{TM}, PPM^{TM} (P), NaDCC, and NaDCC (P)] in eliminating contaminants was insignificant ($p>0.05$), and further optimization approaches need to be investigated. Benlate, alcohol, and TWEEN 20 did not provide any effective outcome to remove possible endogenous and surface contaminants. Bacterial (BC), fungal (FC), and aseptic (ACS) cultures were lost at 120 d due to obstinate microorganisms.

CHAPTER 1

1. Introduction

Sub-Saharan Africa has the world's greatest prevalence of undernutrition, with one in every three individuals suffering from chronic hunger (FAO 2008; Boliko 2019). Unemployment, the deepening of the HIV/AIDS epidemic, and natural catastrophes are all linked to increasing poverty in the region (Banda Chitsamatanga and Malinga 2021; Lombe, Carvalho and Rosa-Santos 2024). The global food insecurity risks imposed by the recent COVID-19 pandemic have perpetuated food scarcity and distribution worldwide particularly in Sub-Saharan Africa and South Asia (Laborde *et al.* 2020). In extreme poverty, especially in Africa, it is highly unlikely people will afford nutritious foods, leading to a reliance on cheaper and less nutritious foods to avoid hunger and undernourishment (Pollard and Booth 2019). The social and economic changes demand an exploration of food substitutes like underutilized crops such as *Colocasia esculenta* to be used as a dual-purpose (root crop and leafy vegetable) alternative.

Colocasia esculenta (L.) Schott commonly known as taro and as amadumbe in South Africa is mainly grown for its starchy corm (Chivenge *et al.* 2015), but the utilization of its leaves as a green leafy vegetable in South Africa is insignificant. Green leafy vegetables (GLVs) offer variation to cereal and starch-based staple diets while also providing an abundance of critical micronutrients such as Ca, Fe, Cu, P, Cl, Na, and Zn (Sarker, Hossain and Oba 2020). They also supply a wide array of vitamins that are essential for normal growth, and development, especially for individuals that have marginal nutritional status (Kumar *et al.* 2020). Ghosh (2019) revealed vitamin A supports the prevention of cataracts and macular degeneration. Vitamin B6, thiamine, niacin, and riboflavin aid in proper development of fetal brain while strengthening and protecting the nervous system (Prajapati *et al.* 2011; Kalariya *et al.* 2023) Moreover, leafy vegetables are the only natural sources of folic acid, which are considerably high in leaves of lettuce, mustard green, *Colocasia*, green leaf spinach, asparagus, and turnip green plants as compared to other leafy and non-leafy vegetables (Kumar *et al.* 2020). In addition, folic acid retention in amadumbe leaves, despite the leaching (39%) into boiling water the gravy is subsequently consumed as gravy compensating for the loss as compared to other green leafy vegetables (Mitharwal *et al.* 2022). Despite South Africa being considered to be food secure at the national level (de Clercq, de Witt and Laker 2021), most people are food insecure at the household level because they cannot afford to feed their families properly (van

den Berg and Walsh 2023). GLV intake varies by household, and in South Africa, it is influenced by problems such as income, degree of urbanization, proximity to fresh produce markets, and season of the year (Njume, Goduka and George 2014). Poor households, who account for 78.2% of the population (Voster *et al.* 2007), consume more green leafy vegetables than their richer counterparts (Jansen van Rensburg *et al.* 2007). This is because, when GLVs are compared to exotic vegetables like spinach (*Spinacea oleracea*) and cabbage (*Brassica oleracea*), they are typically thought to be inferior in taste and nutritional content (Patricia *et al.* 2014). The corms are also known to have anti-nutritional factors such as calcium oxalate crystals. The needle shaped calcium oxalate crystals called raphides are typical of the aroid family. When *Colocasia esculenta* is consumed raw, causes the swelling of the lips, and throat. The reduction of the calcium oxalate crystals is obtained through cooking (frying and boiling) the leaves and corms for increased palatability (Mitchell *et al.* 2019; Siener, Seidler and Honow 2021).

While the corms of *Colocasia esculenta* have been found to provide important micronutrients and therapeutic properties, diminutive information is known about the leaves. It is envisaged that by providing information on the nutritional qualities of leaves collected from the same corm, South Africans will be encouraged to use *Colocasia esculenta* in their diets as a natural and economical whole food that contains carbohydrates (in the corms) and critical micronutrients in the leaves. However, the promotion of the consumption of leaves as a green vegetable reduces the bulking of the corms underground and subsequently a substantial loss in the mother stock plant. To maximize production of the corms an alternative propagation technique must be implemented to ensure continuous supply.

The conventional method of propagation of amadumbe is by exchanging vegetative propagules between farmers. This increases the risk of disease transmission between farms. Furthermore, the availability of planting materials limits commercialization. Additionally, ineffective sterilization approaches of underground food storage organs (geophytes) bring challenges of high contamination. If decontamination is successful, the output will be improved, and the tissue culture of the most promising landraces would allow for the bulk production of pathogen-free planting materials, year-round production, speeds up new variety production, maintain virus-free stock, crop improvement and may improve production and commercialization.

1.1. Objectives of the study

- To determine the nutritional (and anti-nutritional) status of amadumbe leaves of varying maturity collected from two popular landraces for use as green leafy vegetables
- To develop tissue culture propagation protocol for the two popular landraces to provide *en masse* clean planting materials.

1.2. Research Questions and Hypothesis

- What is the nutritional quality of the leaves of amadumbe as a green leafy vegetable?
- Are there any significant differences among the different landraces in nutritional factors found in the leaves?
- Are there any significant differences in nutritional (and antinutritional) between leaves of different maturity?
- Is there any correlation in the nutritional (and anti-nutritional) factors between the corm and the leaves of the same plant?
- Is there any correlation/interaction between the nutritional (and anti-nutritional) factors in the leaves and corms, and the soils where the plants are grown?
- Are there any anti-nutritional factors of health concern in the leaves of amadumbe?

CHAPTER 2

2. Literature review

2.1. Abstract

This review investigates the nutritional quality of *Colocasia esculenta* (amadumbe) and the development of an efficient tissue culture propagation protocols. Emphasis is placed on the mineral content of amadumbe leaves, highlighting their abundance of essential micronutrients when compared to conventional green leafy vegetables such as spinach (*Spinacia oleracea*) and cabbage (*Brassica oleracea*). The review explores the significance of amadumbe as a nutrient-dense food source, discussing its role in addressing malnutrition and enhancing food security, particularly in KwaZulu-Natal. Additionally, it evaluates the neutral detergent fiber (NDF), acid detergent fiber (ADF), and oxalate composition in amadumbe leaves and corms, which are critical for understanding their overall nutritional value and potential health implications. Green leafy vegetables play a crucial role in global nutrition due to their high micronutrient density, yet their mineral composition is often influenced by factors such as geographical location and growing soil conditions. While amadumbe is traditionally propagated vegetatively, challenges such as maintaining healthy planting material during dry seasons, susceptibility to diseases, and difficulties in achieving large-scale production inhibit its commercialization. These propagation impediments accentuate the need for alternative propagation methods. Plant tissue culture emerges as a promising alternative solution, offering a stable and efficient method for producing disease-free planting materials at a larger commercial scale. However, propagating geophytes like amadumbe presents unique challenges due to soil-residing microorganisms that complicate the tissue culture process. This review underscores the potential of tissue culture to revolutionize amadumbe cultivation and highlights the importance of further research to overcome existing propagation barriers.

2.2. Characteristics of (amadumbe) *Colocasia esculenta (L.) Schott*

2.2.1. Morphological structure and anatomy

Colocasia esculenta (L.) Schott commonly known as amadumbe, elephant's ear, or cocoyam, is an emergent, semi-perennial, aquatic, and semi-aquatic stemless herbaceous plant (Fig 1a) cultivated mainly for its starchy corms. *Colocasia esculenta* is a member of the Araceae family of monocots (Lebot *et al.* 2009). Approximately 144 genera and 3 645 widely dispersed species

belong to the Arum family (Croat and Ortiz 2020). The species is polymorphic and reproduction occurs through different species (Ivancic, Garcia and Lebot 2003). There are numerous cultivated varieties of amadumbe, but they may be divided into categorical species: *viz C. esculenta* var. *esculenta*, dasheen, containing miniature corm and massive cormels, and *C. esculenta* var. *antiquorum*, eddoe, with an enormous corm, and insignificant cormels (Onwueme 1999). The starchy, cylinder- or orbicular-shaped amadumbe corms measure up to 15 cm and up to 20 cm in diameter (Strauss 2021). Despite hydration, size, color, and chemistry, it has been discovered that these characteristics are highly varied (Strauss 2021). The corm is made of scales and concentric rings of scarred leaves on the outside (Fig 1). Amadumbe is characterized by a fibrous and adventitious root system that facilitates numerous plant-soil relationships (Purseglove and Sheard 1972; Onwueme 1978).

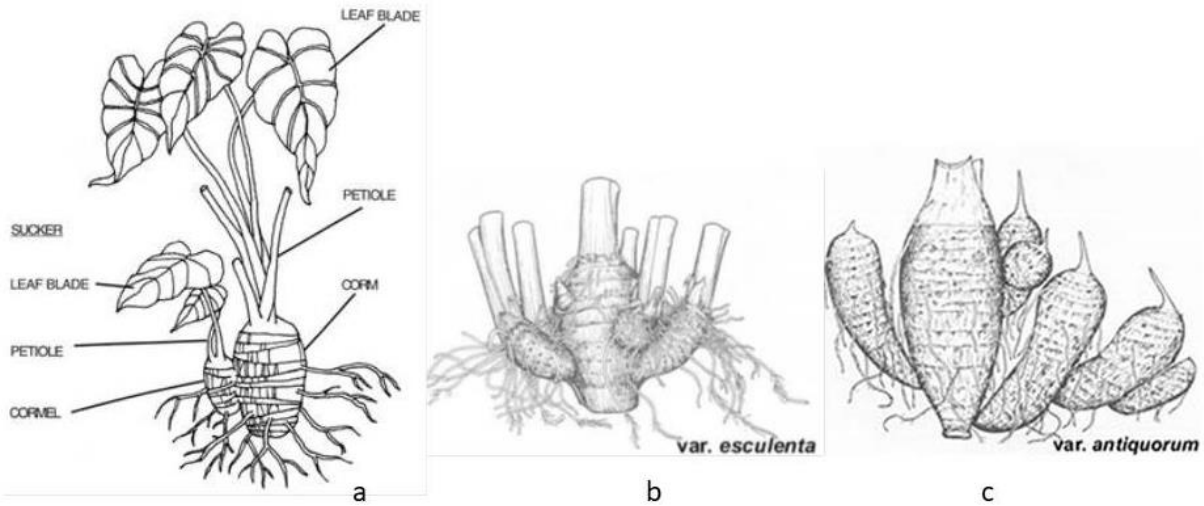

Figure 1: *Colocasia esculenta* (L) Schott: Illustrations of the full plant (a) and two main botanical varieties (b and c).

Source: (Sukal 2014)

2.2.2. Origin and distribution

According to (Chair *et al.* 2016) amadumbe evolved in tropical America and eventually expanded east to the rest of South East Asia, China, Japan, and the Pacific Islands. An alternate origin for amadumbe than the Asian core may have been the island of New Guinea, according to some experts. *C. esculenta* materialized on the east coast of Africa over 2000 years ago probably through voyagers crossing the continent to West Africa and later, by slave ships to

the Caribbean (van Wyk and Gericke 2000). West Africa has the largest area of cultivation, constituting the highest production tonnage, and the greatest amount of production (Otekunrin *et al.* 2021; Oladimeji *et al.* 2022). The global production of amadumbe in tonnes is illustrated in (Fig 2).

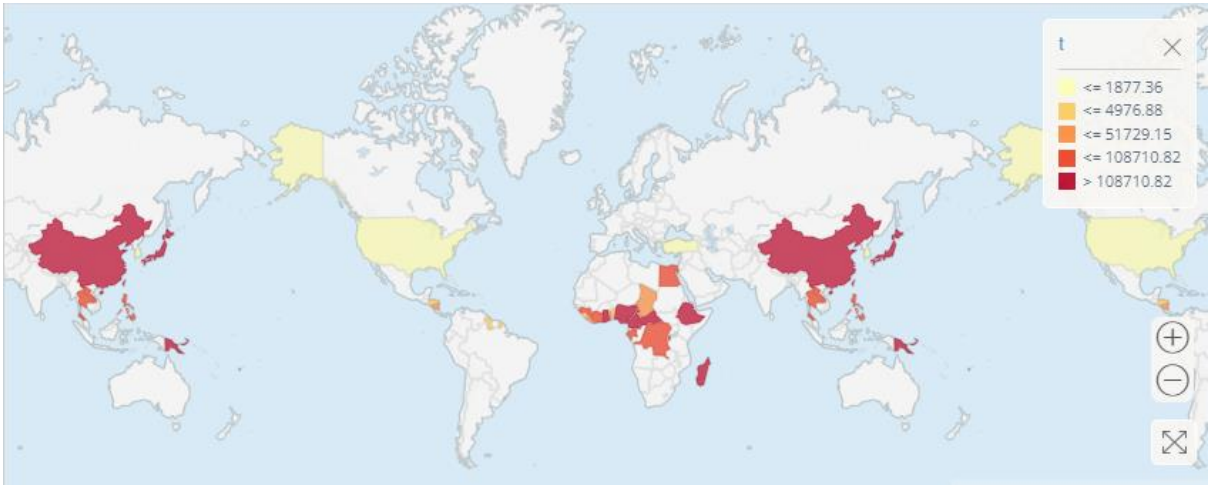

Figure 2: Top global countries producing amadumbe in million tonnes (period: 2000-2021)
Source: (FAO 2023)

2.3. Cultural practices

2.3.1. Fertilizer applications

The three basic nutrients used for the fertilization of amadumbe are nitrogen, phosphorus, and potassium to maintain plant growth, yield, and quality either individually or in combination (Rajičić *et al.* 2019). Considering that soils contain limited concentrations of phosphorus and potassium, additional nutrients are supplemented to achieve optimal yields and quality (Selim 2021). Fertilization affects the plant's access to water and nutrients, which may have an impact on the nutritional value of any harvested plant part (Grzebisz *et al.* 2013). Nonetheless, the implications of the mineral and elemental uptake by plants from fertilizers are significant and variable (Thomas *et al.* 2019) as its nutritional requirements differ considerably in its stage of development (John 2013).

2.3.2. Production systems

2.3.2.1. Flooded/Wetland amadumbe

In the development of amadumbe crops, there are two basic production methods: flooded or wetland (Fig 3a), and dryland (unflooded) or upland production (Fig 3b) amadumbe production (Zungu *et al.* 2016). Flood-prone cultivation occurs in locations with an excessive amount of water either directed from tributaries and streams, marshy land, or irrigation (Rashmi *et al.* 2018a). The soil must be dense enough to accommodate the impounding of water with little percolation loss (Rashmi *et al.* 2018a). The water in which the amadumbe is growing has to be temperate and continuously flowing for it to have the most dissolved oxygen possible to avoid amadumbe's basal rotting (Rashmi *et al.* 2018a). Flooded amadumbe is often farmed monoculture for several years as not many crops can tolerate the flooded environment (FAO 2015).

Figure 3: Conventional practices of amadumbe production - a) represent dryland/unflooded or rain-fed amadumbe production system, b) Wetland/flooded amadumbe production system.

2.3.2.2. Wetland Dryland/Unflooded amadumbe

Production of amadumbe in arid areas uses the dry land mode of cultivation. The timing of planting is crucial since dry-land amadumbe cultivation is rain-fed. Dry-land amadumbe can be cultivated on flat ground if the soil is deep and friable; otherwise, ridges of between 50 and 90 cm are formed and planting is done in the furrows unlike flooded amadumbe, dry-land amadumbe is often intercropped (FAO 1999).

2.4. The role of amadumbe leaves in dietary intake

2.4.1. Background of green leafy vegetables

Plant species that produce leafy vegetables include young succulent stems with blooms and very young fruits. Leafy components of these plants are consumed as vegetables (Hiscock *et al.* 2018). Green leafy vegetables (GLVs) are collectively referred to as "*morogo*" or "*imifino*" by South Africans (Faber *et al.* 2010b) (Table 1). GLVs have a very low level of acceptance due to their classification as foods for poor people experiencing poverty, and knowledge about them is considered to be outdated (Faber *et al.* 2010a). Therefore, South Africa, like many other nations, depends mostly on a restricted number of crops (Bjornlund, Bjornlund and Van Rooyen 2020), while neglecting traditional green leafy vegetables (Nlovu and Afolayan 2008).

Table 1: Commonly consumed green leafy vegetables in Sub-Saharan Africa.

Botanical Name	**Common name**	**Family name**
Amaranthus dubius	Red spinach, Chinese spinach	Amaranthaceae
Amaranthus viridis	Green amaranthus	Amaranthaceae
Basella alba	Malabar spinach	Basellaceae
Crassocephalum crepidioides	Ebolo, Red flower ragleaf	Asteraceae
Launaea taraxacifolia	African lettuce	Asteraceae
Amaranth gangeticus	Elephant head amaranth	Amaranthaceae
Chenopodium album	Baconweed	Amaranthaceae
Trigonela foenum-graecum	Fenugreek	Fabaceae
Brassica juncea	Chinese mustard	Brassicaceae
Spinacea oleracea	Spinach	Amaranthaceae
Amaranthus hybridus	Smooth pigweed	Amaranthaceae
Celosia argentea	Cockscomb	Amaranthaceae
Corchorus olitorius	Jutesmallow	Malvaceae
Solanum macrocarpon	African eggplant	Solanaceae
Solanum americanum	American black nightshade	Solanaceae

Source: (USDA 2014; Longvah *et al.* 2017; Ejoh *et al.* 2019)

2.4.2. Mineral composition in amadumbe leaves

GLVs are a significant source of essential minerals such as phosphorus, sodium, potassium, calcium, magnesium, iron, and zinc (Sarkar *et al.* 2022). Besides their metabolic function, their secondary role is acting as co-factors in enzymatic reactions (Mitharwal *et al.* 2022). Compared to vitamins, minerals tend to be more thermal and processing resistant (Van der Walt *et al.* 2009; Kilic-Akyilmaz *et al.* 2022). The variation in mineral concentration is attributed to differences in cultivars, agronomic factors, geographical location, genetics, soil composition, climatic conditions, and the maturity of the leaves. Amadumbe however, has sufficient

nutritional concentrations required to maintain a balanced healthy diet (Ndabikunze *et al.* 2011; Gerrano *et al.* 2021). Amadumbe leaves are an excellent source of a wide variety of micronutrients such as iron and zinc. The iron (3.41 – 5.12 mg/100 g) and zinc (0.82 – 1.14 mg/100 g) concentrations in fresh leaves of amadumbe were found to be significantly higher than spinach (iron 2.95 – 3 mg/100 g; zinc 0.46 – 0.55 mg/ 100g) (Longvah *et al.* 2017; USDA 2023). Amadumbe leaves contain essential sources of iron (2.25 – 5.12 mg/100 g), zinc (0.41 – 0.59 mg/100 g), manganese (0.71 - 1 mg/100 g), and copper (0.27 – 0.40 mg/ 100 g) (USDA 2023). The most abundant micronutrients in amadumbe dry weight (DW) basis consisted of zinc (0.25 mg/100 g), sodium (79.52 – 82 mg/ 100 g), and iron (0.7 – 25 mg/100 g) (Amagloh and Nyarko 2012) (Table 2). Furthermore, (Table S2) shows the mineral composition of commonly consumed green leafy vegetables.

Macronutrients are also abundantly rich in amadumbe leaves, calcium (216 mg/100 g), and phosphorus (57.88 mg/100 g), compared to spinach (calcium, 82.29 mg/100 g; phosphorus, 32.59 mg/100 g) (Table S2). The (USDA 2023) reported the values of amadumbe leaves (calcium, 107 mg/100 g; magnesium, 45 mg/100 g, phosphorus, 60 mg/100 g) (Table S2) (Longvah *et al.* 2017; USDA 2023). Furthermore, a study conducted by (Sayeed *et al.* 2021) observed significantly higher macronutrient concentrations in amadumbe leaves (calcium, 238 mg/100 g; magnesium, 64 mg/100 g; 38 mg/100 g; potassium, 768 mg/100 g). Moreover, (Amagloh and Nyarko 2012) found that the most abundant macronutrients in amadumbe leaves were phosphorus, magnesium, and sodium (76, 7.03-30, 79.52-82 mg/100 g), respectively (Table 2).

Table 2: Mineral composition of amadumbe leaves fresh weight (FW) basis.

Parameter	**Concentration (mg/100 g)**			
Macro minerals	**Ca**	**P**	**Mg**	**K**
	0.19 - 18	76	7.03 – 30	0.15 – 0.19
Micro minerals	**Fe**	**Zn**	**Mn**	**Cu**
	0.7 – 2.25	0.27 - 26.02	0.68	dna

*DNA = No Data Available

(Amagloh and Nyarko 2012; USDA 2021)

2.4.3. Proximate composition

Amadumbe leaves are rich in proteins, fats, and carbohydrates. However, the content of these organic molecules depends on the maturity stage, plant variety, and climatic conditions (Mitharwal *et al.* 2022). Table 3 shows how these properties influence the proximate composition of amadumbe leaves comparatively to commonly consumed green leafy vegetables. Fresh leaves of amadumbe contain around 82.7–92.4 g/100 g moisture on a fresh weight (FW) basis which is comparable to other commonly consumed leafy vegetables such as fenugreek, mustard, and spinach (Table 3). The dry weight (DW) basis of amadumbe leaves ranged between 3.44 -3.73 g/100 g of moisture (Temesgen and Retta 2015). Oliveira and de Carvalho (1975) documented moisture content of 87 g/100 g, Awasthi and Singh (2000) reported mean values ranging from 66.15 – 66.45 g/100 g, while Ejoh *et al.* (2019) found amadumbe leaves to contain 90.6 g/100 g moisture.

Plant proteins are typically incomplete, lacking one or more essential amino acids; consequently, the essential amino acid requirements can be supplied by combining complementary plant proteins. Proteins must constitute approximately 12% of our total daily calorie requirement (Lehnen *et al.* 2015). Amadumbe leaves were documented by Oliveira and de Carvalho (1975) containing 26.84 g/100 g of protein, Ejoh *et al.* (2019) reported protein content of 30 g/100 g, and Awasthi and Singh (2000) found a concentration of 22.25 g/100 g. Generally, green leafy vegetables, are poor in fats, thus contributing low calories (Obeng *et al.* 2020). Many reports recorded low values of fat in amadumbe leaves compared to other commonly consumed green leafy vegetables (Table 3), although some studies recorded higher fat between 3.16 -7.1 g/100 g (Awasthi and Singh 2000; Temesgen and Retta 2015; Ejoh *et al.* 2019; Obeng *et al.* 2020) while (Oliveira and de Carvalho 1975) documented a significantly higher concentration (8.02 g/100 g) of fat in amadumbe leaves.

The assessment of total ash is a valuable criterion for determining the nutritional content of many foods and feeds (Glencross, Booth and Allan 2007). Various studies investigated ash content in amadumbe leaves and observed significantly higher content compared to other commonly consumed green leafy vegetables (Table 3). Oliveira and de Carvalho (1975) documented concentrations of (15.49 mg/100 g DW), while (Ejoh *et al.* 2019) recorded (9.8 mg/100 g), (Awasthi and Singh 2000) found the ash content to be (11.3 mg/100 g), and (Temesgen and Retta 2015) documented ranges between (2.706 and 2.876 g/100 g).

Table 3: Proximate composition of amadumbe leaves and commonly consumed green leafy vegetables.

Name (GLVs)	Moisture (g/100 g)	Protein (g/100g)	Fat (g/100g)	Crude fibre (g/100 g)	Ash (g/100g)	Reference
Colocasia esculenta	83.61	3.42	1.38	n.d.	2.3	(Longvah *et al.* 2017)
	82.7	3.9	1.5	2.9	2.2	(Gopalan, Rama Sastri and Balasubramanian 1971)
	90.6a	30.7	7.1	n.d.	9.8	(Ejoh *et al.* 2019)
	92.4a	6.75	3.73	6.32	13.50	(Singh *et al.* 2011)
	85.66	4.98	0.74	–	1.92	(USDA 2023)
Amaranthus gangeticus	11.29	13.56	7.02	6.7	2.88	(Maurya and Arya 2018)
Chenopodium album	87.5	3.7	1.16	0.81	2.07	(Poonia and Upadhayay 2015)
Trigonella foenum-graecum	86.36	28.76	5.60	7.30	9.10	(Sinha 2018)
Brassica juncea	89.8	0.85	0.33	0.75	1.6	(Mobeen *et al.* 2021)
Spinaceae oleracea	87.5	0.83	0.44	0.82	1.2	(Mobeen *et al.* 2021)

2.4.3.1. Nutrient Detergent Fibre (NDF) and Acid Detergent Fibre (ADF)

A conventional quality standard test for a roughage source is to determine its acid-detergent fiber (ADF), non-detergent fiber (NDF), crude protein (CP), and dry matter (DM) content (Putnam, Summers and Orloff 2007). Crude fiber previously was primarily used to determine the fiber content. Other indicators, including NDF and ADF, are now being identified to better represent the fiber component of meals (Magaña *et al.* 2009). One issue with using NDF as the sole evaluation of fiber quality is that NDF is a chemical attribute of fiber and does not account for physical factors such as particle size and density (Mertens 1997). Fibre, and thus NDF, is critical to long-term health and human productivity (Mertens 1997). Kaensombath and Frankow-Lindberg (2012) investigated the effect of harvesting intervals on biomass yield and chemical composition of different parts. He discovered that NDF in amadumbe leaves increased with harvesting intervals (548 – 648 g/kg DM) for treatments H4 and H24, respectively. In contrast, ADF decreased from (416 g/kg), (294 g/kg), and (191 g/kg) as defoliation of the leaves preceded treatment H24. Another study explored by (Giang, Preston and Ogle 2010) reported NDF and ADF content (37.8 mg/kg and 7.70 mg/kg), respectively.

Additionally, (Gupta *et al.* 1989) estimated NDF content in amadumbe leaves (37.0 g/100 g) comparatively lower than pumpkin leaves (44.2 g/100 g), amaranth (42.6 g/100 g), and neem (38.2 g/100 g). In contrast, NDF levels in amadumbe leaves were significantly higher than in drumstick (28.8 g/100 g), and fenugreek (21.2 g/100 g). Additionally, ADF concentration of amadumbe leaves was recorded (21.7 g/100 g) considerably lower compared to pumpkin (33.3 g/100 g), and neem (26.8 g/100 g). However, amaranth (21.0 g/100 g), fenugreek (19.9 g/100 g), and drumstick (13.9 g/100 g) contained much lower concentrations compared to amadumbe leaves.

