EFFECT OF THE METER SQUARE ULTRA-HIGH DENSITY PLANTING TECHNOLOGY ON GROWTH, YIELD AND PHYTO-NUTRITIONAL COMPOSITION OF *MORINGA OLEIFERA*

by

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Declaration

I declare that the dissertation hereby submitted to the University of Limpopo, for the degree Master of Agricultural Management (Plant production) has not previously been submitted by me or anybody for a degree at this or any other university; that it is my work in design and execution, and related materials contained herein had been acknowledged.

_____ _____

Magabe R.C **Date**

Dedication

To my dear family (Bakone), your continuous support and confidence in me have been the driving force behind my perseverance and achievements. This work stands as a testament to your eternal trust in my aspirations.

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Abbreviations

%	Percentage
C	Degrees Celsius
ABTS	2,2'-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid
Ala	Alanine
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
ARC	Agricultural Research Council
Arg	Arginine
Asp	Aspartate
C	Carbon
Ca	Calcium
CAF	Central Analytical Facilities
cm	Centimetre
CRD	Completely Randomized Design
Cu	Copper
DAFF	Department of Agriculture, Fishery, and Forestry
DPPH	2, 2-Diphenyl-1-picrylhydrazyl
DSI	Department of Science and Innovation
FAO	Food and Agricultural Organization
Fe	Iron
FRAP	Ferric Reducing Antioxidant Power
g	Gram
GBRCE	Green Biotechnologies Research Centre of Excellence
h	Hour
H_2SO_4	Sulfuric Acid
ha	Hectare
His	Histidine
HNO_3	Nitric Acid
ICPE	Inductively Coupled Plasma Optical Emission Spectrometry
K	Potassium
kg	Kilogram
Leu	Leucine

Lys	Lysine
Met	Methionine
mg	Milligram
mg	Milligram of Catechin Equivalent per Gram
mL	Millilitre
mmol	Millimole per Liter
Mn	Manganese
Na	Sodium
NaNO	Sodium Nitrite
NaOH	Sodium Hydroxide
NRF	National Research Fund
PCA	Principal Component Analysis
Phe	Phenylalanine
PLS-DA	Partial Least Squares Discriminant Analysis
Pro	Proline
RCBD	Randomized Complete Block Design
Ser	Serine
Thr	Threonine
Tyr	Tyrosine
UPLC	Ultra-Performance Liquid Chromatography
UPLC	Ultra-Performance Liquid Chromatograph Mass Spectrometer
Val	Valine
WAT	Weeks After Transplanting
Zn	Zinc

Abstract

Moringa oleifera Lam., is a versatile, nutrient-dense crop that has drawn a lot of interest due to its ability to combat hunger and food insecurity. Despite many uses of *Moringa oleifera*, little is known about how ultra-high density planting (ultra-HDP) technology affects its growth, yield, nutrition and phytochemical composition. The aim of this study was to develop scientific information on how meter square ultra-HDP technology would affect growth and yield attributes (plant height, stem diameter, leaf yield), nutrition (proximate, mineral and amino acid composition) and phytochemical (total phenols, total carotenoids, antioxidant activity and Ultra-Performance Liquid Chromatograph Mass Spectrometer (UPLC-MS) untargeted polar metabolites) attributes in both young and mature leaves of *M. oleifera*.

An experimental trial was established in an open field condition to accommodate four planting densities: ultra-HDP (10 x 10 cm), high (30 x 30 cm), medium (50 x 50 cm), and conventional (100 x 100 cm) in a Randomized Complete Block Design (RCBD) with three replications. During growth of seedlings, growth parameters including plant height and stem diameter, were recorded weekly. At harvest (Week 18) some growth, yield and physico-chemical components such as leaf yield, root length, root-to-shoot ratio, chlorophyll SPAD index, proximate composition, amino acids, minerals, bioactive compounds antioxidants (total phenols, total carotenoids, antioxidants activity: , 2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), and Ferric Reducing Antioxidant Power (FRAP), and UPLC-MS untargeted polar metabolites were determined. Data were analyzed using Statistix 10.0, with mean separation by Least Significant Difference on growth and yield parameters and Tukey's Honestly Significant Difference on all other evaluated parameters at a 5% significance level.

The results revealed that ultra-HDP (10 x 10 cm) significantly ($p \leq 0.05$) enhanced leaf yield per meter square in comparison to other densities. However, this outcome was influenced by the unequal number of plants per metre square, with ultra-HDP having the highest plant density. While ultra-HDP increased total leaf yield, the conventional density (100 x 100 cm) achieved superior growth parameters, including stem diameter,

plant height, Conventional density also attained higher plant leaf yield (390.7 ± 1.59 g) and some nutrients such as calcium (1.22 ± 0.02%), potassium (2.38 ± 0.01%), and glutamic acid (4.88 ± 0.04%) when compared to the other studied densities. In contrast, ultra-HDP favored zinc accumulation. Young leaves exhibited higher protein (28.36 ± 0.1%), carbohydrates (43.52 ± 0.2%), and essential amino acids, including leucine and phenylalanine, while mature leaves were richer in fat. The results further revealed that young leaves grown at conventional density contained higher levels of protein (31.00 ± 0.01%), carbohydrates and essential amino acids, such as histidine (0.77 ± 0.02%), arginine (2.62 ± 0.04%), threonine (1.63 ± 0.04%), and lysine (2.11 ± 0.03%). In contrast, young leaves under ultra-HDP showed significantly elevated proline levels (1.46 ± 0.01%), likely as a response to stress.

Untargeted metabolites, total phenols content, total carotenoids, and antioxidant activity were also evaluated and compared across all planting densities. Methanol extracts were analyzed for untargeted metabolites using UPLC-ESI-QTOF-MS. Untargeted metabolite profiling identified 96 polar analytes, including members of the glucuronic acids, flavonoid-O-glycosides, and terpene glycosides. Principal component analysis (PCA) revealed distinct clustering of metabolites based on planting density and leaf age. Ultra-high density enhanced the accumulation of flavonoids, such as quercetin derivatives, which significantly contributed to antioxidant activity. Ultra-high density elevated total phenols content (1260.9 ± 3.12 mg GAE/g) compared to 804.6 ± 1.98 mg GAE/g under conventional density. Additionally, antioxidant activity, as determined by DPPH, ABTS, and FRAP assays, increased under ultra-HDP giving a result of 51.96 ± 0.4, 3.85 and 0.29 ± 0.01 µmol TE/g DW respectively. Flavonoids were identified as the primary contributors to total antioxidant activity, with ultra-HDP promoting the accumulation of most metabolites including kaempferol derivatives, quercetin 3-O-(6''-acetyl-glucoside), quercetin 4'-glucoside and rutin.

This study highlights the potential of ultra-HDP to optimize *M. oleifera* cultivation by improving both yield and nutritional quality, particularly zinc content, while enhancing the concentration of bioactive metabolites. Additionally, the study demonstrated that ultra-high density planting and young leave markedly increased the concentrations of phytochemicals, such as total phenols, total carotenoids, and antioxidant activity,

which are non-nutritive substances with significant antioxidant and medicinal qualities. These results have great potential to advance sustainable farming methods and encourage the wider use of *M. oleifera* as a nutrient- and phytochemical-rich crop for food and medicinal applications.

Chapter 1: Introduction

1.1 Background

1.1.1 Description of the research problem

Moringa oleifera Lam., often referred to as the "miracle tree," is an exceptionally versatile species that is valued for its numerous uses in medicine, and nutrition (Milla *et al*., 2021). It is part of the monogeneric Moringaceae family, which covers thirteen distinct species over the world. It is recognized for its adaptability to marginal growth conditions and higher nutritional content essential for human health (Rathnayake *et al*., 2019). *Moringa oleifera* originates from South Asia, particularly in the Himalayan foothills, yet it is now widely distributed across tropical and subtropical regions worldwide, including Limpopo Province, South Africa, where it is predominantly grown by smallholder farmers (Sharma *et al*., 2011; Lekgau, 2011; Gandji *et al*., 2018).

In South Africa, *M. oleifera* has become a vital resource for combating malnutrition due to its rich contents of vitamin C, minerals (Zn, K, Mg and Fe), protein, carotenoid (beta-carotene), and essential amino acids (histidine, arginine, threonine, lysine, methionine, valine, isoleucine, leucine and phenylalanine) (Busani *et al*., 2011; Dhakad *et al*., 2019). Additionally, the *M. oleifera* leaves includes other useful compounds such as antioxidants, phenolics, and isothiocyanates, which possess bioactive and anti-inflammatory properties (Anwar and Rashid, 2007; Waterman *et al*., 2014). These phytochemicals are plant-derived, non-nutritive chemical components which protect plants from microbial diseases and pest infestations, while also offering health benefits to human (Nweze *et al*., 2004; Doughari *et al*., 2009; Lin *et al*., 2018; Arora and Arora, 2021).

Urbanization and climate change have restricted agricultural land availability, emphasizing the need for effective cultivation techniques (Usha *et al*., 2015; Applequist *et al*., 2020; Trigo *et al*., 2021). Conventionally, Gadzirayi *et al*. (2013) and Mabapa *et al*. (2017a) reported that *M. oleifera* is mostly planted at 1 x 1 m spacing,

such practices require more land, prompting a shift towards high density planting (HDP) methods. High density planting involves planting crops closer together (e.g., 0.3 x 0.3 m), with the goal of maximizing yield per unit area while maintaining quality (Usha *et al*., 2015).

Ultra-high density planting (ultra-HDP) extends this concept by spacing crops even closer together (0.1 x 0.1 m), which could potentially enhance yield and improve economic returns per unit area (Reddy, 2004). However, the competition for light, nutrients, and space under ultra-HDP may cause environmental challenges that can encourage the accumulation of antioxidants, phytochemicals, and amino acids like proline, which assist plants cope with stress (Abdel *et al*., 2009; Rajashekar *et al*., 2009; Meng *et al*., 2014). Consequently, field conditions, especially planting density, can significantly influence crop quality factors such as mineral and protein content (Deressegn and Telele, 2017).

1.1.2 Impact of the research problem

Since the 1990s, experts have been engaged in a discussion about whether there will be sufficient land to satisfy the future demands of a rapidly expanding population (Gomiero, 2016). By 2050, global agricultural production is anticipated to expand by 70-110% from 2005 levels to accommodate population growth and changing consumption preferences (Bruinsma, 2011; Tilman *et al*., 2011). To meet this predicted demand, additional annual production increases of 1-1.5% will be necessary for staple crops such as *Oryza sativa* L., *Triticum aestivum* L., and *Zea mays* L. (Cassman, 1999). This may necessitate expanding arable land in developing countries by almost 13% (FAO, 2002). Researchers like Godfray *et al*. (2010) and Tilman *et al*. (2011) argue that achieving such expectations is already difficult given current yield patterns, posing serious challenges to global food security. Food security refers to a condition where individuals have reliable social, physical, and economic access to food that is sufficient, safe, and nutritious, enabling them to meet their dietary requirements and preferences, thereby promoting a healthy and active life (FAO, 2013).

In the present era, food security concerns are more pressing than ever before, with over 800 million individuals experiencing chronic hunger and millions more facing food insecurity (FAO, 2013). As the population continues to rise, food prices are anticipated to increase, worsening the nutritional well-being of vulnerable groups, particularly children under five, as many households may face difficulties affording adequate food (Arndt *et al*., 2016). Malnutrition causes an estimated 3.5 million deaths annually among children under the age of five (Black *et al*., 2008). Furthermore, malnutrition is not restricted to undernutrition; many people around the world, including some who are overweight, lack critical nutrients for healthy growth, such as vitamin A, folate, iron, and calcium (Garnett, 2013).

The use of *M. oleifera* leaves as a dietary supplement has been found to offer protection from oxidative stress and iron deficiency in humans (Saini *et al*., 2014). The incorporation of *M. oleifera* leaf powder into foods including yoghurts, porridge, bread, cereal, and cheese improves their flavour and prolongs their shelf life. Additionally, this fortification increases consumers' natural antioxidant capacity, which may help neutralize free radicals and minimize the risk of diseases (Oyeyinka and Oyeyinka, 2018).

1.1.3 Possible causes of the research problem

The global population is outpacing the carrying capacity of available energy-producing crops (Achten *et al*., 2010). With the quickly expanding population and disease prevalence, there is a greater need for *M. oleifera*, demanding large-scale agricultural production and additional land for cultivation. According to Achten *et al*. (2008), high density planting of *M. oleifera* could potentially increase biomass yield. Although *M. oleifera* food products are available in selected South African marketplaces, production remains limited and inconsistent as reported by Mabapa *et al*. (2017a). Moreover, *M. oleifera* commercialization is still in its early stage, posing challenges in quantifying national production hectares, product value and volume (Lekgau, 2011; Tshabalala *et al*., 2020). Therefore, the shift from backyard, small-scale production to commercial-scale economy is imperative.

Moringa oleifera has become increasingly popular, particularly in Limpopo, KwaZulu-Natal, Mpumalanga and Gauteng (Lekgau, 2011). Furthermore, Tshabalala *et al*. (2020) indicated that the cultivation *M. oleifera* is also possible in various South African regions, including the Free State, North-West, Western cape, Northern cape and Eastern Cape. In Limpopo Province, where cultivation is most common, farmers often produce *M. oleifera* at the household level, using small plots of 0.25 to 1 hectare or backyard spaces with varied management practices (Mabapa *et al*., 2017a).

Moringa oleifera growers generally use plant spacings ranging from 0.3 to 5 meters, with 1 meter being the most common spacing. A survey carried out by Mabapa *et al*. (2017a), showed that most Limpopo growers prefer row planting, while fewer opt for random planting with no spacing. Row growers typically use inter-row spacing of 1-2 meters (Mabapa *et al*., 2017a). However, limited land remains a substantial barrier to increasing production. Brodrick *et al*. (2013) found that Wider spacings may result in inefficient use of resource including water and fertilizer, emphasizing the need for more effective planting strategies.

1.1.4 Possible solutions

The study intends to present new insights into ultra-HDP technology as a viable alternative for improving the growth, yield, phytochemicals, and nutritional composition of *M. oleifera*. This technology is particularly suitable for *M. oleifera* leaf production since it improves leaf yield and returns per unit area. Ultra-high density planting technology not only provides high initial yield and net returns, but it also allows for more efficient utilization of irrigation, fertilizers and other resources (Ladaniya *et al*., 2020). This new technique, which is widely used in global fruit growing, can produce up to 200% more than conventional growing methods. Although Ultra-HDP may reduce plant lifespan, this is less of a concern for *M. oleifera*, which focusses on leaf production.

Cultivation methods strongly influence the accumulation of phytochemicals and the action of antioxidants (Ou *et al*., 2002). Secondary metabolites have an important effect on plant pharmacology and quality, making environmental manipulation a viable

technique for increasing biomass and metabolite (Mehdi *et al*., 2018). High density planting promotes interplant competition for resources, which may result in resource depletion and abiotic stress conditions that might boost antioxidant activity and phytochemical accumulation (Jiang *et al*., 2013). For instance, it has been shown that salt stress increased spinach's polyphenol concentration (Shimomachi *et al*., 2008), implying that this strategy may also improve *M. oleifera*'s medicinal value in worldwide markets.

The findings of this study will encourage efficient land use, thereby improving food security and public health. The insights gained may encourage *M. oleifera* growers to use ultra-HDP technology, enhancing productivity on limited land and ensuring a steady supply of *M. oleifera* leaves for the growing population (Mishra and Goswami, 2016).

1.1.5 General focus of the study

Many farmers in developing countries, including South Africa, do not have adequate resources such as land, water, and fertilizers to sustain crop production. This limitation makes it difficult for small-scale farmers to establish productive farms, especially when they rely on limited land. These limitations make it difficult to produce enough crops to feed the expanding population. Given the recent effects of climate change, *M. oleifera* is the suitable crop to be produced as it can withstand harsh conditions such as extreme temperatures (Elayaraja *et al*., 2017). *Moringa oleifera* also provides considerable health benefits, emphasizing the need for increased production in order to meet demands of an increasing population.

Most growers, especially in rural areas use wider spacing levels for M. oleifera production. However, in order to improve M. oleifera production, a more effective and less demanding crop-producing technique is needed. The focus of this study was to maximise M. oleifera growth, yield, nutrients, and phytochemical composition by introducing a technique called metre square ultra- HDP technology, which requires less water, land, and other resources for production, while maintaining the quality of the crop.

1.2 Problem statement

As the global population continues to urbanize, there is growing concern over the loss of important agricultural land due to urban expansion, which presents a substantial threat to food security (Godfray *et al*., 2010; Gu *et al*., 2019). Previous studies, for example, suggest that worldwide urban expansion might reduce agricultural land by 1.8-2.4% by 2030, which results in a 3-4% drop in world food output, with the greatest impact expected in Africa and Asia (Bren d'Amour *et al*., 2017).

Urbanization, coupled with rapid population growth and economic development, has led to an increase in population-related diseases, thereby driving a higher demand for medicinal plants. This rising demand puts significant strain on the environment (Jiang and Hardee, 2011; Molotoks *et al*., 2018), hence most farmers prioritize staple crops such *Triticum aestivum*, *Oryza sativa*, *Zea mays*, and *Solanum tuberosum* to meet these demands. As a result, *M. oleifera* receives less or no attention in terms of its agronomic requirements for optimum land use and leaf yield. Moreover, farmers often cultivate *M. oleifera* without recognizing that planting density significantly impacts not only leaf yield but also its phytochemical composition, including compounds like antioxidants, flavonoids, and phenolics (Anwar and Rashid, 2007; Waterman *et al*., 2014). Although these phytochemicals are essential to the plant's nutritional and therapeutic benefits, poor planting techniques may limit their potential. In addition to its nutritional and therapeutic advantages, *M. oleifera* shows potential as a long-term remedy to lessen the environmental problems brought on by population expansion and climate change.

Population growth also has a substantial impact on climate change, as it leads to increased greenhouse gas emissions from activities such as fossil fuel consumption, deforestation, and certain agricultural practices. *Moringa oleifera* is increasingly promoted as a dual-purpose crop that can mitigate the impacts of global warming while also giving families another revenue stream at the same time (Trigo *et al*., 2021). Despite all that have been mentioned, *M. oleifera* still has the ability to absorb carbon dioxide twenty times more than the general vegetation, highlighting its importance for climate resilience (Villafuerte and Villafurte-Abonal, 2009; Daba, 2016; ICRAF, 2020).

1.3 Rationale of the study

Since *M. oleifera* cultivation is still in its early stages in South Africa, it is important to motivate producers in provinces with favourable agricultural conditions to cultivate it and to educate them of its possible benefits (Mabapa *et al*., 2017b). Furthermore, research into how ultra-HDP can optimize land use by increasing yield per unit area without compromising crop quality is urgently needed. It has been demonstrated that ultra-HDP increases production in both short-cycle and perennial horticulture crops by making more efficient use of land and resources, making canopy management easier, and enabling mechanized farming and cultural techniques. Its advantages include higher yield quality, better pest, and weed management, and easier harvesting, making ultra-HDP a viable alternative to conventional planting methods (Singh and Singh, 2018).

Studies have indicated that nutrient accumulation in crops varies greatly depending on planting density. Nutrients and planting density may have a complex relationship. Increasing planting density is not always correlated with improvements in nutritional quality (Giordano *et al*., 2021). For example, while low-density planting in cucumbers resulted in higher amounts of proteins, sugars, carotenoids, chlorophyll, flavonoids, and phenols; medium-density planting in red cabbage was associated with enhanced ascorbic acid and antioxidant activity (Phahlane *et al*., 2018; Ding *et al*., 2022). Yet, such studies are still lacking in *M. oleifera* planted on ultra-HDP. In this study, meter square ultra-HDP technology is used to maximise production of *M. oleifera*. No information is available on effect of meter square ultra-HDP technology on growth, yield *M. oleifera* on nutrients, phytochemical composition, general growth, and plant development.

Further highlighting the crop's value, the Department of Science and Innovation (DSI) a national department of South Africa, through its Indigenous Knowledge-based Technology Innovation office, has identified *M. oleifera* as a promising crop for improving food and nutritional security in South Africa. This study is consistent with government objectives to promote *M. oleifera* for commercialization and addresses a

key need for sustainable farming techniques in the face of increasing land scarcity and resource constraints.

1.4 Purpose of the study

1.4.1 Aim:

The aim of the study was to develop scientific information on the meter square ultra-high density planting (ultra-HDP) technology and its effect on the yield, growth, plant stress, nutrients, and phytochemical composition of *M. oleifera*.

1.4.2 Objectives:

i. To investigate effect of the meter square ultra-HDP technology on growth and yield parameters of *M. oleifera*.

ii. To investigate effect of the meter square ultra-HDP technology on nutritional composition of *M. oleifera*.

iii. To test effect of the meter square ultra-HDP technology on phytochemical composition of *M. oleifera.*

1.4.3 Null Hypotheses:

i. Meter square ultra-HDP technology would not affect the growth and yield parameters of *M. oleifera.*

ii. Meter square ultra-HDP technology would not affect the nutritional composition of *M. oleifera.*

iii. Meter square ultra-HDP technology would not affect the phytochemical composition of *M. oleifera.*

1.5 Validity, Reliability and Objectivity

In this research, validity was attained by controlling more factors, repeating the experiment in time, and the treatments were replicated to increase the range of validity when measuring treatment effects. Data was analyzed using Statistix 10.0 at a significance level of $p \leq 0.05$ to establish reliability. Objectivity was achieved by interpreting findings using empirical data and statistical analysis, with results investigated for alignment and divergence from earlier studies to minimize subjectivity (Leedy and Ormrod, 2005).

1.6 Bias

Replications were conducted to minimize experimental errors in each trial, and treatments were randomly allocated to experimental units to prevent bias (Leedy and Ormrod, 2005).

1.7 Scientific significance of the study

The results of this research will contribute to a better understanding of how high-quality *Moringa oleifera* can be cultivated on limited land, offering valuable insights to optimize farmers' decision-making and resource efficiency. The use of meter square ultra-high planting technology will result in effective utilisation of the available natural resources and other inputs, which lead to enhanced yield of high-quality *M. oleifera*. With lower labour costs, reduced water consumption, earlier production and high yield, growers, especially in rural areas, will generate higher revenue per unit area. Since *M. oleifera* has abundant nutrients and phytochemicals, this technology will assist growers, to produce more trees which will be beneficial to humans, as well as livestock. Additionally, the analysis presented in this study will offer valuable information for future research on the therapeutic advantages of *M. oleifera* and other medicinal plants.

1.8 Structure of the Dissertation

The first chapter of this dissertation presents an introduction and a detailed overview of the study problem. The second chapter includes a literature review, which covers previous research and identifies gaps relevant to the topic. Chapter 3 focuses on the effect of meter square ultra-HDP technology on growth and yield parameters of *M. oleifera*. Chapter 4 focuses on the impact of this technology on the nutritional composition of *M. oleifera*, while Chapter 5 examines its effect on the phytochemical composition. In Chapter 6, the findings from each chapter are synthesized and summarized to highlight their significance, along with providing recommendations for future research. The conclusion provided a summary of the study's findings. References and in-text citations were formatted in Harvard style, utilizing author-alphabetical order, as per the guidelines approved by the University of Limpopo Senate.

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Chapter 2: Literature review

2.1 Introduction

Plants have historically been crucial to human survival, providing resources such as food, medicine, clothing, and shelter (Jamshidi-Kia *et al*., 2017). Plants are still vital to numerous industries today, including medicines, healthcare, and cosmetics (Khanuja, 2012), making them critical for environmental, economic, and cultural sustainability (Arora *et al*., 2013). Plants produce secondary metabolites that possess antibacterial and anti-inflammatory properties, playing an important role in both traditional and modern medicine (Škrovánková *et al*., 2012; Xego *et al*., 2016).

Plant competition for resources like light, water, and nutrients is influenced by planting density, making it essential for increasing productivity in agricultural systems (Lauer, 1994; Adegun and Ayodele, 2015). While both high and low densities can lower total yield, proper spacing enables plants to utilize environmental resources to their fullest potential (Zhang *et al*., 2006). It has been demonstrated that high density planting, particularly in *Moringa oleifera*, increases biomass production by expanding the leaf surface available for photosynthesis, which is necessary for greater yields (Sánchez *et al*., 2006). Studying the effects of density and spacing is essential for developing high-yielding, sustainable production methods, especially in consideration of the growing interest in *M. oleifera* (Adegun *et al*., 2013).

2.2 Work done on *Moringa oleifera*

2.2.1 Physical characteristics of *Moringa oleifera*

Moringa oleifera is a fast-growing tree that may reach heights of 10 to 12 meters. Depending on the climate, it can be evergreen or deciduous, with pale grey bark and delicate, spreading branches (Foidl *et al*., 2001; Saini *et al*., 2016). Its feathery, tripinnate leaves, which can reach a maximum length of 45 cm, are composed of tiny green leaflets that are smooth on top and slightly hairy on the underside (Saini *et al*.,

2016). The green twigs are covered in fine hairs (Paikra and Gidwani, 2017). The tree bears fragrant, yellowish-white flowers in hanging clusters, with lengths ranging from 0.1 to 0.25 m. Each flower has five irregular, thin-veined petals and is between 0.007 and 0.2 m (width) and 0.1 m (length). These blooms have both male and female reproductive organs, including five fertile and five smaller sterile stamens, as well as a single-celled ovary with a slender style (Paikra and Gidwani, 2017).

The fruits of *M. oleifera* are brown, three-sided pods that hang downward and break open lengthwise as they dry. They are about 1.8 cm wide and range in length from 0.30 to 1.20 m. These pods, which contain around 26 seeds each, usually reach maturity in March and April. The pods are green while young and turn brown when ripe. The seeds are circular, about 0.01 m in diameter, and wrapped in a semi-permeable hull. They are typically dark to black but may seem white if less viable. A mature tree generates between 15,000 and 25,000 seeds annually, with each seed weighing around 0.3 g, and viable seeds germinate within two weeks (Paikra and Gidwani, 2017).

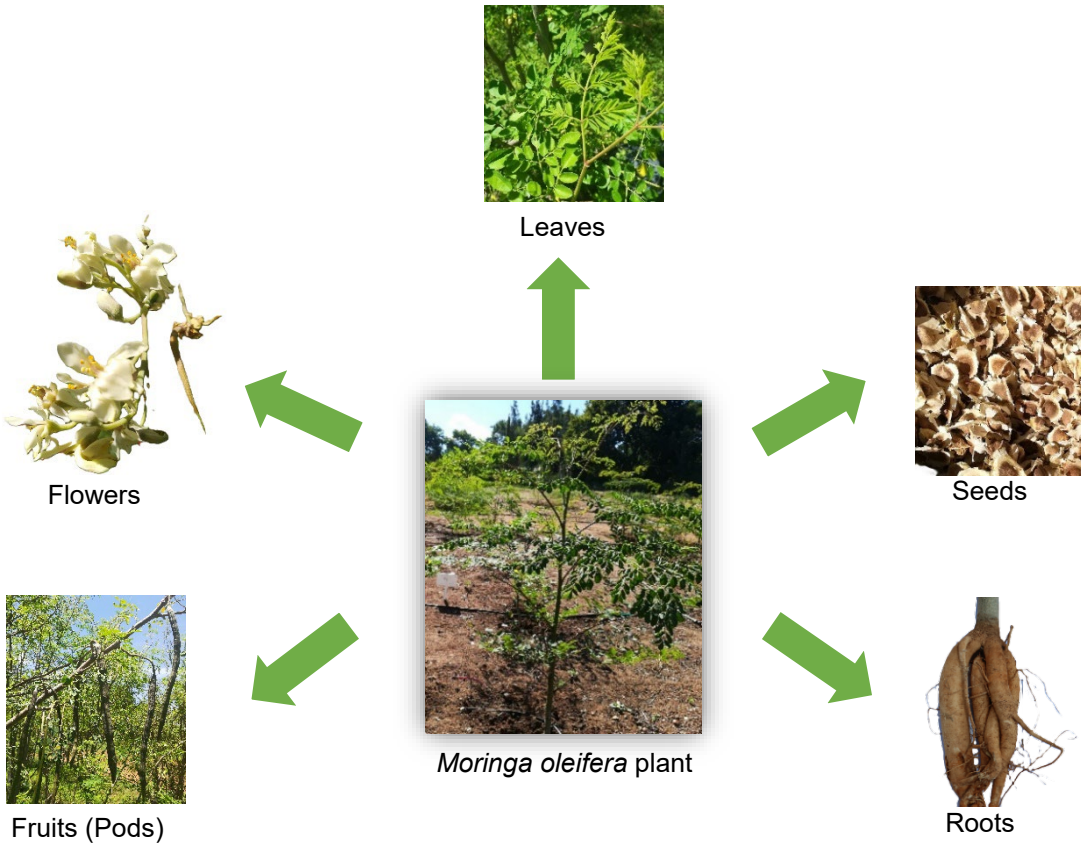

Figure 2.1: Parts of *Moringa oleifera*

2.2.2 Soil conditions and cultivation

Moringa oleifera is capable of flourishing in both tropical and subtropical regions across the globe, where temperatures generally range from 25 to 35°C. However, it can tolerate mild frosts and temperatures as high as 48°C (Pokhrel *et al*., 2016). This plant thrives in loamy or sandy soils with a pH level ranging from slightly acidic to slightly alkaline. It needs 250–3000 mm of rainfall annually; however, it is susceptible to waterlogging (Prince, 2007; Thurber and Fahey, 2010; Kumar *et al*., 2019). *Moringa oleifera* can be grown through direct seeding, which normally leads to high germination rates, but this method may produce lower-quality fruits (Ramachandran *et al*., 1980). The ideal depth for seeding is 2 cm, and seeds usually germinate 5-12 days after sowing. In addition, seedlings can be transplanted once they have grown to a height of 30 cm (Ojiako *et al*., 2011). *Moringa oleifera* can be grown from cuttings of 4-5 cm in diameter and 1 m in length, but these plants might not have strong, deep root systems compared to those grown from seed, making them more susceptible to drought and wind (Gopalakrishnan *et al*., 2016).

2.2.3 Cultivation practices for *Moringa oleiferaMoringa oleifera* can be cultivated using various techniques to enhance yield, optimize resource use, and improve soil fertility. One such method is intercropping, where *M. oleifera* is grown alongside drought-tolerant crops like Phaseolus vulgaris, Capsicum frutescens, and Allium cepa (Ponnuswami, 2012). While Zea mays and Helianthus can also be interplanted, some crops like Solanum melongena may yield less when cultivated with *M. oleifera*. The large taproot of *M. oleifera* reduces nutrient competition, while its fast-growing, protein-rich leaves help enrich the soil (Zohry and Ouda, 2017).

Another method is alley cropping, where *M. oleifera* is planted in hedgerows to provide shade for compatible crops. The pruned leaves serve as organic mulch, enhancing soil fertility. This approach is commonly used in Southern India, particularly with annual *M. oleifera* (PKM 1), to support sustainable agriculture and animal fodder production (Ponnuswami, 2012).

For higher yields, high-density planting is practiced, with spacing as close as 15 × 15 cm, producing approximately 43–52 tonnes of fresh leaves per hectare with up to seven harvests per year. For optimal foliage production, a spacing of 60 × 30 cm, accommodating about 55,000 plants per hectare, is recommended (Jin *et al*., 2017; Sangtam *et al*., 2017).

Additionally, agri-silvi-horticulture systems integrate *M. oleifera* with other crops, trees, and horticultural plants. A common model includes *Casuarina equisetifolia* as the tree species and *Zea mays* as the agricultural crop (Ponnuswami, 2012). Similarly, *M. oleifera* can be intercropped with *Zea mays* L., *Sorghum bicolour* L., and *Arachis hypogaea* L., in rainfed environments, often alongside casuarina or acacia trees, to improve yields and economic returns. Beyond agricultural uses, *M. oleifera* is also utilized as windbreaks, providing structural support for boundary lines around fields and gardens. Its ability to grow rapidly and form dense foliage makes it an effective windbreak and soil erosion control measure.

2.2.4 Uses of *Moringa oleifera*

The *M. oleifera* plant is commonly consumed by humans, and its nutritional and therapeutic properties are highly valued. It serves various culinary purposes, from a main ingredient in dishes to an addition in sauces and salads. Among the best tropical perennial vegetables, *M. oleifera* is recognised for its high nutritional content and role in promoting general health and body balance (Benítez, 2012). Beyond food, it is also used in water treatment, pharmaceuticals, cosmetic products, biodiesel production and functional meals (Gharsallah *et al*., 2023) (Figure 2.2).

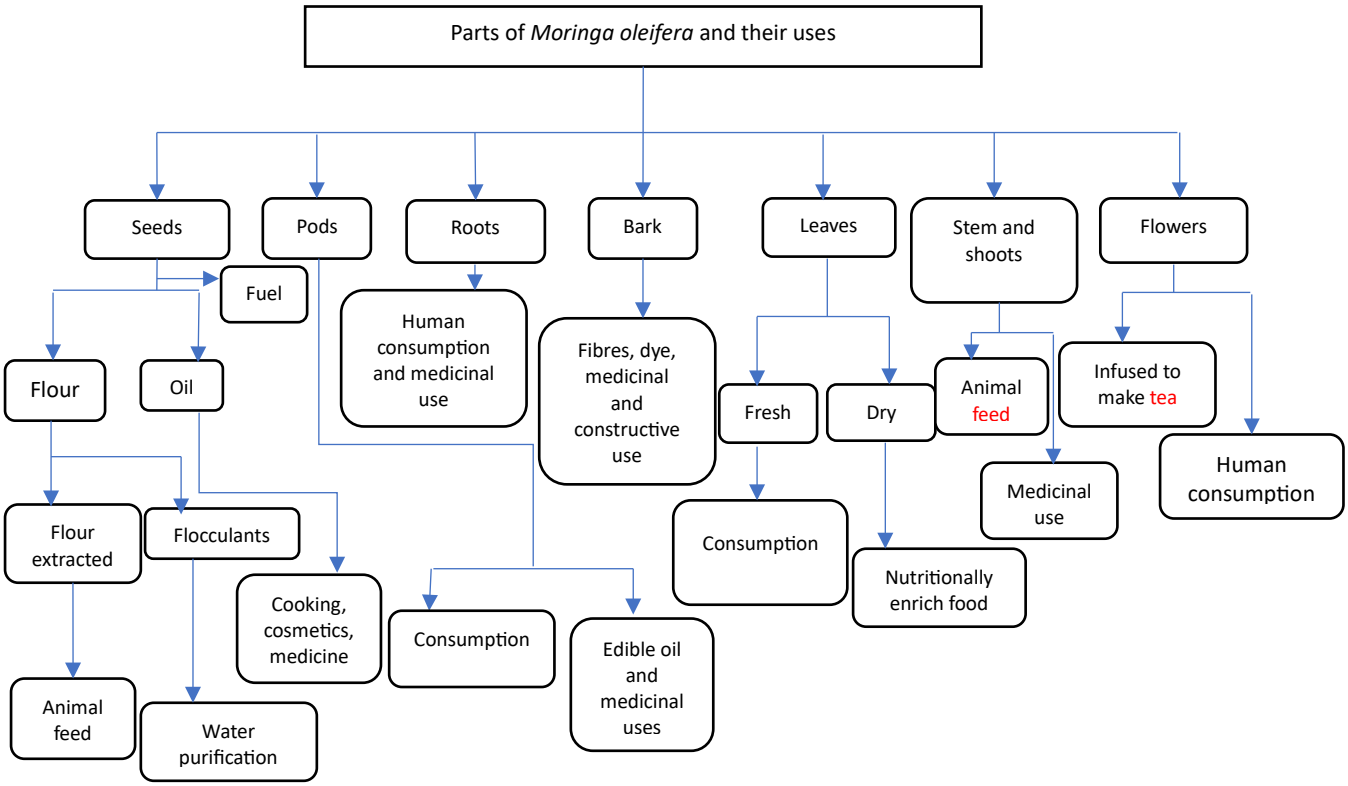

Figure 2.2: Schematic representation of *Moringa oleifera* parts and their uses

Source: Canett-Romero (2014)

2.2.5 Nutritional composition of *Moringa oleifera*

The *M. oleifera* tree provides multiple health benefits from its fruits, seeds, leaves, flowers, bark, and roots, with certain parts providing several benefits (Zaku *et al*., 2015). The leaves, in particular, are abundant with critical minerals, including iron and zinc, as well as crucial macro-minerals like as calcium and magnesium, which are usually lacking in diets in developing nations (Leone *et al*., 2015; Masitlha *et al*., 2024). They also contain significant amounts of vitamins B, C, and E (Mbikay, 2012), and their low-calorie content makes them an excellent nutritional choice for people dealing with obesity and related illnesses.

Compared to other edible leaves, *M. oleifera* leaves are particularly abundant in protein, including significant amounts of provitamin A and important amino acids. *Moringa oleifera* leaves have a protein content of 19 to 29% and a fibre content of 16 to 24% (Maheshwari *et al*., 2014; Teixeira *et al*., 2014). Kashyap *et al*. (2022) state that 100 g of fresh *M. oleifera* leaves supply 17.5% of the required daily protein intake. *Moringa oleifera* leaf powder is also high in carbohydrates, contributing to the body's caloric requirements; carbohydrates should account for half of our daily calorie intake for a balanced diet (Sultana, 2020). An adequate intake of zinc is essential for the proper production and development of sperm cells, as well as the synthesis of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). Additionally, *M. oleifera* leaves meet daily dietary zinc requirements with a zinc content of 25.5 to 31.03 mg/kg (Barminas *et al*., 1998).

Many vital elements essential for growth and development are found in *M. oleifera*, with calcium being especially crucial for human health (Gopalakrishnan *et al*., 2016). A 237 mL glass of milk provides approximately 300–400 mg of calcium, whereas *M. oleifera* leaves contain around 1000 mg per 100 g, and its powder can offer up to 4000 mg per 100 g. Anaemia can be effectively treated by substituting powdered *M. oleifera* leaves for iron supplements. For example, beef contains only 2 mg of iron per 100 g, while *M. oleifera* leaf powder offers 28 mg per 100 g (Gopalakrishnan *et al*., 2016). Additionally, *M. oleifera* seed oil contains polyunsaturated fatty acids (PUFAs), such as oleic, linolenic, and linoleic acids, which aid in cholesterol regulation. According to

Lalas and Tsaknis (2002), *M. oleifera* seed oil cab be a viable alternative to olive oil, considering that it contains about 76% PUFA.