2.4.4. Antinutrient factor (Oxalates)

Oxalic acid is present in various green leafy vegetables and plant food products. Oxalic acid or oxalates can appear as soluble potassium and sodium salts, insoluble calcium, magnesium, or iron salts, or as a mixture of both soluble and insoluble salt concentrates, depending on the type of the plant. Whereas soluble salts are absorbed by the body, insoluble salts are primarily eliminated through feces (Sood, Ohdar and Mahapatra 2012). Strong chelates from this absorption may be produced, and calcium may prevent absorption. Kidney stones can form as a result of consuming excessive oxalates. Mineral supplements are necessary to prevent deficiencies in diets high in oxalic acid (Uusiku *et al.* 2010). Abundant in plant and vegetable diets, oxalic acid produces calcium ions and insoluble calcium oxalate, which can modify the metabolism of calcium (Fig. 4a). Compared to steaming, boiling vegetables has been demonstrated to reduce dietary soluble oxalate (Kouba *et al.* 2014). In a wide array of anti-nutritional factors, oxalates dominate amadumbe leaves. The documented antinutrient content (oxalate; Fig. 4b) in amadumbe leaves is presented in (Table 4). Temesgen and Retta (2015) explored antinutrient factors of amadumbe leaves grown in Ethiopia and recorded oxalate ranges (198.61 – 257.92 mg/100 g). Ejoh, Mbiapo and Fokou (1996) demonstrated 2.41 g/kg of oxalates in amadumbe leaves and 1.17 g/kg in the inflorescence. Additionally, (Savage and Mårtensson 2010) estimated the oxalate concentration of amadumbe leaves grown in New Zealand and compared with numerous Indian green leafy vegetables such as spinach, coriander leaves, fenugreek, purple amaranth, drumstick, and green amaranth. The results indicated that oxalate concentration of amadumbe leaves grown in New Zealand ranged from (3792 – 4108 mg/100 g) was comparable to amadumbe leaves from India (5138 mg/100 g), however, the concentration was comparably lower than commonly consumed green leafy vegetables such

as spinach (12, 576 mg/100 g) and amaranth (8106 – 10056 mg/100 g). It is significant to note that the oxalate content declines with leaf maturity (Sunell and Healey 1985).

Table 4: Antinutrient composition of amadumbe (*Colocasia esculenta*) leaves.

Antinutrient (oxalate)			References
Total (mg/100 g)	**Soluble (mg/100 g)**	**Insoluble (mg/ 100 g)**	
241	113	n.d.	(Ejoh *et al.* 2019)
206	90	116	(Hang, Vanhanen and Savage 2013)
198.61 – 257.92	n.d.	n.d.	(Temesgen and Retta 2015)
701	208	492	(Longvah *et al.* 2017)
479	n.d.		(Singh *et al.* 2015)
589	442	147	(Oscarsson and Savage 2007)
443	334	110	(Oscarsson and Savage 2007)
524.2	241.1	283	(Savage, Mårtensson and Sedcole 2009)
5138	1294	3844	(Radek and Savage 2008)
n.d.	n.d.	n.d.	(Eleojo, Charles and Nimat 2020)

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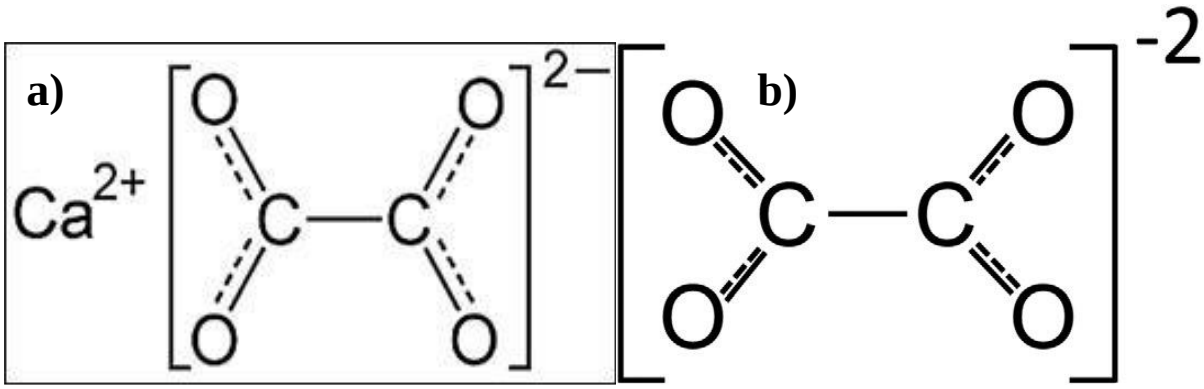

Figure 4: a) Molecular structure of calcium oxalate b) Molecular structure of oxalate ion
Source: a) (Gananca *et al.* 2018; Thomas and Adarsh 2020)

A report by (Oscarsson and Savage 2007) documented the concentrations of total and soluble oxalate content of young leaves (589 mg/100 g and 442 mg/100 g), respectively. The investigation indicated significantly ($p<0.05$) higher concentrations than in mature leaves (443 mg/ 100 g and 334 mg/100 g), respectively. A notable reduction of a staggering 24% of total oxalate at maturity. A report on an evaluation by (Radek and Savage 2008) revealed that amadumbe leaves contained substantial amounts of total oxalate (5138.0 mg/100 g), soluble (1294 mg/100 g), and insoluble (3844.1 mg/100 g), which was significantly ($p \leq 0.05$) lower than spinach (12576.2 mg/100 g, total oxalates, 11899.8 mg/100 g, soluble oxalates; 676.3 mg/100 g, insoluble oxalates) and amaranth leaves (10056.3 mg/100 g, total oxalates, 4674.7 mg/100 g, soluble oxalates; 5381.7 mg/100 g, insoluble oxalates).

2.5. Factors influencing the use of green leafy vegetables

In a study of ten Sub-Saharan African nations, the mean intake was substantially below the minimum recommended level in all countries, with 85% of households failing to meet the required 400g of fruits and vegetables per day in eight of them (Hall *et al.* 2022). In a more recent study, men and women in various Sub-Saharan African nations like Burkina Faso, Côte d'Ivoire, Senegal, Zambia, and Zimbabwe had a high rate of inadequate fruit and vegetable consumption of more than 70% (Wallace *et al.* 2020). Low consumption of green leafy vegetables increased with age and the degree of urbanization (Wallace *et al.* 2020). The accessibility, availability, nutrition knowledge, competitiveness among unhealthy foods, affordability, and parental influence are major home-related influences of green leafy vegetable consumption (Lachat *et al.* 2012). South Africa is faced with issues such as water restrictions (Mabhaudhi and Modi 2014) and population growth which further increases the potential of

growing and sustaining quality and nutritious green leafy vegetables for research, cultivation, and distribution (Mabhaudhi and Modi 2014).

2.6. The role of green leafy vegetables in nutrition and food security.

Food security is defined as having physical, social, and economic access to adequate, safe, and nutritious food that satisfies one's nutritional requirements and preferences for an active and balanced existence consistently (Pinstrup-Andersen 2009). A nutritious and diverse diet is an important part of maintaining good health. Several countries today consume fewer than 200 g of fresh vegetables per person per day, and this low amount, usually in conjunction with poverty and inadequate medical facilities, is associated with obscene levels of mortality and malnutrition among younger kids and other disadvantaged individuals (Keatinge *et al.* 2011). To substantially counteract the malnutrition pandemic, a transition in avoiding unhealthy foods and replacing them with nutritionally rich green leafy vegetables that are economical, abundant, and relatively simple to cultivate for human use is required (Keatinge *et al.* 2011). Globally, the proportion of malnourished individuals exceeds >2 billion (FAO 2009). Around one billion inhabitants of Southeast Asia and Sub-Saharan Africa are malnourished, with diets inadequate in carbohydrates, vitamins, proteins, and minerals (FAO 2009). Consuming extremely fat-rich diets contributes to non-communicable illnesses like hypertension, diabetes, and coronary heart disease (Yach, Stuckler and Brownell 2006).

The advancement of education and commercialization of green leafy vegetable consumption has significant benefits that could ameliorate malnutrition and ensure a global standard in nutrition and food security index, primarily because green leafy vegetable (GLV) consumption is essential to maintaining food and nutritional security. Green leafy vegetables are abundant in both macro and micronutrients, including proteins, lipids, minerals, and vitamins, as well as antioxidants, phytochemicals, and a plethora of other biologically active metabolites. As a result, it is critical to bio-prospect the beneficial compounds and antioxidant properties of locally accessible green leafy vegetables (Anju *et al.* 2022).

2.7. Scope of malnutrition

2.7.1. Undernutrition

Undernutrition is defined as “an insufficient intake of energy and nutrients to meet an individual's needs to sustain good health". Throughout many publications, undernutrition is used interchangeably with malnutrition. Malnutrition, in the strictest definition, refers to both under-nutrition and over-nutrition. To address this, terminologies including protein-energy malnutrition, specific micronutrient deficiencies, and descriptive names like kwashiorkor and marasmus have been introduced. However, because protein energy malnutrition cannot exist in isolation from specific micronutrient deficiencies, neutral terminology like undernutrition is used because they contain both protein energy malnutrition as well as micronutrient inadequacies. Furthermore, over-nutrition applies to an excess of macronutrients and micronutrients (Maleta 2006). Additionally, it affects individuals who are underweight for their age, too short for their age, dangerously thin for their height, and low in vitamins and minerals. Malnutrition transpires in diverse variations and can result in a decline in immunity and resultant mortality (França *et al.* 2009). Approximately >50% of mortalities present data of children under the age of five. Fatalities are attributed to malnutrition, which claims 3 million lives yearly (Lawn *et al.* 2014). Malnourishment has both direct and indirect economic consequences (Nugent *et al.* 2020). It directly promotes disbursement in the healthcare system and indirect costs are incurred in the loss of efficiency (Aklilu and Wekesa 2002). Adult underweight is caused by a combination of low intake, poor absorption, disrupted transit, and altered nutrition absorption, which collectively results in a weight reduction. Adult malnutrition is also associated with increased morbidity and mortality (White *et al.* 2012).

There was a decline in undernourishment from 2003 to 2013. Stabilization of food production in the world occurred from 2013 to 2018, sustainable development goals imposed by the United Nations to fight against global hunger occurred during this period hence, the constant stabilization of malnutrition in the world. Between 2018 and 2021 the prevalence of malnutrition gained momentum probably due to the COVID-19 pandemic and wars (FAOSTAT 2023b) (Fig 5).

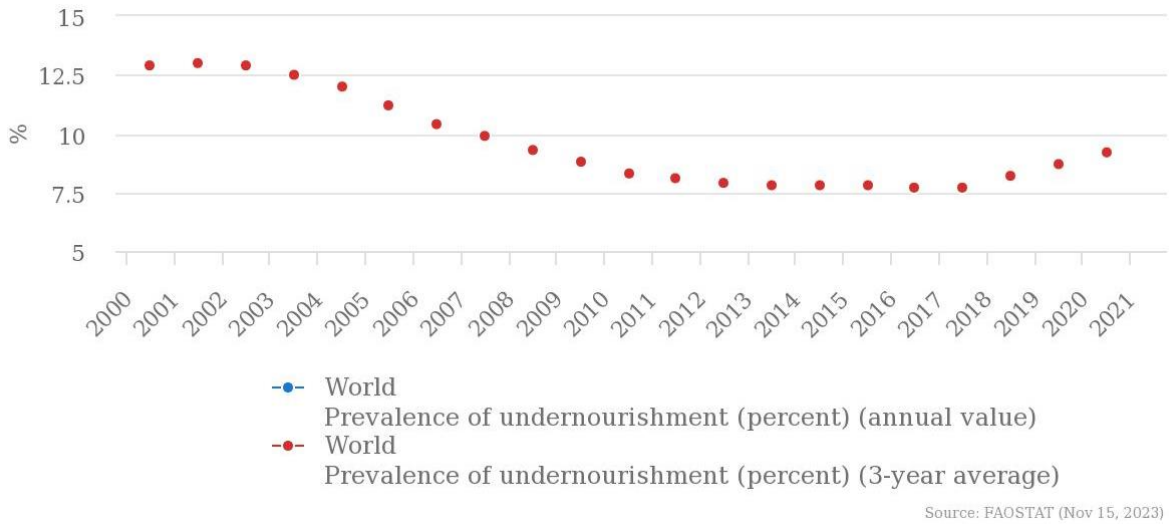

Figure 5: Global prevalence of undernourishment

Source: (FAOSTAT 2023b)

2.8. Micronutrient deficiencies

Throughout infancy development until adolescence and adulthood, humanity is plagued with recurring micronutrient imbalances (Alt, Al-Ahmad and Woelber 2022). The health consequences of inadequacies vary by generation and affect people in different ways. Figure 6 depicts the cycle of micronutrient insufficiency over generations (Bailey, West and Black 2015). Micronutrient inadequacy results from the body's inability to absorb critical vitamins and minerals and can occur at any stage of life. Micronutrients are essential for normal growth and development as well as excellent health (Bailey, West and Black 2015).

Minerals and vitamins such as sodium (Na), copper (Cu), zinc (Zn), and vitamins A and C, are categorized as micronutrients (Bailey, West and Black 2015; Godswill *et al.* 2020). Micronutrient insufficiency has substantial health consequences and affects people in the world's most vulnerable areas. Children under the age of five, together with pregnant women, are the most vulnerable (Bailey, West and Black 2015) and approximately 11% of the SSA deaths of children under five relate to micronutrient deficiency (Krebs, Miller and Michael Hambidge 2014). A lack of micronutrients is a considerable and hazardous health risk factor (WHO 2016). A study by (Bailey, West Jr and Black 2015) indicated that micronutrient deficiency decreases learning ability and limits potential income-earning capacity. Micronutrient insufficiency, on the other hand, is avoidable. In pregnant women, vitamin A deficiency causes blindness and miscarriage and a quarter of a million children are vitamin A

deficient, according to (WHO 2016). Iron deficiency is the most frequent deficit worldwide. Around 30% of the world's population suffers from anemia (WHO 2016).

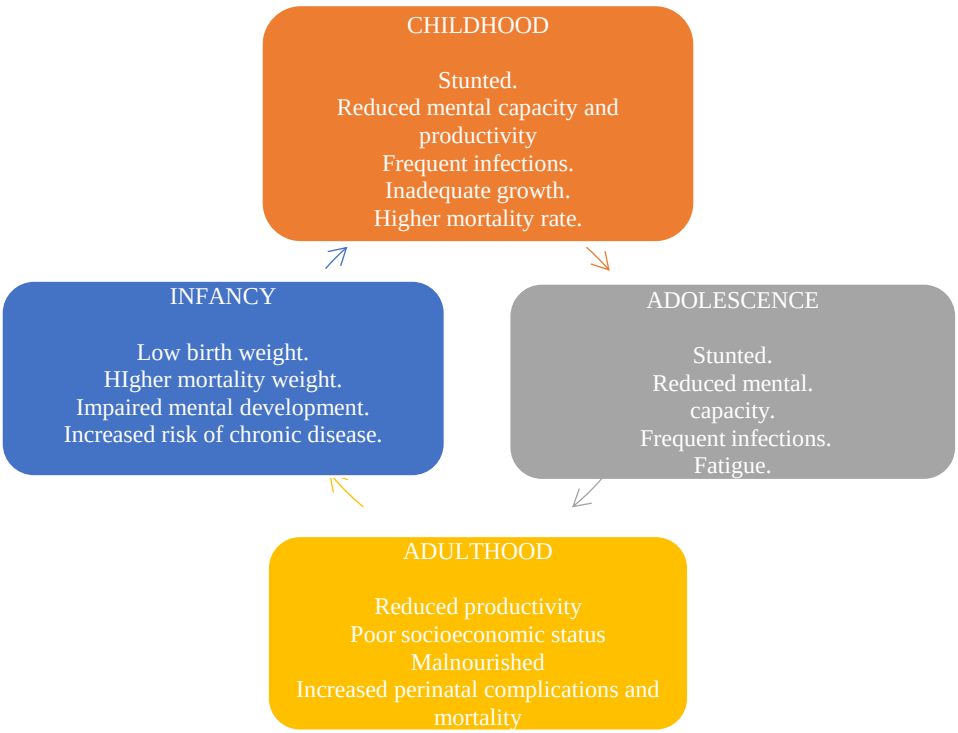

Figure 6: A life-span depiction of micronutrient deficiency perpetuated across generations.
Source: (Bailey, West and Black 2015)

Due to poverty and hunger, children's and adults' diets are deficient in essential nutrients (Muthayya *et al.* 2013; Hameed, Padda and Salam 2021). Vitamin A, iron, and zinc deficits are all problems that require attention (Labadarios *et al.* 2005). At the national scale, two out of three and one out of four women, accordingly, had low levels of vitamin A status although Vitamin A deficiency was also frequent in children (McLean *et al.* 2009; Alaofè *et al.* 2017). The preponderance of women with severe vitamin A inadequacy resided in the province of KwaZulu-Natal (Labadarios et al. 2005; Fiedler et al. 2014). According to (Allen *et al.* 2014) one of the most efficient methods for controlling micronutrient deficiencies would be through dietary intervention, supplementation, and fortification.

2.9. Soil fertility for plant production

2.9.1. Plant production in soils with low nutrient phyto-availability

One of the most important elements influencing crop production and quality is soil fertility and nutrient management. Soil nutrient monitoring, which is performed yearly, is essential for effective plant development. Annual soil testing determines the condition of the soil and its appropriateness for plant growth (Reddy *et al.* 2011). Soil test results assist in determining pH, soil organic matter (SOM), electrical conductivity (EC), cation exchange capacity (CEC), and levels of essential macronutrients including magnesium, phosphorus, calcium, potassium, and magnesium, as well as micronutrients like Mn, Br, Zn, Fe, and Cu (Chatterjee *et al.* 2018). Soil nutrient status and management influence not only the crop yield but also the concentration of nutrients in plant parts consumed as food. As a result, the status of soil nutrients has a significant impact on human health response (Brevik *et al.* 2020). Internationally, around one-third of agricultural soil is low in micronutrients, especially zinc (Zn) (Schjoerring, Cakmak and White 2019), which has an impact on human nutrition (Ackland and Michalczyk 2016). Micronutrient deficiency is a major concern in Sub-Saharan Africa (SSA) (Hengl *et al.* 2017) and this is mostly due to periods of soil deterioration, as well as low and imbalanced fertilizer application, primarily of nitrogen (N), phosphorous (P), and potassium (K). Food consumption trends in Africa are dominated by primary cereals, particularly among resource-constrained small-scale local farmers. Micronutrient deficiency in humans, on the other hand, is most common in communities where cereals cultivated in micronutrient-deficient soils dominate the diet (Karim *et al.* 2012; Monreal *et al.* 2016). Consequently, zinc and iron are the most typically deficient micronutrients in human diets (White and Broadley 2009; Ruscio *et al.* 2010).

2.9.2. The impact of location on the mineral composition of green leafy vegetables

A study by (Oluwole *et al.* 2020) determined that differences in soil properties such as soil water, soil minerals, and soil microorganisms influence the morphological appearance and variation in proximate and mineral compositions directly influenced two leafy vegetables *C. argentea* and *A. cruentus* seedlings. According to Oluwole *et al.* (2020) verified by Odiaka (2001) when they reported that *Telfairia occidentalis* grown in clayey soil were less healthy and stayed considerably shorter in height, root length, shoot length, and leaf areas compared to those grown in humus and loam soil respectively. The findings also agreed with the work of

(Oluwole, Ogun and Dajakpome 2020) when they reported a variation in the growth pattern of *Telfairia occidentalis* grown on different soil types. They reported that soil types have some impact on plant growth; thus, promoting microorganisms found in the root region, increasing plant nutrients and water uptake efficiencies, and production of plant hormones affects plants' shoot and root biomass, nutrient uptake efficiencies, and plant chemical contents.

2.9.3. The relationship between soil moisture and plant growth

Insufficient moisture can affect agricultural practices by reducing production, and crop yield, whereas root disease and limited oxygen availability are brought on due to excessive moisture (Singh and Singh 2022). Since accurate moisture measurement is important for plant growth, achieving the best moisture management practices fosters efficient nutrient management (Havlin 2020; Mitra *et al.* 2020). Soil type has a significant impact on how well water can permeate and be retained in soil (Arenghi, Perra and Caffi 2021). When water infiltrates soil containing large sandy soil particles, barely any fraction remains adsorbed onto soil colloids, whilst the rest percolates aided by a gravitational pull. On the contrary, a proportion of clay soil has a significant number of macropores with a large surface area. When water enters clay soil, surface tension binds it to the soil particles through adhesion, allowing only a fraction to drain downward, therefore clay has a considerable capacity for storing water. Water and nutrients can readily drain away from plants. A sandy soil has a low capacity to retain water, although the water is easily accessible to plants. However clay soil may hold a considerable amount of water, the soil is under tension because of how firmly it holds onto the water (Hamamoto *et al.* 2009). Since clay soil tightly binds water to soil particles, it deprives plants of accessing available water at field capacity it can also retain nutrients out of reach of plants by restricting the amount of water readily available to plants. A loamy soil with a diversity of particle sizes and enough structure that can contain a significant amount of water that is simple for plants to remove is excellent for the majority of growing conditions (Tsai, Lai and Hsien 2003). The soil texture triangle in (Figure 7) illustrates how soil type, water holding capacity, and water availability interact. Therefore, understanding the different soil types is essential and allows for differentiating between the soil agroecological variations of locations and their impact on plant growth (Gerrano *et al.* 2021).

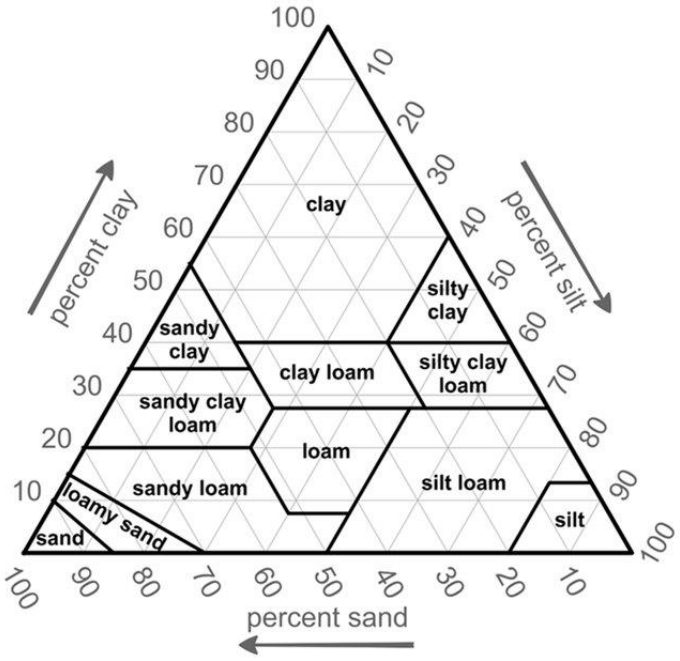

Figure 7: Soil texture triangle illustrating soil types and their textural classification.
Source: (USDA-NRCS)

2.9.4. Plant-soil interaction effect on nutrient uptake

The ability of soil to generate a specific quantity of crops or other plants under a specified management system is known as soil productivity (Fageria and Baliger 2005). Mineral nutrients are typically taken up by plant roots from the soil, although a variety of conditions might influence how effectively nutrients are taken up. Certain soils' chemical makeup can make it challenging for plants to absorb nutrients. In some soils, the nutrients might not be present, or they might be in an undetectable form that the plants can't assimilate. Challenges like these can be aggravated by soil characteristics including water content, pH, and compaction (Ruwanza and Dondofema 2020; Oladosu *et al.* 2022). Several plants have processes or structural traits that help them thrive in specific kinds of nutrient-poor soils. To overcome nutrient shortages, the majority of plants have developed nutrient uptake mechanisms that are tailored to their local soils (Bennett and Klironomos 2019; Trivedi *et al.* 2021). A modification in root structure, which may increase the root's overall surface area to maximize nutrient uptake or may lengthen the root system to reach additional nutrient sources, is one of the most common responses to nutrient-limited soils. These adjustments may result in a larger allocation of resources to total root growth, which would raise the ratio of roots to

shoots in nutrient-limited plants (de Britto Costa *et al.* 2021). Figure 8 illustrates the mechanisms of mineral uptake on different types of soil types.

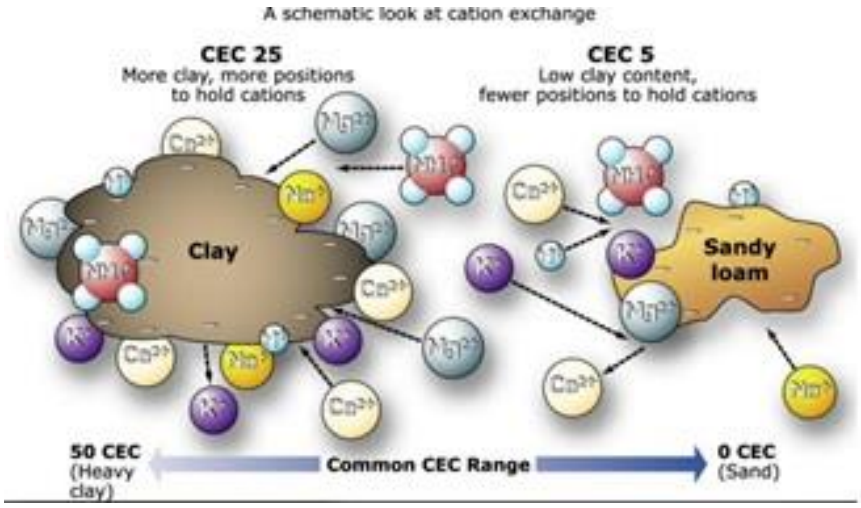

Figure 8: Mechanisms of nutrient adsorption onto different soil properties.
Source: https://labserve.net/the-concept-of-cation-exchange-capacity/

2.10. Plant Propagation

Plant propagation is when the new individual plant develops from any vegetative component of the parent (root, stem, leaf, and other organs) and shares all of the same traits as the parent plant from where it was obtained (Megersa 2017). Any portion of the organism may be able to reproduce vegetatively in higher plants. For natural vegetative propagation, many plants develop modified stems, roots, and leaves. Plants can be propagated vegetatively using a variety of techniques, the most popular of which include cuttings taken from the stem, leaf, or root, layering, grafting, modified specialized organs, and plant tissue culture techniques (Megersa 2017; Chauhan *et al.* 2021).