Table 2.1: The nutritional composition of the Moringa oleifera plant parts

	mg/100 g plant materials				
Nutrients	**Fresh leaves**	**Dry leaves**	**Leaf powder**	**Seed**	**Pods**
Calcium (mg)	440	15.8-17.3	2003	45	30
Calories (cal)	49.5-92	205-329	205	–	26
Carbohydrate (g)	8.633-13.4	38.2-41.2	38.2	8.67 ± 0.12	3.7
Copper (mg)	0.07	0.49	0.57	5.20 ± 0.15	3.1
Fat (g)	1.4-2.1	2.3-9.75	2.3	38.67 ± 0.03	0.1
Fibre (g)	0.9	7.48-30.97	19.2	2.87 ± 0.03	4.8
Iron (mg)	0.85	25.6	28.2	–	5.3
Magnesium (mg)	42	15.8-17.3	368	635 ± 8.66	24
Phosphorus (mg)	70	252	204	75	110
Potassium (mg)	259	1236	1324	–	259
Protein (g)	5.5-9.4	27.1-33.5	27.1	35.97 ± 0.19	2.5
Sulphur (mg)	–	–	870	0.05	137
Vitamin B1 (mg)	0.06	2.02-2.64	2.64	0.05	0.05
Vitamin B2 (mg)	0.05	20.5-21.3	20.5	0.06	0.07
Vitamin B3 (mg)	0.8	7.6-8.2	8.2	0.2	0.2
Vitamin C (mg)	51.7-220	15.8-17.3	17.3	4.5 ± 0.17	120
Vitamin E (mg)	448	108-113	113	751.67 ± 4.41	–

Source: Fuglie (2005); Dhakar *et al*. (2011); Olagbemide and Alikwe (2014); Islam *et al*. (2021)

The leaves of *M. oleifera* are also abundant in essential and non-essential amino acids (Swanepoel *et al*., 2010; Ndubuaku *et al*., 2015), with concentrations varying with leaf age. Young leaves contain more amino acids than mature leaves (Table 2.2). Each amino acid serves different purposes within the body. Generally, amino acids play an important role in enzyme production, immune function, hormone synthesis, growth, tissue repair, and maintaining the red blood cell structure (Brisibe *et al*., 2009; Ndubuaku *et al*., 2014).

Table 2.2: Composition of amino acids in Moringa oleifera plant parts

Essential amino acids (%)	**Young**	**Older**
Argenine	1.72	1.85
Histidine	0.53	0.4
Isoleucine	1.53	1.40
Leucine	2.31	2.16
Lysine	2.00	1.95
Methionine	0.68	0.60
Phenylalanine	1.98	1.52
Threonine	1.52	1.06
Tryptophan	0.82	1.14
Valine	1.73	1.64
Non-essential amino acids (%)		
Alanine	1.19	0.83
Asparagine	1.99	1.79
Aspartic acid	0.46	0.49
Glutamine	0.63	0.55
Glycine	1.94	1.41
Glutamic acid	0.50	0.47
Proline	0.49	0.48
Serine	0.77	0.87
Trymethyl	0.52	0.47
Tyrosine	0.74	0.82

Source: (Ndubuaku *et al*., 2014)

2.2.6 Phytochemical composition of *Moringa oleifera*

Moringa oleifera is a plant with numerous medicinal applications that can aid with a variety of medical ailments, as shown in Table 2.3. This plant contains a wide variety of phytochemicals, including reducing sugars, anthraquinones, sterols, terpenoids, flavonoids, tannins, and alkaloids (Kalauni *et al*., 2023). It also contains anti-cancer substances like glycerol-1-9-octadecanoate, glycoside chemicals, isothiocyanates,

and glucosinolates. *Moringa oleifera* contains a high quantity of ascorbic acid, which increases insulin production and contributes to maintaining normal blood sugar levels (López-García, 2016). Additionally, *M. oleifera*'s pterygospermin and spirochin components give it antibacterial efficacy against both gram-positive and gram-negative bacteria. Figure 2.3 below presents various phytoconstituents extracted from *M. oleifera*.

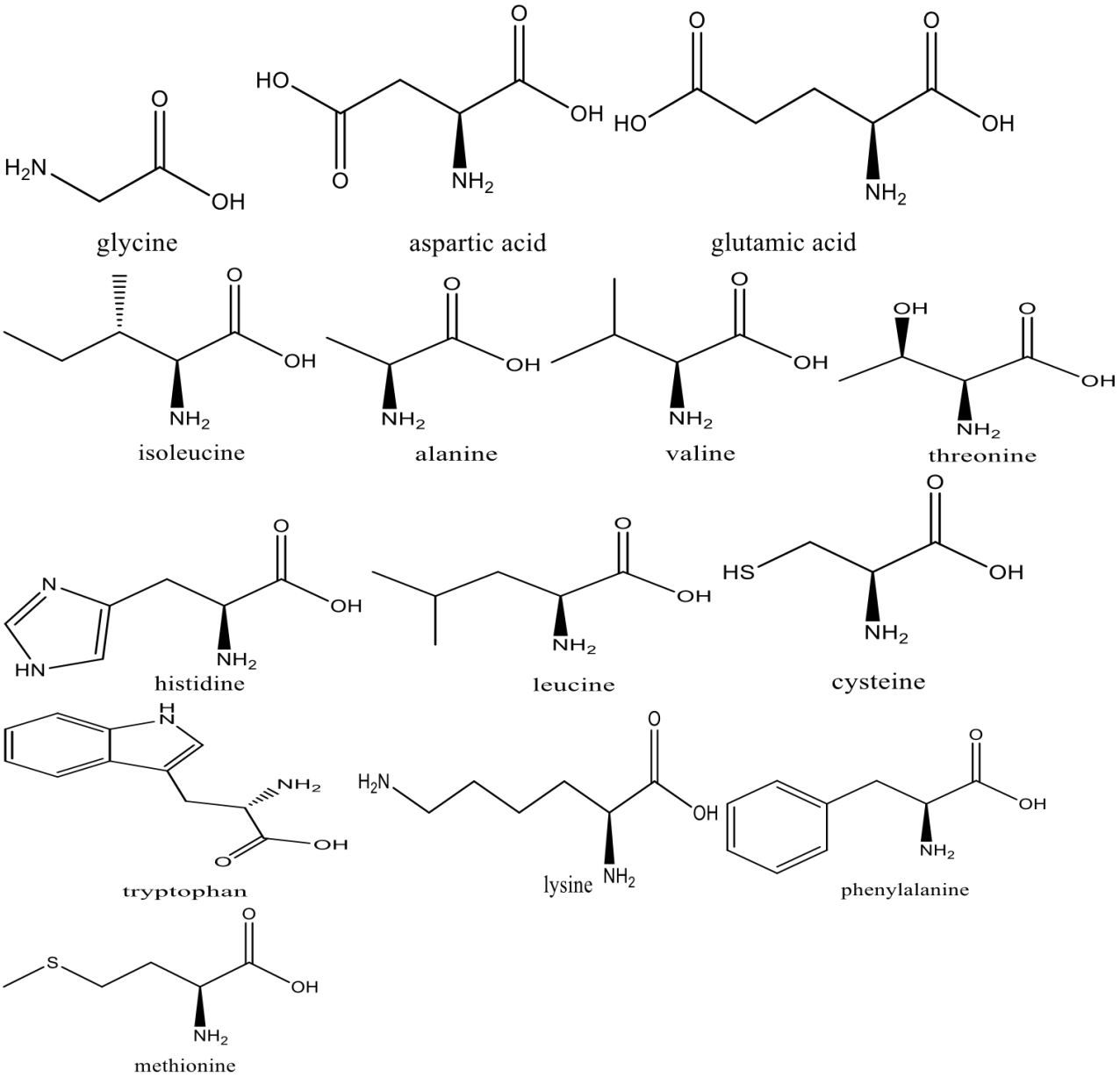

Figure 2.3: Structures of various phytoconstituents extracted from *Moringa oleifera*

Source: (Mishra *et al*, 2011)

Table 2.3: Medicinal properties of *Moringa oleifera* by plant part consumed

Plant parts	Medicinal use	Authors
Roots	Envenomation, antibacterial, antiulcer, stomatitis, antispasmodic, cholesterol-lowering effect, antifungal, bronchitis, constipation, anti-inflammation, anticancer, antidiabetic, antidiuretic and antihypertensive and analgesic.	Liu *et al*. (2018) López-García (2016) Dixit *et al*. (2016)
Leaves	Cholesterol-lowering effect, antioxidant, glandular swellings, anti-inflammatory and anti-hyperthyroidism, colitis and rheumatism, antidiabetic, anti-scurb, hemorrhoids, antihypertensive, antiproliferative, anxiolytic, Pharyngitis, diuretic and pharyngitis.	Liu *et al*. (2018) López-García (2016) Dixit *et al*. (2016) Pal *et al*. (1995)
Flowers	Anti-inflammation, antipsychotic and anti-tumour, bacterial infections, common colds, external sores/ulcers, throat infections, muscle diseases and cholera.	Liu *et al*. (2018) Dixit *et al*. (2016)
Seeds	Skin disorders, antibacterial, genitourinary, abdominal tumours, schistosomes, antidiuretic, antitumor, anti-tuberculous, anti-asthmatic and hepatoprotective.	Padmarao *et al*. (1996) Meireles *et al*. (2020)
Bark	Reduced blood pressure, decreased toothache, antidiabetic, alleviated earaches, antiulcer, anti-inflammation, eye disorders, dyspepsia, spleen enlargement, antihypertensive, treats headaches, hysteria, digestive disorders, colds and sores.	Siddhuraju and Becker (2003) Popoola *et al*. (2013) Adeyemi, *et al*. (2014)
Twigs	Wound healing, anti-inflammatory and anti-bacterial	Agnes *et al*. (2014)
Gum	Abortifacient, rubefacient, and astringent. used to treat fever, diarrhoea, dysentery, toothaches, headaches, and asthma, rheumatism and syphilis.	Padayachee *et al*. (2020)

2.3 Work done on the research problem

2.3.1 Effect of plant spacing on growth and yield of *Moringa oleifera*

2.3.1.1 Plant height

According to a number of studies, the tallest plants were produced by closer plant spacing because of higher competition. For example, when Abdullahi *et al*. (2021) investigated several spacing levels (15 × 15, 15 × 20, 20 × 20, and 20 × 30 cm), they found that the tallest plants were produced at the closest spacing of 15 x 15 cm. Amaglo *et al*. (2006) found similar results in a study conducted in Ghana, which indicated that plant height decreased with increasing spacing and reached its highest at the narrow spacing of 5 x 5 cm, followed by slightly wider spacing (50 × 10 and 50 × 15 cm).

Other studies such as those of Adegun and Ayodele (2015), Sutarno and Rosyida (2020) and Pradhan *et al*. (2023) supported these findings, demonstrating that the closer spacings produced the tallest plants when compared to wider spacings. Testa *et al*. (2016) also confirmed these findings by demonstrating that plant height tends to increase as planting density rises which can be attributed to competition for light among closely planted plants (Adegun and Ayodele (2015). According to Amaglo *et al*. (2006), higher planting density promotes more rapid growth, explaining why plants grow taller at closer spacing.

On the other hand, Abdullahi (2013) and Adekola and Abdulrahaman (2017) found that planting density had no significant effect on plant height, indicating that plant height remained consistent across different densities. This reduced height may be a result of fertilizers application, which increased soil nutrient availability and reduced plant competition. Pradhan *et al*. (2023) supported these findings by pointing out that plant growth is closely related to the availability of soil resources, implying that adequate nutrient supply can mitigate the effects of density on plant height.

2.3.1.2 Stem diameter

Sutarno and Rosyida (2020) found that wider spacing resulted in larger stem diameters than narrow and medium spacing. Similarly, Amaglo *et al*. (2006) found that wider spacing resulted in higher stem diameters than both narrow and medium spacings, although medium spacing remained greater than narrow spacing. The findings are further supported by the results of Adekola and Abdulrahaman (2017) and Pradhan *et al*. (2023) who found that stem diameter was higher at the widest spacing compared to other densities that were utilized.

Adekola and Abdulrahaman (2017) and found no significant difference in stem diameter across different densities four weeks after planting. However, stem diameter increased with time, especially after the sixth week. The population with the lowest density (wider spacing) resulted in maximum stem diameter, whereas the highest density (closer spacing) obtained the lowest stem diameter as indicated in Table 2.4. Adekola and Abdulrahaman (2017) further reported that these findings might be due to less competition among the plants under the lowest density.

Table 2.4: Effect of plant population on mean stem diameter (cm) of *Moringa oleifera*

	Plant population/ha		
	1 000 000	250 000	111 111
4 WAP	1.64	1.55	1.69
6 WAP	2.64	2.90	2.93
8 WAP	4.00	2.93	6.63

Source: Adekola and Abdulrahaman (2017) WAP weeks after planting

2.3.1.3 Leaf biomass production

Studies showed that closer plant spacing can improve branching and biomass production, with certain limitations. Abdullahi *et al*. (2013) found that a narrow spacing produced more branches per plant than wider spacing. This branching advantage led

to higher biomass production in terms of leaf area index despite the reduced spacing. Interestingly, the same study found that the total number of branches did not differ significantly between different plant populations, highlighting the fact that increased densities might lead to greater leaf biomass despite the competition. Amaglo *et al*. (2006) confirmed this by demonstrating that, while wider spacing produced more leaves per plant, narrower spacing produced more on a hectare basis. For example, wider spacing increased fresh and dry shoot weights per plant, yet closer spacing increased yields per unit area. This demonstrates the compromise between individual plant output and overall yield in densely planted systems.

Numerous studies have shown that closer planting densities may also have negative effects on biomass production. Amaglo *et al*. (2006) demonstrated a critical threshold at which intense competition for resources causes lower leaf mortality and significant plant loss, affecting up to 49.36% of plants at extremely close spacing (5 x 5 cm). Similarly, Adekola and Abdulrahaman (2017) reported that young plants with higher densities (1,000,000 plants/ha) initially had more leaves, but over time, the negative effects of competition became evident highlighting the significance of establishing a balance between density and spacing for long-term sustainability.

Santos *et al*. (2021) reported that wider spacing (1.66 x 1.0 m) enabled more lateral development and denser canopies due to reduced competition and improved nutrient availability. Even the number of branches was reported to be higher. These findings further support the concept that reduced competition can result in enhanced growth. On the other hand, narrower spacing reduces lateral growth, which in result limits biomass output, as highlighted by Adegun and Ayodele (2015). This is in accordance with research by Pradhan *et al*. (2023), which showed reduced number branches at closer spacings, supporting the concept that competition inhibits the growth of individual plants.

There is frequently a clear pattern in the yield per hectare in relation to planting density. According to Ella *et al*. (1989), competition for essential nutrients resulted from high density plantings produce lower yields per individual plant but higher total yields per unit area. According to Norman (1992) and Foidl *et al*. (2001), individual plant development remains constant until competition thresholds are reached, after which

yields gradually decrease. Figure 2.4 below indicates how yield per plant is compromised for yield per unit area.

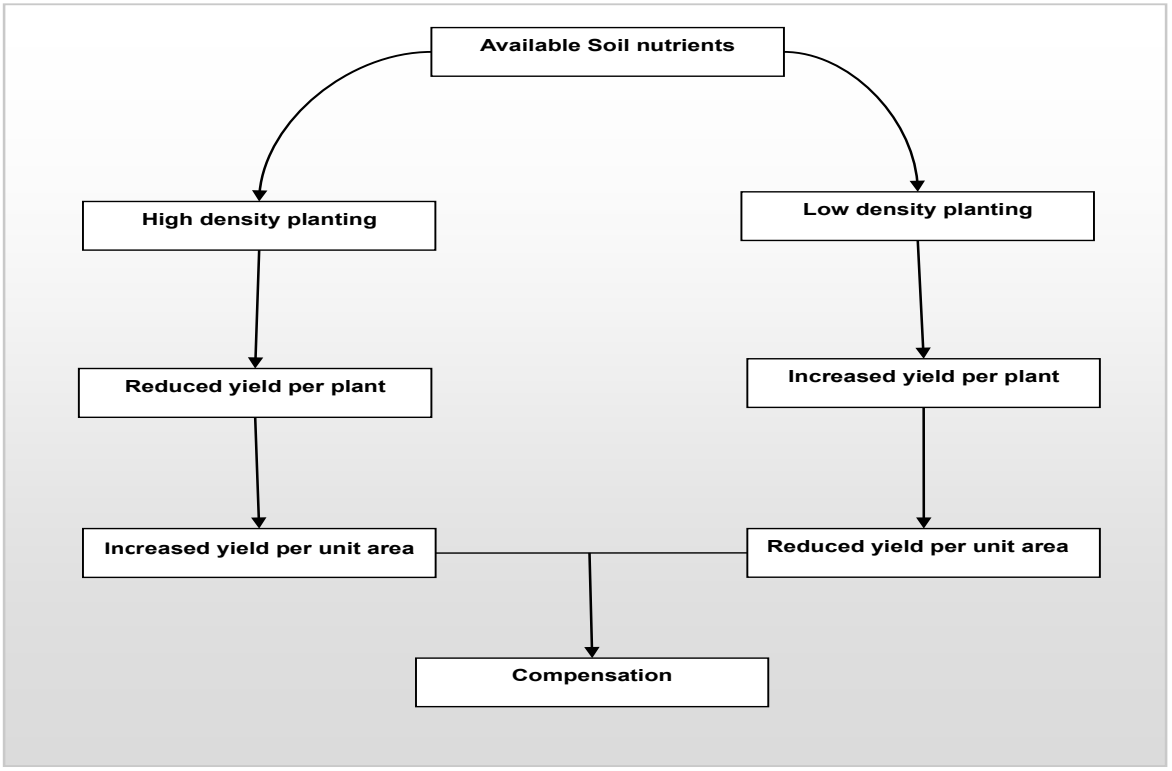

Figure 2.4: Compensation effect: balancing yield per plant with yield per unit area

2.3.2 Effect of plant spacing on nutritional composition of *Moringa oleifera*

Studies revealed that plant spacing significantly affects nutritional composition of *M. oleifera*. Newton *et al*. (2006) emphasized how plant spacing affects mineral content. Comparing narrow spacing (15 cm × 30 cm) to wider spacing (15 cm × 60 cm), it was found that the narrower increased leaf nutritional content (Basra *et al*., 2015). On the other hand, when plants were closely spaced, Sánchez *et al*. (2006) found that the mineral composition decreased. Additionally, Basra *et al*. (2015) noticed that the nutritional composition decreased as planting density increased. This conclusion was supported by El-Morsy (2009), who found that higher planting densities were related to lower nitrogen content. The competition between plants for nutrients is the reason

behind these phenomena. The chemical composition of *M. oleifera* leaves may also be impacted by the interval between harvests.

During the wet season, Zheng *et al*. (2016) found that planting density had a significant impact on both crude protein (CP) and in vitro dry matter digestibility (IVDMD). However, other researchers found no differences in CP and IVDMD among planting densities, which could be attributed to seasonal variations in analysis (Manh *et al*., 2005; Sánchez *et al*., 2006 and Mendieta-Araica *et al*., 2013) (Table 2.5).

Table 2.5: Effect of plant spacing on chemical composition of *Moringa oleifera*

Spacing (cm)	**Ash**	**CP**	**NDF**	**ADF**	**EE**
40 x 20	8.57	25.4	21.50	17.06	10.22
40 x 30	7.91	26.39	24.09	18.26	11.04
40 x 40	8.38	25.92	22.83	17.78	11.47

Source: Manh *et al*. (2005)

Mommer *et al*. (2006) found that the dry season had higher concentrations of ash, crude fibre (CF), neutral detergent fibre (NDF), dry matter (DM) and acid detergent fibre (ADF) than the rainy season. This might be due to differences in plant growth patterns. Plants grow thinner and more lignified in dry conditions, which increases the amount of dry matter they produce (Ella *et al*., 1989). Similarly, Zheng *et al*. (2016) found that planting density did not significantly impact NDF, ADF, and ether extract (EE), aligning with Al-Masri (2003).

Al-Masri (2003) and Sánchez *et al*. (2006) discovered that planting density did not significantly affect the amount of ADF and NDF in *M. oleifera* during the first and second years of growth. They did, however, note that, while ADF was largely affected in the second year, cutting frequency had a significant impact on NDF in both years. Although planting density did not influence ash content, cutting frequency did (Sánchez *et al*., 2006; Al-Masri, 2003).

2.4 Work done on other crops regarding the research problem

2.4.1 Effect of spacing on growth and yield of other crops

Wu *et al*. (2020) investigated perilla sprouts with different planting densities of 575, 1012, 1450, 1887, and 2325 plants per square meter. The findings indicated that, in comparison to the other densities, a plant population of 1450 plants per square meter produced the most fresh and dry matter, with 1887 plants per square meter following closely. This demonstrates how a population density that is higher than optimal can result in unfavourable ventilation and light exposure circumstances, which in turn reduces light energy utilization and compromises biological and economic production (Wu *et al*., 2020).

2.4.2 Effect of plant spacing on chemical composition of other crops

Studies on a variety of crops, including trees like *Morus alba* and shrubs like *Manihot esculenta*, showed that planting density had minimal effect on the concentration of crude protein (CP) (Ventura and Pulgar, 1997; Boschini *et al*., 2000). Additionally, Liu *et al*. (2021) discovered that high planting densities of *Panax notoginseng* caused environmental stress because of increased plant competition, which was associated with a notable rise in the relative amount of antioxidants. An increasing amount of research highlights that the production and accumulation of phytochemicals are key mechanisms through which medicinal plants respond to environmental stress (Guo *et al*., 2020). However, medicinal plants may not produce as many or accumulate secondary metabolites if they are subjected to extreme environmental stress (Huang and Guo, 2007). It is well known that the energy and fundamental elements required for the synthesis of secondary metabolites are provided by primary metabolism (Ramakrishna and Ravishankar, 2011; Caretto *et al*., 2015; Liu *et al*., 2017).

2.4.3 Effect of plant spacing on amino acids content of other crops

Planting density can have significant effects on the amino acid composition of plants, affecting the amount and quality of protein produced. Research suggests that plant spacing affects the levels of essential amino acids in various ways. Wider spacing, for example, has been associated with increased methionine but reduced lysine levels, resulting in a lower protein quality due to the diminished lysine/protein and tryptophan/protein ratios (Scott *et al*., 2006). Higher planting density may have a positive impact on some protein characteristics, such as amino acid composition. According to research by Yuan *et al*. (2021), which shows that as planting density increases, the amount of soluble protein increases while the amount of free amino acids decreases.

Proline, a proteinogenic amino acid, is essential for plant stress tolerance, especially under abiotic stress conditions like drought, salinity, and extreme temperatures (Anjum *et al*., 2011). By storing carbon and nitrogen, assisting in ammonia detoxification, and preserving protein hydration under dehydration stress, it serves as an osmoprotectant, reducing cell damage and promoting cellular functioning (Patil *et al*., 2011). Proline accumulation is shown across plant species as an early adaptation response to environmental stress, allowing drought-tolerant cultivars to store proline for longer than more vulnerable varieties (Saruhan *et al*., 2006).

Given that plant spacing may influence nitrogen content in crops (Seginer, 2004), it is reasonable to suggest a link between plant spacing and proline accumulation. Singh *et al*. (2015) found that plant spacing had a direct effect on proline levels in cotton. The study used three spacing levels: 40 x 40, 60 x 20, 50 x 50 cm, and discovered that a 60 x 60 cm spacing resulted in the highest proline content at the square formation stage, whereas a 50 x 50 cm spacing resulted in peak proline levels during the flowering and boll bursting stages (Figure 3.4). These findings highlight the need for optimizing plant spacing to successfully manage proline levels, which can lead to increased stress resilience and crop performance.

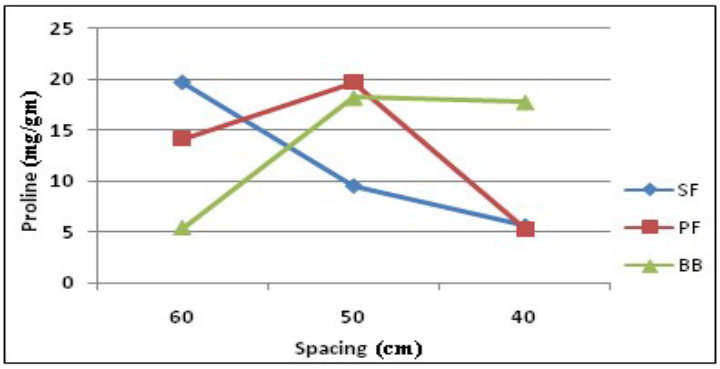

Figure 2.5: Proline levels at various spacing across different growth stages, Abbreviations: SF= square formation; PF= Peak flowering; BB= Boll bursting

Source: Singh *et al*. (2015)

2.4.4 Effect of planting density on water use efficiency of other crops

Planting density is essential for agricultural productivity and water use efficiency (WUE), especially in arid areas with limited irrigation resources (Djaman *et al*., 2022; Manibharathi *et al*., 2024). Optimizing WUE is critical for maintaining agricultural productivity in these conditions.

According to studies on poplar trees, environmental factors including water availability and soil fertility significantly influence WUE. In favourable soil conditions, higher planting density primarily intensifies competition for light, resulting in taller trees with smaller diameters and lower WUE (Zhang *et al*., 2022). On the other hand, higher planting densities promote competition for water when soil water availability is limited, which results in lower plant height but higher WUE (Toillon *et al*., 2013).

Food security depends on water security; a lack of water resources may result in fluctuating grain production and even cause severe food crises (Kang *et al*., 2017). Thus, it is acknowledged that increasing WUE and conserving irrigation water are effective strategies for sustainable agricultural development (Zhang *et al*., 2017). In order to balance the need for high and consistent agricultural yields with water scarcity, WUE improvement has become more and more important (Zhang *et al*., 2021).

Reducing the amount of water used to produce each unit of grain or improving the yield per unit of water used are two ways to increase water usage efficiency (Kang *et al*., 2017). Increasing planting density is an easy method to boost yield because it can improve the crop canopy's ability to intercept light, raise canopy productivity, encourage the accumulation of dry matter, and ultimately boost grain production (Assefa *et al*., 2016; Li *et al*., 2018). For example, research found that a 25% increase in planting density may result in a 5.59% increase in *Zea mays* yields in China (Hou *et al*., 2020).

2.5 Addressing the identified gaps

Numerous studies have investigated high density planting and its effects on the growth and yield of *M. oleifera*. However, there is insufficient research on the effects of ultra-HDP technology on the phytonutritional composition of this plant. Existing literature have shown that when plants are planted very close to each other, they grow taller at a fast rate and then develop thinner stems. For *M. oleifera*, which is grown largely for its nutrient-dense and phytochemical-rich leaves, stem thickness is less important than leaf quality. Abiotic stress, such as nutritional competition in ultra-HDP systems, increases the synthesis of phytochemicals and antioxidants, both of which are important in combating malnutrition and other health related issues. Despite the fact that this stress-induced response can be advantageous for medicinal purposes, little is known about how planting densities and short cultivation periods affect the leaves' nutritional and phytonutritional quality. Furthermore, frequent cutting reduces *M. oleifera's* growth capacity, while prolonged cultivation periods lead to fibre buildup and a decline in quality. This study addresses these gaps by determining how planting density influences the phytonutritional composition of *M. oleifera* grown under ultra-high-HDP technology, with the goal of enhancing leaf yield and medicinal benefits.

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Chapter 3: The influence of ultra-high density planting technology, implemented per meter square, on the growth and yield of *Moringa oleifera*

Abstract

Moringa oleifera Lam., is a plant that is regarded to have potential to improve food nutrition security and sustainable agriculture in South Africa. However, optimizing its farming techniques to improve yield while maintaining its quality remains a challenge. The objective of this study was to investigate effect the of meter square ultra- high density planting technology on growth and yield parameters of *M. oleifera*. Seeds (cultivar-PKM1) were sown in seedling trays, and seedlings were transplanted into the field after a one-week hardening period. A randomized complete block design with three replications was used to arrange the field experiment, which was conducted at the University of Limpopo's Green Biotechnologies Research Centre of Excellence. Four spacing levels, namely, ultra-high density (ultra-HDP) (10 x 10 cm), high density (30 x 30 cm), medium density (50 x 50 cm), and a conventional density (100 x 100 cm) were established, and each spacing level gave a total population of 100, 16, 9 and 4 plants m^{-2}, respectively. Plant height and stem diameter were recorded weekly, whilst root length, root-shoot ratio, fresh leaf yield, dry leaf yield, leaf chlorophyll SPAD index and colour were determined at harvest. Data were analysed using analysis of variance with Statistix 10.0. Least Significant Difference was used for growth and yield parameters, while Tukey's Honestly Significant Difference was applied to physiological characteristics, both at a 5% significance level.

Results indicated that the growth and yield parameters, including plant height, stem diameter, and leaf yield, were significantly influenced ($p \leq 0.05$) by the planting density treatments. Among the different planting densities, conventional density resulted in increased plant height (261.5 ± 1.24 cm), stem diameter (51.60 ± 1.04 mm), fresh leaf yield per plant (261.5 ± 1.24 cm) and reduced leaf yield per meter square (51.60 ± 1.04 mm). On the contrary, plants grown at ultra-HDP produced more fresh leaf yield per meter square (9293.6 ± 2.9 g), but less per plant (93.23 ± 0.72 g). Ultra-HDP planting maximises leaf production per meter square whereas conventional density

promotes greater individual plant growth. For increased leaf production, ultra-HDP is recommended.

3.1 Introduction

Moringa oleifera Lam., has received a lot of interest because of its wide range of applications and excellent nutritional value. The demand for products of *M. oleifera* is rapidly increasing, particularly in underdeveloped countries where it is promoted as a solution to malnutrition and hunger (Ma *et al*., 2020). Although there is still a lot of interest in this plant worldwide and locally, production levels have not yet met the demand, which presents an excellent opportunity for expansion (Mabapa *et al*., 2017; Ojo *et al*., 2022). For instance, it is anticipated that by 2025, the market for *M. oleifera* leaf powder alone will exceed USD 6 billion, primarily due to its use in food products and dietary supplements (Ahuja and Mamtani, 2019). In South Africa, *M. oleifera* is mainly grown by smallholder farmers, particularly in Limpopo Province (Lekgau, 2011).

Improved agronomic techniques are required to meet the growing demand for *M. oleifera*, especially increasing planting density, which has a significant impact on crop output (Adegun and Ayodele, 2015). High density planting systems improve biomass by optimizing leaf area for photosynthesis and light interception, resulting in increased crop development and production. Various planting densities have been studied and found to have different effects on *M. oleifera* leaf production, plant height, and stem diameter. For example, high density planting has been demonstrated to provide more leaf yield per unit area than lower densities (Mabapa *et al*., 2017). Similarly, high density planting typically results in taller plants with smaller stem diameters, resulting in higher yield because of higher number of plants per unit area (Adegun and Ayodele, 2015). A similar pattern has been observed in other crops; for example, high and medium soybean densities produced higher seed yields than low and traditional densities (Matsuo *et al*., 2015).

In addition to growth and yield, leaf colour is an important quality factor that determines consumer preference. According to the International Commission on Illumination, colour characteristics are measured using the CIELAB (Lab) colour space, where L

represents lightness, a* indicates the red-green axis, and b* represents the blue-yellow axis (Chen *et al*., 2011). Chen *et al*. (2011) and Kasim and Kasim (2017) stated that colour is darker when the L* value is lower and lighter when it is greater. Similarly, the b* value denotes yellowness when positive and blueness when negative, whereas the a* value indicates greenness when negative and redness when positive (Lancaster *et al*., 1997; Chen *et al*., 2021). Colour intensity is further measured by chroma (c*). Significantly, higher planting densities can lower chlorophyll content by damaging thylakoids and disrupting the structure of chloroplasts, which results in less chlorophyll per unit leaf area (Ren *et al*., 2017).

Optimizing cultivation techniques to promote the development of *M. oleifera* plants and maximum output becomes crucial as the demand for *M. oleifera* continues rising. One method for increasing the number of plants per unit space is ultra-high density planting (ultra-HDP), which involves closely spacing plants. Particularly when narrower spacing is utilized for leaf production, this technique has the potential to greatly increase leaf biomass (Hossain, 2021). Thus, the objective of this study was to investigate effect of the meter square ultra-high density planting technology on growth and yield of *M. oleifera*.

3.2 Materials and methods

3.2.1 Description of study location

This study was carried out in an open field at the University of Limpopo's Green Biotechnologies Research Centre of Excellence (GBRCE), located in the South African province of Limpopo at 23°53'10"S, 29°44'15"E latitude and 1200 m altitude above sea level. This region's climate is categorized as semi-arid, with day/night temperature ranging between 38^{o}C and 28^{o}C during summer period. Mostly during the summer, the region receives less than 500 mm of rainfall on average (Mokgalabone *et al*., 2023).

3.2.2 Treatment and research design

The study used three replications and a Randomized Complete Block Design (RCBD). Treatments included four planting densities: ultra-high density (10 x 10 cm), high density (30 x 30 cm), medium density (50 x 50 cm), and conventional density (100 x 100 cm, used as the control). Each planting density gave plant population of 100, 16, 9 and 4 plants m^{-2} respectively. The experiment consisted of 12 plots, each measuring 1 m x 1 m, and separated by 2 m walkways.

3.2.3 Research procedures

Seeds of *M. oleifera* Lam. (cultivar-PKM1), obtained from World Vegetable Centre (Taiwan), were sown in sterile 200-cell polystyrene seedling trays filled with Hygromix® growth media (Hygrotech SA Pty Ltd, Pretoria). The seedlings were raised following standard protocols and hardened off outdoors for one week before transplanting into to the field. To minimize variation in growth, seedlings were selected based on uniform height measurements taken prior to transplanting. Each plot was prepared with a 30-cm-deep pit, into which soil from the plot and Culterra compost (30 dm^3 COMPO0030 Pty Ltd, containing blended organic materials) were mixed in a 1:1 ratio. Spacing of plants in the plots was marked using a measuring tape according to the densities outlined in the experimental design, and *M. oleifera* seedlings were transplanted into each plot. Irrigation was provided through a drip system (Figure 3.1). Plots were well-maintained throughout the study; weeds were removed manually.

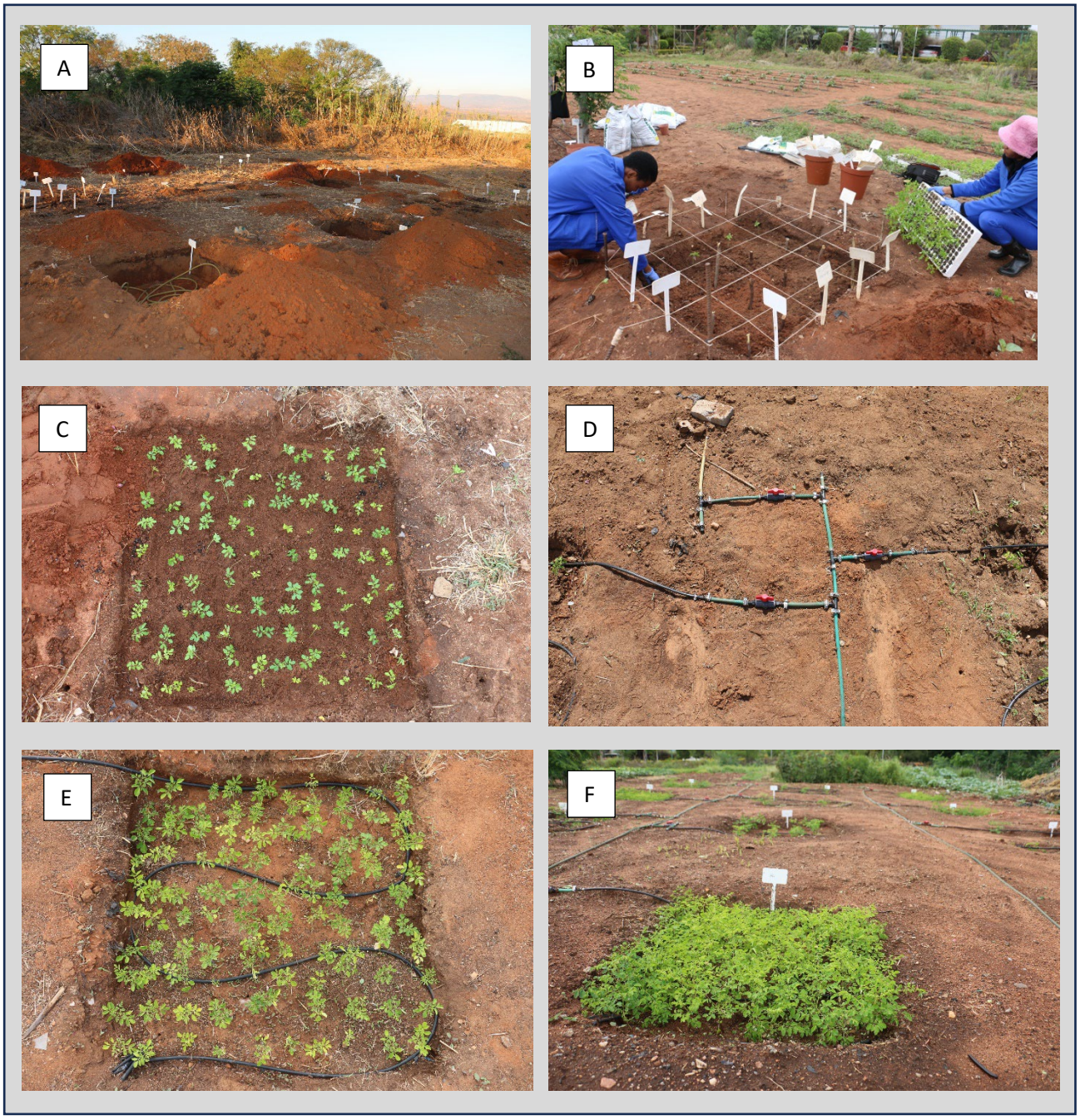

Figure 3.1: Illustration of the establishment of the meter square technology focusing on the ultra-high density planting (ultra-HDP) technology. (A) – 1 m x 1 m marking of plots and digging; (B) – planting; (C) - Day-1 aerial view of the UHD plantlets; (D) – Drip irrigation system set-up; (E) – Day-7 arial view of the ultra-HDP plants; (F) – Day-7 side view of the ultra-HDP plants. Photographs by Keratilwe Mokoditoa (2023).

3.2.4 Data collection

Growth and yield measurements were undertaken on 50% of the plants that were randomly selected and tagged as representative samples within each population. In populations of 100, 16, 9, and 4 plants, the number of randomly selected plants was 50, 8, 5, and 2, respectively. Stem diameter and plant height were measured weekly from day zero (post-transplanting) and continued until the termination date (day 180). For plant height, measurements were taken from the base of stem to apex with a measuring tape (Mokgalabone *et al*., 2023). Stem diameter measurements were taken 5 cm above the soil level with a digital Vernier calliper (KTV 150-major Tech) and recorded in mm (Magwele *et al*., 2024). Root length was measured at termination date using a ruler (cm). The root-shoot ratio was calculated as follows (Wang *et al*., 2020):

$$\text{Root} - \text{shoot ratio} = \frac{\text{Root biomass}}{\text{Shoot biomass}}$$

Leaf biomass, chlorophyll SPAD index and colour were recorded at termination of the experiment (week 18). Fresh weight of leaves harvested from randomly selected plants within a m² area was measured immediately after harvest using an electronic weighing balance and recorded in grams, then averaged. Leaves were then shade-dried at room temperature until they reached a constant weight, after which dried leaf weight was measured. Leaf yield per plant was calculated by averaging the total leaf weight from two sampled plants per plot. Each planting density treatment had three replicates, with two plants per plot randomly selected for leaf yield measurements. Leaf yield per plant was calculated as:

$$\text{Leaf yield per plant (g)} = \frac{\text{Total leaf weight from sampled plants}}{\text{Number of sampled plants}}$$

To estimate leaf yield per metre sqaure, the average leaf yield per plant was multiplied by the total plant population per m² in each density treatment:

Leaf yield (g/m^2) = Average leaf yield per plant × Plan population per meter square

Chlorophyll SPAD index was measured using a CCM-200 plus chlorophyll meter. Young leaves were defined as newly matured leaves located at the tips of the branches, while mature leaves were older, fully developed leaves from the lower parts of the branches. The average SPAD value per plant was used in statistical analysis to ensure consistency across treatments. Leaf colour characteristics (L* - Lightness; a* - Red-green; b* - Blue-yellow; c* - Chroma; h* - Hue) were assessed using a portable chroma meter (CR-400 and 410), calibrated against a white standard plate for accuracy and reliability, following the method outlined by Chen *et al*. (2021).