2.11. Conventional methods of propagation

In the majority of nations all over the globe, amadumbe are typically grown during the start of a wet season (Tembe 2008; Lewu, Adebola and Afolayan 2010). Two to three weeks after planting, new roots and leaves start to grow, and two months later, suckers (bulking) start to develop. Except for production in flooded environments, where the development of the center main corm takes 2 to 5 months, central corm growth begins in around two months. Amadumbe

leaves exhibit exceptional growth and turnover, reaching their maximum leaf area in 4 to 5 months. At reaching this point of development, leaf stalks get shorter and there are fewer leaf blades overall. Amadumbe rarely blooms, but the majority of clones never do. Applications of the growth regulator gibberellic acid (GA) are required to accelerate the blooming process. When a plant has six or more blades of leaves, leaf harvesting can begin (approximately, three months after planting). The size of the corm, the number of suckers, and the output yield are all significantly reduced by aggressive leaf harvesting (Nyathi *et al.* 2019). In dryland systems, corms reach maturity between 8 and 10 months, but in wetland environments, corms reach maximum mass in 11 months (Macharia *et al.* 2014; Sibiya 2015). Since amadumbe is propagated vegetatively, it can be challenging to maintain planting material healthy during the dry season and prolonged drought. When cultivating amadumbe conventionally, disease issues and mass production are quite common, making commercialization unfeasible.

2.12. Plant tissue culture

Plant tissue culture is a technique that enables plants to develop *in vitro* into fully-fledged higher plants. Through growing plant parts (cells, tissues, and organelles) in artificial media under sterile and controlled conditions. Some plant cells maintain their embryonic potential and remain totipotent (Raghavan 2006; Linskens and Heslop-Harrison 2012). This demonstrates that one cell can transform into an entire plant (Moyo *et al.* 2011; Gupta *et al.* 2020). Competent cells in plant tissues are those that react to outside cues to follow a certain developmental course. Biological structures (organs) are produced directly or indirectly by meristematic tissues, which are collections of cells with the ability to divide, throughout the process of plant growth and development (Ascough, Erwin and Van Staden 2009; Ascough *et al.* 2009). Plant tissue culture results in cell division, which causes adventitious shoots to first emerge before adventitious roots are formed (De Klerk 2002; Ribeiro *et al.* 2022).

Shoot organogenesis *in vitro* is a novel method for the generation of shoot meristems from somatic cells rather than embryonic cells. Somatic cells react to plant growth regulators (PGRs), the reactive cells divide, and new shoot induction and proliferation are all a component of the process (Mercier *et al.* 2003; Gaba 2005). PGR applications accelerate the cell cycle and program the resulting cells to have a distinct developmental outcome (Pierik *et al.* 1987). The rate of shoot induction in plant tissue culture varies significantly from species to species, although the variation is within the same family. Essential factors that determine this feature

include the type of explant, orientation, growth media, plant age, time of the plant material collection, and environment to which the explants are exposed (Pierik *et al.* 1987).

2.12.1. Stages in plant tissue culture

2.12.1.1. Selection and preparation of stock plants

The mother stock plant's genetic make-up, physiological state, and phytosanitary status all have a significant impact on the quality of the plant and its subsequent response *in vitro* (Debergh and Maene 1981; del Castillo *et al.* 2008; Núñez-Cárdenas *et al.* 2022). Before undertaking culture establishment, considerable deliberation is paid to the selection and maintenance of the stock plants used as the source of explants. Stock plants that have been properly managed and kept in a hygienic, controlled environment encourage growth and reduce the possibility of diseases developing. Pathogen-tested stock plants cultivated in low-humidity conditions with drip irrigation and antibiotic sprays have decreased the possible exposure of explants to contaminants. Managing and promoting phyto-sanitization permits significantly bigger and more responsive explants to prevent contamination risks (Trigiano *et al.* 2011).

2.12.1.2. Initiation and establishment of aseptic culture

Initiating and growing an aseptic establishment of disease meristematic explants is the primary goal of this stage. To prevent pathogen multiplication, the main explants obtained from the parent plants must have shoot apical meristems, meristem tips, or shoot tips from terminal or lateral buds. Shoot survival, growth, and multiplication rate can all be adversely affected by the presence of microbial pollutants. Even in cultures that on the surface seem to be devoid of contaminants, bacterial and fungal pollutants frequently persist. So, before multiplication proceeds, stage 1 cultures must be enumerated (screened) for the possibility of endogenous microbiological contaminants. Meristematic explants in stage 1 can be successfully established depending on several variables, including explantation time, explant position on the stem, and explant size. Explantation timing can influence protoplast responsiveness *in vitro*, especially with herbaceous perennials that produce storage structures such as corms and tubers; when explants are dissected at the ending of dormancy and after emergence, the best *in vitro* outcomes can be achieved (Isah 2015; Gregory *et al.* 2019). Explants grown *in vitro* have varying qualities depending on the position of the mother stock plant. Terminal bud explants, for example, often surpass lateral bud explants in terms of survival and growth. *In vitro*,

equivalent lateral meristem explants from the top and bottom of a single shoot frequently react differently. Young explants from woody plants that demonstrate phase development are frequently more responsive than mature tissues from the same plant, which are frequently non-responsive. Root suckers, basal sections of older plants, and lateral shoots obtained from extensively trimmed plants are also sources of new explants (Kane 2011).

2.12.1.3. In-vitro shoot multiplication

This stage is distinguished primarily by the recurrent development of axillary shoots from shoot tips or the establishment of lateral buds. Buds are often cultivated in a medium supplemented with a high concentration of cytokinin to destabilize the apical dominance of the shoot tip. Many crops grown by shoot tip culture have a four-week sub-culture interval with a three- to eightfold rise. With these rates of multiplication, a single beginning explant may produce more than 4.3 × 107 shoots every year (Kane 2011).

2.12.1.4. In-vitro rooting

Stage 3 is distinguished by the effective transfer of stage II shoots to growth media. To promote survivability, the technique involves elongating shoots preceding rooting, rooting individual shoots or shoot clumps, or completing dormancy requirements of storage organs through cold treatment or pre-hardening cultures. Commercial facilities have developed methods for transferring stage II cuttings to growing media while avoiding stage III roots. Herbaceous plants may usually root in a medium without the presence of auxin. Hardwood perennials require either (IBA – NAA). The medium in Stage III is improved to encourage the growth of adventitious roots. The optimal auxin concentration is determined by the percentage of rooting, root number, and length of the plant. When selecting an auxin for stage III rooting, more caution is required because NAA has been known to reduce survival rates or suppress post-planting growth (Conner and Thomas 1982).

2.12.1.5. Acclimatization

To achieve success, careful transfer and re-establishment of developing plants from cultured cells to ex-vitro environments are required. Poor re-establishment can result from the acclimation of plants to surroundings with decreased humidity levels, significantly higher light

levels, and/or poor water loss, among other key causes. Inevitably, plants must quickly switch from a heterotrophic to a photoautotrophic condition to survive during the acclimatization process (Morgan-Kiss *et al.* 2006; Hüner *et al.* 2013; Demmig-Adams, Stewart and Adams III 2017). Unfortunately, there is a delay in the changeover. Cauliflower species, for instance, did not exhibit an increase in CO_2 uptake until 14 days after transplantation (Pospóšilová *et al.* 1999). New leaves only develop once this process takes place; older leaves that were generated *in vitro* in sucrose never became capable of photosynthesizing. As some micropropagated plants continue to have photosynthetic leaves following acclimation, this idea does not, however, apply to all micropropagated plants. Plantlets are transplanted into a sterile, well-drained growing medium that is initially maintained at relative humidity and low light, typically light at (40 - 160 µmol x m^{-2} x s^{-1}) at 20° - 27°C, to overcome such barriers. To encourage brisk growth, this progressive exposure to relative humidity must occur at regular intervals throughout one to four weeks (Kane 2011).

2.13. Factors influencing successful plant tissue culture

2.13.1. Microbial contaminants in explants

Unsanitary laboratory manipulations, ineffective plant tissue culture techniques, and infected motherstock plants may harbor microbial contaminants (Ray *et al.* 2017). The presence of these microorganisms leads to an increase in plant death, they also create variation in growth (reducing the rate of shoot growth and rooting), and tissue necrosis (Jaskelioff *et al.* 2011; Thomas 2013). Predominantly, fungal and bacterial contaminants remain problematic microbial contaminants in plant tissue culture techniques (Shen *et al.* 2010).

2.13.2. Bacterial contaminants

Based on how they react to gram staining, two types of bacteria are associated with bacterial contaminations (Leifert and Cassells 2001; Moyes, Reynolds and Breakwell 2009). Gram-negative bacteria, particularly *Pseudomonas fluorescens*, *Erwinia*, and *Agrobacterium* species, are strongly associated with ineffective initial disinfection procedures. Gram-positive bacteria are a sign of insufficient growth media sterilization and aseptic technique training for tissue culture propagators. Bacterial contamination can actively enter cultures because of all these vectors (Cassells 1991). After being injected into plant tissue cultures, bacterial contamination may become obvious right away or may take a long time to manifest any symptoms

(Orlikowska, Nowak and Reed 2017). Although some environmental bacteria, such as *Xanthomonas*, *Pseudomonas*, and *Corynebacteria*, are difficult to identify or exhibit any symptoms in culture medium but can inhibit plant development and rooting. Numerous environmental bacteria can reproduce quickly in plant tissue culture media and have the potential to become extremely pathogenic. Bacterial contamination frequently requires minerals and vitamins that are lacking in plant tissue culture media (Basu *et al.* 2015; Orlikowska, Nowak and Reed 2017). The accuracy of the data is decreased by latent contamination, which is more likely to occur in plant tissue culture methods (Ray *et al.* 2017). The application of antibiotics is one method of lowering bacterial vectors, although this method can also decrease the proliferation of contaminants while causing latent persistence of contaminants in plant tissue cultures. (Wagih, Adkins and Attia 2009).

2.13.3. Fungal contaminants

Numerous measures have been taken to eliminate microorganisms that are detrimental to plant tissue culture systems (Reed, Buckley and DeWilde 1995; Harabi *et al.* 2016). Despite the continuing presence of endophytic persistent microorganisms that compromise tissue cultures, surface sterilization of the explant to eradicate epiphytic pathogens is routinely performed (Cassells 1991; Arab *et al.* 2014). It's occasionally challenging to maintain contamination-free, healthy tissue for an extended period in some tissue cultures because they regenerate more slowly and include endophytic organisms (Nair and Padmavathy 2014). In contrast to bacterial proliferation, controlling fungal populations below the specified threshold in plant tissue culture is of major importance. It is still crucial to include an effective fungicide in the plant tissue culture mix. Plant fungicides made of synthetic materials are widely used in plant tissue culture, although they may have hazardous and carcinogenic side effects (Gond *et al.* 2007).

2.14. Plant Growth Regulators

“Plant Growth Regulators (PGRs) are organic molecules that affect different plant processes such as growth and morphogenesis in small concentrations” (Gaba 2005; Beyl 2011; Łyczko *et al.* 2020). Naturally occurring PGRs are referred to as phytohormones or hormones and synthetics are more resistant to enzymic breakdown and thus display intensified efficacy (Aslam, Maqbool and Cengiz 2015). Growth regulators are substances possessing the capability of utilizing great effects in plants and influencing development, gene expression,

and growth despite their low concentrations. Plants biosynthesize their endogenous PGRs called hormones. Hormones control various processes of ripening, cell division, and enlargement, flowering, dormancy, root and bud initiation. Plant propagators utilize characteristic features of artificial growth regulators (AGRs) for their exogenous application generating desired responses in plants. Five categories of hormones are widely used in the horticultural manipulation of growth responses; auxins, gibberellins, cytokinins, abscissic acid, and ethylene. Each of these PGRs retains unique effects with a capability to function together synergy or antagonistic to deploy other responses within a plant. Plant growth regulators function as a combination or only one PGR may be required at a specific stage of growth and development and a diverse one at another stage of growth. In particular, auxins and cytokinins may be essential in stage I, the initiation phase; in stage II, the multiplication stage, cytokinins are applied to stimulate shoot proliferation; and in stage III, auxins are applied to encourage root growth (Beyl 2011; Cao *et al.* 2019).

2.14.1. Auxins

Auxins are involved in root induction, terminal dominance, geotropism, and phototropism. Auxins are used commercially for root stimulation on cuttings, the induction of parthenocarpy to avoid fruit abscission, and as a herbicide, if manufactured in a potent synthetic form. An aromatic ring that is isolated from a carboxyl group is an attribute of auxins. The most prevalent naturally occurring auxin is indole acetic (IAA) acid. However, due to its intense oxidizing capability in culture conditions or metabolism by plant tissues, as well as its sensitivity to light, its application in plant tissue culture is restricted. Phenylacetic acid (PAA) and indole-3-butyric acid (IBA) are two additional naturally occurring auxins that are frequently employed to stimulate the production of roots. Strong auxins 2, 4-dichlorophenoxy acetic acid (2, 4-D) and naphthalene acetic acid (NAA) are synthetic auxins that are frequently applied as herbicides in horticulture (Guan, Huisman and de Klerk 1997; De Klerk, Van Der Krieken and de Jong 1999; Beyl 2011).

2.14.2. Cytokinins

Cytokinins play several roles in plant development, including the delaying of senescence, cell division and specialization, chloroplast formation, vascular progression, and the beginning and growth of shoots. They are utilized in synergistic capacities in plant tissue culture propagation

systems to reduce apical dormancy, accelerate shoot proliferation, and stimulate cell division. Adenine-type cytokinins and phenylurea cytokinins are two major divisions of cytokinin groups. KIN, zeatin (ZEA), and benzyladenine are the different forms of adenine (BA). Adenine moiety is further divided according to the type of side nature (isoprenoid or aromatic) on the N6 terminal. ZEA and isopentnyladenine are two types of isoprenoid (IPA). A couple of aromatic kinds are BA and meta-topolin (mT). Cytokinins like diphenylurea and thidiazuron are phenylurea kinds (TDZ) (Beyl 2011).

2.15. Environmental factors in plant tissue culture

2.15.1. Light

Although plants are sessile, environmental influences that affect their morphological traits can alter their growth and development. As a result, plants can recognize this signal since it provides them with energy for physiological activities like photosynthesis (Hart 2012), and the Light Emitting Diode (LED) lamp functions as an appropriate source of artificial lighting (Darko *et al.* 2014). According to research by (Bula *et al.* 1991) employing LED lights for the cultivation of plants, *Lactuca sativa* plants grown under red LED combined with blue fluorescent lamps had foliage with the same form, colour, and quality as those grown under white fluorescent and incandescent lamps. Commercial plant tissue culture has experienced increased growth in the use of LED lights as a result of their greater advantages over the traditional fluorescent system (Dutta Gupta and Agarwal 2017). The multiple wavelengths that the LED lights provide—400 nm or less ultraviolet, 400–450 violet, 450–500 blue, 500–570 green, 590–610 orange/amber, 610–760 red, and > 760 nm infrared colour—can be used either independently or in combination to maximize photosynthesis (Serbin *et al.* 2015; Shengxin *et al.* 2016). High humidity inside the tubes during plant tissue culture is a circumstance to which plants are exposed. As a result, plant tissue culture explants may experience poor CO_2 concentration, restricted gas exchange, low photosynthetic active radiation (PAR), and excessive exposure to carbohydrates, nitrogen, and growth regulators (Xiao *et al.* 2011; Nguyen *et al.* 2016). All of these elements will have a direct impact on the physiological and biological functions of plants, including *in vitro* photosynthesis. On the contrary, better light quality and gaseous exchange, either through forced or natural ventilation, can be adjusted with the use of LED lights to enhance plant performance in plant tissue culture. Plant tissue culture facilities are moving away from conventional fluorescent lamps with LED due to increased

technological advancements and falling LED prices, as various studies reveal more robust *in vitro* plants grown under LED lighting (Hung *et al.* 2015; Ferreira *et al.* 2017).

2.15.2. Temperature

To achieve optimum growth and development, and high production in plant tissue culture, temperature conditions need to be precise for that particular plant species being propagated (Murashige 1977). In the natural environment, there are wide fluctuations in temperatures particularly, day and night temperatures. The possibility of mimicking these diurnal variations internally has many advantages (George 1996). Another advantage of alternating temperatures is the facilitation of the exchange of gases in culture tubes (Chalupa 1987). Maintaining temperatures at optimum levels promotes enzyme activity and many other biological processes that aid in plant growth (Amoo, Finnie and Van Staden 2009; Veselova, Nuzhnaya and Maksimov 2015). Generally, a growth room temperature for *in vitro* culture is often maintained at a constant temperature of approximately 25ºC in many plant tissue culture protocols. On the contrary, low temperatures can modify levels of PGRs through the stimulation of cytokinins (Bulgakov *et al.* 2005). An experiment that was conducted on four mint (*Mentha* spp.) accessions (Islam, Ahmed and Mahaldar 2005) discovered that terminal and axillary explants cultured at 25ºC showed better growth *in vitro* than 20ºC cultured explants.

2.15.3. Humidity

In vitro, cultivated plants are grown in tiny vessels with a restricted air exchange rate between the inside and outside air. For cultivated explants, an adequate supply of water and nutrients recommends a very high relative humidity. To keep the equilibrium between the two habitats, the microclimate must be modified. Proper laboratory modifications are essential to ensure that air temperature (AT) and relative humidity (RH) are adjusted to promote plantlet growth since they are necessary for favorable culture growth (Dalina and Sobejana 2019). (Gaspar *et al.* 1987) According to Gaspar et al. (1987), high relative humidity in culture vessels led to morphological and physiological problems with cultures.

A study by (Teixeira da Silva *et al.* 2020) suggested that elevated relative humidity and excessive water availability in the tube are the primary causes of physiological and morphological abnormalities in cultures. Control of relative humidity or water in a culture

vessel has only been the focus of a small number of investigations, in contrast to the numerous studies that have been performed on the impact of relative humidity on growth and development. There are also not many studies on determining the relative humidity in a culture vessel. Although many studies suggested that high relative humidity in culture vessels caused physiological and morphological disorders of cultures, it is crucial to lower or manipulate relative humidity to the proper levels to achieve adequate levels of culture growth and development for different species.

2.16. Plant tissue culture of amadumbe

2.16.1. *In-vitro* culture

The development of an effective strategy to exponentially propagate *Colocasia esculenta* in-vitro utilizing various decontamination techniques has been the subject of numerous types of research. Using an apical meristem produced from the base of leaf petioles and sections of the corm, (Kepue *et al.* 2021) reported on an efficient plant tissue culture approach for Kenya Purple Wild and Dasheen genotype landraces found in Kenya. For improved shoot and root development, the study optimized the concentrations of 6-benzylaminopurine (BAP) and indole-3-butyric acid (IBA).

When 2.0 mg/L^{-1} of BAP was administered to Murashige & Skoog (MS) media, the maximum shoot initiation was observed in both genotypes. Murashige and Skoog's media augmented with 0.50 mg/L^{-1} of IBA and half-strength, both varieties maximally responded to rooting. For the development of shoots and roots, the investigation discovered significant interactions between a specific IBA x variety x BAP levels at ($p<0.05$), respectively. The plant tissue culture technique proposed in this study is suitable for establishing amadumbe transformation protocols for the efficient multiplication of genetically pure planting materials for commercial usage.

A report by (Verma 2017) using axillary meristem explants, successfully improved direct somatic embryogenesis and organogenesis of (*Colocasia esculenta var. esculenta*). Plantlets were produced in significant numbers and rapidly and underwent acclimatization and field transfer as a result of both direct somatic embryogenesis and organogenesis. Direct somatic embryogenesis and organogenesis *in vitro* propagation approach for amadumbe is considerably more dependable than the standard techniques used for other cultivars and offers sustainable

methods for producing high-quality amadumbe. Additional plant tissue culture research studies in each successive phase are discussed in the next sections:

2.16.1.1. Explant initiation

The two cytokinin groups that are crucial for culture initiation are kinetin and BAP. For several species, the comparative significance of BAP for the regeneration of shoots has been extensively described (Cheruvathur, Abraham and Thomas 2015). In contrast to BAP, Kinetin was only marginally effective in promoting shoot proliferation. According to (Bogale 2018) the initiation culture medium assessment with IAA exhibited supplementary healthy and robust development containing a maximum proportion of roots and foliage. Furthermore, experimentation showed media augmented with IAA at concentrations above 15.0 mg/L^{-1} discontinued support for the growth of explants as contrary to basal MS media. According to another study by (Naik, Pattnaik and Chand 1999), explants cultivated on modified MS medium + 1.0 mg/L BA developed more rapidly than on media containing TDZ. According to (Ko, Kung and McDonald 2008) BA at an 8 mg/L^{-1} concentration was paramount for the initiation of corm explants *in vitro*.

2.16.1.2. In vitro shoot multiplication.

For several species, the comparative importance of BAP for the multiplication of shoots and elongation is comprehensively reported (Rai, Jaiswal and Jaiswal 2009; Bekele, Abera and Getahun 2013). Different amadumbe studies were undertaken on innumerable cultivars. According to (Ko, Kung and McDonald 2008), full MS media comprising BA and IAA could produce the greatest shoot growth and development frequency of 30.5-99.5% and the maximum amount of shoots recorded. An investigation by (Hossain 2012) demonstrated that an appropriate combination of NAA and BAP promoted effective shoot regeneration and was particularly suitable for in-vitro growth at a temperature of 24°C-2°C with a photoperiod of 16 hours. Additionally, according to (Hussain and Tyagi 2006), MS media supplemented with 3% (30.0 g/L^{-1}) sucrose + 0.5% (5.0 g/L^{-1}) BAP + 0.1% (1.0 g/L^{-1}) NAA and 0.8% (8.0 g/L^{-1}) gelatine, produced the highest rate of shoot number. Higher concentrations of BAP with low auxin concentration are additionally efficient in providing superior outcomes on proliferation.

2.16.1.3. In vitro rooting

Auxins have been utilized in tissue cultures for root differentiation and cell division. IAA, IBA, and NAA (indole-3-acetic acid, indole-3-butyric acid, and naphthalene acetic acid, respectively) are the auxins most frequently manipulated for root induction. IAA and IBA are particularly utilized in the rooting phase and occasionally interact with cytokinins applied for root expansion (Feyissa, Welander and Negash 2005; Bekele, Abera and Getahun 2013). According to (Demeke 1991), auxins like IAA, IBA, and NAA increased the root development of micropropagated shoot tips culture. A high rooting percentage (%) was acquired in a large percentage of the treatments, except for the control and IAA at 0.57 g/mL^{-1}. Although, the callus was observable in the majority of the treatments. With reduced callus and a lower concentration (0.49 g/mL^{-1}), IBA showed a decent rooting percentage.

In particular, root initiation percentage and quantity of roots per culture of (*C. esculenta*) increased with higher levels of IAA up to 0.50 g/mL^{-1} before declining (Bhuiyan *et al.* 2011). Further investigative research on plantlet establishment through somatic embryogenesis and organogenesis in plant tissue culture by (Verma and Cho 2007) reported 100% root induction in a week from MS medium augmented with 2µM IAA and 10.0±5.0 roots per shoot with 8.0-15.0 cm length after four weeks of planting. Researchers suggested plant tissue culture protocols for various *C. esculenta* genotypes, but none dealt with or reported on the selected South African variety *Colocasia esculenta* var. *esculenta* (L.) Schott growing in completely different geographical regions with varying rainfall patterns, climatic conditions, unique genetics encoding, agro ecological environment, and soil edaphic factors.

2.16.1.4. Acclimatization

It is the last stage of plant tissue culture in which plantlets are relocated to their natural habitats. Plantlets grown from plant tissue culture are often acclimatized or hardened in a greenhouse or other similar environment before being transplanted to the field (George, Hall and Klerk 2008). Plantlets undergo acclimatisation or hardening processes in greenhouse structures to establish vigorous root systems, and leaf support structures, to survive the natural harsh environment ex-vitro. The process of acclimatization is accomplished by gradually decreasing relative humidity (RH) and increasing light intensity. The greenhouse chamber requires bright lighting (approx. 4,000-10,000 lux), relatively high humidity (90-100%), ventilation, and heating equipment

(Feyissa, Welander and Negash 2005; George, Hall and Klerk 2008). According to (Verma and Cho 2007) 100% survival of plantlets and vigorous development was observed on completely acclimatized plants (15.0-20.0 cm). The surviving plants were transported to the ex-vitro environment four weeks after planting.

2.17. Decontamination procedures

A crucial technique in plant tissue culture is the decontamination procedure and use of sterile chemicals to eliminate surface (exogenous) opportunistic contaminants and endogenous contaminants. Plant tissue culture media rich in sucrose/sugar and organic nutrients provides a conducive environment that supports the growth of various pathogens such as fungi, bacteria, and viruses. Microbiological agents potentially proliferate exponentially surpassing cultured explants, beyond recoverable and eventually death (Bekele, Abera and Getahun 2013) Plant tissue culture medium can be contaminated through multiple sources: i) culture pathways, ii) media, iii) culture explants, iv) instruments for handling the explants (Hailu, Abera and Mariam 2014; Kebede and Abera 2015). Except for the explants, the instruments used in the decontamination procedure must be sterilized by autoclaving for 20 minutes at 121°C and 103.4 KPa. Before inserting the tissue into the nutritional medium, it must be rigorously surface disinfected to eliminate the broad spectrum microbiological contaminants potentially latent (Leifert, Morris and Waites 1994; Bhojwani and Dantu 2013).

Sterilization of plant tissues, several disinfecting agents including calcium hypochlorite ($Ca(ClO)_2$, 9.0-10.0%), sodium hypochlorite (NaOCl, 0.025%-0.25%), hydrogen peroxide (H_2O_2) solution (3.0%-10.0%), additionally, mercuric chloride (HgCl, 0.10%-1.0%), silver nitrate (AgNO3, 1.0%), and bromine water (Br_2, 1.0%-2.0%) had been utilized (Feyissa, Welander and Negash 2005). A diluted regular bleach can be employed, which typically includes 5.25% NaOCl (Bekele, Abera and Getahun 2013). Similar to this, a study by (Bogale 2018) on the disinfection of amadumbe explants with NaOCl demonstrated an efficient result in the composition of 2% NaOCl except for damaging the intracellular tissue and produced the highest overall survival rates and percentage of buds developed approximately five weeks. According to (Bogale 2018) 2.5% NaOCl provided better results than 2.0% NaOCl at eradicating all surface contamination, nevertheless, it was detrimental to the plant's cellular tissue. Sodium hypochlorite is predominantly used as a disinfectant in plant tissue culture to promote surface sterilization to ameliorate aseptic culture establishment, however, its

commercial use is becoming widely rejected (Bogale 2018). This is due to NaOCl's capability to degrade fatty acids and lipids, potentially causing bio-synthetic modifications, destruction of phospholipids, and inactivation of enzymic bacteria through oxidative action (Estrela *et al.* 2002).