3.2.5 Statistical analysis

Statistical analyses were performed using Statistix 10.0. A one-way analysis of variance (ANOVA) was used to analyse growth and yield parameters, while a two-way ANOVA was used to evaluate physiological characteristics (chlorophyll SPAD index and colour), considering both planting density and leaf age as factors. Means for growth and yield parameters were separated using Least Significant Difference (LSD), while means for physiological characteristics were separated using the Tukey's Honestly Significant Difference test, both at a 5% significance level. Graphs were generated using GraphPad Prism version 9.5.0.

3.3 Results and discussion

3.3.1 Effect of different planting densities on the growth parameters of *Moringa oleifera*

Planting density significantly influenced the height of *M. oleifera* (Figure 3.2). Uniform growth was observed during the first 28 days post transplanting. The height generally increased over time across all densities, with significant differences observed from the 5^{th} week onwards. Between the 5^{th} and 11^{th} week, plants under the ultra-HDP (22.93 ± 1.02 and 66.94 ± 1.00 cm) attained the tallest height, significantly outstanding those in medium and conventional densities. The lowest plant height was observed in the conventional density (58.7 ± 1.48 cm).

About the 12th week, plants under the ultra-HDP (68.67 ± 2.04 cm) and high density (70.07 ± 1.89 cm) had decreased growth rate. In contrast, in the 15th week, plants under the conventional (85.97 ± 1.98 cm) and medium (83.52 ± 2.66 cm) densities began to attain greater heights compared to other densities. At the end of the experiment, plants under the conventional density significantly achieved the greatest height. However, there was no significant difference in plant height between conventional and medium densities. The observed results suggest that, initially, as planting density increases, plant height also increases. However, over time, lower planting densities tend to achieve greater heights.

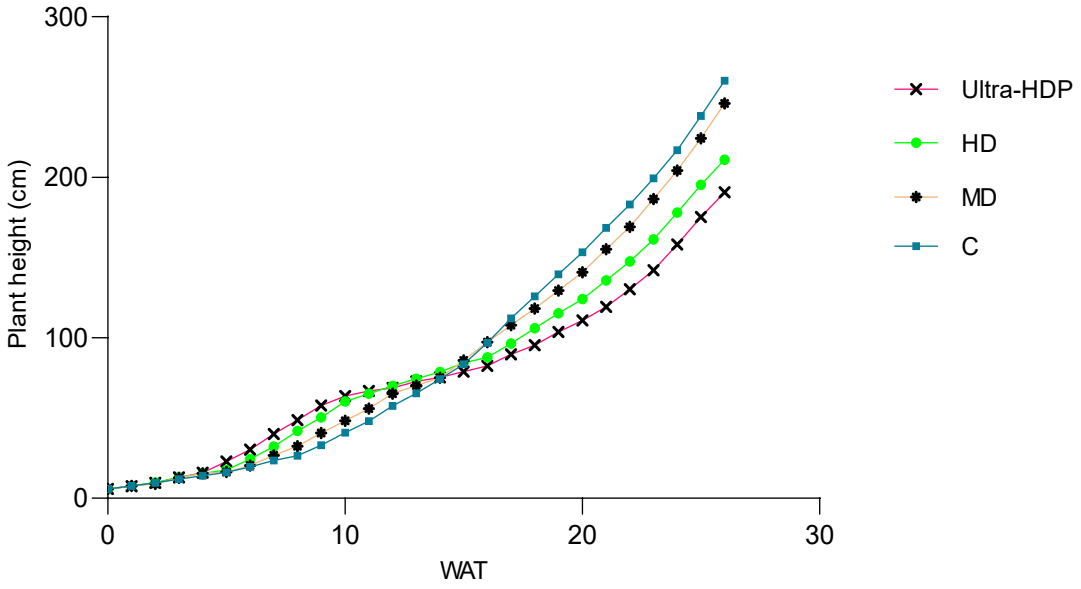

Figure 3.2: Height of M. oleifera plants grown under varying planting densities. Abbreviations: Ultra-HDP= Ultra-high density planting; HD= High density; MD= Medium density; C= Conventional density; WAT= Weeks after Transplanting.

Planting density significantly influenced the stem diameter of *M. oleifera* (Figure 3.3). Initially, during the 3 weeks after transplanting, stem diameter remained uniform across all densities. However, from the 4th week onwards, differences were observed. At the end of the experiment, plants under the conventional density (51.60 ± 1.04 mm) obtained the largest stem diameter, significantly outperforming the other planting densities (Figure 3.3 and 3.6). This suggests that lower planting densities encourage greater stem diameter development, with the conventional density being the most advantageous for stem growth.

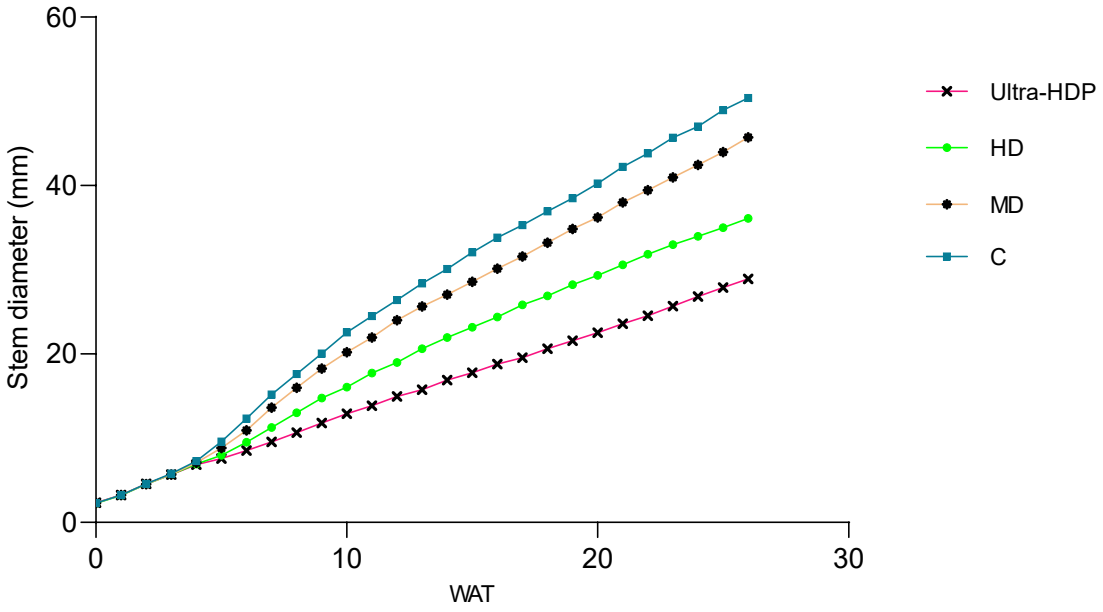

Figure 3.3: Stem diameter of *Moringa oleifera* grown under varying planting densities. Abbreviations: Ultra-HDP= Ultra-high density planting; HD= High density; MD= Medium density; C= Conventional density; WAT= Weeks after Transplanting.

Planting density significantly influenced the root length of *M. oleifera* (Figure 3.4). Individual plants at high density (35.0 ± 0.3 cm) developed significantly longer root systems compared to the other planting densities including conventional and medium densities. Ultra-HDP (16.3 ± 0.2 cm) produced the shallowest root systems (Figure 3.6).

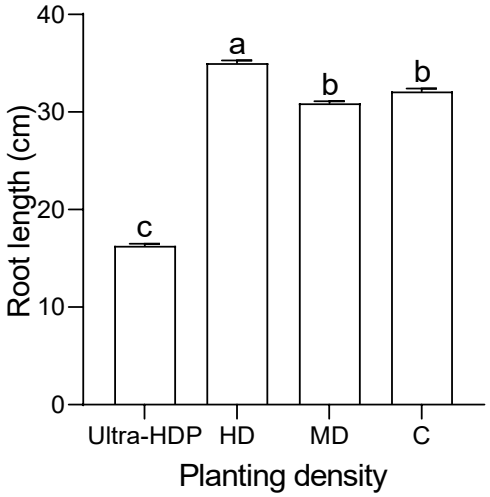

Figure 3.4: Root length of *Moringa oleifera* grown under varying planting densities. Bars represent means ± standard error, and different letters indicate significant differences ($p \leq 0.05$). Abbreviations: Ultra-HDP= Ultra-high density planting; HD= High density; MD= Medium density; C= Conventional density.

Planting density significantly influenced the root-shoot ratio of *M. oleifera* (Figure 3.5). It was found that the root-shoot ratio decreased as planting density increased. Plants under high density (0.68 ± 0.01) gave significantly higher root-shoot ratio compared to other planting densities including medium and conventional densities. The ultra-HDP gave the lowest root-shoot ratio. The results suggest that ultra-HDP appear to inhibit overall growth, resulting in less developed root systems and lower root-shoot ratios, however, high planting density increase root-shoot ratio.

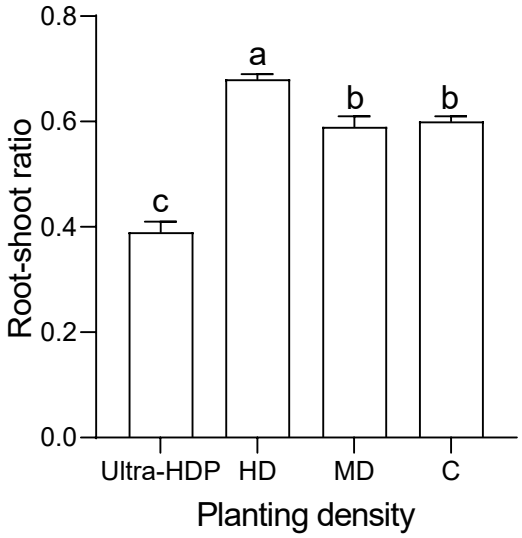

Figure 3.5: Root-shoot ratio of *Moringa oleifera* grown under varying planting densities. Bars represent means ± standard error, and different letters indicate significant differences ($p \leq 0.05$). Abbreviations: Ultra-HDP= Ultra-high density planting; HD= High density; MD= Medium density; C= Conventional density.

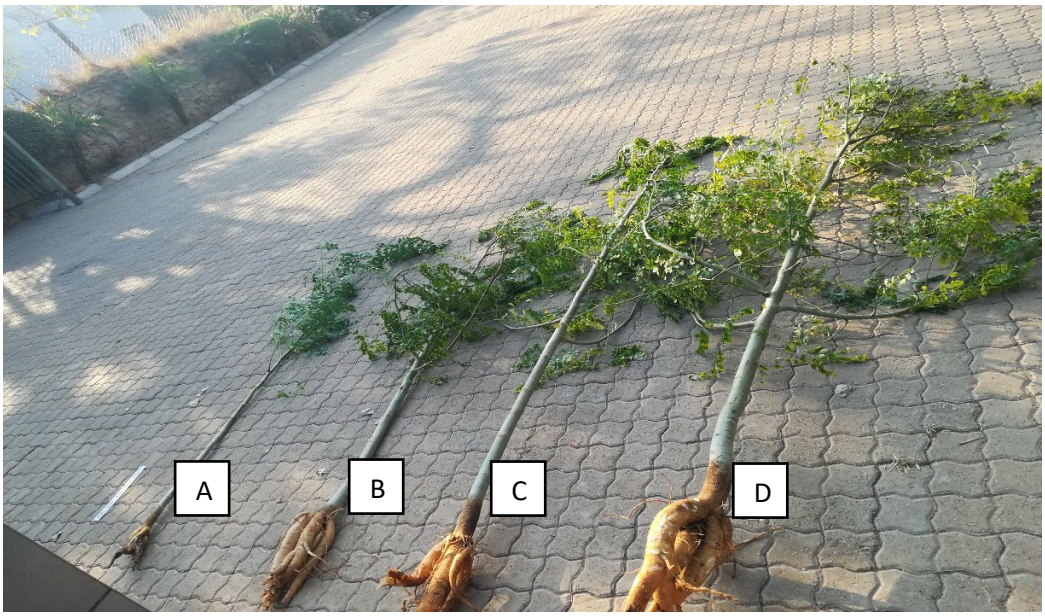

Figure 3.6: *Moringa oleifera* plants harvested at termination (Week 18) from four distinct planting densities. Note: A= Ultra-high density planting; B= High density; C= Medium density; D= Conventional density.

3.3.2 Effect of different planting densities on the yield parameters of *Moringa oleifera*

Planting density significantly influenced the fresh and dry leaf yield per plant (Figure 3.7). The increase in planting density resulted in decreased fresh and dry leaf yield per plant. Plants under conventional density produced significantly higher fresh (390.7 ± 1.59 g) and dry (22.67 ± 1.0 g) leaf yield per plant in comparison to the other planting densities, followed by the medium and high densities, while the ultra-HDP produced the lowest fresh (93.23 ± 0.72 g) and dry (22.67 ± 1.0 g) leaf yield per plant. Since the conventional density obtained the highest leaf yield per plant, the results suggest that when planting density is reduced, individual plants develop more robustly and produce more leaves.

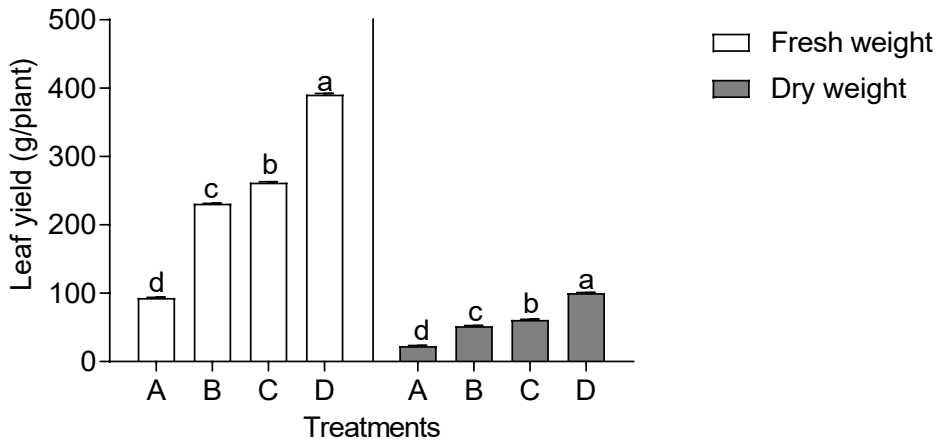

Figure 3.7: Fresh and dry leaf yield per plant of *M. oleifera* plants grown at various planting densities. Bars represent means ± standard error, and different letters within each trait indicate significant differences ($p \leq 0.05$). Ultra-HDP= Ultra-high density planting; HD= High density; MD= Medium density; C= Conventional density.

Planting density significantly ($p= 0.00$) influenced the fresh and dry leaf yield per meter square (Figure 3.8). Higher planting density increased fresh and dry leaf yield per meter square (Figure 3.9). Plants under ultra-HDP gave significantly higher fresh (9293.6 ± 2.9 g) and dry (2300.40 ± 3.2 g) leaf yield per meter square compared to other densities such as high and medium densities. The conventional density

produced the lowest fresh (1562.0 ± 3.02 g) and dry (400.07 ± 2.87 g) leaf yield per meter square.

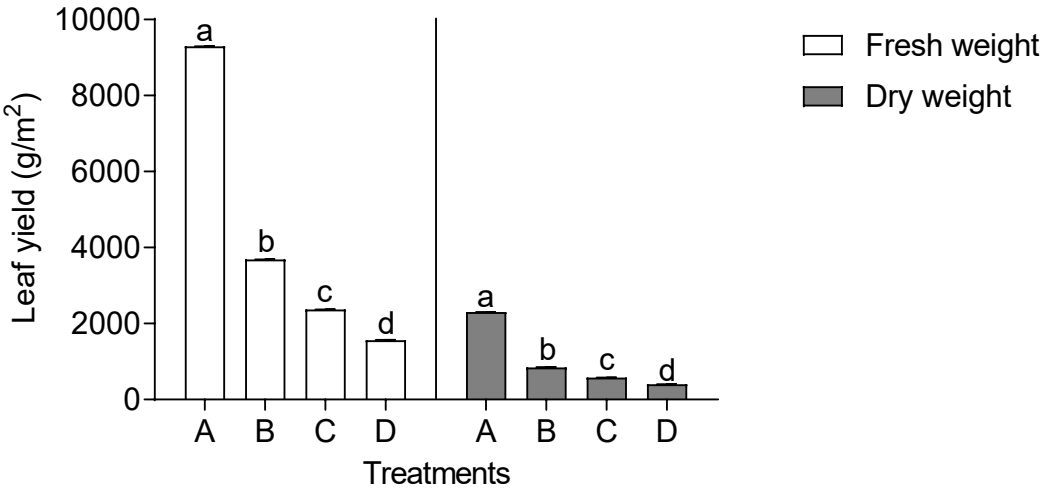

Figure 3.8: Fresh and dry leaf yield per meter square of *Moringa oleifera* grown under varying planting densities. Bars represent means ± standard error, and different letters within each trait indicate significant differences ($p \leq 0.05$). A = Ultra-high density planting; B = High density; C = Medium density; D = Conventional density. Fresh and dry weight: analysed independently

Figure 3.9: Leaf yield per meter square of *Moringa oleifera* grown under varying planting densities. Note = Ultra-high density planting; B = High density; C = Medium density; D = Conventional density.

3.3.3 Effect of planting density, leaf age, and their interaction on physiological characteristics of *Moringa oleifera*

Leaf chlorophyll SPAD index and colour were significantly influenced by planting density (Table 3.1). The chlorophyll results among the planting densities revealed that conventional density (67.0 ± 1.38) obtained the highest content compared to medium,

high and ultra-HDP densities (Table 3.1). In contrast, conventional density produced the lowest b* (blue-yellow) coordinate, in comparison to medium, high and ultra-HDP densities. A similar pattern was observed on the L* (lightness) coordinate, where the conventional density (43.01 ± 0.99) produced the lowest, in comparison to medium, high and ultra-HDP. The c* coordinate also followed this trend whereby the conventional density produced the lowest c* (chroma) coordinate, in comparison to medium, high and ultra-HDP densities. On the contrary, the conventional density (128.58 ± 1.10) obtained the highest h coordinate, in comparison to the other studied densities (Table 3.1).

The comparison between leaf ages also revealed a significant difference between mature and young leaves in terms leaf chlorophyll SPAD index and colour (Table 3.1). The Chlorophyll SPAD index was more in mature leaves than young leaves. In terms of colour, the a* (red-green) coordinate in mature leaves was higher than in young leaves. In contrast, the b* coordinate was lower in mature leaves compared to young leaves. The L* coordinate also followed a similar pattern whereby mature leaves produced lower L* coordinate compared to young leaves. The c* coordinate was also lower in mature leaves than young leaves. Conversely, mature leaves obtained higher h (hue angle) coordinate than young leaves.

The interaction between the planting density and leaf age was significant, as samples from the combination of conventional density and mature leaves contained the highest chlorophyll SPAD index, whereas the combination of ultra-HDP and young leaves produced the lowest chlorophyll SPAD index (Table 3.1). The h coordinate was higher under the combination of conventional density and mature leaves than the combination of ultra-HDP and young leaves. A lower L* value, which indicates darker leaf colour, is associated with the higher chlorophyll SPAD index.