Moreover, NaOCl damages plant tissue and causes death, therefore sodium dichloroisocyanurate is administered as a surface disinfectant due to its reliability and non-corrosive property (Caton 2008). The protocol consists of a 2-step process 1) rinsing the explants in a detergent solution to remove contaminants and subsequently break the surface tension on the explant tissue and 2) draining the detergent solution and then immerse explants in a 5.0 mg/L^{-1} solution of NaDCC. Explants in solution are constantly shaken in solution (NaDCC) (Simran Chandrahas and Narasimhan 2021). Additionally, NaDCC is not comparatively noxious compared to NaOCl, and rinsing in double deionized water (DDW) is not vital before placing the explants into culture (Simran Chandrahas and Narasimhan 2021). Hypochlorous acid is synthesized by reacting NaDCC and NaOCl (HOCl) in an aqueous solution. Due to the compound's ability to dissociate and maintain a consistent concentration of HOCl in solution, NaDCC has an effective sterilizing reaction. Compared to NaOCl, which has a pH of 10, NaDCC has a pH of 6.8, physiologically not detrimental to plant's cellular structures. The main advantage of using NaDCC is its low toxicity, permitting the culture of explants without rinsing the chemical. Thus, the disinfectant remains in contact with the explant for an extended duration. Additionally, due to its high stability, dichloroisocyanuric acid has an extended shelf life in a sealed container stored at room temperature (Caton 2008).

2.17.1. In-vitro culture of geophytes

Generally, plants with food-storing modified underground stems (rhizomes, bulbs, tubers, corms) are called geophytes. The plant families such as *Amaryllidaceae*, *Liliaceceae*, *Iridaceace,* and *Araceae* contain mostly geophytes. In Turkey, recent reports indicated 688 geophyte species estimated at 6% of overall vegetation (Kamenetsky and Okubo 2012; Sagiroglu 2020). Propagation of geophytes using traditional methods is used in some species such as *Cyclamen*, *Galanthus*, *Leucojum*, *Arum*, *Lilium*, and *Fritilallia*. However, problems are encountered with such methods, and pests and diseases proliferate rapidly. Therefore, plant tissue culture of geophytes gained traction over the years and laboratories perform in-vitro

culture-producing seedlings free from disease, viruses, and bacteria (Sevgin and Karatas 2022). Microbiological contamination is a major problem in plant tissue culture of geophytes, as a consequence of underground modified stems used for inoculation, the rate of contamination is exceedingly high. To optimize geophytes in plant tissue culture careful consideration of maturity, and physiological stage is critical. Certain maturity is suitable for propagating geophytes in-vitro, determined by physiological stage (Sevgin and Karatas 2022). *Ornitagalum maculatum* was reported to be suitable for in-vitro culture during the first flowering period. Additionally, geophytes used as explants must be stored in cold before being cultured to prevent dormancy during in-vitro. Pre-chilling treatment for approximately 8 weeks in garlic explants increased shoot formation 4-5 times (Ayabe and Sumi 1998). The temperature and duration of pre-chill conditions effective in some geophytes are outlined in (Table 5).

Table 5: Pre-chill temperatures and times applied in plant tissue culture of geophytes

Species	**Treatment**	**Reference**
Fritillaria cirrhosa	4°C (3 months)	(Chen *et al.* 2020)
Fritillaria meleagris	4°C - 15°C (6 weeks)	(Petrić *et al.* 2013)
Fritillaria thunbergii	10°C (6 weeks)	(Paek and Murthy 2002)
Saffron	15°C at dark for (1-3) weeks	(Bhagyalakshmi 1999)
	4°C (3 months)	(Azadi *et al.* 2017)
Garlic	4°C (8 weeks)	(Ayabe and Sumi 1998)
Lilium longiflorum	2°C (6 weeks)	(Ayabe and Sumi 1998)
Lilium nepalense	4°C (8 weeks)	(Wawrosch, Malla and Kopp 2001)
Lilium speciosum	Storage at -1°C, thawing at 5°C	(Langens-Gerrits and De Klerk 1999)

Explants obtained from different sections of the plant such as an immature embryo, root sections, mature seeds, leaves, stem nodes, and onion scales can be manipulated in the plant tissue culture of geophytes (Mirici *et al.* 2005). Although the growth potential quotients documented in onion explants are higher in comparison to roots, stems, and leaves. Furthermore, different sections of the plant may exhibit unique growth potential quotients, moreover, different explants may contain different endogenous hormones. The regeneration potential of explant types through somatic embryogenesis and organogenesis also differ (Table 3).

Table 6: Demonstration of different geophytic explant types (%) in somatic embryogenesis and organogenesis.

Explant	Somatic embryogenesis	Direct organogenesis	Indirect organogenesis
Above ground parts			
(a) Axillary bud and shoot tip	22	10	17
(b) Embryo, anther, carpel, flower bud, flower parts, flower stalk, and stem.	31	14	26
Below ground parts			
(c) Shallot, false onion	8	14	12
(d) Root	8	0	0
(e) Shoot base, tuber pieces, basal plates, double onion scaly leaves, meristematic zone.	15	33	12
(f) Onion scale leaves, leaf base, main onion fleshy leaves.	15	29	31

Source: (Zaidi *et al.* 2000)

Since *Colocasia esculenta* (L.) Schott is a geophyte a new approach to decontamination procedures using environmentally friendly alternatives NaDCC and PPM will be investigated. If decontamination is successful further plant tissue culture stages will be implemented to produce safe, clean material for self-sustenance farmers and commercialisation purposes.

CHAPTER 3

3. Nutritional analysis of *Colocasia esculenta* (L.) Schott

3.1. Abstract

Green leafy vegetables (GLVs) are good sources of vital nutrients for human health sustenance. In this study, the nutrient compositions of *Colocasia esculenta* L. (Schott) leaves and corms from both Southern and Northern locations of KwaZulu-Natal province were investigated. Total, soluble, and insoluble oxalates and soil minerals were analyzed to establish nutrient-plant organ correlations. Substantial variations were observed in nutrient contents among plant parts and locations. Young leaves in both locations showed higher phosphorus content and the northern location had the highest potassium levels in both young and mature leaves. Zinc and copper contents of leaf tissues were relatively low, ranging from 1.9- 4.7 mg/100 g and 0.48 to 1.46 mg/100 g FW, respectively, whereas manganese (2 - 64 mg/100 g FW) and iron (13 – 88 mg/100 g FW) showed higher variability. While oxalate levels showed minimal location effects, corms had more than double the total oxalate content (35-39 g/100 g FW) of leaf tissues. Despite variations in mineral profile, *Colocasia esculenta* leaves can serve as a valuable source of essential micronutrients to address micronutrient needs in vulnerable communities.

3.2. Introduction

Global food systems rely on the consumption of a limited number of species. Cassava, millet, sorghum, wheat, rice, sugar, and maize are critical human calorie sources contributing about 70-90% (Noort *et al.* 2022). Global food security is at risk due to the decreasing diversity of crop species and hence a decline in genetic heterogeneity (Khoury *et al.* 2014). In addition, a combination of different economic, political, and environmental crises has led to increased poverty, and hence food insecurity, particularly among rural communities in Sub-Saharan Africa including South Africa (Abukutsa-Onyango *et al.* 2010; Seekings and Nattrass 2015).

Although South Africa is food secure at a national level, food insecurity at the household level is imminent. About 20% of South African households had inadequate or severely inadequate access to food in 2017 (STATS 2019). This situation has recently been aggravated by the coronavirus pandemic (STATS 2020). Food insecurity is defined as a situation where an individual or household has limited access to enough, safe and nutritious food or consumes

food that falls below 80 percent of the daily minimum recommended allowance of caloric intake for normal growth and development (Mohamed 2017). Food insecurity and hidden hunger affect poorer households a hundred times more than their wealthier counterparts (Schönfeldt, Gibson and Vermeulen 2010).

Vitamin A-rich green leafy vegetables are the least consumed by South African adults (Labadarios, Steyn and Nel 2011). Moreover, 64% of 1- to 9-year-old children are vitamin A deficient, 28% are anemic, 13% have poor iron levels, and 45% have low zinc levels. Green leafy vegetables (GLVs) are abundantly high in minerals such as Ca, Fe, Cu, P, Cl, Na, and Zn (Kumar *et al.* 2020). Some of these nutrients enhance the absorption of other nutrients in the body (Melse-Boonstra 2020). GLVs also form the cheapest and most readily available sources of important fibers, vitamins, and essential amino acids and are the only natural source of folic acid (Randhawa *et al.* 2015). They are low in lipids and hence ideal for weight management (Kumar *et al.* 2020). GLVs are also an excellent source of antioxidants (Odhav *et al.* 2007), with beta-carotene being the most crucial pro-vitamin. Hence diets supplemented with GLVs have the potential to not only improve nutritional security (Toledo and Burlingame 2006) but also boost immunity (Ghosh 2019; Kumar *et al.* 2020).

Colocasia esculenta (L.) Schott commonly known as amadumbe is an emergent, semi-perennial, aquatic, and semi-aquatic stemless herbaceous plant cultivated for its starchy corms. It belongs to the Araceae family of which about 140 genera and about 3750 extensively distributed species are known (Christenhusz and Byng 2016). The species is allogamous and polymorphic (Rashmi *et al.* 2018b). The corm is a reliable source of starch (70–80 g/100g dry weight), fiber (0.80%), ash (1.2%), and fat (0.20%) but low in protein (1.5%) similar to many other tuber crops (Rashmi *et al.* 2018b). The leaves are rich in nutritional and phytochemical compounds; however, they are greatly underutilized. Numerous studies depict that leaves of amadumbe have a high nutritional composition in comparison to green leafy vegetables generally consumed by masses regularly (Table S2). They're also an essential source of thiamine, riboflavin, iron, phosphorus, and zinc and an exceptionally good source of vitamin B6, vitamin C, niacin, potassium, copper, and manganese (Magbalot-Fernandez and Umar 2018).

Amadumbe *Colocasia esculenta* (L.) Schott, is an archaic crop cultivated in the tropical and subtropical regions of the world, particularly in the Pacific and Caribbean islands, parts of Central and South America, and in isolated regions of the United States (Quero-Garcia, Ivancic and Lebot 2010). Amadumbe is a widely cultivated root crop in 50 countries

worldwide. Nigeria is the largest producer of amadumbe, followed by Cameroon, China, and Ghana, and collectively, they account for over half of the global amadumbe production. In 2021, global amadumbe production reached 12,396,248.5 tons, with Africa contributing 76.8% of the total output. Asia contributed 19.3%, while Oceania and the Americas contributed 3.3% and 0.5%, respectively (FAOSTAT 2023a). In South Africa, amadumbe is mainly grown as a subsistence starchy staple. The main growing areas include KwaZulu Natal, Mpumalanga, Eastern and Western Cape provinces, where it is popularly known as amadumbe, *madumbis, mufhongwe,* amadumbe*, and dasheen* (Shange 2004; Louw 2022).

Although grown for its corm, all other parts of the plant including the leaves, petiole, and flowers are edible (Chivenge *et al.* 2015) and have antioxidant, anticancer, and antidiabetic activity (Mitharwal *et al.* 2022). However, according to (FAOSTAT 2023a) South Africa's amadumbe production is minor compared to other major African amadumbe producers. It is therefore postulated that the promotion of consumption of leaves as a green leafy vegetable in South Africa will supplement and complement the nutrients available in the corms. Consequently, provincial and national subsistence farmers can grow and use amadumbe leaves as a 'complete' food source to alleviate food insecurity. However, the complete nutritional information must be known before the crop can be promoted as a green leafy vegetable (Maseko *et al.* 2017).

Typical of the Aroid family (Kumar, Patel and Gupta 2017), all parts of amadumbe contain needle-like calcium oxalate crystals or raphides (Du Thanh *et al.* 2017). These crystals produce a sharp irritation and burning sensation in the mouth and throat resulting in low preference and consumption of this crop (Du Thanh *et al.* 2017). Oxalates occur in plants in either soluble or insoluble forms, each of which impacts differently when consumed (Holmes and Assimos 2004; Nakata 2012). Because amadumbe in South Africa is grown under different growing conditions with different soil types, this may subsequently influence the nutrient uptake and nutritional (including antinutritional) quality of the harvested crop (Raju and Byju 2018).

The objectives of the current study, therefore, were: 1: to determine the nutritional quality of leaves (young and mature) and compare them to that of the corms of the same plants 2: to compare the nutritional quality of two landraces collected from two regions in KwaZulu Natal province of South Africa, 3: to assess the correlation between soil nutrient status and nutritional quality of the plant tissues for the two landraces and 4: to assess the level of oxalates in young and mature leaves and compare to that of the corms.

3.3. Materials and methods

3.3.1. Study sites

Two different sites located in eThekwini Municipality in the KwaZulu-Natal province of South Africa were used for the study: Snembe farms (29° 34' 48" S; 31° 7' 12" E) in Tongaat located in North Coast, about 40 km north of Durban, and farms in Umbumbulu (29° 59' 0" S; 30° 42' 0" E) about 45 km south-west of Durban (Fig 9). They use the upland (non-flooded) growing system. Five farms were randomly selected from the two sites and 20 plants were randomly collected along a transect in each farm.

Figure 9: Map showing selected sampling sites in Snembe and Umbumbulu in eThekwini Municipality in KwaZulu-Natal.
Source: (Shih 2017).

3.3.2. Chemical and plant materials

Three types of tissues were analysed: young newly emerged succulent leaves of approximately 20 cm × 10 cm, mature fully expanded leaves of about 50 cm × 25 cm, and mature corms were prepared for nutrient analysis. Farms were visited regularly in the

autumn (April – May 2018) season during leaf growth. Young and mature leaves (excluding the petiole) were harvested from the plant using a pair of scissors. Corms were harvested from the ground and the soil debris was removed using Bunty's Dish Cloth – Honey-Comb DC. For each farm, all mature leaves were pooled together to make a composite sample from which 20 random samples were drawn for analysis. This was repeated for young leaves and corms. Samples were packed in Leo Manilla Plain Wage Gummed C4 envelope 297 mm × 210 mm and immediately transported to Cedara College of Agriculture and Rural Development, Kwa-Natal, South Africa for nutritional analysis (mineral content and proximate composition). Analytical reagents (AR) were supplied by Merck Sigma-Aldrich, South Africa, all AR grade (≥99.5%). No additional purification was undertaken in any reagents.

3.3.3. Soil sampling technique

Soil samples from the same five farms in Umbumbulu and Snembe sites were also collected for soil nutrient and pH analysis. All soil sample collection was carried out during the autumn-winter period and soil was collected between the rows of amadumbe plants in a *zig-zag* pattern across the farm to ensure homogeneity. Samples from each farm were independently homogenized for analysis. The surface was cleared of all the litter using a Lasher 26 – Tooth Steel Garden Rake and a Stainless-Steel Soil Sampler Probe 25 cm × 52 cm (Johnson Soil Augers, Johannesburg, South Africa) was driven to a plow depth of 30 cm.

Fifteen samples were collected from each sampling unit and placed in a high-density polyethylene bucket (HDPE) 25 L, and all foreign materials such as decomposing leaves, leaf stalks, and stones were removed before covering the bucket with a lid. The procedure was repeated for each farm in the two sites and all samples were labeled and immediately transported to the South African Sugar Research Institute (SASRI) for analysis of pH, macronutrients, and micronutrients.

3.3.4. Mineral analysis

Analysis of the mineral elements calcium, magnesium, potassium, phosphorus, copper, iron, manganese, and zinc content followed (Allen *et al.* 1974). Pieces of leaf samples were

chopped using an Eiger - Bruno 4PC Stainless Steel Knife Set EG-SKKS01 (Yangjiang Smartwife Kitchenware Manufacturing Co. LTD, Yangjiang, China) and acid digested using nitric (HNO_3), sulphuric (H_2SO_4), and perchloric ($HClO_4$) acid mixtures (5:1:0.1). A 0.5 g of sample mixed with 6.5 ml acid mixture was digested on the digital hotplate E-PT1000-23 (Masiye Labs, Johannesburg, South Africa), at 80°C and gradually increased to 250 °C until it turned into thick viscous material. After cooling, the volume was raised to 20 ml using 0.2 N nitric acid, and the contents were filtered (Whatman No. 42) and analyzed with a flame through an atomic absorption spectrophotometer (Shimadzu, Kyoto, Japan) for elements Ca, K, P, Cu, Fe, Mn, and Zn. The instrument was calibrated by a series of standard solutions including a blank, prepared for each nutrient.

3.3.5. Proximate composition

3.3.5.1. Moisture content

Moisture content (%) was determined according to the method by (Osborne and Voogt 1978). The porcelain crucibles were thoroughly scrubbed and allowed to dry in an air oven at 110°C for 10 min to obtain a constant weight. The crucibles were subjected to cooling in a desiccator for 30 min, labeled, and weighed (W_1). A 2.0 g of each plant tissue sample was precisely weighed in the crucibles and re-weighed (W_2). The crucibles containing the samples were placed in an oven maintained at 105°C for 14 hrs. The crucibles were removed and transferred to desiccators to cool and obtain the final weight (W_3). The percentage of weight loss between the wet weight and dry weight of samples was calculated.

3.3.5.2. Protein content

Protein analysis followed the Dumas method (Simonne *et al.* 1994) of total combustion using the LECO- Trumac instrument model FP – 528 (Leco, St Joseph, USA). The plant tissue samples, leaves (young and mature), and corms were isolated. The samples were then dried in an air-forced oven (G.C.A. Corporation, Chicago, USA) at 70°C for 72 h and ground in a Wiley mill (Arthur H. Thomas Co., Philadelphia, USA) to pass through a 20-mesh screen. A 0.2 g of dry tissue was wrapped in tin foil, weighed, and introduced into the induction furnace of an FP-428 N Analyser (LECO Inc., St Joseph, USA). Nitrogen oxide gas (NO_2) formed during combustion was reduced to N_2, which was then quantified by

thermal conductivity. Nitrogen (N_2) was calculated by dividing protein by 6.25 and expressed directly as g N/100 g of dry matter (% N).

3.3.5.3. Fat content

Fat content was determined using the ether extraction Soxhlet method (Nielsen 1998). Two grams (2 g) of sample pre-dried at 100°C to constant weight under pressure was weighed into a pre-dried extraction thimble, ensuring porosity permits a rapid flow of ethyl ether. The sample in the thimble was then covered with glass wool. The pre-dried boiling flask was weighed, and anhydrous ether was added to the boiling flask. The boiling flask, Soxhlet flask, and condenser were assembled simultaneously. The extract was removed at a rate of six drops per second by condensation for about 4 hours by heating the solvent in a boiling flask. The boiling flask with extracted fat was air dried in an oven at 100°C for 30 min, thereafter, cooled in a desiccator and weighed. Fat was calculated with the following formula:

$$\%\ Fat\ on\ a\ dry\ weight\ basis = \frac{g\ of\ fat\ in\ sample}{g\ of\ dried\ sample} x100$$

3.3.5.4. Ash content

The determination of ash content followed the AOAC method (Horwitz 1980). The porcelain crucibles were thoroughly washed with distilled water and dried in an oven to a constant weight at 110°C for 10 min. The crucibles were cooled in a desiccator and weighed (W_1). A 2.0 g of each plant tissue was weighed into the crucibles and reweighed (W_2). The crucibles containing the samples were transferred into a muffle furnace set at 550°C for 8 h to ascertain adequate ashing. The crucibles were removed and allowed to cool in desiccators and weighed (W_3). The percentage of ash was calculated.

3.3.5.5. NDF and ADF content

Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined using (Goering and Van Soest 1970). A 0.5 g of air-dried sample was ground to a fine powder to allow passing through a 1 mm mesh into a beaker of the refluxing apparatus. A 100 ml cold (room temperature) neutral detergent solution was added to the beaker, 2 ml

decahydronaphthalene, and 0.5 g of sodium sulfite using a calibrated measuring instrument. The solution was heated to boiling for 10 min and intermittently reduced the heat to prevent foaming. The solution was adjusted to a constant boiling temperature and refluxed for 6 min. The tared Gooch crucibles were placed on filter manifolds while simultaneously swirling the beaker to suspend solids and fill crucibles. The sample was rinsed with 100°C hot water, filtered the liquid, and duplicate the washing procedure. Wash twice with acetone and dry the crucible at 100°C for 8 h and weigh. NDF was calculated by subtracting the weighted value from 100. The residue was ashed for 3 h at 500°C, weighed, and presented as insoluble NDF.

ADF was repeatedly washed with acetone until no color was removed. The remaining lumps were broken to permeate the contact of particles of the fiber with the solvent. Suck the ADF and dry at 100°C for 8 h and weigh. ADF was calculated using the following equation:

$$ADF = \frac{W0 - W1}{S} x100$$

Where, W_0 = Weight of oven dry crucible including fibre;

W_1 = Tared weight of the oven-dry crucible;

S = Oven dry sample weight.

3.3.6. Determination of oxalate content

Oxalates analysis was conducted at the Department of Chemistry Laboratory at the Durban University of Technology, Kwa-Zulu Natal, South Africa. Leaves and corms were oven-dried at 60˚C for 48 h and powdered separately in a 1.5 L, 400W blender (Sunbeam, Florida, USA). To determine total oxalate and soluble oxalate, 36 Erlenmeyer (100 ml) flasks were first rinsed with distilled water. A 0.5 g of powdered leaf powder sample was weighed into each of the 18 Erlenmeyer flasks and 0.5 g of powdered corm was weighed into the other eighteen Erlenmeyer flasks. Total oxalates were extracted by diluting the powdered leaf or corm samples with 50 ml of distilled water. The flasks were placed in a shaking water bath at 80ºC for 30 min. After filtration using 90 mm Whatman filter paper, the extracts were diluted with 50 ml of distilled water.

From each diluted extract, 25 ml aliquots were titrated with standard 0.02 mol^{-1} of potassium permanganate solution standardized against calcium oxalate. To acidify the extracts dissolved in distilled water (soluble oxalate), 20 ml of 0.02 mol^{-1} sulphuric acid was added to the solutions. Titrations were performed in triplicate at 50˚C using a hotplate KT6301-56

(Science Lab, Navasota, USA) and the titration endpoint of the solution was considered when the colorless solution turned pink for more than 30s. The insoluble oxalate content was calculated by the difference between the total oxalate and soluble oxalate. Oxalate concentrations were expressed as g/100 g dry matter (Sá *et al.* 2019).

3.3.7. Soil analysis

3.3.7.1. Instrumentation

A true-simultaneous Agilent 5110 Vertical Dual View (VDV) ICP-OES was used. The sample introduction system consisted of a double–pass glass cyclonic spray chamber, a OneNeb Series II nebulizer, and a standard 1.8 mm ID injector standard one-piece easy-fit torch. An Agilent SPS 4 autosampler was used for the fast and automated delivery of the samples to the ICP-OES. The 5110 uses a solid-state radio frequency (SSRF) system operating at 27 MHz to produce a robust plasma that is capable of excellent long-term analytical stability during the analysis of soil extracts. The high-speed (1 MHz) VistaChip II CCD detector enables fast warm-up, fast analysis, and high sensitivity as required by soil testing laboratories. The VistaChip II measures the full wavelength range simultaneously from 167 to 785 nm from a single entrance slit without the need for multiple detectors or slits. The detector also has a large dynamic range of up to eight orders of magnitude allowing for both macro- and micro-nutrients to be analyzed from a single sample preparation method (FAO 2019).

3.3.7.2. AB-DTPA extraction solution

The AB-DTPA universal method extraction solution was composed of 0.005 M of diethylenetriamaineacetic acid (DTPA) and 1 M ammonium bicarbonate (NH_4HCO_3). To make the solution, 1.97 g of DTPA and 79.06 g of NH_4HCO_3 were dissolved in distilled water. The pH was maintained at 7.6 while making up the volume to 1000 ml using distilled water.

3.3.7.3. Extraction procedure and analysis

The soil was collected from the northern and southern regions of KwaZulu-Natal. The soil was air-dried and sieved (2 mm). 10 g samples of soil were weighed into 250 ml conical

flasks and 20 ml of AB-DTPA solution was added. The flasks were shaken for 15 min at 180 rpm, then filtered through 2.5µm filter paper (Whatman grade 42, Sigma Aldrich) and diluted 1:1 using distilled water. The samples were spiked with known concentrations of analytes to test the recoveries of the required elements. The samples and spiked samples were loaded on the SPS 4 autosampler for analysis. Calibration standards for all the required elements were prepared using Agilent single-element standards (1000 mg/l). The standards were diluted using the AB-DTPA extraction solution to ensure matrix matching. Linear calibrations were obtained for all analytes and wavelengths, with calibration coefficients greater than 0.999. The samples were analysed by ICP-OES for elements, potassium, calcium, magnesium, zinc, copper, manganese, and iron. Phosphorus was determined using the Resin ion exchange method, the ion exchange membrane simulates the interaction between the plant roots and a soil/water mixture (FAO 2019).

3.3.8. Data analysis

All plant nutrient (antinutrient) data for the three distinct types of plant tissue collected from the two sites were subjected to R Statistics R version 4.0.0 (Auckland, New Zealand). Two-way analysis of variance (ANOVA) and the means for all analyses of variance were further separated using Tukey's posthoc HSD (honestly significant difference) test. Soil nutrient data were independently analyzed using the T-Test MS XLX statistical analysis to determine the means, S±D, and *p* values. The significant differences were determined at $p < 0.05$.

3.4. Results and Discussion

3.4.1. Macronutrient content

Young and mature leaves from Umbumbulu and Snembe did not differ significantly in calcium and magnesium content, but young leaves contained significantly higher ($p<0.05$) phosphorus and potassium than mature leaves in Umbumbulu and Snembe, respectively, (Table 7). All macronutrient content in young and mature leaves was significantly higher than in corms from both sites. The insignificant differences in the macronutrient content of calcium and magnesium in both young and mature leaves indicate that the maturity of leaves has no significant impact on the availability of the two macronutrients. Since young leaves had significantly higher phosphorus and potassium content than mature leaves, the maturity of leaves needs to be considered when harvested as a GLV.

The high phosphorus concentration in young and mature leaves from both cultivation sites indicates higher sources of nutritional phosphorus that can supplement the low phosphorus content in the corms from Umbumbulu and Snembe, respectively (Table 7). *Colocasia* leaves have the potential to meet the recommended daily allowance of 1.250 mg/day (Table S4) (IM 1997) if adequate servings of 300 g per person/day are consumed providing a minimum of 72% and (>100%) of the RDA of this mineral. The results indicated a higher phosphorus content compared to spinach (32.59 mg/100 g), mustard (71.62 mg/100 g), fenugreek (53.05 mg/100 g), amaranth (73.22 mg/100 g), and bathua (37.55 mg/100 g) leaves (Table S4) (Longvah *et al.* 2017; USDA 2021).