Table 3.1: Chlorophyll and leaf colour parameters of *Moringa oleifera* leaves under varying planting densities and leaf ages

		Leaf colour					Chlorophyll
		L*	**a***	**b***	**c***	**H**	
Planting density (A)	Ultra-HDP	46.56 ± 0.67^{a}	-9.97 ± 0.84^{a}	13.34 ± 1.32^{a}	16.78 ± 1.15^{a}	123.15° ± 1.21^{c}	43.80 ± 1.47^{c}
	HD	45.44 ± 0.95^{b}	-9.64 ± 0.61^{a}	12.91 ± 1.05^{b}	16.23 ± 1.32^{b}	125.24° ± 0.58^{b}	52.72 ± 0.94^{b}
	MD	43.26 ± 1.02^{c}	-8.59 ± 1.45^{b}	11.97 ± 0.89^{bc}	14.72 ± 1.50b^{b}	127.99° ± 1.34^{a}	65.05 ± 1.99^{a}
	C	43.01 ± 0.99^{c}	-7.04 ± 0.96^{c}	10.54 ± 1.03^{c}	12.68 ± 1.26^{c}	128.58° ± 1.10^{a}	67.00 ± 1.38^{a}
	HSD	0.87	0.69	0.77	0.73	1.03	3.64
Leaf age (B)	Young leaves	47.22 ± 0.58^{a}	-10.20 ± 0.88^{a}	15.08 ± 0.53^{a}	18.20 ± 1.12^{a}	125.68° ± 0.39^{b}	47.58 ± 0.87^{b}
	Mature leaves	41.91 ± 0.69^{b}	-7.42 ± 0.67^{b}	9.30 ± 0.41^{b}	12.00 ± 1.04^{b}	126.79° ± 0.64^{a}	66.73 ± 1.23^{a}
	HSD	0.45	0.36	0.40	0.38	0.54	1.90
Young leaves	Ultra-HDP	50.07 ± 0.76^{ab}	-10.67 ± 0.68^{a}	17.23 ± 0.68^{a}	20.30 ± 1.33^{a}	121.72° ± 1.32^{f}	37.23 ± 0.68^{e}
	HD	47.52 ± 1.00^{b}	-10.63 ± 0.22^{b}	15.25 ± 0.75^{b}	18.55 ± 0.58^{b}	126.25° ± 0.40^{cd}	43.63 ± 1.12^{d}
	MD	45.73 ± 0.44^{bc}	-10.46 ± 0.78^{c}	14.35 ± 0.94^{bc}	17.73 ± 1.18b^{b}	127.33° ± 2.16^{bc}	53.57 ± 1.01^{c}
	C	45.57 ± 0.53^{c}	-9.31 ± 0.44^{c}	13.47 ± 0.68^{c}	16.23 ± 0.76^{c}	127.44 ± 2.02^{bc}	55.80 ± 0.56^{bc}
Mature leaves	Ultra-HDP	43.47 ± 0.49^{d}	-9.30 ± 0.78^{d}	10.57 ± 0.40^{d}	13.26 ± 0.75^{d}	124.58° ± 0.39^{de}	50.37 ± 3.84^{c}
	HD	43.05 ± 1.45^{d}	-8.38 ± 0.60^{d}	9.58 ± 0.84^{d}	13.91 ± 0.10^{d}	124.22° ± 0.64^{e}	61.80 ± 2.19^{b}
	MD	40.80 ± 0.67^{d}	-6.79 ± 0.60^{e}	9.44 ± 1.64^{d}	11.70 ± 1.63^{e}	128.64° ± 1.40^{ab}	76.77 ± 2.63^{a}
	C	40.45 ± 0.67^{d}	-5.49± 0.99^{e}	7.62 ± 0.82^{e}	9.14 ± 1.15^{f}	129.72° ± 1.06^{a}	78.20 ± 2.14^{a}
HSD	Planting density (A x B)	0.86		0.77	0.73	1.03	3.64

Values are expressed as means ± standard deviations (n = 3). Within each column, different superscript letters indicate significant differences ($p < 0.05$). Abbreviations: Ultra-HDP= Ultra-high density planting; HD= High density; MD= Medium density; C= Conventional density; L*= Lightness; a*= Red-green; b*= Blue-yellow; c*= chroma; h= Hue.

3.3.2 Discussion

3.3.2.1 Effect of planting density on plant height

The results indicated that planting density did not significantly affect plant growth at early stages, suggesting that initial height development is largely independent of competition. Similar trends have been observed in *M. oleifera* and other species, where uniform growth was reported during the initial weeks after planting, regardless of spacing (Walker, 2007; Adekola and Abdulrahaman, 2017). However, as the plants matured, those grown at wider spacing achieved greater height compared to those in high density plantings. Similar results were reported in *Moringa stenopetala*, where seedlings grown at wider spacing attained better height growth at the end of the experiment, while high density planting restricted height development (Eshete *et al*., 2022). Wider spacing in *M. oleifera* has also been associated with increased average plant height, reinforcing the findings of this study (Ponnuswami and Rani, 2019; Yilma *et al*., 2021). In contrast, some studies have reported no significant effect of planting density on final plant height (Abdullahi *et al*., 2013), while others have noted that high density planting initially promotes taller plants due to competition for light but ultimately results in shorter final height (Kaggwa-Asiimwe *et al*., 2013). These variations highlight the role of species-specific responses to planting density and the importance of optimizing spacing to enhance plant growth.

The initial increase in plant height observed at ultra-HDP likely resulted from competition for light, which stimulated vertical growth as plants elongated to maximize light interception. High density planting systems are known to encourage taller growth due to limited lateral space, forcing plants to grow taller in search of light (Adegun and Ayodele, 2015). This response was evident in this study, where ultra-HDP restricted lateral development and promoted stem elongation.

The subsequent decrease in growth rate at ultra-HDP and the higher final plant height at lower densities may be attributed to multiple factors. High density planting is associated with early maturity and rapid canopy closure, which can limit prolonged vegetative growth and lead to mutual shading (Manibharathi *et al*., 2024). Shading within the canopy in high density systems reduces light penetration, preventing efficient light capture and ultimately restricting plant growth and development (Djaman *et al*., 2022). Additionally, shading effects at high densities have been reported to have a greater impact on plant performance than nutrient depletion, though limited nutrient availability can also contribute to reduced plant growth (Postma *et al*., 2021).

3.3.2.2 Effect of planting density on stem diameter

The results indicated that ultra-HDP resulted in reduced stem diameter, while other densities promoted thicker stems. This trend has been observed in *M. oleifera*, where high density planting limited stem development due to increased competition for resources such as light, nutrients, and water (Goss, 2012; Olawepo *et al*., 2020). Similarly, studies have shown that stem diameter increases over time when plants are spaced further apart, as reduced competition allows for better resource allocation (Chau and Sam *et al*., 2021; Eshete *et al*., 2022).

The variation in stem diameter observed in this study may be attributed to the reduced competition at lower planting densities, which allows plants to access more nutrients, facilitating greater stem growth (Khan and Chaudhry, 2007; Eshete *et al*., 2022). Similar results has been reported in previous studies, where the largest stem diameters were observed in the lowest plant densities, supporting the perception that wider spacing supports thicker stem development (Goss, 2012). This suggests that lower planting densities may be more suitable when the objective is to produce seed and firewood, as they promote faster and more substantial individual plant growth, ultimately leading to larger stem diameters and higher seed yields (Goss, 2012).

3.3.2.3 Effect of planting density on root length

The reduced root length observed under ultra-HDP suggests that increased planting density does not necessarily promote larger root systems. Higher densities have been associated with shorter root lengths, as competition for resources limits plant growth and development (Zhang *et al*., 2006; Shao *et al*., 2018). This reduction may be attributed to decreased light interception per plant, leading to lower whole-plant photosynthesis and biomass accumulation. With less carbon available for root growth, plants in high density conditions tend to develop shorter root systems (Mi *et al*., 2016).

In contrast, this study found that high density planting produced the largest root length compared to lower densities, particularly conventional density. When plants have adequate moisture and nutrients, extensive root expansion may not be necessary, which could explain the more compact root systems observed in conventional and medium densities (Abdullahi *et al*., 2021). In high density environments, root systems may need to extend deeper to access resources in response to increased competition (Postma *et al*., 2021). Similar adaptive responses have been observed in plants exposed to stress conditions such as water scarcity or nutrient depletion, where longer root systems develop to access deeper soil layers (Manschadi *et al*., 2008). Competitive environments also trigger modifications in root architecture to enhance survival, which may explain the increased root length observed at high planting densities in this study (Grime, 2001).

3.3.2.4 Effect of planting density on root-shoot ratio

The findings of this study indicated that root-shoot ratio decreased under ultra-HDP, suggesting that higher planting densities promote greater shoot growth at the expense of root development. A similar trend has been observed in other species, where increased planting density reduces the allocation of biomass to roots while favoring shoot elongation to enhance light capture (Shao *et al*., 2018; Abdullahi *et al*., 2021). This may be due to increased competition for light, which forces plants to prioritize shoot growth, leading to a lower proportion of resources being directed toward root development. Furthermore, as

plant size increases, mutual shading reduces light penetration to the middle and lower canopy layers, restricting the availability of photosynthates for both shoots and roots (Demotes-Mainard and Pellerin, 1992; Hebert and Bertholeau, 1992; Li *et al*., 2014).

However, the observed increase in root-shoot ratio at high density compared to ultra-HDP may indicate a shift in resource allocation under reduced competition. In competitive environments, plants may invest more in root expansion to enhance access to water and nutrients, leading to a higher root-shoot ratio (Mašková and Herben, 2018). This may explain the larger root systems observed in high density planting in this study, where less extreme competition than in ultra-HDP allowed for greater root proliferation and resource acquisition.

3.3.2.5 Effect of planting density on plant height on leaf yield per plant

The findings of this study indicated that ultra-HDP resulted in lower dry leaf yield per plant, whereas lower densities promoted greater yield per plant. Similar trends have been observed in *M. oleifera*, where high density planting led to reduced leaf biomass per plant, while wider spacing allowed for increased yield (Adegun *et al*., 2015; Eshete *et al*., 2022). A comparable pattern has also been reported in *Moringa stenopetala*, where the highest biomass yield was obtained at low densities (Hamore *et al*., 2022).

The observed increase in fresh and dry leaf yield per plant at lower planting densities may be attributed to improved lateral growth, which enhances canopy expansion and branch development. Wider spacing between plants has been shown to facilitate greater vegetative growth, leading to denser canopies, thicker stems, and an overall increase in branching (Kumalasari *et al*., 2017). This response is likely due to reduced competition, allowing for better access to soil nutrients and water. Interestingly, previous reports indicate that high density planting has little impact on individual plant yield until a critical competition threshold is reached (Norman, 1992; Foidl *et al*., 2001). Beyond this threshold, intensified resource competition leads to a decline in yield per plant, which aligns with the reduced yield observed under ultra-HDP in this study.

3.3.2.6 Effect of planting density on leaf yield per meter square

The findings of this study indicated that ultra-HDP resulted in increased total leaf yield per unit area, despite a decrease in yield per individual plant. Similar trends have been observed in *M. oleifera*, where an increase in planting density led to greater biomass production per hectare (Mabapa *et al*., 2017). Higher planting densities have also been associated with increased leaf yield, as the cumulative production from multiple plants compensates for the reduced yield per plant (Foidl *et al*., 2001; Adegun *et al*., 2015).

The results of this study clearly demonstrate that as planting density increases, individual plant yield declines, but the total leaf yield per unit area increases. This trade-off occurs because while each plant produces less biomass under competitive conditions, the greater number of plants per area offsets this reduction (Ball *et al*., 2000; Chau and Sam, 2021). Consequently, higher plant densities effectively enhance total yield per unit area, as observed in other crops where increased densities result in greater overall production (Ella *et al*., 1989; Ball *et al*., 2000; Chau and Sam, 2021).

3.3.2.7 Effect of planting density on leaf chlorophyll SPAD index

The results indicated that ultra-HDP resulted in reduced chlorophyll SPAD index, while lower densities obtained the highest chlorophyll SPAD index. These observations are consistent with “Mondal and Puteh (2013), who reported an increase in chlorophyll content of modern rice under wider plant spacing. Similarly, Ren *et al*. (2017) reported that chlorophyll content in *Zea mays* Lam,decreased significantly with increased planting densities.

These results may be due to the results of Brahim *et al*. (1998) and Aminifard *et al*. (2010), who stated that shading within the lower canopy reduces the amount of photosynthetically active radiation absorbed by the leaves, thereby limiting chlorophyll synthesis. However, the lower chlorophyll content in the young leaves aligns with the findings of Datt (1998), who reported that new leaves were found to have lower pigment content compared to

mature leaves. High density planting likely intensifies competition for water and essential nutrients, especially nitrogen, which is important for chlorophyll synthesis due to the shallow root system typical of high density planting (Brahim *et al*., 1998). Young leaves are therefore more susceptible to these conditions, but high density planting affects both young and mature leaves.

The results show that planting density and leaf age are important factors in determining chlorophyll SPAD index, with mature leaves having higher chlorophyll levels as a result of lower densities. Bote and Struik (2011) also suggested that dark green leaves, which contain more chlorophyll, are more efficient in photosynthesis because of their increased ability to absorb light and use chloroplasts more effectively. This could explain why conventional density, with less shadowing, produced the highest chlorophyll SPAD index and darkest leaf colour, absorbing more sunlight than ultra-HDP.

3.4 Conclusion

Planting density has significant effects on *M. oleifera* growth, yield, and quality parameters. The ultra-HDP resulted in significantly higher total leaf production per unit area, despite smaller stem diameters, shorter plant heights, and shallower root systems. This ultra-high density planting approach was shown to be helpful in increasing leaf production, especially in situations where maximum yield per unit area is needed. The increased number of plants per unit area in this density is more than compensated for the reduced size of individual plants, resulting in a much greater total leaf yield than at lower densities. Given the growing global population, limited land resources, and increased demand for sustainable agricultural practices, *M. oleifera* has considerable potential for cultivation even in limited areas. The study's findings indicated that farming *M. oleifera* at a 10 x 10 cm density is recommended for maximum leaf yield. This method is especially beneficial for intensive farming systems with limited land, making it appropriate for high demand markets and small-scale farmers hoping to increase production. Furthermore, *M. oleifera*'s nutritional and economic benefits make it an important crop for both local communities and business investors. Studies in the future should focus on improving this

planting density by investigating the long-term effects on plant health and potential applications to additional high-value crops. Understanding these factors could improve productivity in areas of limited resources, providing a long-term solution for increasing crop yields in densely populated or land-scarce areas. Furthermore, investigating the impact of ultra-HDP on nutrient content and crop quality may provide additional insights into optimizing *M. oleifera* agriculture for a variety of agricultural situations.

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Chapter 4: Impact of meter square ultra-high density planting technology on the nutritional composition of *Moringa oleifera*

Abstract

Moringa oleifera Lam., is widely recognized for its potential to alleviate malnutrition in communities with limited resources, and it is critical to understand the optimum production methods for maximizing its nutrients. This study evaluated effect of the meter square ultra-high density planting (ultra-HDP) and leaf age on the nutritional composition of *M. oleifera*. After harvest, leaves were classified as young and mature per planting density and were shade dried until constant dried weight were obtained. The samples were subjected to the proximate, mineral, energy and amino acid analyses. Data were analyzed using a two-way ANOVA with Statistix 10.0 to evaluate the effect of planting density and leaf age on the nutritional composition of *M. oleifera*. Mean comparisons were performed at a 5% significance level using the Tukey's Honestly Significant Difference (HSD) test.

Moringa oleifera leaves grown under conventional density (100 x 100 cm) showed the highest levels of protein (29.12 ± 0.3%), carbohydrates (49.67 ± 0.3%), fat and ash, In contrast, ultra-HDP produced the lowest protein, carbohydrates and ash levels. Additionally, young leaves significant showed higher protein (28.36 ± 0.1%), carbohydrates (43.52 ± 0.2%), energy (588.25 ± 3.4 kJ/100 g) and amino acid contents such as arginine, lysine and threonine, compared to mature leaves, which had lower levels of these nutrients.

Conventional density and young leaves obtained higher energy content while the ultra-HDP and mature leaves produced the lowest energy content. Interestingly, young leaf under ultra-HDP obtained significantly higher Zn (34.35 ± 1.0 mg/kg) content. Essential amino acids, including histidine, arginine, threonine, and lysine, were more abundant in conventional density young leaves. On the other hand, the young leaves under the ultra-

HDP resulted in significantly higher proline (1.40 ± 0.03%) levels likely as a stress response. These results indicated that ultra-HDP resulted in lower nutritional composition though it was able to maintain high Zn levels compared to the other densities. Young leaves on the other hand showed an abundance of most nutrients compared to mature ones. These findings further highlight the importance of carefully selecting plant density and leaf age in *M. oleifera* cultivation strategies. Further research is recommended to investigate techniques for reducing nutrient loss in ultra-HDP systems while increasing yield.

4.1 Introduction

Moringa oleifera Lam., stands out as one of the most promising plants for use as a functional food to address the need for well-balanced nutrients for humans (Padayachee and Baijnath, 2020). The leaves of *M. oleifera* serve as the main source of essential macro and micronutrients contributing significantly overall health (Peñalver *et al*., 2022). Fresh leaves of *M. oleifera* were found to have a protein content of 6.7 g/100 g, which is twice as high as that of milk, while dry leaves had a protein content of 27.1 g/100 g (Andrew *et al*., 2018; Arora *et al*., 2021). Essential minerals present in *M. oleifera* leaves include potassium, zinc, calcium, phosphorus, magnesium, iron and copper. The leaves also provide 17 amino acids, including tryptophan, an essential amino acid often deficient in other vegetables, indicating their potential to increase dietary diversity and promote human health (Badwaik *et al*., 2020; Mutar *et al*., 2021).

Further analyses revealed that *M. oleifera* leaves are good for treating malnutrition, especially in nursing mothers and infants, because they contain a significant amount of ash (8.05-10.38%), moderate levels of fibre (6.00-9.60%), low fat (4.03-9.51%), and high protein (22.99-29.36%) (Moyo *et al*., 2011; Sultana, 2020). The leaves have an average mineral content of 520 mg/100 g calcium and 7.7 mg/100 g iron, including trace minerals such as magnesium, aluminum and chromium (Masitlha *et al*., 2024). This nutrient profile

establishes *M. oleifera* as an important dietary supplement for addressing diet-related health issues (Olusanya *et al*., 2020).

A number of variables, including soil type, harvesting stage, growth environment, and processing techniques, may affect the nutritional composition of *M. oleifera* plants (Ahmed *et al*., 2023; Yang *et al*., 2023). Particularly, leaf maturity has significant effects on nutritional levels (Dhakad *et al*., 2024). According to research, mature leaves have the highest levels of ash, protein (50.3–56 mg/100 g), and amino acids such as phenylalanine, leucine, alanine, and valine (Qadir *et al*., 2022). Furthermore, mature leaves were found to have 1.5 times more iron than young leaves and three times higher calcium levels (1.58%) than young leaves (Agamou *et al*., 2015).

There are still inconsistencies and gaps in the information regarding the nutritional composition of *M. oleifera*, despite the growing interest in this nutrient-dense food source (Lamidi *et al*., 2017). Although earlier research has looked at growth and production at various planting densities, little is known about how planting density and leaf age affect the nutritional composition of *M. oleifera*, particularly in meter square ultra-HDP systems. It is anticipated that higher planting densities may lead to variations in nutrient allocation, potentially reducing macronutrient content due to competition for resources, while younger leaves might exhibit higher protein and amino acid concentrations compared to mature leaves. The objective of this chapter was to investigate the impact of meter square ultra-high density planting technology on the nutritional composition of *M. oleifera*, providing insights to optimize cultivation strategies.