Potassium content was significantly higher ($p<0.05$) in young and mature leaves (Table 7) from the Snembe population, contrarily young and mature leaf tissues from Umbumbulu demonstrated no significant difference *($p>0.05$),* (more than double) compared to the corms (Table 7). The abundance of potassium in leaves is similar to the study by (Azubuike *et al.* 2018) who also found potassium to be the most abundant mineral in fresh leaves of *Colocasia*. A high intake of dietary K potentially reduces the risk of coronary heart disease and other chronic illnesses (Asyira *et al.* 2016). Young leaves alone from the Snembe population could be sufficient for satisfying the maximum recommended daily allowance of 3.400 mg/day per 100 g servings (Table S4) (IM 1997; Sawka 2005) of 100 g portions per person contributing (>100%) of the RDA of potassium.

Young leaves from Umbumbulu had a lower calcium content compared to mature leaves from the same site. Similarly, Snembe had a higher content of calcium in mature than in

young leaves. In both population sites, leaf tissues indicated no significant difference (*p>0.05*) in calcium content. Moreover, corm tissues displayed no significant difference (*p>0.05*) and contained the lowest calcium concentration compared to leaf tissues. Young and mature leaves from Umbumbulu and Snembe have the potential to supply 1.4 – 1.5 g/100 g and 1.2 – 1.3 g/100 g, respectively, (Table 1).

Contrarily, corms contain 0.08 – 0.09 g/100 g of calcium requiring a colossal intake of 1.700 g of the starchy corm to satisfy recommended daily allowance. The maximum recommended daily intake of calcium is 1.300 mg/day (Table S4) (IM 1997; Sawka 2005), and about 100 g of *Colocasia* leaves can potentially provide a complete nutritional value to satisfy the RDA (Ross *et al.* 2011) if sufficient portions are consumed contributing (>100%) of RDA of calcium. Calcium improves bone health, facilitates growth and development, and maintains the human skeletal system and supporting structure (Flynn 2003; Sicinska *et al.* 2020). Magnesium plays a protective role against cardiovascular diseases, strokes, and diabetes (Barbagallo, Veronese and Dominguez 2021).

Young and mature leaves from both sites had a high magnesium content (Table 7). Young and mature leaves from Umbumbulu indicated no significant difference (*p>0.05*), both sites had no significant difference (*p>0.05*), except mature leaves from Umbumbulu with a higher concentration (0.48 g/100 g). While corms from Umbumbulu and Snembe had a lower concentration (*p<0.05*) (Table 7). The highest daily requirement for magnesium is 360 mg (Table S4) (IM 1997; Sawka 2005) which can be satisfied by consuming 100 g of *Colocasia* leaves per person contributing >100% of RDA indicating that *Colocasia* leaves can be considered a high-content source of magnesium.

According to (Longvah *et al.* 2017), numerous other commonly consumed green leafy vegetables such as *Amaranth*, *Spinacea*, *Brassica*, and *Trigonella* contain considerably lower magnesium content in leaves (Table S2). Jalali and Fakhri (2021) Discovered that fresh wild edible plants, *Taraxacum vulgar* and *Fumaria parviflora* supplied between 146.9 and 203.9 mg/100^{-1} g of Mg, providing 10-14%, thus none of the WEPs can be considered a good source of Mg. Longvah *et al.* (2017) Reported Mg concentrations of *Spinacea oleracea*, *Brassica juncea*, and *Chenopodium gangeticus* as 24%, 14%, and 13% indicating the superiority of *Colocasia* leaves as a contributor of Mg (Table S2).

Table 7: Macronutrient content in the tissue of amadumbe plants collected in Umbumbulu and Snembe sites.

	Umbumbulu			**Snembe**		
Macronutrients (g/100g FW)	*Young*	*Mature*	*Corms*	*Young*	*Mature*	*Corms*
Phosphorus (P)	0.43 ± 0.02^{c}	0.30 ± 0.09^{b}	0.14 ± 0.02^{a}	0.43 ± 0.02^{c}	0.42 ± 0.02^{c}	0.24 ± 0.01^{b}
Potassium (K)	3.5 ± 0.09^{b}	3.7 ± 0.25^{b}	2.2 ± 0.11^{ab}	5.1 ± 0.24^{d}	4.5 ± 0.32^{c}	2.0 ± 0.11^{a}
Calcium (Ca)	1.4 ± 0.02^{bc}	1.5 ± 0.21^{c}	0.08 ± 0.01^{a}	1.2 ± 0.08^{b}	1.3 ± 0.18^{bc}	0.09 ± 0.03^{ab}
Magnesium (Mg)	0.40 ± 0.03^{bc}	0.48 ± 0.09^{c}	0.15 ± 0.02^{ab}	0.36 ± 0.02^{b}	0.39 ± 0.05^{bc}	0.14 ± 0.01^{a}

Mean ± Standard Deviation, n = 5 farms per site. Values followed by the same letter in a row are not significantly different at $p<0.05$.

Table 8: Micronutrient content in the tissue of amadumbe plants collected in Umbumbulu and Snembe sites.

	Umbumbulu			**Snembe**		
Micronutrients (mg/100g)	*Young*	*Mature*	*Corms*	*Young*	*Mature*	*Corms*
Zinc (Zn)	4.7 ± 2.7^{b}	3.7 ± 20^{ab}	1.9 ± 1.5^{a}	5 ± 4.5^{b}	5.1 ± 7^{b}	4 ± 21.5^{ab}
Copper (Cu)	1.46 ± 0.89^{d}	1.16 ± 3.1^{c}	0.62 ± 0.45^{ab}	0.78 ± 0.45^{b}	0.82 ± 0.45^{b}	0.48 ± 0.45^{a}
Manganese (Mn)	64 ± 73^{d}	45 ± 158^{c}	3 ± 6^{ab}	14 ± 40^{b}	17 ± 39^{b}	2 ± 2.5^{a}
Iron (Fe)	24 ± 120^{a}	13.4 ± 26^{a}	48 ± 170^{b}	23 ± 105^{a}	13 ± 11^{a}	88 ± 149^{c}

Mean ± Standard Deviation, n = 5 farms per site. Values followed by the same letter in a row are not significantly different at $p<0.05$.

3.4.2. Micronutrient content

Leaves had higher levels of the micronutrients Zn, Cu, and Mn than the corms in both sites, with young leaves containing higher micronutrient concentrations (Table 8). In both sites, although Zn content was higher in younger leaves, there was no significant difference *($p>0.05$)* in Zn content for the young and mature leaves, and hence both can be good sources of nutritional zinc. Both population sites displayed no significant differences ($p>0.05$) in zinc concentration in corms.

The RDA of Zn is 11 and 9.0 mg/day (Trumbo *et al.* 2001a) for adult males and females, respectively. Studied *Colocasia* leaves can potentially contribute 3-4% of the RDA, suggesting that leaves are a poor source of Zn. To satisfy daily Zn rations about 2.100 g (> 2 kg) of portions must be consumed. Furthermore, supplementation is required for pregnant women of up to 11 mg/day (Table S3) (Silva *et al.* 2019; Carducci *et al.* 2021). Unlike the Snembe population which exhibited no significant differences in micronutrient content between young and mature leaves, young leaves from the Umbumbulu population were significantly higher in copper ($p<0.05$) and manganese ($p<0.05$) content compared to mature leaves (Table 8). Since copper is essential for several physiological processes, it is needed in human diets. Both young and mature leaves from the Umbumbulu population contained adequate concentrations, whilst the corms retained considerably low concentrations (Table 8). The consumption of leafy parts of *Colocasia* has the potential to contribute effective copper concentrations if portions of 100 g are served. The RDA of Cu is (0.9 mg) and (1.3 mg) for pregnant and lactating females (USDA 2014). *Colocasia* leaves can supply a maximum of 15% of the RDA of Cu. Jalali and Fakhri (2021) Indicated the leaves could contribute 14% of the RDA of Cu.

Manganese is essential for bone development and amino acids, lipid, protein, and carbohydrate metabolism and is also needed for the proper functioning of several metalloenzymes (Islam *et al.* 2023). Young and mature leaves from Umbumbulu contained Mn in significantly high amounts ($p<0.05$), in contrast, leaf organs from Snembe exhibited no significant difference ($p>0.05$), respectively, compared to corms from both sites ($p<0.05$) (Table 8). The Mn in leaves may supply from 71% - (>100%) of the RDA. The RDA is 2 mg/day (Trumbo *et al.* 2001b) for adults, rations of 100 g/day may provide the necessary daily allowance to avoid toxicity.

The excessive amounts of Mn in leaves would be expected to cause manganese toxicity (He *et al.* 2022). However, since no evidence of manganese toxicity from dietary Mn intake has been documented (Greger 1998; Finley *et al.* 2003; Evans and Masullo 2020) and further, humans can maintain stable tissue levels of Mn through homeostatic control of both its absorption and excretion, this ensures only a small percentage of dietary manganese is absorbed and the excess is excreted very rapidly into the gut via bile (Silva *et al.* 2019). Hence, the high concentrations of Mn reported for leaves or corms in this study may not have negative health implications if consumed.

Iron content in leaves showed no significant difference ($p>0.05$) for young and mature leaves from Umbumbulu, similarly with Snembe leaf tissues ($p>0.05$). However, young leaves contain more iron than mature leaves since they have double the iron content of mature leaves. Iron content in leaves (Table 8) may potentially provide about 13 - 30% of the required RDA 600 g – 1.800 g of servings for males (8 mg) and females (18 mg) of all ages, respectively (Piskin *et al.* 2022). (Jalali and Fakhri 2021) studied *Allium ampeloprasum* and *Taraxicum vulgare* and discovered that these wild edible plants (WEPs) supply approximately 29% and 47% of the RDA for males and 13% and 21% for females, respectively. Additionally, (Longvah *et al.* 2017) documented potential RDA values of *Colocasia esculenta* (3.41 mg/100 g), *Spinacea oleracea* (2.95 mg/100 g), and *Brassica juncea* (2.84 mg/100 g) as 43%, 37%, and 36% for males and 19%, 16%, and 16% for females respectively.

Interestingly, unlike other microelements, the corms from both sites had higher iron content ($p<0.05$) (Table 8) than leaves with an excess of four times that of leaves and therefore would be a better source of iron to meet the RDA, more especially for pregnant women who require at least 27 mg/100 g in their daily diet (Muriuki, Sila and Onyango 2014). Iron is essential for DNA synthesis and the production of hemoglobin, necessary for oxygen transport, and therefore necessary for preventing anemia (Abbaspour, Hurrell and Kelishadi 2014).

Iron transportation within various parts of the plant is linked to different complex biochemical processes, genetic codes, and protein mechanisms (Kobayashi and Nishizawa 2012). The higher Fe concentration in corms than in leaves is possibly linked to Heat Shock Cognate Protein B (*At*HSCB) which regulates iron translocation from roots to shoots (Upadhyay 2022). Overexpression of *At*HSCB causes over-accumulation of Fe in roots rather than in leaves

(Mahawar *et al.* 2022). It is therefore possible that the corms have a higher concentration of *At*HSCB, or the protein could be overexpressed.

These results showed leaves to have significantly higher proportions of macro and micronutrients than reported for exotic leafy vegetables like cabbage or spinach (Hussain *et al.* 2009; Butnariu and Butu 2015; Lewu and Kambizi 2015; Bwembya *et al.* 2018).

3.4.3. Effect of harvesting of leaves on corm production

Colocasia leaves have the immense potential to contribute to alleviating micronutrient deficiencies and hence the overall mitigation of food insecurity. However, when utilizing *Colocasia esculenta* leaves as a green leafy vegetable premature harvesting of the leaves must take place. (Shields and Wyman 1984) determined that defoliation of the upper leaves of 'Superior' and 'Russet Burbank' cultivars potatoes during full bloom resulted in significant yield loss while 10% of lower leaf loss during full bloom on 'Russet Burbank' plants resulted in tuber yield loss. (Dukuh 2011) examined the effect of defoliation on *Ipomoea batatas* L. sweet potato tubers and found a significantly high effect on tuber sprouting in the field. The number of days of defoliation also had an immense impact on increasing tuber sprouting.

Other studies have also shown that increasing the frequency of *Ipomoea batatas* var. Bophelo vine harvesting improved leaf yield, but total storage root yield decreased (Gomes and Carr 2001; Gomes and Carr 2003; Dukuh 2011). Further, (Nyathi *et al.* 2019) investigated the effect of vine harvesting on the storage root yield of orange-fleshed sweet potato and found that vine harvesting reduced storage root yield and reduced micronutrients such as zinc and iron concentrations in the storage root thus decreasing the potential of utilizing the orange-fleshed sweet potato as a dual-purpose food crop for commercialization. However, on a subsistence scale, dual-purpose food crop practice is recommended as the availability of edible nutritious leaves would supplement micronutrients that are low in the starchy underground storage roots. Therefore, the harvesting of young and mature leaves of *Colocasia esculenta* may potentially reduce underground production of root crops but improve nutritionally balanced diets for impoverished communities.

3.4.4. Proximate composition

Moisture content in young and mature leaves from Umbumbulu did not differ significantly ($p > 0.05$), but young leaves had a higher moisture content than the corms from Snembe ($p < 0.05$) (Fig. 10a). High moisture content is indicative of freshness but is also associated with low shelf life (Gogo *et al.* 2017). The high moisture content observed in amadumbe tissues is within the range documented for amadumbe (60 – 83%) and comparable to other leafy vegetables (Temesgen and Retta 2015; Azubuike *et al.* 2018).

Young and mature leaves from both populations contained significantly higher amounts of protein than corms ($p<0.05$) (Fig 10b). Proteins facilitate the body to combat infections and are a major energy supplier (Temesgen and Retta 2015). (Temesgen and Retta 2015) showed that the essential amino acids, methionine, lycine, cystine, phenylalanine, and leucine are relatively more abundant in the leaves than in the corms. The results of this study demonstrated that leaves have higher protein content than the previously cited amount in amadumbe (23%) (FAO 1999). Communities consuming both the leaves and corms will ideally receive higher protein compared to people including only corms in their diets.

Mature leaves from both populations had significantly ($p>005$) higher fat content than young leaves (Fig 10c). Further, compared to other organic components, the percentage of fat was also significantly ($p<0.05$) lower in both the leaves and corms (Fig 10c). The low-fat content in the leaves is advantageous for people suffering from obesity (Lewu, Adebola and Afolayan 2009). The mature leaves from the Umbumbulu population had significantly ($p<0.05$) higher ash content than young leaves, but the opposite was true for the Snembe population (Fig 10d). Ash content is directly correlated to mineral content and therefore mature leaves from the Umbumbulu generally contained higher mineral content than young leaves, contrary to the young leaves from the Snembe population (Fig. 10d; Table 7; Table 8). The ash content of the leaves is above previously documented ranges for *C. esculenta* tissues (3.5 – 7.8%) (Temesgen and Retta 2015), confirming their suitability as a mineral-rich leafy vegetable.

Young and mature leaves from both the Umbumbulu and Snembe populations had no significant differences ($p<0.05$) in ADF (Fig 10e) and NDF (Fig 10f). However, ADF and NDF content was significantly lower in corms than in leaves from both populations. ADF and NDF values are inversely related to digestibility (Stergiadis *et al.* 2015); therefore, leafy vegetables

with low ADF concentrations are usually more digestible thus releasing more nutrients. Further, NDF and ADF digestibility depends on the age of the organ (Salama 2019). According to (Van Soest, Robertson and Lewis 1991), NDF increases as forage matures. While the results conform to (Van Soest, Robertson and Lewis 1991) in NDF, these values are not significantly different between young and mature leaves and are even lower than previously cited levels of 38% (Giang *et al.* 2010). Hence both young and mature leaves indicate similar and appreciable digestibility. However, corms would be more digestible than leaves based on their low ADF and NDF values. In addition to aiding in digestion, leaves have been found to support the growth of beneficial gut and intestinal microbes such as *Escherichia coli* and *Lactobacillus acidophilus*, that help digestion and fight against harmful microbes (Saenphoom *et al.* 2016).

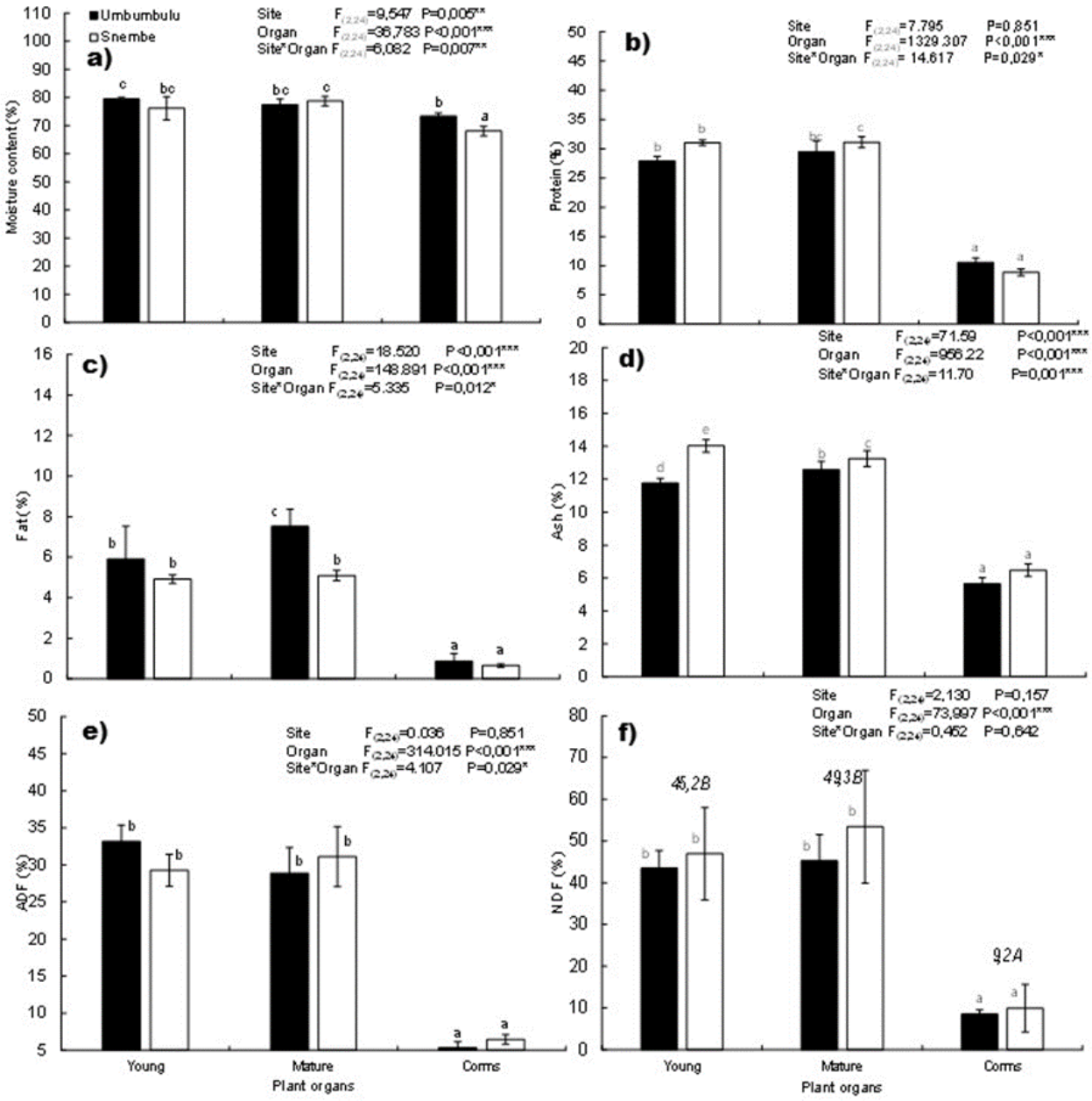

Figure 10: Proximate composition of different tissues of *Colocasia esculenta* from Umbumbulu and Snembe sites of KwaZulu-Natal province of South Africa. a) Moisture content in leaf and corm tissues; b) Protein content availability in *Colocasia* leaves and corms; c) Fat concentrations in leaves and corm tissues; d) Ash content; e) ADF content; f) NDF content. Bars represent mean±standard error. Different letters denote significantly different means at $p<0.05$ using Tukey HSD.

3.4.5. Oxalate content

Young and mature leaves from both sites showed no significant difference in total oxalate content (Fig 11). The total oxalate content in leaves from both sites was significantly lower ($p<0.05$) than in corms, with corms from the Umbumbulu site having more than two times higher oxalate content and corms from the Snembe site having up to three times more oxalate than leaf oxalate content (Fig 11). Similarly, for insoluble oxalate content, no significant differences ($p>0.05$) were observed in young and mature leaves from both sites. Leaves contained lower amounts of insoluble oxalate compared to corms with more than five times higher concentrations. Soluble oxalates were lowest in the corms. No significant differences were observed for soluble oxalates in leaf and corm tissue except for corms from Snembe that contained significantly ($p>0.05$) lower soluble oxalates (Figure 11).

The presence of oxalates in food is considered antinutritional due to its negative effects on the consumer after consumption (Samtiya, Aluko and Dhewa 2020). Oxalates are present in almost all plants as a natural defense mechanism against grazing animals by producing acridity, causing lip and mouth swelling (Mitharwal *et al.* 2022). Total oxalate is always the sum of both dietary soluble and insoluble oxalates. Soluble oxalates bind with minerals such as K^+, and Na^+ rendering them unabsorbed through the intestinal wall into the bloodstream. Hence excessive intake of soluble oxalates can lead to the formation of kidney stones (Savage and Dubois 2006; Mitharwal *et al.* 2022).

Insoluble oxalates however occur as salts of CaOx, MgOx, and FeOx which are usually excreted, and therefore more preferred than soluble oxalates. The results indicated that both young and mature leaves had significantly lower total and insoluble oxalates than corms from both sites and indeed lower than in spinach which is among leafy vegetables with the highest oxalate content of 658 mg/100g; (Bargagli *et al.* 2009). These results also corroborate those of (Mitharwal *et al.* 2022) who observed higher total and insoluble oxalate content in corms than in leaves. Cooking and other processing methods have been used to reduce the negative effects of oxalates in corms e.g., boiling amadumbe corm at 90˚C for 30 minutes and steeping in water at 30˚C for 24 hours can reduce the oxalate-salt content to 32.7% and 56.7%, respectively, of its original content (Temesgen and Retta 2015). Similarly, boiling the leaves was found to significantly reduce oxalate content (Du Thanh *et al.* 2017).

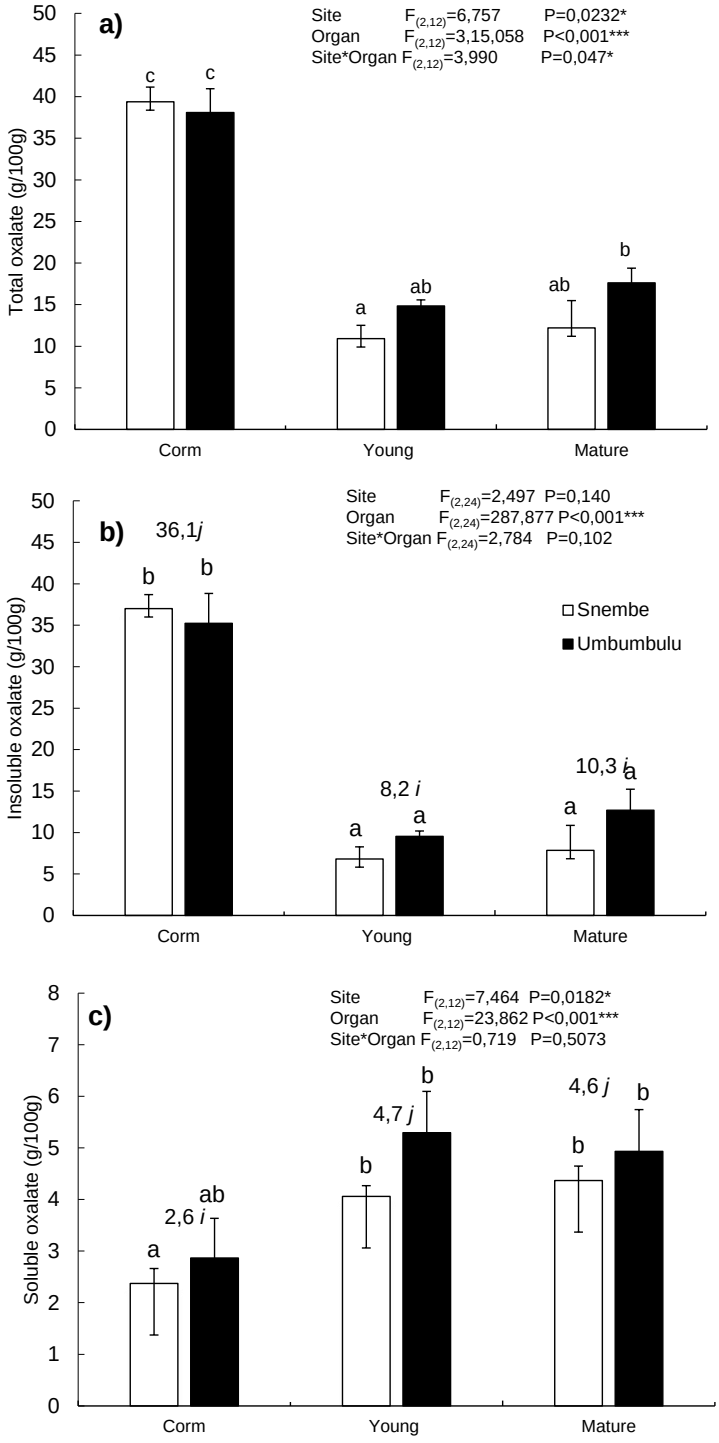

Figure 11: Oxalate content in different tissues of *Colocasia esculenta* from Umbumbulu and Snembe sites of KwaZulu-Natal province of South Africa. A) Total oxalate in leaf and corm tissues; B) Insoluble oxalate content availability in *Colocasia* leaves and corms; C) Soluble oxalate in leaves and corm tissues. Bars represent mean±standard deviation. Different letters denote significantly different means at $p<0.05$ using Tukey HSD.