4.2 Materials and methods

4.2.1 Laboratory analysis

The evaluation of leaf mineral content of *M. oleifera* plants was performed at the Agricultural Research Council (ARC)-Natural Resources and Engineering division in

Pretoria, South Africa. Evaluation of other nutritional components, such as moisture, crude protein, fat, ash, carbohydrate content, and energy value, was carried out at the ARC-Irene Analytical Research Laboratory, also located in Pretoria. Meanwhile, the amino acid profile was examined at Stellenbosch University through the Central Analytical Facilities.

4.2.2 Treatments, design, and sample preparation

The samples utilized for nutritional analyses were obtained following the procedures outlined in Section 3.2.3. Prior to the analysis of nutritional composition, leaves of *M. oleifera* were harvested from trees planted in a Randomized Complete Block Design (RCBD) experiment. Then for analysis, a Completely Randomized Design was followed with a 4 x 2 factorial trial to carry out four planting densities (ultra-HDP, high, medium, and conventional densities), and two leaf ages (young and mature as outlined in section 3.2.4). For each planting density, 2 trees were harvested per plot, ensuring that both young and mature leaves were included. Once the leaves were picked, they were washed several times using tap water and dried with a paper towel before shade drying. Shade drying was performed up to two weeks until a fixed weight was achieved, and samples were then ground to powder and kept under 4°C cold storage until analysis. In total, 8 samples (n = 8) were analyzed, representing 4 planting densities with 2 leaf age categories per density.

4.2.3 Proximate analysis

4.2.3.1 Moisture content

Moisture content analysis was performed based on the AOAC (2000) method, with adjustments described by Rak *et al*. (2020). A precise balance was used to weigh empty porcelain crucibles after they had been pre-dried for 30 minutes in an oven and then

cooled. The samples were finely ground to promote consistent heat exposure during drying. Each crucible received about 5 g of the sample, and then oven-dried at 105°C for 24 hours. The samples were dried and cooled in a desiccator before their final weights were noted. The percentage of dry matter (DM) and moisture content was calculated using a standard formula indicated below:

$$\%\text{Moisture content} = \frac{Initial\ Weight\ of\ Sample - Weight\ of\ Dried\ Sample}{\text{Initial Weight of sample}} x100$$

4.2.3.2 Determination of protein content

The analysis of protein content was performed utilizing the micro-Kjeldahl protocol as outlined in the AOAC (1990) guidelines. The procedure involved placing 2 g of the sample, 10 mL of concentrated sulfuric acid (H_2SO_4), and a selenium catalyst tablet into a heating tube. Following heating in a fume hood, the mixture was transferred to a 100 mL volumetric flask and diluted with distilled water to a final volume of 100 mL. A 10 mL sample of the solution was mixed with an equal amount of 45% sodium hydroxide (NaOH) and distilled using a Kjeldahl apparatus. Three drops of an indicator solution were added to a 4% boric acid solution in order to collect the distillate. After that, the distillate (50 mL) was collected and titrated. All analyses were performed in duplicate, and the average result was used. In order to convert the nitrogen content to protein %, a factor of 6.25 was used.

$$\%\text{Nitrogen} = \frac{(100\ x\ W\ x\ N\ x\ 14\ x\ Vf)\ T}{100\ \text{x Va}}$$

Where: W = Sample weight, N = Titrate normality (0.1N), Vf = Digest total volume = 100 mL, T = Titre value and Va = Aliquot volume distilled.

4.2.3.3 Determination of fat content

Fat content was determined by extracting the sample using petroleum ether, in accordance with the soxhlet extraction technique specified in AACC method 30-25 (1983). Following extraction, low-temperature evaporation was used to remove the petroleum ether from the collection flask. After 30 minutes of drying at 100°C in an oven, the remainder of the fat residue was measured using the following formula to determine the fat percentage:

$$\%Fat = \frac{[(mass\ of\ beaker\ +\ mass\ of\ extracted\ fat) - (mass\ of\ beaker)]\ \times\ 100}{\text{mass of sample}}$$

4.2.3.4 Crude fibre determination

Crude fibre content was measured by first weighing (W_1) the filter bag, followed by placing 0.5 g of dried, finely ground sample (W_2) that passed through a 1 mm screen into the bag. Each bag was sealed with a heat sealer about 0.5 cm from the edge, placed in a holder, and submerged in about 2000 mL of preheated acid detergent solution inside an ANKOM Fibre Analyzer (ANKOM Technology, Macedon, NY, USA) tank. A blank bag was also weighed and then processed for weight correction (C1). The vessel was automatically stirred for 60 minutes, then rinsed with 2000 mL of hot water. In order to achieve a neutral pH of neutral, the samples were shaken once more and washed for 3 to 5 minutes. After carefully removing any excess water from the bags, they were immersed in acetone for 3 minutes to get rid of any remaining moisture. Following the removal of the acetone, the bags were cooled to ambient temperature, dried at 105°C for at least 2 hours, and then weighed once more (W_3).

$$\%ADF = \frac{=\ [W3 - (W1\ x\ )]\ x\ 100}{\text{W2}}$$

4.2.3.5 Determination of ash content

Ash content was measured according to the AACC method 08-01 (1999), which involves burning organic material in the leaves through oxidative combustion. Prior weighing, crucibles were allowed to cool after the oven drying set at 100°C for 5 hours. Following a period of heating over a tripod until the samples were charred, 2 g of each sample were placed in crucibles. Thereafter, crucibles were subjected to heat treatment in a muffle furnace, maintained at 550°C for 5 hours. Once the heating process was finished, the samples were taken out of the furnace, cooled in a desiccator, and subsequently weighed. The ash percentage was then determined using the given formula:

$$\%Ash = \frac{(mass\ ash\ +\ crucible) - (mass\ crucible)\ x100}{(\text{mass food}\ +\ \text{crucible}) - (\text{mass crucible})}$$

4.2.3.6 Determination of carbohydrates

The available carbohydrate content of the samples was calculated by difference using the following formula:

% Carbohydrate = 100 – (% moisture + % ash + % crude protein + % crude fat + % crude fibre).

4.2.4 Determination of minerals

The mineral composition of the *M. oleifera* leaf samples was determined following the protocol outlined by Mafokoane *et al*. (2019). For digestion, dried leaf material (10 g) was mixed with 40 mL of 4% nitric acid (HNO_3) and vigorously mixed using a vortex. Following magnetic stirring, the mixture was incubated for 90 minutes at 95°C in a water bath. The solution was filtered once it had cooled to room temperature and then put into 50 mL

tubes, which were then wrapped in foil to protect the sample. Analysis of nutrient elements was performed using an ICPE-9000 inductively coupled plasma optical emission spectrometer (ICP-OES).

4.2.5 Determination of amino acids

The amino acid composition of dried *M. oleifera* leaves was determined according to the procedure described by Mpai *et al*. (2018). First, 0.5 mL of 6 N HCl was added to 100 g of leaf material, which was then incubated for 18 hours at 110°C. The supernatant was subsequently filtered to eliminate any remaining particulates before undergoing vacuum drying under reduced pressure to concentrate the sample and remove excess solvent. The dried residue then was then dissolved in 70 µL of borate buffer for subsequent derivatization after cooling. The derivatization process was carried out using the accQ-Tag ultra-amino acid kit, which included AQC (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate), dry acetonitrile, and a 0.2 M sodium borate buffer (pH 8.8). Initial analyses were performed on undiluted samples, followed by a tenfold dilution to quantify higher amino acid concentrations. For derivatization, 20 µL of AQC was mixed with 10 µL of the sample, which contained norvaline (20 µL/L) in 80 µL, then vortexed and incubated at 55°C for 10 minutes. After cooling, the derivatized samples were prepared for analysis using Ultra Performance Liquid Chromatography (UPLC).

The separation of amino acid and detection were performed utilizing the Waters Aquity UPLC system, equipped with a photodiode array detector. A 1 µL aliquot was injected into a Waters Ultra Tag C18 column (2.1 x 50 mm, 1.7 µm), maintained at a temperature of 60°C. The chromatographic run was performed over 9.5 minutes with a gradient flow rate of 0.7 mL/min, using eluent A (99.95% water) and eluent B (1% acetonitrile) as the mobile phases. Amino acid concentrations were calculated by analyzing the peak areas of standards and samples.

4.2.6 Determination of energy

The caloric value of the sample was determined using the Atwater factor method, where the contents of crude protein, lipid, and carbohydrate were multiplied by their respective energy conversion factors (3.99, 9.1, and 3.99 kcal/g). The resulting products were then summed to calculate the total caloric value (Onwuka, 2005; Sultana, 2020).

4.3 Statistical analysis

Data were analyzed using a two-way analysis of variance (ANOVA) with Statistix 10.0 to assess the effects of planting density and leaf age, as well as their interaction, on the studied variables. Mean comparisons were performed at a 5% significance level using Tukey's Honestly Significant Difference test. Graphs were generated using version 9.5.0 of GraphPad Prism.

4.4 Results and discussion

4.4.1 Results

4.4.1.1 Effect of planting density and leaf age on proximate composition of *Moringa oleifera*

The influence of planting density and leave age was significantly different across all studied proximate analysis including moisture, protein, carbohydrates, fat and ash contents. A comparison of each proximate analysis among planting densities and between leaf ages revealed significant differences in proximate composition.

The moisture results among the planting densities revealed that conventional density (7.38 ± 0.1%) obtained the highest moisture content in comparison to other studied densities such as medium density, high density and ultra-HDP (Figure 4.1). A similar trend of the results was observed on protein content, whereby the conventional density (29.12 ± 0.3%) recorded the highest protein content in comparison to the medium, high and ultra-HDP densities. Similarly, the conventional density obtained the highest fat content compared to medium, high and ultra-HDP densities. Crude fibre content also followed this trend, with the conventional density leading compared to the medium, high and ultra-HDP densities. A similar pattern was observed for ash content, where the conventional density obtained the highest ash content (9.5 ± 0.3%) in comparison to medium, high and ultra-HDP densities. Conventional density also recorded the highest carbohydrate content (49.67 ± 0.3%) compared to medium, high and ultra-HDP densities. *Moringa oleifera* leaves indicated higher nutrient in conventional density compared to other densities confirming a notable effect of planting density on nutrient composition (Figure 4.1).

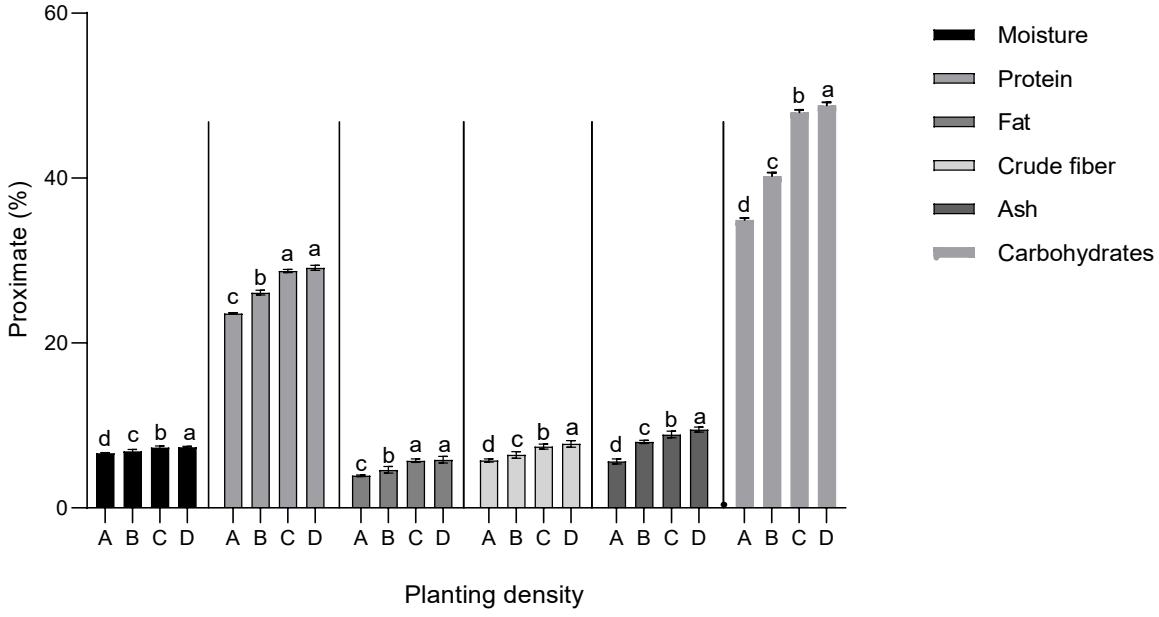

Figure 4.1: Proximate composition of *Moringa oleifera* leaves grown under varying planting densities. Bars represent means ± standard error, and different letters within each

component indicate significant differences ($p \leq 0.05$). Note: A= Ultra-high density planting; B= High density; C= Medium density; D= Conventional density.

The comparison between leaf ages also revealed a significant effect between mature and young leaves in terms of proximate composition. Mature leaves obtained a higher moisture content than youth leaves. Protein content peaked in young leaves (28.36 ± 0.1%) compared to mature leaves. In contrast, fat content was more in mature leaves in comparison to young leaves. Similarly, crude fibre was higher in mature leaves compared to young leaves. A similar trend was found for ash content whereby mature leaves (9.23 ± 0.2%) obtained the highest ash content than young leaves. However, carbohydrate content was found to be higher in young leaves (43.52 ± 0.2%) than in mature ones (Figure 4.1E).

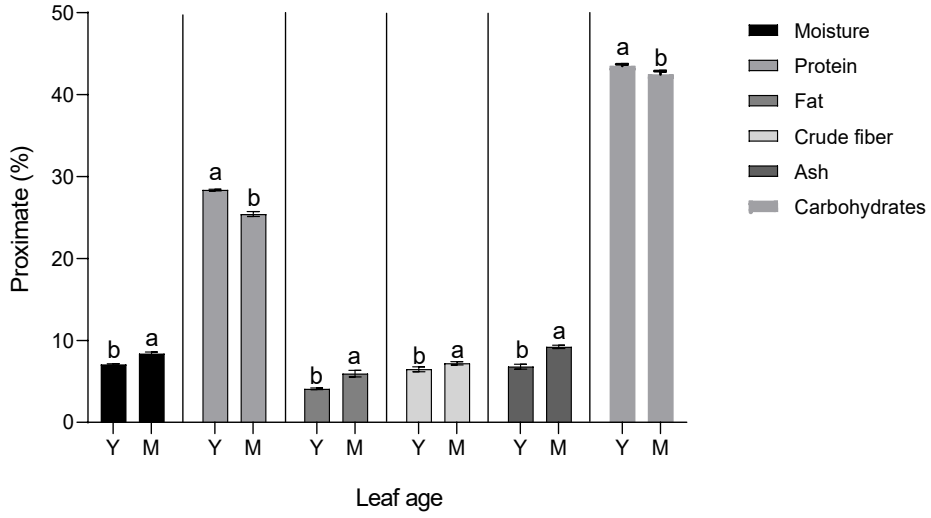

Figure 4.2: Proximate composition of *Moringa oleifera* leaves grown under various planting densities. Bars represent means ± standard error, and different superscript letters within each component indicate significant differences ($p \leq 0.05$). Note: Y= Young leaves; M= Mature leaves.

The interaction between the planting density and leaf age was significant, as samples from the conventional density and mature leaves obtained the highest moisture content (8.55 ± 0.1%), while the ultra-HDP and young leaves produced the lowest moisture content (Figure 4.3A). For protein content, the conventional density and young leaves (31.00 ± 0.01%) obtained the highest content, while the combination of ultra-HDP and mature leaves produced the lowest protein content (Figure 4.3B). Fat content was higher in the combination of conventional density and mature leaves than in ultra-HDP and young leave (Figure 4.3C). Similar results were observed in crude fibre content whereby high content was achieved in the combination of conventional density and mature leaves than in ultra-HDP and young leaves (Figure 4.3D). Ash content followed a similar pattern, where the combination of conventional density and mature leaves (10.66 ± 0.1%) recorded the highest ash content compared to ultra-HDP and young leaves (Figure 4.3E). In contrast, carbohydrate content was higher under the combination of conventional density and young leaves than the combination of ultra-HDP and mature leaves (Figure 4.3F).

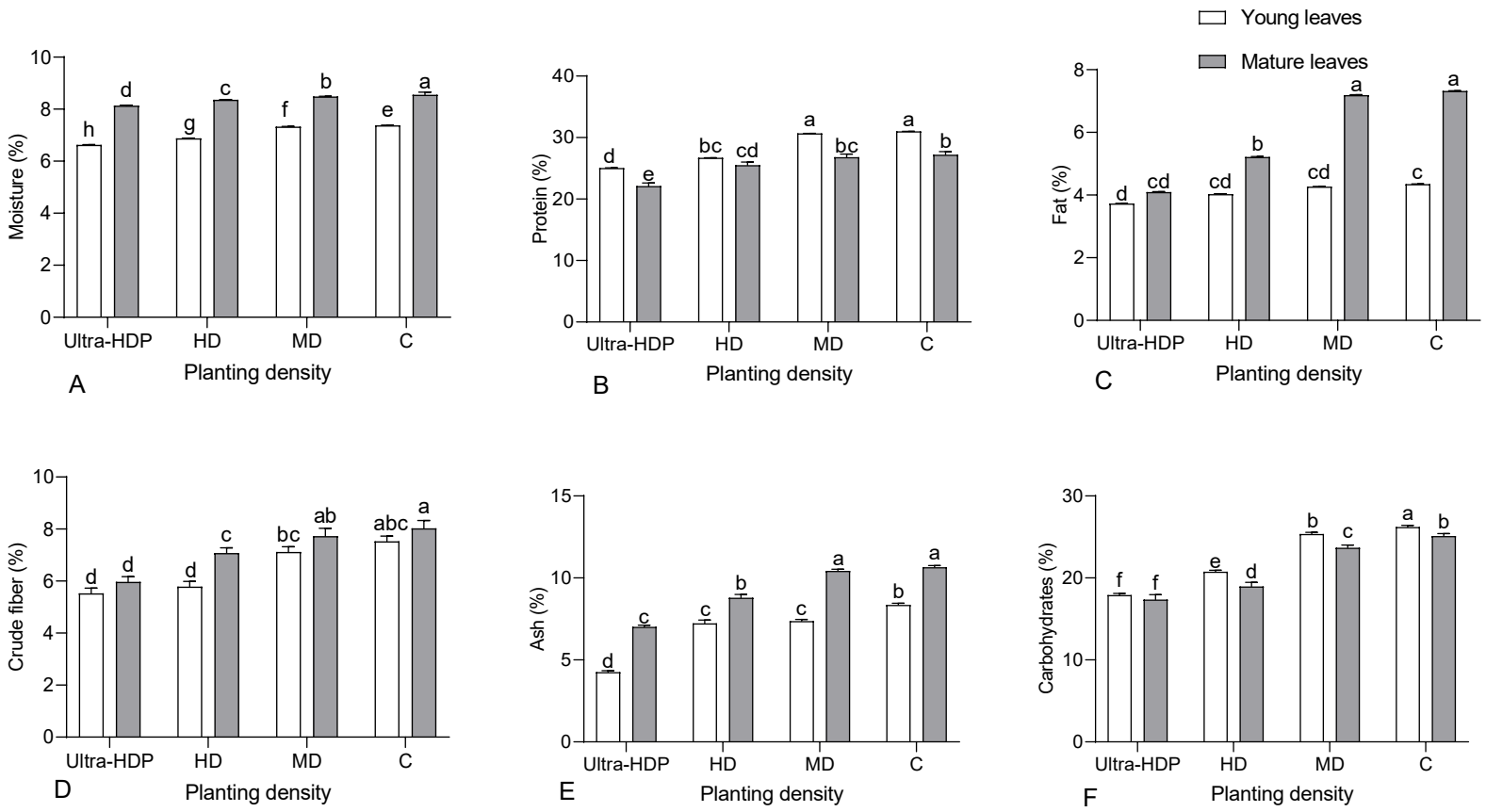

Figure 4.3: Interaction effect of planting densities and leaf ages on the proximate composition of *Moringa oleifera* leaves. Bars represent means ± standard error, and different superscript letters indicate significant differences ($p \leq 0.05$). Abbreviations: Ultra-HDP= Ultra-high density planting; HD= High density; MD= Medium density; C= Conventional density; A= Moisture; B= Protein; C= Fat; D= Crude fiber; E= Ash; F= Carbohydrates.