3.4.6. Plant-soil Interactions

The pH of the soil from the Snembe site was 5.42, significantly ($p<0.05$) higher than the soil pH of 4.76 at the Umbumbulu site. Apart from magnesium which was significantly ($p<0.05$) higher in Umbumbulu, Snembe soil was significantly higher in all other macronutrients (Table 9). While a higher level of nutrients in the Snembe soil appeared to correspond to higher nutrient levels in the plant tissues for some elements, in others there was no clear direct relationship. For example, phosphorus was 6 times higher in the Snembe soil corresponding to significantly higher levels of phosphorus specifically in mature leaves and corms, compared to mature leaves and corms from the Umbumbulu site.

Potassium was 2.5 times higher in the Snembe soil and this corresponded to a significantly higher content of potassium in both young and mature leaves but not in the corm (Table 7; Table 9). However, calcium was 1.8 times and magnesium 0.4 times more abundant in the Snembe soil, significant differences ($p<0.05$) were observed in the magnesium content in the plant tissues from both sites. Nutrient uptake and translocation are influenced by several factors (Malik 2009). While nutrients may be abundant in the soils they must be in a readily absorbable form (Bhatla and Lal 2018). In addition, several mechanisms enable plants to maximize their nutrient acquisition from the soil and at the same time also protect against the accumulation of these nutrients at toxic levels (Osvalde 2011). These mechanisms are influenced by factors such as soil properties (e.g., structure, composition, pH), plants' characteristics (e.g., root structure, high or low-affinity transport systems), microorganisms (e.g., symbiotic bacteria, mycorrhiza), and the presence of synergistic or antagonistic minerals (Osvalde 2011).

Similarly, for micronutrients, despite Zn being exceptionally higher (28-fold) in the Snembe soil than in the Umbumbulu soil, this only resulted in slightly higher but insignificant differences in Zn content in young and mature leaves from the Snembe site. Copper was also significantly ($p<0.05$) higher in Snembe soil, but plant tissues showed significantly lower levels compared to the same tissues collected from Umbumbulu (Table 8; Table 9). An exception was in manganese concentration which was significantly higher in the Umbumbulu soil and appeared to directly influence significantly higher levels of manganese in both young and mature leaves from Umbumbulu. Manganese solubility in soil solution is dependent on pH, organic matter, and the presence of antagonistic minerals (Ghasemi-Fasaei and Ronaghi 2008).

Acceptable pH ranges for Mn availability in the soil are 5 – 6.5. Lower ranges such as 4.76 that of soil in Umbumbulu promote excessive Mn^{2+} availability.

The significantly ($p<0.05$) higher iron concentration in the Snembe soil did not appear to cause significant differences in leaf tissue iron content but resulted in considerably high amounts of iron in corms (Table 7) collected from Snembe. Iron uptake from the soil and translocation from the corm to the leaves may be linked to Heat Shock Cognate Protein B (*At*HSCB) (Leaden *et al.* 2016) and hence higher soil iron levels in Snembe may have translated to the high iron levels in Snembe corms.

Table 9: Soil nutrient content in Umbumbulu and Snembe sites.

Parameters (mg/kg)	**Umbumbulu**	**Snembe**
P	69 ± 24^{a}	412 ± 80^{b}
K	1521 ± 336^{a}	3785 ± 231^{b}
Ca	18694 ± 4451^{a}	33301 ± 6056^{b}
Mg	6315 ± 984^{a}	2556 ± 371^{b}
Zn	20 ± 8^{a}	553 ± 104^{b}
Cu	97 ± 10^{a}	166 ± 39^{b}
Mn	154 ± 28^{a}	108 ± 16^{b}
Fe	2467 ± 308^{a}	3475 ± 213^{b}

Mean±SD, n = 5 farms per site. Values followed by the same letters in a row are not significantly different at ($p<0.05$).

3.4.7. Conclusion

Establishing the nutrient profile is essential due to the importance of green leafy vegetable consumption in the human diet and the impact on disease prevention. Our findings strongly suggest that the plant parts are promising sources of essential nutrients. Specifically, potassium (K), calcium (Ca), manganese (Mn), and iron (Fe) were the most abundant among both macro and microelements. *Colocasia* leaves can contribute significantly to meeting the requirements for potassium and calcium, manganese, and iron, respectively, provided that factors such as oxalates are processed thoroughly to avoid mineral binding and unavailability. Suitable cooking methods that would minimize excessive leaching of minerals should be adopted. The consumption of green leafy vegetable species can potentially provide health benefits owing to their nutritional profile. These vegetables offer a cost-effective source of nutrition, particularly for rural communities. Since amadumbe is a minor crop in South Africa, and production is mainly subsistence, interval harvesting of leaves is not recommended for commercial production but rather supported for small-scale self-sustenance farmers to balance trade-offs.

CHAPTER 4

4. Plant Tissue Culture

4.1. Abstract

The aim of this study was to evaluate the effect of NaDCC (Sodium Dichloroisocyanurate), PPM^{TM} (Plant Preservative Mixture), and further assess the effect of pre-chilling explants before treating with NaDCC and PPM^{TM} on the elimination of contaminants in amadumbe explants from Umbumbulu (South) and Snembe (North) of KwaZulu-Natal, South Africa. A 0.2% BAC Tween 20 (60 min), 70 % ethanol (4 min), 30% ethanol (4 min), and 0.2% Benlate (30 min) was employed to optimize the surface decontamination of explant groups [PPM^{TM}, PPM^{TM} (P) (Plant Preservative Mixture containing explants refrigerated at 2 degrees Celsius), NaDCC, and NaDCC (P) (Sodium Dichloroisocyanurate solution with explants refrigerated at 2 degrees Celsius)]. PPM^{TM} and PPM^{TM} (P) were evaluated at 5 ml l^{-1} (in media) and NaDCC and NaDCC (P) at 0.5% concentration in solution under a laminar airflow chamber. Explant response to treatments was monitored on a 30 -120-day observation and bacterial, fungal, aseptic, and shoot development parameters were recorded. The results highlighted no significant differences ($p>0.05$) in treatments with slight variations where ($p<0.05$). NaDCC (P) and PPM^{TM} (P) effectiveness were observed at (BC30) in Umbumbulu and Snembe respectively. Contamination persisted at (BC120 - FC120), and aseptic culture loss at (ACS120) in Umbumbulu except Snembe had a survival rate of NaDCC (P) and PPM^{TM} (P) (43±39 and 7±12 %), respectively. Shoot development was higher at (SD30) and gradually decreased as it reached (SD120). NaDCC, NaDCC (P), PPM^{TM,} and PPM^{TM} (P) were found to be ineffective in eliminating all contamination, although slight variations were observed.

4.2. Introduction

The world's food security is under threat attributed to several causes, such as poor food production, climate change, and an exponentially expanding population. As a result, it is necessary to explore and identify alternative food sources, such as underutilized crops like *Colocasia esculenta* (L.) Schott *(*amadumbe) (WHO 2020). In South Africa, amadumbe is conventionally propagated by sharing vegetative propagules (suckers/stolons, corms, and cormels) among farmers under flooded (wetland cultivation) and unflooded (dryland-rain fed)

cultivation practices (Opara 2003). Umbumbulu (South) and Snembe (North) amadumbe growing regions of KwaZulu Natal, practice the conventional methods of propagating, under unflooded growing conditions. This practice is, however, challenging to subsistence farmers aiming at commercializing the crop as the exchange of propagules results in a higher intra-clonal variation in the growth rate and final yield (Lebot *et al.* 2017). The exchange of propagules also increases the risk of transmitting various pests and diseases affecting the quality and yield of amadumbe (Ravi *et al.* 2021). Furthermore, this method of cultivation affects the shape and yield of amadumbe corms (Onwueme 1999).

Pests and disease outbreaks are major factors contributing to inadequate amadumbe production in several countries; therefore, amadumbe has been mainly replaced by crops such as cassava and sweet potato (Deo *et al.* 2009). For example, the outbreak of amadumbe leaf blight (TLB) (*Phytophthora colocasiae*) in West Africa in 2009 accounted for more than US$1.4 billion in economic loss annually. TLB not only led to genetic erosion of the gene pool in the region (Onyeka 2014) but has continued to impact negatively on the yield and quality of corms and subsequently commercializing the crop in most amadumbe producing countries (Mbong *et al.* 2013; Oladimeji *et al.* 2022). Commercial cultivars produced conventional through vegetative propagation are also susceptible to *Pythium myriotylum* caused by cocoyam root rot, and dasheen mosaic virus (DMV), a pathogenic strain that is detected in the foliage, corms, and cormels, reducing quality, yield, and production (Reyes Castro and Ronnberg-Wastljung 2006; Omoboye 2019). Viruses are among the dangerous pathogenic microorganisms that can negatively impact amadumbe production causing reduced yields and in some instances complete loss of a crop (Sibiya 2015). In addition, the propagules used in conventional propagation are bulky and low in throughput as one plant may produce only a few suckers, and the availability of these planting materials is also seasonal, thus compromising steady high production rates (Kepue *et al.* 2021). These drawbacks highlight the need to use technology that will produce disease-free high-quality amadumbe. Plant tissue culture technique, also known as *in vitro* culture offers a sustainable solution to problems associated with conventional propagation (Espinosa-Leal, Puente-Garza and García-Lara 2018). This method exploits the totipotent nature of plant cells: i.e., the genetic ability to produce a complete plant (Haberlandt 1902; Fehér 2019; Long *et al.* 2022). As a result, a single plant can produce a large number of plants within a short time and space (Samantara *et al.* 2022). Propagation via tissue culture, the plant parts such as cells, tissues, and organs are cultured in artificial aseptic media under highly

controlled laboratory conditions, that allow rapid production of superior quality, disease-free, and identical planting materials (Suman 2017) that can be achieved all year round. The plant tissue culture technique has been successfully applied to thousands of plant species (Hussain *et al.* 2012) including a study by (Kepue *et al.* 2021) and (Alam and Kadir 2022) on *C. esculenta*.

The ability to regenerate plants by tissue culture is affected by multiple factors such as the type of explant (initial plant part used for regeneration) (Minutolo *et al.* 2020), plant growth regulators (PGRs) (Gerdakaneh *et al.* 2020), the composition of growth medium (Chimdessa 2020) and explant type (Dhar and Joshi 2005), culture conditions and maintenance of aseptic conditions throughout the culture period (Gammoudi, Nagaz and Ferchichi 2022) to end up with clean planting material. *In vitro* culture environment offers a conducive environment for microorganisms including filamentous fungi, yeasts, bacteria, viruses, viroids, and micro−arthropods (mites and thrips) that compete with explants for nutrients, which negatively impact the subsequent growth and development of the explant. The microorganisms grow faster than the explant and and outcompete the explants for the nutrients available in the medium and the explant eventually dies within days without sufficient nutrients available (Gammoudi, Nagaz and Ferchichi 2022).

Hence, successful culture initiation requires all microbial contaminants to be removed (da Silva *et al.* 2016). Surface disinfectants including (NaOCl), hydrogen peroxide (H_2O_2), mercuric chloride ($HgCl_2$), silver nitrate ($AgNO_3$), calcium hypochlorite ($Ca(OCl)_2$), and various antibiotics are used to remove microbial contaminants from the explant before *in vitro* initiation of the explant. Sodium hypochlorite is the most preferred chemical surface disinfectant in tissue culture due to its broad antimicrobial activity, rapid bactericidal action, solubility in water, and relative stability (Fukuzaki 2006). However, there is no standard disinfection protocol as disinfection efficiency depends on the contamination type (epiphytic or endophytic and expressed or latent), the explant type, age or size as well on the disinfection procedure (da Silva *et al.* 2016). For example, amadumbe was successfully regenerated from apical meristems tips after surface disinfecting using alcohol and sodium hypochlorite (Manju *et al.* 2017) while (Adelegn 2018), obtained 83% of the sprout tip and 67% of the corm explant after disinfecting using different concentrations of NaOCl at different exposure times of 15 and 20 min. Shoot tip explants excised from the cormels were successfully surface disinfected with NaOCl for

direct regeneration of the amadumbe cultivars Wangi and Putih (Alam and Kadir 2022). An investigation by Cavusoglu, Sulusoglu and Erkal (2013) explored different decontamination treatments (mercuric chloride ($HgCl_2$), NaOCl, hydrogen peroxide (H_2O_2) of saffron (*Crocus sativus*) a geophyte to overcome persistent endophytic contamination. Furthermore, (Nath *et al.* 2012) successfully surface-disinfected apical meristems excised from the corm using 70% ethyl alcohol, and 0.1% $HgCl_2$ and then initiated the excised meristems *in vitro.* Bhagavan *et al.* (2013) investigated and effectively developed a protocol for large-scale regeneration and development of amadumbe through organogenesis from shoot apices and axillary buds. Explants used in the experiment were disinfected with 0.1% carbendazim, 70% ethyl alcohol, and 0.1% $HgCl_2$. However, (Pradhan and Deo 2016) reported that using $HgCl_2$ proved to be extremely hazardous and environmentally damaging whereas NaOCl is corrosive and detrimental to plants' intra- and intercellular tissues reducing explant sizes and leading to cessation (Bogale 2018). Additionally, (Srivastava *et al.* 2020) devised a strategy to eliminate endophytic bacteria using antibiotics and re-establishment of plant tissue culture-generated corms *in vitro* after *ex vitro* hardening of micro corms. Although antibiotics were unsuccessful, they observed that hardening mitigated endophytic bacteria through periodically transferring tissue-cultured derived corms to *ex vitro* condition and then re-introducing the bacteria-free shoot tip explants *in vitro.*

Sodium dichloroisocyanurate (NaDCC) is an eco-friendly chemical alternative for explant surface sterilization in plant tissue culture as it does not accumulate in the natural environment, retains stability, and can be reused over extended periods (Simran Chandrahas and Narasimhan 2021). The Plant Preservative Mixture^{TM} (PPM^{TM}) is also described as an environmentally friendly effective and phytologically safe broad-spectrum microbiocide for use in plant tissue cultures (PCT 2023). Therefore, to reduce environmental degradation and possible explant tissue damage this study investigated the use of NaDCC and PPM^{TM} as an alternative disinfecting agent to eliminate surface and endogenous contaminants such as endophytic bacteria and fungi. The objectives of the study therefore were: 1) To determine the effect of NaDCC on eliminating contaminants in amadumbe explants. 2) To determine the effect of PPM on eliminating contaminants in amadumbe explants. 3) To further evaluate the effect of pre-chilled explants at 2˚C on eliminating contaminants in amadumbe before treating with disinfectants NaDCC (P), and PPM (P).

4.3. Materials and Methods

4.3.1. Plant Material

4.3.1.1. Harvesting and storage of corms

Corms of two landraces of amadumbe (Fig 12) were collected from two locations in the KwaZulu-Natal Province of South Africa: Snembe in Tongaat (North Coast, 29° 34' 48" S; 31° 7' 12" E.) and Umbumbulu (South Coast, 29° 59' 0" S; 30° 42' 0" E). The corms were harvested in autumn, cleaned to remove excess soil from the corms, and then packed in High-Density Polyethylene (HDPE) 350 mm (height with lid) × 311 (top lid diameter) × 275 mm (base diameter) buckets (Household Plastics, South Africa). The buckets were labeled and transported to the Durban University of Technology (DUT) Nursery Section of the Horticulture Department for further processing. The corms were dried in direct sunlight for 8 h to eliminate excess water from the plant material.

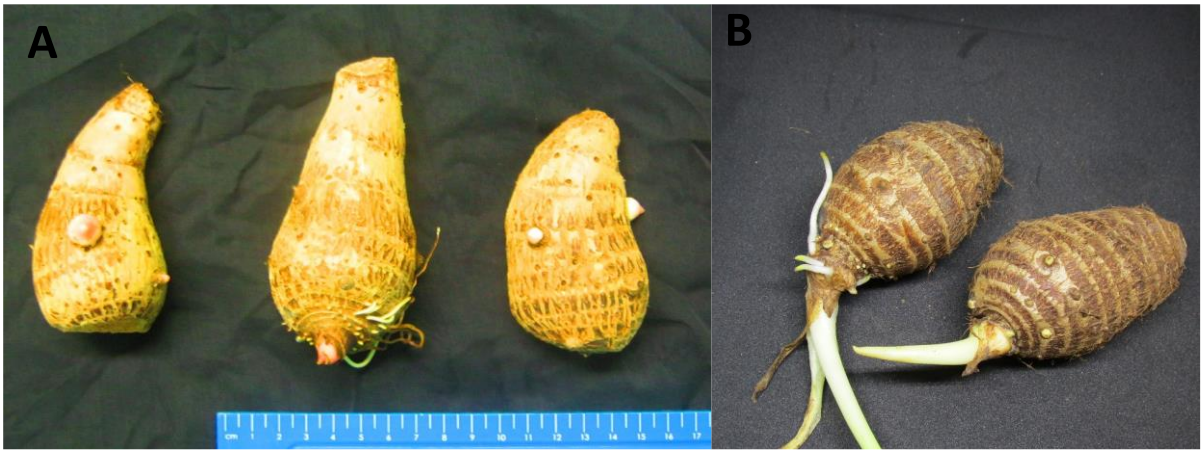

Figure 12: a) Mother corms harvested from the field, from which b) bud explants were excised for *in vitro* initiation.

Each corm was carefully scrubbed using a terry dish drying cloth of 100% cotton (Volkem, South Africa) to remove soil residue on the surface and carefully screen and discard cormels that rot tissues. Thereafter, the mother stock was transported in ventilated 595 mm × 525 mm × 170 mm crates (Supplywise, South Africa) and stored in a single layer in each crate. To facilitate the development of apical and axillary buds for culture induction, the crates containing the corms were completely covered with uncoated brown craft paper (Supplywise, South Africa) and stored in a dark ventilated room at ambient temperature at the eThekwini

Municipality Tissue Culture Laboratory (Durban Botanical Gardens - Production and Display Section). The corms were monitored weekly for bud emergence to be used for *in vitro* initiation.

4.3.2. Explant preparation

After 30 d of consistent monitoring of corms stored in the dark, the roots, and mother-stock corms marked by changes in the color of the flesh to brown, fungal growth, a decrease in hardness, and odour during storage were removed and discarded. Corms that developed apical and axillary meristematic buds were selected for surface disinfection and processing for *in vitro* initiation after 30 d. The corms were first washed under running tap water for 30 min and intermittently stirred. Depending on the size (8 × 5 mm, and 5 × 3 mm) of the mother corm, the washed corms were then trimmed into smaller pieces: 10 × 3 mm, 15 × 3.5 mm, and 20 × 4 mm) containing the apical or axillary buds (Fig 13). Only corm-derived explants treated with NaDCC (P) or PPM (P) were refrigerated (pre-chilled) overnight at 2°C for 17 h until further decontamination. Explants were categorized according to the type of treatment [Control, NaDCC, PPM, NaDCC (P), and PPM (P)] (Table 10).

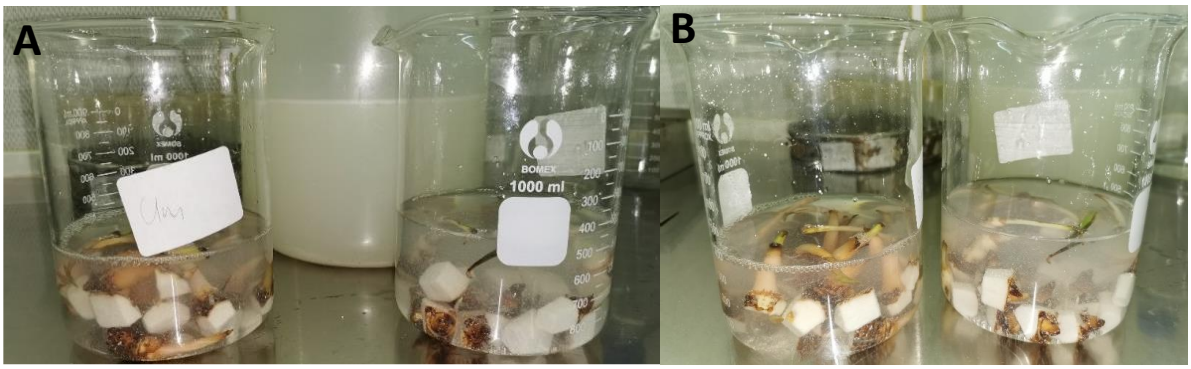

Figure 13: Excised corm explants from Umbumbulu and Snembe rinsed with sterile deionized water A) non-chilled and B) Pre-chilled explants.

4.3.3. Media preparation

A full-strength MS (Murashige and Skoog, 1962) basal salt medium with vitamins supplemented with 30 g l^{-1} sucrose (w/v) and 5 ml l^{-1} 6-benzylaminopurine and 3 g l^{-1} Gelrite™ (Merck® Sigma-Alrdich, South Africa) dissolved in distilled water was prepared. Five (5 ml l^{-1}) PPM™ was added in MS media for PPM™ and PPM™ (P) treatments. The pH was adjusted to 5.8 using 0.1 N HCL (v/v) and 0.1 N NaOH (v/v). Using a sterile pipette, 10 ml of the clear medium was dispensed into 100 mm × 13 mm culture tubes before autoclaving for 30 minutes at 121° C and 103.4 KPa. The medium for the control experiment contained water and Gelrite with the exclusion of all treatment variables. The medium was allowed to cool and solidify before inoculation. The medium was modified according to the type of treatment. The analytical reagents (AR) were supplied by Merck® Sigma-Aldrich, South Africa, all AR grade (≥99.5%). No additional purification was undertaken in any of the reagents.

4.3.4. Sterilisation of explants

Trimmed corm explants were washed under running tap water for 30 minutes. Thereafter, immersed in a surfactant of 0.2% TWEEN 20 (Merck® Sigma-Aldrich, South Africa) for 60 minutes with periodical swirling and rinsing under running water for an additional 30 minutes. In the laminar air-flow chamber, the non-chilled and pre-chilled explants were surface disinfected in 70% (v/v) and 30% (v/v) ethanol concentration respectively, for 4 minutes (Table 11). Thereafter, decanted, and immersed in 0.2% Benlate® (Merck® Sigma-Alrdich, South Africa) for surface sterilisation for 30 minutes (Fig 13). Subsequently, the Benlate® solution was decanted and explants rinsed five times with autoclaved sterile deionized water (DH_2O). PPM and PPM (P) explants were isolated and only NaDCC and NaDCC (P) isolates were treated in sodium dichloroisocyanurate (NaDCC) solution for 30 minutes, gently swirled at intervals and rinsed five times with autoclaved sterile (DH_2O) in a laminar airflow chamber. Explants containing excessive outer leaf-covering tissue were excised and carefully trimmed to a length of about 1 cm – 1.5 cm without damaging the tissue and blotted on sterile paper towels before detached from the dome for inoculation in the 100 mm × 25 mm test tubes containing the initiation medium.

Table 10: Treatment and parameter indicator for corm explants from Umbumbulu and Snembe monitored for 120 d.

Treatment type	Treatment indicator	Measured Parameters	Parameter indicator
Control	Control	Aseptic cultures	ACS
Plant Preservative Mixture	PPM™	Shoot development	SD
Plant Preservative Mixture (Pre-chilled explants	PPM™ (P)	Bacterial contamination	BC
Sodium dichloroisocyanurate	NaDCC	Fungal contamination	FC
Sodium dichloroisocyanurate (Pre-chilled explants)	NaDCC (P)		

Table 11: Decontamination treatment for explants from Umbumbulu and Snembe.

Explant groups	Treatments			
	0.2% Tween 20 60 min	70% ethanol 4 min	30% ethanol 4 min	0.2% Benlate 30 min
Control	×	×	×	×
PPM (5 ml)	√	√	√	√
PPM (P)	√	√	√	√
NaDCC (5 g)	√	√	√	√
NaDCC (P)	√	√	√	√

4.3.5. *In vitro* initiation

The surface-sterilized explants were inoculated in 100 mm × 25 mm culture tubes containing 10 ml of full-strength MS media, while the control explants were initiated a water-based media solidified with Gelrite®. Ten explants were initiated individually in the culture tubes and sealed with a PARAFILM (Merck® Sigma-Alrdich, South Africa) for each treatment. All treatments were replicated thrice, and cultures were maintained in a growthroom. The Growth room was maintained at 24 ± 2°C and 16/8-hour light/dark photoperiod provided by cool white fluorescent lights (≈ 45 μmol m^{-2} s^{-1} light intensity) and 95% relative humidity.

4.3.6. Sub-culturing

Aseptic explants that indicated basal bud and shoot growth were sub-cultured in full-strength MS medium in (110 mm × 60 mm, approx. 300 ml volume) jars, maintained in the growth room, and further monitored for 60 d to evaluate aseptic culture growth (ACS), bacterial (BC), fungal contamination (FC), and shoot development (SD). Cultures that survived contamination were further sub-cultured in fresh MS medium in jars for the next 90-120 d with constant monitoring and monthly recording of contaminated and aseptic cultures. To calculate mean of ACS, BC, and FC, the number of aseptic and infected tubes was recorded and divided by the number of tubes per treatment to determine the average mean multiplied by 100 to find a percentage i.e.

$$Px = \frac{Ncl}{Ntb}\ x\ 100$$

Where, *Ncl* = Number of clean/aseptic cultures

Ntb = Mean number of tubes in a treatment

P*x* = Parameter (ACS, BC, FC)

4.3.7. Data analysis

The cultured explants from all five experiments were monitored weekly and data was initially collected at 30 days (d) intervals. Explants were individually assessed for aseptic culture growth (ACS), bacterial contamination (BC), fungal contamination (FC), and shoot development (SD). All cultured explants contaminated with bacteria and fungi were removed and discarded. After the 30 d, the number of developed shoots (t) and expressed as the average mean value per treatment i.e.

$$\text{Mean}_{SD} = \frac{Nst}{Ntb}$$

Where, *Nst* = Number of shoots per tube

Ntt = Number of tubes in a batch of treatment

Mean_{SD} = Mean number of shoots developed in a batch

Only clean explants cultures were transferred to the subsequent multiplication stage.

The data collected for all treatments, Control, NaDCC, PPM, NaDCC (P), and PPM (P) from 30 to 120 d and analysed with SPSS v29.0.0.0 (IBM, USA) using a non-parametric test for non-normal distributed data. The Kruskal-Wallis one-way ANOVA was performed on the dataset to statistically compute significant differences at ($p<0.05$) between all groups. Subsequently, Dunnet's post-hoc test for multiple comparisons between all combinations of groups was performed to determine which groups were ($p\leq0.05$) and ($p\geq0.05$).

4.4. Results and Discussion

4.4.1. The effects of surface decontamination on *in vitro* initiation of C. *esculenta* explants.

Surface decontamination using NaDCC and PPM produced lower contamination rates in inoculated explants from both population sites (Table 12). Although no specific trend was observed, NaDCC (P) and PPM (P) indicated some contamination suppression at 30 d (BC30). Bacterial proliferation was observed in the control explants within five days (5 d) and in the treated explants within fifteen days (15 d) of initial *in vitro* culture. The bacterial contamination observed in the Umbumbulu explants was mostly pink and purplish around the base (Fig 14A). The bacterial contamination for the explants from the Snembe population changed the color of the media to rusty brown, and some cultures were contaminated by a combination of the bacterial and filamentous web-like sporangia surrounding the base of the explant (Fig 14B). The effect of decontamination treatments on bacterial contamination is presented in (Table 12). In Umbumbulu explants, no consistent trend was observed, but interestingly at (BC60) NaDCC and PPM^{TM} treatments were observed to be significantly different ($p<0.05$) with NaDCC exhibiting 0% contamination and PPM^{TM} with 16% bacterial contamination (Table 12). There is a possibility that NaDCC concentration in conjunction with the quality of the cultured explants, suppressed the proliferation of any endogenous contaminants. Previous studies show that *Colocasia esculenta* is a geophyte and the likelihood of contamination is >60-80% certain depending on the type of cultivar. Non-chilled explants treated with NaDCC generally appeared more effective than PPM^{TM} for Snembe explants. Notably, NaDCC and PPM^{TM} treatments on pre-chilled explants (NaDCC (P) and PPM^{TM} (P)), respectively, from both sites, effectively eliminated bacterial contamination at 30 d (BC30), but thereafter bacterial contamination continued to increase and almost exponentially after 90 d (BC90) to 120 d (BC120) (Table 12). Multiple factors could cause this observation, including the geographical area where the mother plant was obtained, cultural practices, and cultivated variety. Therefore, the combination of all the environmental and intrinsic factors including the dominant pathogenic agent (bacterial strain) determines how an explant behaves during inoculation.

The control explants from both sites had high bacterial contamination at 30 d (BC30). This is because the control treatment was not supplemented with any disinfecting agent such as Benlate, alcohol, NaDCC, or PPM^{TM}. Although not significant ($p>0.05$), Umbumbulu control explants exhibited a lower mean bacterial contamination of 40% at (BC30) compared to

Snembe 67% (Table 12). The surviving explants were sub-cultured and transferred to a clean medium for further observation for 60 d. At (BC60), Umbumbulu and Snembe control treatment had varying contamination of 31% and 22%, respectively though non-significant (*p>0.05*). After (BC60), no clean cultures could be sub-cultured.

Surface decontamination using NaDCC (P) and PPM (P) showed effective results compared to other treatments in producing cultures with lower contamination rates from both population sites, even though overlapping occurs between low contamination rates and type of treatment (Table 13). The fungal signs of contamination were observed ten days (10 d) in culture in the control and fifteen (15 d) days in treated explants. The contamination appeared as hyphae surrounding the base of the explant in both Umbumbulu (Fig 14C) and Snembe (Fig 14D). The effect of decontamination treatments on fungal contamination is presented in (Table 2). There appeared to be no consistent trend in fungal contamination in explants from Umbumbulu for non- and pre-chilled explants during the entire culture period, however, NaDCC and NaDCC (P) exhibited low fungal contamination of 3% and 10% respectively, at (FC30) compared to 30% and 23% from Snembe.

Generally, non-chilled explants from Snembe, treated with NaDCC showed less but non-significant (*p>0.05*) fungal contamination than those treated with PPM^{TM} (Table 13), although none of the explants from both treatments survived for subculture beyond 90 d (FC90). On the contrary, there were surviving explants at 120 d in culture (FC120) from pre-chilled explants of both treatments: NaDCC (P) and PPM^{TM} (P) from both sites (Table 13). Similar to bacterial contamination, control explants from both sites exhibited high fungal overgrowth at 30 and 60 d (FC30 and FC60, respectively), with Umbumbulu exhibiting generally higher but not significantly different (*p>0.05*) mean fungal contamination than explants from Snembe (Table 13). No clean cultures were sub-cultured beyond the 60 d in both sites due to fungal overgrowth.

Contamination of plant cell and tissue cultures remains a persistent problem despite taking stringent measures to use sterile techniques and maintain aseptic conditions (Orlikowska et al. 2016). Chemical decontamination of explants in plant tissue culture is the most important process *in vitro* cultures as explants cannot be autoclaved like culture media and culture equipment. In this study, the effectiveness of two disinfecting agents: Sodium dichloroisocyanurate (NaDCC) and Plant Preservative Mixture (PPM) on explants collected

from two diverse sites in KwaZulu Natal was investigated. Further, the effect of explant pretreatment (pre-chilling overnight at 2°C for 17h) was also evaluated. For non-chilled explants from Snembe, NaDCC was more effective than PPM in controlling bacterial and fungal contamination but in Umbumbulu there was no consistent trend. But interestingly while NaDCC eliminated bacterial contamination at BC60 in Umbumbulu, fungal contamination proliferated at the same observation time. NaDCC is a safe disinfectant that has been used for water disinfection, surface disinfection, as a broad-spectrum disinfecting agent (Shetty and Narasimhan 2021) achieving similar disinfecting efficiency than the commonly used sodium hypochlorite but less corrosive (Artasensi, Mazzotta and Fumagalli 2021). The disinfecting property of NaDCC is due to the hydrolytic release of chlorine, which changes the permeability of the bacterial cell membrane leading to leakage of cell contents thus causing the death of bacteria (Venkobachar, Iyengar and Rao 1977). NaDCC has been documented to have antibacterial activity against several bacteria species: (gram-positive -seven species and gram-negative -nine species), and only six species of fungi (Shetty and Narasimhan 2021). Plant preservative mixture (PPM^{TM}) is also rated as a broad-spectrum microbicide effective against bacteria and fungi targeting multiple enzymes and hence a low chance of developing resistance. PPM is effective against airborne, waterborne, and endogenous microbial contaminants at culture initiation and during tissue (PCT 2023) and that is why it was incorporated into culture media at 0.5%. For example (Nowakowska, Marciniak and Pacholczak 2022) successfully established *in vitro* cultures of other rare orchid species by supplementation of 0.1-0.2% PPM™ to culture media.

In the current study, PPM^{TM} appeared to be more effective on bacteria than fungi in both sites and both NaDCC and PPM^{TM} did not eliminate bacterial and fungal contamination. Contamination continued to increase gradually after the initial suppression at BC30 and FC30. Similar to the current study (Thomas, Agrawal and Bharathkumar 2017) did not eliminate bacterial and fungal contamination even after testing three different levels of the Plant Preservative Mixture^{TM} (PPM^{TM}) in establishing *in vitro* cultures of papaya (*Carica papaya*) from field-grown trees. Hence endogenous contaminants appear to be a nuisance for all micropropagated plant species regardless of the treatment used unless critically calculated steps are established to eliminate intrinsic opportunistic infections, particularly in geophytes (underground storage structures), such as corms and bulbs which carry a huge load of microorganisms, to prevent culture loss (Thomas, Agrawal and Bharathkumar 2017). The slight differences between sites may be attributed to agronomic and microbial abundance in

the soil in the two sites (Waheeda and Shyam 2017) at the time the corms were harvested and carried to the laboratory initiation stage. Pre-chilled explants treated with NaDCC and PPM^{TM} (NaDCC (P) and PPM^{TM} (P) from Umbumbulu and Snembe showed no contamination at BC30. This may be due to the effect of the cold treatment immobilizing endophytic bacteria (Kowalski and van Staden 1998). In developing a plant tissue culture protocol for five pecan cultivars (Vahdati *et al.* 2020) found that a pre-chilling treatment at 4°C for 48 h before cutting nodal segments improved explant establishment. In both sites, the resurfacing of the endophytic microbial contaminants is recorded at periods (BC60-90), and during the period (BC120). As a result of explant excisions, multiplication, and provision of fresh medium, chronic/endogenous contaminants proliferate causing a high mortal rate in multiple explants. (Habiba *et al.* 2002) investigated the decontamination of *Musa sapientum* cv. chini champa and observed that initial surface sterilization was a success, although microbial contaminants at the base of the explants were observed at 7-15 d after inoculation which destroyed the explants.
The results indicated no significant difference ($p>0.05$) between the Umbumbulu and Snembe sites for the control explants. However, Umbumbulu exhibited generally higher fungal contamination while Snembe had generally higher bacterial contamination.

Table 12: Bacterial contamination (BC) in cultures of Umbumbulu and Snembe over 120 d (%).

Site	**Umbumbulu**				**Snembe**			
Treatment	BC30	BC60	BC90	BC120	BC30	BC60	BC90	BC120
Control	40 ± 0.00^{b}	31 ± 17^{b}	-	-	67 ± 5.8^{b}	22 ± 10^{b}	-	-
NaDCC	30 ± 0.00^{b}	0.00 ± 0.00^{a}	29 ± 13^{b}	33 ± 58^{ab}	6.7 ± 12^{ab}	6 ± 1.0^{ab}	18 ± 17^{ab}	67 ± 58^{b}
PPM	10 ± 10^{ab}	16 ± 3.8^{b}	15 ± 13^{ab}	42 ± 38^{ab}	27 ± 5.8^{ab}	6 ± 6.5^{ab}	47 ± 26^{b}	67 ± 58^{b}
NaDCC(P)	0.00 ± 0.00^{a}	17 ± 7.5^{b}	9.7 ± 4.7^{ab}	86 ± 12^{b}	0.00 ± 0.00^{a}	15 ± 2.1^{ab}	28 ± 12^{b}	76 ± 22^{b}
PPM(P)	0.00 ± 0.00^{a}	25 ± 6.8^{b}	30 ± 20^{b}	55 ± 8.1^{ab}	0.00 ± 0.00^{a}	16 ± 5.1^{b}	50 ± 17^{b}	77 ± 25^{b}

*Mean ± Standard Deviation, n = 5 treatments. Values followed by the same letter in a column are not significantly different at $p<0.05$ according to the Kruskal Wallis-Dunnet multi-comparison test. * (-) represents total contamination in previous observations and no cultures available for subculturing.

Table 13: Fungal contamination (FC) in cultures of Umbumbulu and Snembe over 120 d (%).

Site	**Umbumbulu**				**Snembe**			
Treatment	FC30	FC60	FC90	FC120	FC30	FC60	FC90	FC120
Control	33 ± 5.8^{b}	42 ± 38^{ab}	-	-	23 ± 5.774^{ab}	11 ± 19^{a}	-	-
NaDCC	3 ± 5.8^{b}	65 ± 39^{b}	16 ± 15^{ab}	33 ± 58^{ab}	30 ± 10^{b}	46 ± 25^{ab}	18 ± 17^{ab}	-
PPM	30 ± 17^{b}	37 ± 27^{ab}	23 ± 25^{ab}	25 ± 25^{ab}	40 ± 10^{b}	61 ± 18^{b}	22 ± 20^{ab}	-
NaDCC(P)	10 ± 10^{ab}	36 ± 41^{ab}	17 ± 25^{ab}	14 ± 12^{ab}	23 ± 5.8^{ab}	28 ± 19^{ab}	15 ± 1.7^{ab}	24 ± 22^{ab}
PPM(P)	33 ± 5.8^{b}	12 ± 15^{a}	9.7 ± 17^{ab}	15 ± 2.0^{ab}	13 ± 5.8^{ab}	9 ± 8.5^{a}	25 ± 8.0^{b}	50 ± 44^{b}

Mean ± Standard Deviation, n = 5 treatments. Values followed by the same letter in a column are not significantly different at $p<0.05$ according to the Kruskal Wallis-Dunnet multi-comparison test. * (-) represents total contamination in previous observations and no cultures available for subculturing.

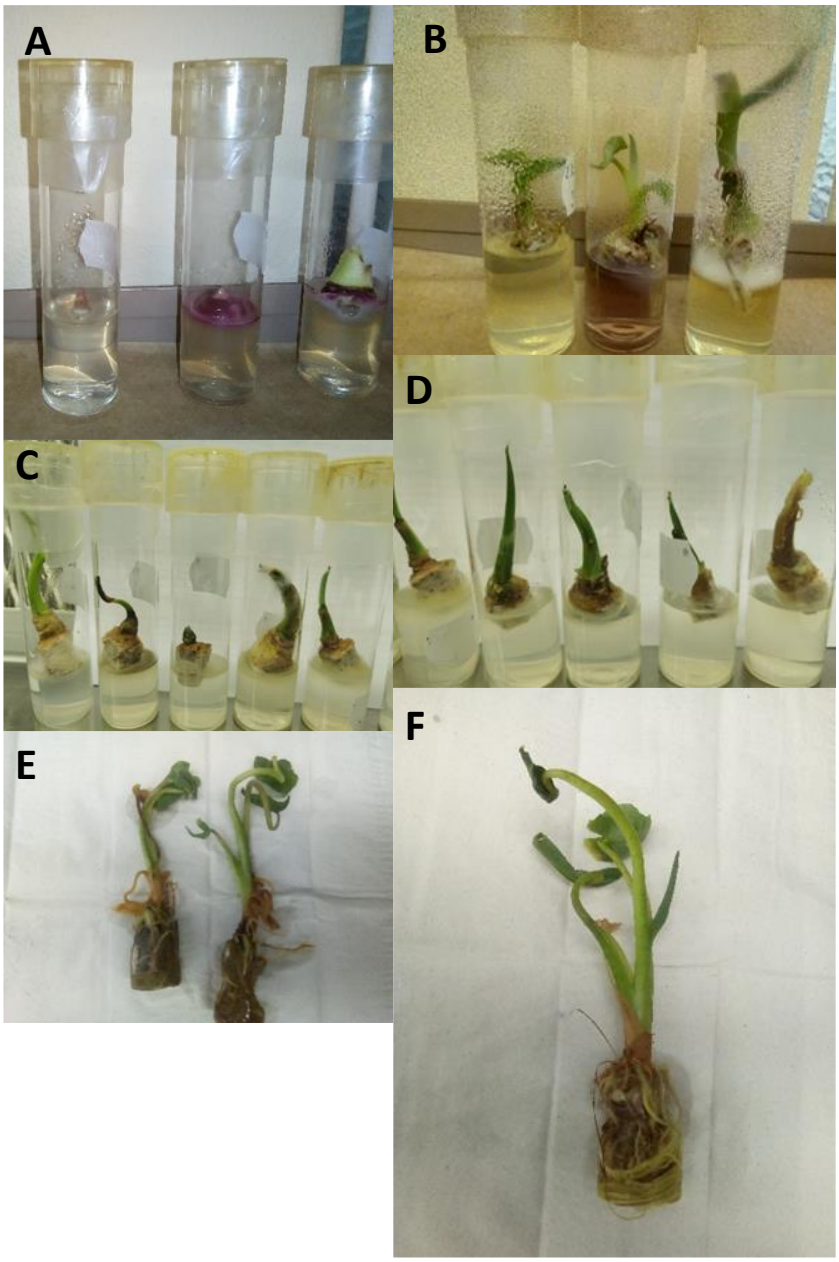

Figure 14: A) Explants from Umbumbulu displaying bacterial contamination. B) Snembe explants with a clear visible purple and cream bacterial contamination and fungal overgrowth. C) A combination of fungal and bacterial contamination excised from mother stock collected from Umbumbulu. D) Snembe explants clearly show a web of filamentous fungal contamination around the medium and base of the explant limiting the growth of explants. E) Aseptic cultures from pre-chilled Umbumbulu explants treated with NaDCC, and (NaDCC (P)) after three months in culture (ACS 90) and F) Aseptic cultures from pre-chilled Snembe explants treated with NaDCC, and (NaDCC (P)) after four months in culture (ACS 120).

4.4.2. Effect of decontamination treatments on subsequent in *vitro* subculture of *C. esculenta*

Decontamination of explants with NaDCC (P) and PPM (P) produced a higher number of aseptic cultures in 30 d of inoculation in both population sites (Table 14). Explants from the Umbumbulu site displayed no significant difference ($p>0.05$) in response to decontamination with NaDCC, PPM, NaDCC (P), and PPM^{TM} (P) treatments at (ACS30) and (ACS60). Similarly, at 90 d (ACS90), treatment of explants with NaDCC and PPM^{TM} no significant difference was observed ($p>0.05$), and when pre-chilled and treated with PPM^{TM} (P) and (NaDCC (P) explants appeared to have a low cumulative effect compared to NaDCC and PPM^{TM}. However, no aseptic cultures were obtained after 120 d in culture (ACS120), as a result of the persistent proliferation of both fungal and bacterial contamination. In contrast, pre-chilling explants from the Snembe site and treating them with NaDCC, PPM^{TM}, NaDCC (P), and PPM^{TM} (P), respectively, yielded significantly higher aseptic cultures than the control at (ACS30) and (ACS60) (Table 14). At ACS90 explants from the Snembe site, had a significant effect from treatment with NaDCC whether non-chilled (NaDCC - 74%) or pre-chilled (NaDCC (P) – 53%). Unlike Umbumbulu explants, where no aseptic explants were obtained from pre-chilled explants at 120 d in culture (ACS120), aseptic cultures were obtained from Snembe explants with NaDCC (P) yielding 43% clean cultures significantly ($p<0.05$) higher than PPM (P) 7% (Table 14; Fig 14F).

In both Umbumbulu and Snembe sites, the control treatment exhibited no significant differences at ($p>0.05$) at the 30 d (ACS30) observation. However, at ACS30, the control explants from Umbumbulu yielded a higher number (27%) of aseptic explants compared to Snembe (13%). All aseptic cultures that survived in each treatment were sub-cultured to a fresh medium and monitored for a further 30 d (ACS60). At ACS60, 22% of Umbumbulu explants were still aseptic while no aseptic cultures were observed from Snembe beyond (ACS30). All control cultures from both sites were contaminated at 120 d in culture (Table 3). The effect of NaDCC and PPM on shoot development from the control, non-, and pre-chilled *C. esculenta* explants is presented in (Table 15). There were no significant differences ($p>0.05$) in shoot development in explants treated with NaDCC or PPM whether chilled or non-chilled from Umbumbulu or Snembe. However, shoot development in non- and pre-chilled explants from both sites treated with NaDCC appeared to increase slightly in 60 d and then gradually decrease thereafter, while shoot development in those treated with PPM generally decreased as days in culture progressed (Table 15) (Fig 15A; Fig 15B). Both populations displayed no significant

difference (*p>0.05*) in the control treatment, and no shoot development was recorded at SD120 from both sites as no shoots were available for subculture beyond 90 d. Shoot development in the cultured explants was solely based on medium supplemented with 5 ml BAP with no variation in concentrations.

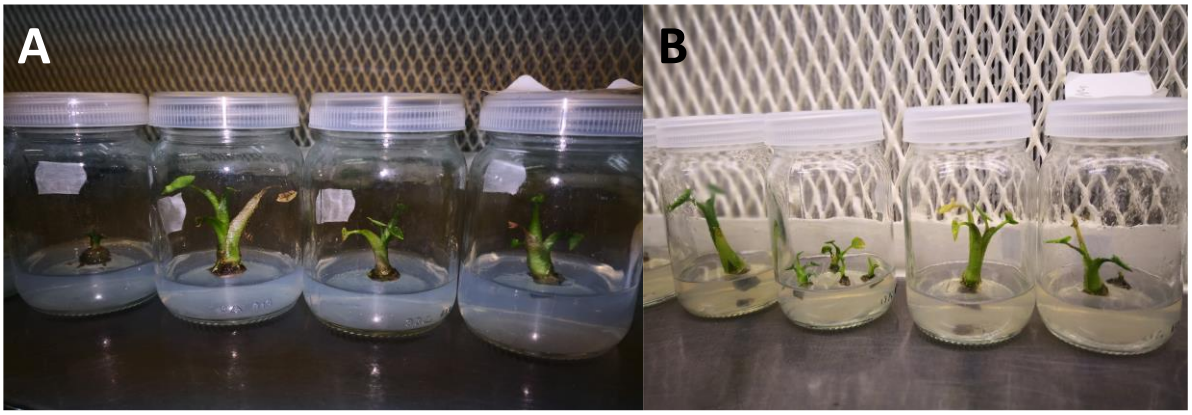

Figure 15: A) Shoot development on Umbumbulu explants after 90 d and B) Shoot development on Snembe explants at 90 d showing yellowing of the leaves and slow growth.

However, a higher percentage of pre-chilled explants, in both NaDCC (P) and PPM (P) collectively, produced shoots. Pre-chilling has been found to break dormancy in some plant species, which can improve the success rate of tissue culture (Dar *et al.* 2021) and synchronize the growth of explants, which can improve the consistency of shoot production (Rezazadeh and Stafne 2018). Treatment with NaDCC and PPM increased the number of aseptic cultures in Umbumbulu more than in Snembe. In both cases, aseptic cultures were obtained up to AC90. Pre-chilling treatment further increased the number of aseptic cultures at ACS120 from Snembe. For example, (Renau-Morata *et al.* 2013) investigated the *in vitro* multiplication of saffron explants by pre-chilling mother-stock corm explants at very low temperatures of 1 - 3°C for 9 months and achieved a high shoot multiplication rate and eliminated contamination**.** After culture initiation, the success or failure of *in vitro* culture depends on the maintenance of aseptic cultures (Espinosa-Leal, Puente-Garza and García-Lara 2018) and shoot development and multiplication. Pre-chilling is known to reduce the risk of contamination by inhibiting the growth of microorganisms (Sevgin and Karatas 2022). Shoot development in control explants was generally higher Umbumbulu than Snembe though not significantly (*p>0.05*) different. Adelegn (2018) explored corm explants for shoot multiplication in a culture medium lacking phytohormones (control) and identified an insignificant shoot multiplication rate with a mean

number of shoot explants of 1.93±0.3, comparable to our results Umbumbulu (2.00 – 2.7 and Snembe 0.67 – 2.67), respectively. There were also no significant differences among the treatments and between sites in shoot development which is expected as these chemical treatments were for decontamination. For this study, a single concentration of 0.5% BAP was supplemented in all treatments, and therefore, deductions could not be made in shoot development based on 6-benzylaminopurine.

Table 14: Aseptic cultures (ACS) (%) from Umbumbulu and Snembe over 120 d.

Site	**Umbumbulu**				**Snembe**			
Treatment	ACS30	ACS60	ACS90	ACS120	ACS30	ACS60	ACS90	ACS120
Control	27 ± 5.8^{ab}	22 ± 5.2^{ab}	-	-	13 ± 5.8^{a}	-	-	-
NaDCC	63 ± 12^{b}	72 ± 2.6^{b}	53 ± 5.8^{b}	-	53 ± 5.8^{ab}	50 ± 0.00^{ab}	74 ± 1.2^{b}	-
PPM	63 ± 12^{b}	40 ± 1.5^{ab}	58 ± 6.8^{b}	-	43 ± 15^{ab}	36 ± 2.5^{ab}	32 ± 1.7^{ab}	-
NaDCC(P)	90 ± 10^{b}	84 ± 3.1^{b}	50 ± 0.00^{b}	-	77 ± 5.8^{b}	87 ± 1.2^{b}	53 ± 2.5^{b}	43 ± 39^{c}
PPM(P)	67 ± 5.8^{b}	57 ± 1.0^{b}	41 ± 1.1^{ab}	-	83 ± 5.8^{b}	62 ± 4.0^{b}	45 ± 1.2^{ab}	7 ± 12^{b}

Mean ± Standard Deviation, n = 5 treatments. Values followed by the same letter in a column are not significantly different at $p<0.05$ according to the Kruskal Wallis-Dunnet multi-comparison test. * (-) represents total contamination in previous observations and no cultures available for sub culturing.

Table 15: Shoot development (SD) in cultures of Umbumbulu and Snembe over 120 d (mean values).

Site	**Umbumbulu**				**Snembe**			
Treatment	SD30	SD60	SD90	SD120	SD30	SD60	SD90	SD120
Control	2.0 ± 0.00^{a}	2.7 ± 0.58^{ab}	2.0 ± 0.00^{a}	-	1.0 ± 0.00^{a}	0.67 ± 0.58^{a}	2.7 ± 0.58^{a}	-
NaDCC	3.0 ± 0.00^{ab}	4.0 ± 1.7^{b}	1.7 ± 1.5^{a}	1.0 ± 1.0^{ab}	3.0 ± 0.00^{ab}	4.0 ± 1.7^{b}	2.0 ± 1.7^{a}	0.67 ± 0.58^{ab}
PPM	4.0 ± 0.00^{b}	3.7 ± 2.1^{ab}	1.7 ± 0.58^{a}	0.67 ± 0.58^{ab}	3.0 ± 0.00^{ab}	2.3 ± 0.58^{ab}	1.7 ± 2.1^{a}	1.0 ± 1.0^{ab}
NaDCC(P)	4.3 ± 0.58^{b}	7.3 ± 0.58^{b}	2.7 ± 3.1^{a}	2.7 ± 1.2^{b}	4.7 ± 0.58^{b}	6.0 ± 1.7^{b}	3.0 ± 2.6^{a}	3.7 ± 0.58^{b}
PPM(P)	4.3 ± 0.77^{b}	4.7 ± 0.58^{b}	1.33 ± 1.2^{a}	1.7 ± 1.2^{ab}	3.0 ± 0.00^{ab}	4.0 ± 2.6^{b}	2.3 ± 0.58^{a}	2.7 ± 1.5^{b}

Mean ± Standard Deviation, n = 5 treatments. Values followed by the same letter in a column are not significantly different at $p<0.05$ according to the Kruskal Wallis-Dunnet multi-comparison test. * (-) represents total contamination in previous observations and no cultures available for sub culturing.

4.5. Conclusion

The NaDCC and PPM disinfecting agents reduced but did not eliminate contamination in tissue-cultured corm explants from both sites though slight site differences were observed. The combination of pre-chilling corm explants with NaDCC and PPM has the potential to substantially reduce contamination if adequately optimized and fine-tuned to explant source. Since some aseptic shoots were obtained in this study more significantly at ACS90 in Umbumbulu with an aseptic correlation of 41 -58% in Umbumbulu and 32 – 74% in Snembe, the optimization of a shoot multiplication media can further enhance the production of shoots *en masse* for rooting and acclimatization stages, for tissue culture of amadumbe which can further be used for its cryopreservation. This is believed to be the first report of *C. esculenta* disinfection with NaDCC and PPM^{TM} *in vitro.*

CHAPTER 5

5. General discussions and conclusions

5.1. Nutritional analysis

Colocasia esculenta leaves utilized as a green leafy vegetable forms an essential nutritive profile that contributes immensely to food security and the provision of significant minerals for human health. Amadumbe landraces grown in two different locations in KwaZulu-Natal showed high proximate and mineral content in the leaves. The phosphorus content in young leaves of Umbumbulu and Snembe was recorded as (0.43 g/100g). This concentration was recorded although the leaves were sampled from different agricultural sites grown in different soil climatic conditions. The mature leaves from both sites indicated significantly different phosphorus concentrations with Snembe showing higher (0.42 g/100g) content. The leaves of amadumbe have the potential to contribute a minimum of 72% and >100% of RDA of phosphorus if adequate servings are consumed. The major differences in soil phosphorus concentration in Umbumbulu and Snembe may be attributed to farming practices, such as the application of fertilizers high in phosphorus such as super phosphates N: P: K (0:18:0) which may be responsible for the levels of phosphorus found in plant tissues. However, other soil limiting factors may cause the immobilization of phosphorus rendering it unavailable in larger quantities.

Potassium was found to be the highest macronutrient in young and mature leaves from both sites, however, Snembe had the highest potassium concentrations in leaf organs compared to Umbumbulu while corms did not differ significantly in both sites. More than (>100%) RDA contribution of potassium could be obtained from the leaves in both sites if sufficient consumption of (approx. 100g/person/servings) is achieved. The differences in potassium content between the sites may be attributed to higher concentrations in soil composition facilitated by high composting and fertilizer applications associated with agronomic practices. The calcium concentration had no significant difference for leaf organs from Snembe while Umbumbulu contained slightly higher concentrations. Corms indicated to have low concentrations of all macronutrients in Umbumbulu and Snembe. More than (>100%) of RDA can be obtained from consuming an adequate 100g of recommended servings. Magnesium showed higher concentrations in Umbumbulu than in Snembe although no significant differences can be observed in young and mature leaves, the possible contributing factor for

the difference is the high magnesium found in the soil composition sampled in Umbumbulu viz, Um >Mg in soil, >Mg in leaf organs, a clear direct proportionality. In Snembe, the relationship was inversely proportional. Therefore, for most macronutrients, the level of concentrations found in the leaves could be attributed mainly to cultivar response to cultivation practices, differential in genetic makeup, and adaptation to local climatic conditions and soil composition. Zinc, copper, and manganese had higher concentrations in leaf organs (young and mature) in both sites compared to corms. Amadumbe leaves are a poor source of zinc, contributing around 3-4% of the RDA. Zinc concentrations reported in leaves of this study indicate that more than (>2 kg) must be consumed to satisfy the RDA of 9 and 11 mg/day. The higher zinc content (553 mg/kg) in soil composition in Snembe might have a direct influence on increased Zn concentrations in (young and mature) leaves and corms. Whereas, in Umbumbulu soils a concentration of 20 mg/kg is responsible for low Zn concentrations in leafy organs and underground corms.

Copper was generally the lowest micronutrient found in leaves and corms of amadumbe ranging from (0.62 - 1.46 mg/100g and 0.48 – 0.78 mg/100g) with corms having the lowest concentration and leaves with the potential of providing >15% of the RDA of copper. The data concentrations reported in this study suggested that there are more complex mechanisms responsible for the absorption and accumulation of copper from the soil to the leaves and corms. This is evidenced in (Table 2) where Snembe had a higher Cu concentration of (166 mg/kg) which did not directly translate to higher Cu in leaves and corms. Moreover, soils in Umbumbulu had (97 mg/kg) Cu concentration but the leaves had more traces of Cu compared to Snembe with a higher Cu concentration in the soil.

In Umbumbulu manganese was 64 – 45 mg/100g in young and mature leaves, respectively. Several studies have investigated the absolute destruction of manganese in the human health system and found no negative health implications of excessive Mn consumption. Therefore, these high levels of Mn would be expected to be directly linked to Mn toxicity, however, human homeostatic controls can maintain Mn levels to desirable levels ensuring that a small percentage of Mn is absorbed, and the excess is excreted into the bile. Nutritionally, manganese has the potential to provide 71% to more than (>100%) of the RDA, if the RDA is 2 mg/day rationed at 100g/person/day of servings. There is a positive correlation between soil Mn (154 mg/kg) in Umbumbulu and the high concentration in (young and mature) leaves and corms. In Snembe (108 mg/kg), translates to lower Mn in plant tissues (leaves and corms). The

application of fertilizers and supplemental feeding of the soil has an overall direct relationship to the level each plant absorbs and accumulates specific minerals. Soil type, cultivation factors, and feeding regimens determine the level of elemental distribution in amadumbe. The leaves (young and mature) from Umbumbulu had (approx. 79 -77%) moisture content of fresh weight respectively and showed no difference from those harvested from Snembe (approx. 76 – 79%). Whilst corms contained (approx. 73% - 68%) moisture content in Umbumbulu and Snembe respectively. This suggests that amadumbe has a high moisture content which may cause severe susceptibility to various pathogenic infections if not properly handled.

These ranges were comparable to previously documented green leafy vegetables studies. The leaves young and mature from both population sites showed higher protein content (approx. 27– 31%) while corms had relatively low (approx. 9 – 11%). Amadumbe corms are a poor source of proteins. Although amadumbe corms are highly nutritious, they do not serve as a nutritionally complete food. In contrast, amadumbe leaves are a good source of nutritious protein, therefore, the utilization of both could provide nutritional quality and combat certain human ills. Fat appeared to be low in young and mature leaves of Umbumbulu, surprisingly these did not show any statistical difference with mature leaves from Snembe. Moreover, the young leaves from Snembe indicated higher fat content ($p>0.05$).

Furthermore, the corms from both sites were extremely low in fat concentration 0.86 – 0.65 for Umbumbulu and Snembe respectively. The ranges recorded in this study are comparable to those published by other reports. Like all other tubers and root crops fat is low consisting mainly of lipids of the cell membrane, although this may also be variable between cultivars. The ash content significantly varied between sites in both young and mature leaves, nonetheless, the corms from both sites showed no major variation, however, Snembe had higher ash content in the corm tissues. The variation may be attributed to soil mineral components that were ($p<0.05$) between sites which led to greater adsorption of mineral ions to soil colloids influencing higher and varied mineral accumulation and distribution in the plant organs. In both population sites ADF and NDF were much higher in young and mature leaves but significantly lower in corms. Although there were no significant variations between sites in ADF and NDF concentration in leaves, ADF had significantly lower values of digestibility, which means higher digestibility and nutrient absorption. Furthermore, since corms have lower ADF and NDF concentrations digestibility would be more prominent when the diet consists of corms. Corms in Umbumbulu and Snembe appeared to have higher total and insoluble oxalate

content whilst young and mature leaves had considerably lower total and insoluble oxalates, respectively. In contrast, corms were shown to have lower soluble oxalates in young and mature leaves. The presence of highly insoluble oxalates in the diet usually binds with CaOx, FeOx, and MgOx. Leaves of *C. esculenta* sampled from Umbumbulu were not significantly different from Snembe leaves, however, Umbumbulu had higher concentrations of soluble oxalates. Corms from Umbumbulu although they were higher in soluble oxalates than in corms from Snembe were not significantly different even with leaves sampled from both sites. The high oxalate content present in the leaves means that potassium and sodium ions will bind with the oxalates rendering these minerals unavailable for absorption in the bloodstream, although, the excessive intake of oxalates can lead to the formation of kidney stones. Many reports have corroborated the findings of this study which observed higher insoluble oxalate content in corms and leaves. Furthermore, to reduce the level of oxalates in the dietary foods certain processing methods are much more effective than others. One of the studies demonstrated that boiling amadumbe corms at 90ºC for 30 min and steeping in water at 30ºC for 24 h can potentially reduce oxalate-salt content to 32.7% and 56.7%, respectively, of its original content.

Apart from magnesium, all other macronutrients were significantly ($p<0.05$) higher in Snembe. The vast difference in soil mineral concentrations in Snembe may be attributed to agronomic factors, different climatic conditions, soil type, and landrace adaptation to local microclimate. There is evidence that the higher soil minerals have a direct influence on the absorption and accumulation of plant nutrients in different plant parts, although this may not be true for other minerals. Phosphorus was 6 folds higher in Snembe than in Umbumbulu possibly resulting in higher phosphorus particularly in mature leaves and corms when compared to plant tissues from Umbumbulu. Another attributing factor may be the absence of antagonistic minerals that prevent the accumulation of phosphorus within the plant.

A similar trend was also observed with potassium which was 2.5 folds higher in Snembe, which corresponds with a higher availability of potassium in young and mature leaves, however, lower in the corms. There is a possibility that potassium is actively adsorbed on exchange sites especially if there is high cation exchange capacity, organic matter, and available water for the translocation of K^+. Therefore, soil type, and water availability (whether flooded or non-flooded amadumbe) may determine the availability of potassium in the leaves of the plant. Calcium was 1.8 folds and magnesium 0.4 in abundance in Snembe soil. Several factors are associated with the presence of these macronutrients. Plants tend to absorb available forms of

minerals in the soil. Several studies have shown that mineral acquisition from the soil depends on various mechanisms. Therefore, plants are engineered to detect absolute limit of absorption to prevent the accumulation of nutrients at toxic levels. A few of the mechanisms that may be associated with variations in the absorption of calcium and magnesium are influenced by factors but are not limited to soil properties, plant characteristics, microorganisms, and the presence of synergistic and antagonistic minerals. The micronutrient constituent zinc and copper were 28 – 2-fold in Snembe, respectively. Although zinc was exceptionally higher in soil mineral composition this resulted in insignificant differences in zinc content in young and mature leaves from Snembe when compared with similar plant tissues from Umbumbulu.

The results of this study correlate with previous reports on zinc and copper absorption. These studies revealed that the plant roots have a comparable mechanism for the absorption of zinc and copper, and when the assimilation of one element is greater than the other, the slacking mineral is excluded from the plant. When we looked closely at the concentrations of zinc and copper in the study it was apparent that the plant favours the uptake of zinc more than copper. This is directly associated with both of the sites studied, even though Snembe had more bioavailable zinc than Umbumbulu. Other studies also confirmed this phenomenon by investigating different plant species. Manganese was an exception and its concentration was significantly higher ($p<0.05$) in Umbumbulu and showed a direct influence of soil Mn concentration and young and mature leaves in Snembe, corms remained exceptionally low in manganese concentration. This distinct manganese behavior may be attributed to factors such as pH, organic matter, and the presence of antagonistic minerals.

The pH ranges that are acceptable for manganese are 5 – 6.5, Umbumbulu showing lower levels of pH may constitute Mn toxicity. Higher iron content detection in soil composition in Umbumbulu and Snembe contributed minimally to the iron content found in young and mature leaves, in contrast, corms accumulated more of the iron. There may be a mechanism that is responsible for the iron-bound concentration in corms preventing its transportation to the other parts of the plant. Iron uptake may be associated with a protein called Heat Shock Cognate Protein B (*At*HSCB). The transport mechanisms linked with this protein may be the block that potentially limits the amount of iron that is released from the corm to other parts of the plant.

The promotion of the utilization of the leaves of *Colocasia esculenta* as a green leafy vegetable carries a multitude of complications. The use of young leaves would require interval harvesting to obtain the nutritious lower leaves, similar, to mature leaves. After each harvesting cycle,

there will be low carbohydrate production as the number of leaves is reduced. The lower number of leaves to perform photosynthesis suggests that less CHO is transported to underground storage organs and therefore there is potential for significant yield loss or production of corms and tubers. In massive intercontinental commercialization, crop defoliation or intermittent harvesting is not recommended as the number of underground crop production is negatively impacted. However, for subsistence farming dual use of the crop may be practiced availability of edible nutritious leaves would supplement micronutrients that are low in the starchy underground storage roots.

5.2. Plant tissue culture

For commercialization, an effective plant tissue culture protocol for decontamination of amadumbe explants for *en masse in vitro* production requires superior laboratory fine-tuning. This study evaluated two disinfecting agents NaDCC and PPM^{TM} and pre-chilling treatments of explants to achieve aseptic cultures *in vitro*, however, during intense experimental approaches endogenous contaminants persisted in cultures as the number of days in culture progressed. For bacterial observations, several species may have caused contamination in culture tubes. As reported by various studies, species of *Acinetobacter, Agrobacterium, Bacillus, Corynebacterium, Enterobacter or Erwinia, Micrococcus, Flavobacterium, Pseudomonas, Staphylococcus, and Xanthomonas* are a group of bacteria isolated in tissue cultures by numerous research approaches. There is a possibility that any of these species could've been a major contributor to contamination in culture. In both sites, the control treatment exhibited a high contamination rate mainly because cultures were not sterilized either with NaDCC, PPM^{TM}, Benlate, or alcohol.

Although the level of contamination was significantly high in the control treatment, Umbumbulu showed lower rates of contamination compared to Snembe. Inoculated cultures were all contaminated at (BC60) in control and none remained for further sub-culturing. Although the cultures in the control treatment were contaminated there was no significant difference when the two sites were compared. Snembe explants generally seemed to respond more effectively to treatment with NaDCC compared with PPM^{TM}. Explant response from Umbumbulu indicated no consistent trend, although there was a significant difference ($p<0.05$) at BC60, NaDCC in Umbumbulu showed no contamination during this observation while PPM^{TM} indicated a 16% rate of bacterial contamination. NaDCC and PPM^{TM} effectively

eliminated bacterial contamination in the first 30 d (BC30) in both sites, however, bacterial contamination exponentially increased from (BC90) to (BC120). The possible attributes for the variation could be the mother stock plant's geographic location, the type of explants used in culture, and possibly the age of the explant. It has been reported that endophyte type and quantity vary considerably throughout the plant's tissues, with underground organs highly susceptible, and heavily colonized by intrinsic microbes, while young soft shoot tips possess fewer possible endophytic contaminants. Furthermore, the main probable factors that contributed to this observation could potentially be endogenous contaminants that reside within plant tissues, and due to favorable nutritional conditions that the media supplements, these colonies proliferate around the explant and eventually destroy the ability for further explant growth as a result of competing for nutrients in the media. Endogenous contaminants are challenging in plant tissue cultures, therefore proper screening and isolation of possible bacteria through various approaches such as the application of several antibiotics, or optimisation of procedures to allow low or zero infection of cultures is highly recommended.

Fungal contamination usually surfaces as white fibers of filamentous hyphae on the media surface. Similarly, with bacterial contamination control explants exhibited high fungal overgrowth in both sites. Although Umbumbulu had higher levels of infection than Snembe, there was no significant difference ($p>0.05$). This early detection of high loss of explants in this particular treatment is probably attributed to a result of the abandonment of sterilization agents. Consequently, beyond (FC60) there were no clean cultures available for sub-culturing probably due to fungal spore germination. Pre-chilled and non-chilled explants showed no apparent trend in fungal contamination for explants from Umbumbulu for the duration of culturing, although NaDCC and NaDCC (P) had low contamination compared to Snembe. There is a probability that by exposing explants to low temperatures, fungal colonies could be mitigated from exponentially proliferating during the duration in culture media. The effect of NaDCC also appeared to have the capability to reduce contamination, however, this requires high optimization and careful selection of healthy explant progeny. The results also correlate with other studies that investigated the possibility of modifying the rate of contaminants in underground storage organs. In both experimental sites, the effect of NaDCC, NaDCC (P), PPM^{TM}, and PPM^{TM} (P) was not significantly different ($p>0.05$) with a considerable loss of explants from (FC30 – FC120) as a result of excessive growth of fungi, in particular, amadumbe leaf blight or leaf blight fungus, *Phytophthora colocasiae*.

Additionally, no significant difference ($p>0.05$) in the control treatment in aseptic cultures for both sites was observed. Umbumbulu had a higher number of clean cultures at (ACS3)0 in comparison to Snembe with no clean cultures for sub-culturing beyond (ACS30). During the observation period (ACS120) all cultures were lost to contamination in both experimental sites. The results were anticipated for the control treatment because explants were not decontaminated. Explants treated with various sterilizing agents (NaDCC, NaDCC (P), PPM^{TM}, PPM^{TM} (P) from Umbumbulu had no significant differences ($p>0.05$) to the control treatment at ACS30 and ACS60, and ACS90. However, no aseptic cultures were maintained after 4 months. In contrast, Snembe treatments yielded much higher aseptic cultures under treatments in comparison to the control treatment after 60 d. Snembe also had aseptic explants surviving after ACS120, unlike Umbumbulu where contamination destroyed all clean cultures. The main possible factors that aggravated the loss of cultures may have been the genotypic makeup of individual cultivars, the location where amadumbe mother corm plants were harvested, rainfall, and the age of the mother stock.

For this study there was no variation in BAP concentrations, all treatments were supplemented with 0.5% BAP. No significant difference ($p>0.05$) was observed in the control treatment. In both populations no healthy shoots were recorded, therefore no material is available for sub-culturing beyond SD90. For treatments NaDCC, NaDCC (P), PPM^{TM}, and PPM^{TM} (P) no significant difference was observed in both sites, however, in the first 60 d shoot development was higher compared to SD90 and SD120. The response of shoot development depends on PGR concentration, concentration of nutrients, and viability of the explant. If factorial considerations are stabilized at optimum levels, greater success rates of shoot development will be achieved.

This proved that amadumbe as a geophyte requires extreme fine-tuning and optimization of decontamination protocols to achieve the best results. The inadequate response of explants from Umbumbulu and Snembe in cultures could be attributed to various factors, such as agroecological conditions, soil profile and composition, intensity of rainfall, and cultural practices. Various reports previously published, correlate with our data specifying the problem associated with the *in vitro* propagation of underground storage organs (corms, cormels, bulbs, rhizomes, and stolons) in tissue culture. Although, some studies have verified some successes in optimization, geographical regions, genotype, cultivar and age of explant type play a significant role in ensuring the success rate of *in vitro* propagation of a particular species.

5.3. Conclusions

5.3.1. Nutritional analysis

Amadumbe [*Colocasia esculenta* (L.) Schott] leaves have the potential to contribute nutritionally high value of health benefits. Amadumbe leaves had significantly high K concentration in both young (3.5 – 5.1 g/100 g) and mature (3.7 – 4.5 g/100 g) leaves, low in corms (2.0 – 2.2 g/100 g) and potentially contributing (>100%) of the recommended daily allowance per 100 g. Ca had the second highest concentration among the macronutrients in leaf tissues: young (1.4 – 1.2 g/100 g) and mature (1.5 -1.3 g/100 g) leaves and corms (0.08 – 0.09 g/100 g) in Umbumbulu and Snembe, respectively. The contribution of bioaccumulative RDA of Ca is (>100%) high. Mg was also significantly higher in leaves compared to corms from both population sites. P concentration in leaves measured higher than in corms. Mn was significantly higher in leaf tissues (young and mature) from Umbumbulu (64 and 45 mg/100 g) than Snembe, corms had the lowest concentration. Iron was significantly higher in corms than in leaves from both sites with a combined potential RDA contribution of 13 – 30%. Amadumbe leaves however are not a very good zinc supplement, although copper was detected in minute concentrations its RDA per 100 g of serving has the potential to supply 15% of the mineral. Amadumbe leaves had significant values of moisture, proteins, ash, fat, ADF, and NDF. Oxalate content varied across plant tissues with total and insoluble oxalate significantly higher in corms than in young and mature leaves. However, soluble oxalates were higher in leaves and corms, and Umbumbulu showed elevated concentrations compared to Snembe. Suitable methods of cooking to eliminate high toxic oxalates should be adopted while ensuring that essential nutrients do not leach out during cooking. The soil nutrient concentrations indicated significant differences and interactions between Umbumbulu and Snembe. Despite, Mg/Mn being higher in Umbumbulu, Snembe had overall high concentrations in soil composition. This is attributed to higher levels of essential nutrients in leaves and corms harvested from Snembe. The promotion of consuming young and mature leaves as an alternative food source brings challenges to international commercialization. Harvesting young and mature leaves for preparation as green leafy vegetables means underground corm production will be reduced due to insufficient carbohydrate production by leaves through photosynthesis. Thus, the yield, and size of corm produced will be greatly reduced. Therefore, the consideration of consuming *Colocasia esculenta* as a green leafy vegetable must be carefully evaluated if the crop will be used for a dual purpose. The practice of interval harvesting will affect South Africa's value chain commercially since the country's amadumbe

production is extremely minor compared to other African countries. Small-holder farmers might be recommended to utilise the crop for subsistence as a dual purpose, however, not highly recommended for commercialisation.

5.3.2. Plant tissue culture

The application of different decontamination approaches for the disinfection of amadumbe explants displayed no significant differences and high bacterial contamination in both sites when cultures were treated with deionized water DH_2O and gelrite for media solidification (control). However, Umbumbulu had higher contamination than Snembe with total contamination beyond BC60. Although treatments NaDCC, NaDCC (P), PPM^{TM,} and PPM^{TM} (P) indicated some significant levels (%) of recordings between treatments whether pre-chilled or non-chilled for 4 months (BC120) NaDCC (P) and PPM^{TM} displayed 100% survival rate during the first month (BC30) of inoculation, with a high rate of bacterial contamination as time progresses. A similar trend is also observed for fungal contamination with a high rate of fungal spore development in control cultures of Umbumbulu, and lower levels in Snembe (FC30 – FC60), respectively. As expected, the control treatment was discarded earlier than the explants exposed to disinfecting treatments. Treatment of explants using decontamination approaches (NaDCC, NaDCC (P), PPM^{TM,} and PPM^{TM} (P)) showed no particular trend, and eventually during the observation period (FC120) all cultures extracted deleterious contaminants in both sites. NaDCC and NaDCC (P) appeared to have some effect in suppressing possible latent fungal overgrowth. In Umbumbulu no treatment was successful in eliminating contamination leading to a total decline of aseptic culture with zero output for sub-culturing (ACS120). However, in Snembe (ACS120) treatment with NaDCC (P) and PPM^{TM} (P) showed the potential of optimizing approaches to achieve a higher aseptic number of cultures. Although shoot development was steady in the control treatment for 90 d, as a result of tissue necrosis and possible high sucrose levels (30g l^{-1}) shoots dried out. While shoot development was stable from (SD30 – SD120) in all treatments; the trend suggests a degenerative effect with a clear low output of shoots at (SD120). NaDCC and PPM^{TM} did not eliminate possible endogenous microbes. A potential effect that was observed requires a thorough fine-tuning of decontamination approaches that would work best for Umbumbulu and Snembe landraces to achieve the best possible output. It is noted that other protocols have been developed globally for various amadumbe cultivars, however, it is apparent that protocols are cultivar and landrace-specific. Therefore, the development of an efficient plant tissue culture

propagation protocol relies heavily upon the optimisation of a decontamination protocol to produce clean cultures for shoot multiplication and en masse production of amadumbe.

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APPENDIX 1: Supplementary material.

Table S1: Calibration curve equations of analyzed compounds

Analysis	**Target compounds**	**Calibration equations**	**Correlation coefficients**
Macronutrients	Phosphorus	y = (139.73x-52.91) / (1 + 0.020x)	R^2 = 1.00000
	Potassium	y = (3103446.427x) / (1 + 0.277x)	R^2 = 0.99903
	Calcium	y = (82097.706x) / (1 + 0143x)	R^2 = 0.99999
	Magnesium	y = (22644273.780x) / (1 + 2.032x)	R^2 = 0.99999
Micronutrients	Zinc	y = (139.73x-52.91) / (1 + 0.00x)	R^2 = 0.99997
	Copper	y = (1222.57x-529.66) / (1 + 0.00x)	R^2 = 0.99998
	Manganese	y = (267.77x-26.51) / (1 + 0.00x)	R^2 = 0.99998
	Iron	y = (108.40x-282.16) / (1 – 0.00x)	R^2 = 0.99975
Soil nutrients	Phosphorus	y = 1593.5x + 66.262	R^2 = 0.9999
	Potassium	y = 830.61x + 193.65	R^2 = 0.9979
	Calcium	y = 6798.8x + 14.965	R^2 = 1
	Magnesium	y = 4079.1x + 294.23	R^2 = 0.9999
	Zinc	y = 1292.6x - 34.906	R^2 = 0.999
	Copper	y = 63027x + 84.026	R^2 = 0.9999
	Manganese	y = 111.42x + 14.571	R^2 = 0.9986
	Iron	y = 12350x + 5.864	R^2 = 0.9999

Table S2: Mineral composition of amadumbe (*Colocasia esculenta*) leaves and commonly consumed green leafy vegetables.

Name	**Minerals (mg/100g edible portion)**						
	Ca	**Fe**	**Mg**	**P**	**K**	**Mn**	**Zn**
Amadumbe leaves (*Colocasia esculenta*)	216	3.41	59.44	57.88	404	1.30	0.82
Amadumbe leaves (*Colocasia esculenta*)	107	2.25	45	60	648	0.71	0.41
Amaranth leaves (*Amaranth gangeticus*)	245	7.25	194	73.22	572	1.24	0.86
Bathua leaves (*Chenopodium album*)	211	2.66	48.41	37.55	438	1.58	0.98
Fenugreek leaves (*Trigonela foenum-graecum*)	274	5.69	63.67	53.05	226	0.84	0.54
Mustard leaves (*Brassica juncea*)	191	2.84	51.63	71.62	403	0.41	0.68
Spinach leaves (*Spinacea oleracea*)	82.29	2.95	86.97	32.59	625	1.12	0.46

Source: (USDA 2014; Longvah *et al.* 2017)

Table S3: Micronutrient recommended daily allowance (RDA-mg).

Macronutrient	**Life Stage (years)**	**Recommended Daily Limit (RDA-mg)**		
		Male	**Female**	**Pregnant**
Zinc (Zn)	9–13	8	8	
	14–18	11	9	12
	19+	11	8	11
Copper (Cu)	9–13	0.7	0.7	
	14–18	0.89	0.89	1
	19+	0.9	0.9	1.3
Manganese (Mn)	9–13	1.9	1.6	
	14–18	2.2	1.6	2.0
	19–50	2.3	1.8	2.0
	51+	2.3	1.8	
Iron (Fe)	9–13	8	8	
	14–18	11	15	27
	19–50	8	18	27
	51+	8	8	

(Trumbo *et al.* 2001b; Carducci *et al.* 2021)

Table S4: Macronutrient recommended daily allowance (RDA-mg).

Macronutrient	Life Stage (years)	Recommended Daily Limit (RDA-mg)		
		Male	**Female**	**Pregnant**
Phosphorus (P)	9–13	1,250	1,250	
	14–18	1,250	1,250	1,250
	19+	700	700	700
Potassium (K)	9–13	2,500	2,300	
	14–18	3,000	2,300	2,600
	19–50	3,400	2,600	2,900
	51+	3,400	2,600	
Calcium (Ca)	9–13	1,300	1,300	
	14–18	1,300	1,300	1,300
	19–50	1,000	1,000	1,000
	51–70	1,000	1,200	
	71+	1,200	1,200	
Magnesium (Mg)	9–13	240	240	
	14–18	410	360	400
	19–30	400	310	350
	31–50	420	320	360
	51+	420	320	

Source: (IM 1997)

Chapter 1 APPENDIX 2: Paper accepted for publication