4.4.1.2 Effect of planting density, leaf age and leaf age on energy composition of *Moringa oleifera* leaves

The energy content of leaves was significantly influenced by planting density. Among the planting densities, the conventional density produced the highest energy content compared to medium, high, and ultra-HDP densities (Figure 4.4A). Energy content was also higher in young leaves (588.25 ± 3.4 kJ/100 g) than in mature leaves (Figure 4.4A). Regarding the interaction between planting density and leaf age, the combination of conventional density and young leaves produced the highest energy content, while the combination of ultra-HDP and mature leaves produced the lowest energy content (Figure 4.4B).

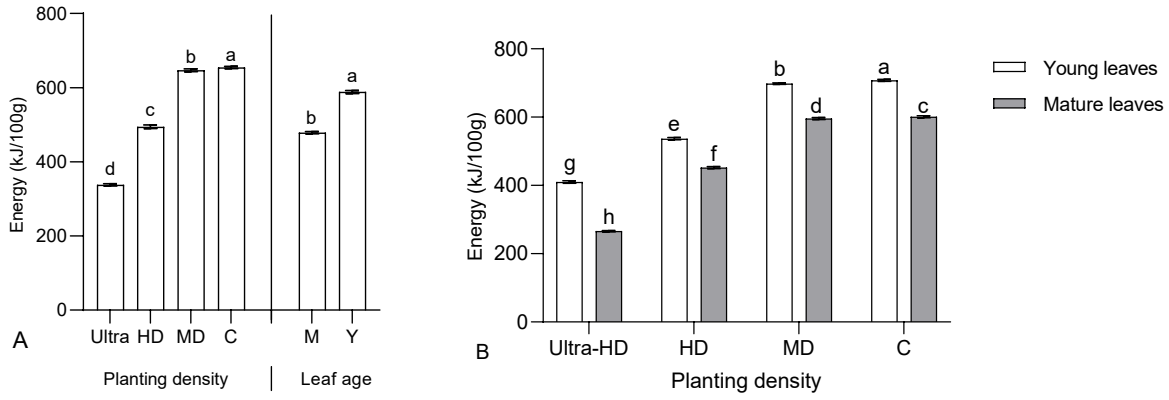

Figure 4.4: Energy composition of young and mature leaves of *M. oleifera* plants grown at various densities. Bars represent means ± standard error, and different letters indicate significant differences ($p \leq 0.05$). Abbreviations: Ultra-HDP= Ultra-high density planting; HD= High density; MD= Medium density; C= Conventional; Y= Young; M= Mature. A= separate effects and B= Interaction effect of planting densities and leaf ages.

4.4.1.3 Effect of planting density, leaf age, and their interaction on mineral composition of *Moringa oleifera* leaves

A comparison of each nutritional analysis among planting densities and leaf age revealed some significant differences in mineral contents. The K results among the planting densities revealed that conventional density (2.38 ± 0.01%) obtained the highest K content in comparison to other studied densities such as medium density, high density and ultra-HDP (Figure 4.5A). A similar trend of the results was observed on Ca performance, whereby the conventional density (1.22 ± 0.02%) recorded the highest Ca content in comparison to the medium, high and ultra-HDP densities. Similarly, the conventional density obtained the highest Mg content compared to medium, high (and ultra-HDP densities (Figure 4.3A). Iron content also followed this trend, with the conventional density leading compared to the medium, high and ultra-HDP densities. Interestingly, the highest Zn content was obtained under the ultra-HDP (34.35 ± 1.0 mg/kg) compared to high, medium and conventional densities (Figure 4.5B).

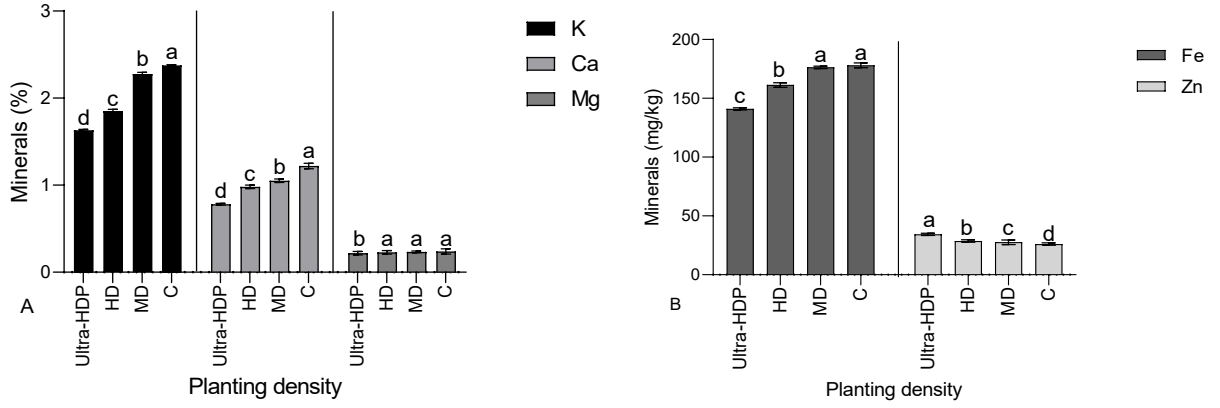

Figure 4.5: Mineral composition of *Moringa oleifera* leaves grown under varying planting densities. Bars represent means ± standard error, and different letters within each component indicate significant differences ($p \leq 0.05$). Abbreviations: Ultra-HDP= Ultra-high density planting; HD= High density; MD= Medium density; C= Conventional density;

K= Potassium; Ca= Calcium; Mg= Magnesium; Fe= Iron; Zn= Zinc; A= Minerals (K, Ca and Mg); B= Minerals (Fe and Zn).

The comparison between leaf ages revealed a significant effect between mature and young leaves in terms of mineral composition. Potassium content was greater in mature leaves (2.09 ± 0.01 mg/kg) compared to young leaves (Figure 4.6A). Similarly, Ca content peaked in mature leaves (1.48 ± 0.02%) than the young ones. In contrast, Mg content was greater in young leaves than in mature leaves. Iron content was greater in mature leaves than in young leaves. However, Zn content was more in young leaves (37.08 ± 2.0 mg/kg) than that of mature leaves (Figure 4.6B).

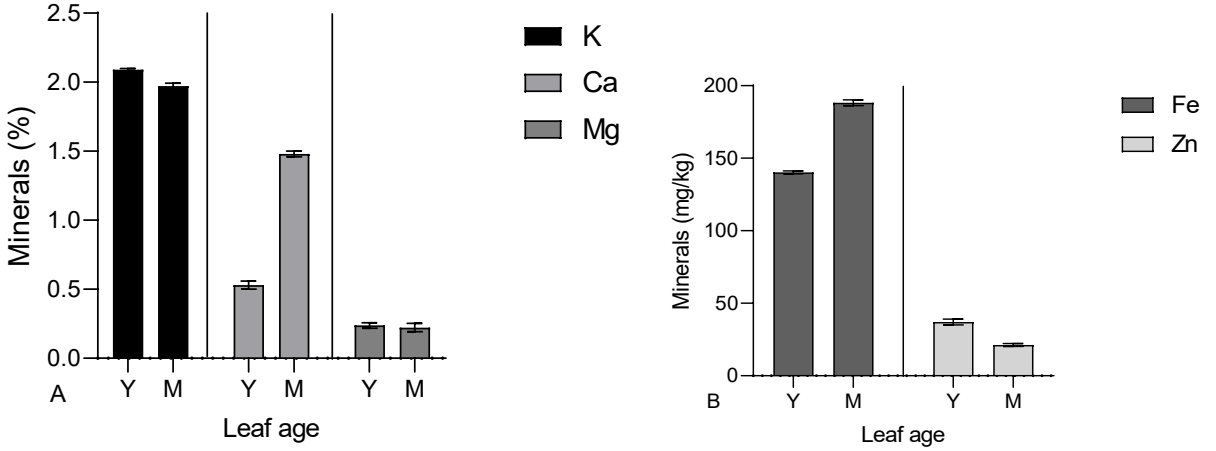

Figure 4.6: Mineral composition of *Moringa oleifera* leaves grown under varying leaf ages. Bars represent means ± standard error, and different letters within each component indicate significant differences ($p \leq 0.05$). Abbreviations: Y= Young leaves; M= Mature leaves; K= Potassium; Ca= Calcium; Mg= Magnesium; Fe= Iron; Zn= Zinc; A= Minerals (K, Ca and Mg); B= Minerals (Fe and Zn).

The interaction between planting density and leaf age revealed that samples from the combination of conventional density and mature leaves contained the highest K content (2.40 ± 0.01%), whereas the combination of ultra-HDP and young leaves produced the

lowest K content (Figure 4.7A). For Ca content, the combination of conventional density and mature leaves obtained the highest content, while the combination of ultra-HDP and young leaves produced the lowest Ca content (Figure 4.7B). A contrasting trend was seen for Mg content whereby the combination of conventional density and young leaves (0.24 ± 0.0%) produced the highest Mg content in comparison to the combination of ultra-HDP and mature leaves (Figure 4.7C). Iron content was higher in the combination of conventional density and mature leaves than in ultra-HDP and young leave (Figure 4.7D). Zinc content was higher under the combination of ultra-HDP, and young leaves compared to the combination of conventional density and mature leaves (Figure 4.7E).

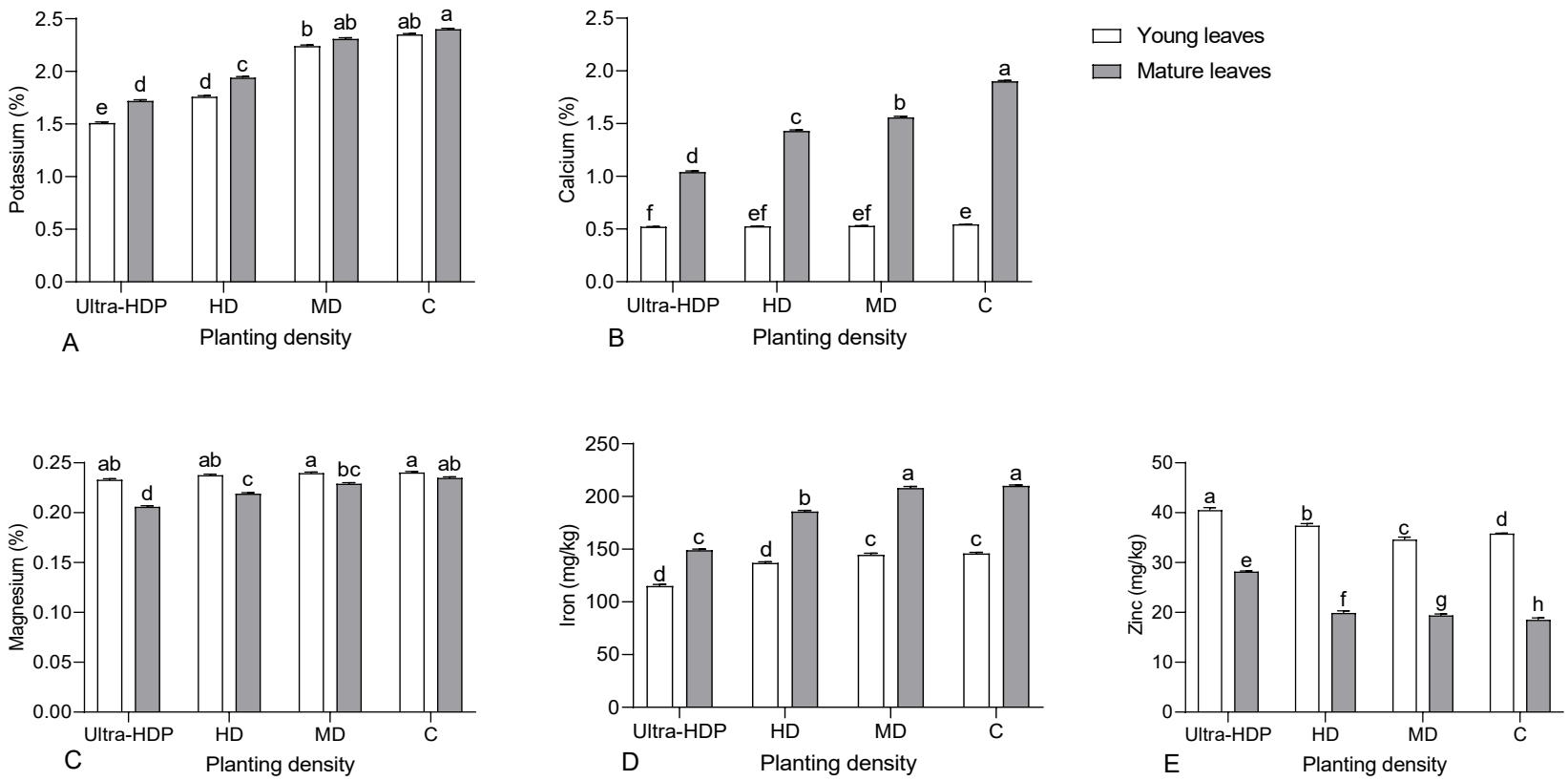

Figure 4.7: Interaction effect of planting densities and leaf ages on the mineral composition of *Moringa oleifera* leaves. Bars represent means ± standard error, and different letters indicate significant differences ($p \leq 0.05$). Abbreviations: Ultra-HDP= Ultra-high density planting; HD= High density; MD= Medium density; C= Conventional density; A= Potassium; B= Calcium; C= Magnesium; D= Iron; E= Zinc.

4.4.1.4 Effect of planting density, leaf age, and their interaction on amino acid composition of *Moringa oleifera* leaves

Amino acid composition of M. oleifera leaves was significantly ($p = 0.00$) influenced by planting density. Conventional density obtained the highest histidine, arginine, threonine, lysine, methionine, valine, isoleucine, leucine, and phenylalanine contents in comparison to medium, high and ultra-HDP (Table 4.1). Non-essential amino acids followed the same pattern whereby conventional density obtained higher serine, glycine, aspartic acid, glutamic acid, alanine, and tyrosine contents compared to medium, high and ultra-HDP densities. Interestingly, proline was an exception, with a higher content attained in plants grown at under ultra-HDP (Table 4.2).

The comparison between leaf ages revealed a significant difference in both essential and non-essential amino acid content, with young leaves exhibiting higher concentrations than mature leaves. Essential amino acids, including histidine, arginine, methionine, leucine, lysine, threonine, valine, isoleucine, and phenylalanine, were more abundant in young leaves (Table 4.1). Likewise, non-essential amino acids such as serine, glycine, aspartic acid, glutamic acid, alanine, proline, and tyrosine were present in greater amounts in young leaves compared to mature leaves (Table 4.2).

The interaction between the planting density and leaf age was also significant as samples from the combination of conventional density and young leaves contained the highest essential amino acid contents, whereas the combination of ultra-HDP and mature leaves produced the lowest essential amino acids (Table 4.1). A similar trend was observed for non-essential amino acids whereby the combination of conventional density and young leaves contained the highest contents compared to the combination of ultra-HDP and mature leaves (Table 4.2).

Table 4.1: Essential amino acid composition of *Moringa oleifera* leaves grown under varying planting densities and leaf ages

		His	**Arg**	**Thr**	**Lys**	**Met**	**Val**	**ILe**	**Leu**	**Phe**
Planting density (A)	Ultra-HDP	0.61 ± 0.05^{c}	1.98 ± 0.06^{c}	1.30 ± 0.04^{d}	1.74 ± 0.00^{bc}	0.32 ± 0.02^{c}	1.55 ± 0.10^{d}	1.25 ± 0.05^{d}	2.42 ± 0.01^{d}	1.72 ± 0.05^{c}
	HD	0.67 ± 0.01^{b}	2.26 ± 0.09^{b}	1.36 ± 0.01^{c}	1.70 ± 0.01^{c}	0.31 ± 0.05^{c}	1.69 ± 0.10^{c}	1.30 ± 0.06^{c}	2.57 ± 0.04^{c}	1.65 ± 0.10^{b}
	MD	0.70 ± 0.02^{b}	2.15 ± 0.08^{c}	1.45 ± 0.04^{b}	1.79 ± 0.03^{b}	0.39 ± 0.02^{b}	1.74 ± 0.07^{b}	1.38 ± 0.04^{b}	2.69 ± 0.04^{b}	1.96 ± 0.07^{b}
	C	0.72 ± 0.02^{a}	2.33 ± 0.11^{a}	1.55 ± 0.03^{a}	1.91 ± 0.04^{a}	0.45 ± 0.02^{a}	1.85 ± 0.06^{a}	1.45 ± 0.06^{a}	2.84 ± 0.02^{a}	2.11 ± 0.09^{a}
	HSD	0.03	0.05	0.06	0.07	0.03	0.03	0.03	0.03	0.03
Leaf age (B)	Young leaves	0.70 ± 0.01^{a}	2.42 ± 0.12^{a}	1.44 ± 0.05^{a}	1.91 ± 0.03^{a}	0.35 ± 0.06^{a}	1.77 ± 0.07^{a}	1.36 ± 0.05^{a}	2.64 ± 0.05^{a}	1.95 ± 0.07^{a}
	Mature leaves	0.65 ± 0.03^{b}	1.94 ± 0.10^{b}	1.39 ± 0.03^{b}	1.66 ± 0.04^{b}	0.31 ± 0.05^{b}	1.65 ± 0.08^{b}	1.33 ± 0.02^{b}	2.62 ± 0.04^{b}	1.91 ± 0.07^{b}
	HSD	0.01	0.03	0.03	0.04	0.01	0.02	0.02	0.01	0.02
Young leaves	Ultra-HDP	0.63 ± 0.02^{d}	2.13 ± 0.00^{c}	1.30 ± 0.03^{d}	1.73 ± 0.05^{de}	0.29 ± 0.02^{e}	1.56 ± 0.09^{e}	1.23 ± 0.05^{d}	2.36 ± 0.02^{f}	1.78 ± 0.02^{d}
	HD	0.68 ± 0.00^{bc}	2.60 ± 0.01^{a}	1.37 ± 0.01^{bcd}	1.85 ± 0.04^{bc}	0.31 ± 0.02^{de}	1.76 ± 0.08^{b}	1.29 ± 0.03^{c}	2.58 ± 0.02^{d}	1.88 ± 0.01^{c}
	MD	0.72 ± 0.01^{b}	2.31 ± 0.05^{b}	1.46 ± 0.04^{b}	1.96 ± 0.03^{b}	0.33 ± 0.02^{de}	1.79 ± 0.12^{b}	1.39 ± 0.04^{b}	2.68 ± 0.04^{c}	2.04 ± 0.03^{b}
	C	0.77 ± 0.02^{a}	2.62 ± 0.04^{a}	1.63 ± 0.04^{a}	2.11 ± 0.03^{a}	0.39 ± 0.01^{C}	1.96 ± 0.08^{a}	1.51 ± 0.05^{a}	2.88 ± 0.02^{a}	2.11 ± 0.03^{a}
Mature leaves	Ultra-HDP	0.58 ± 0.00^{e}	1.83 ± 0.00^{f}	1.29 ± 0.01^{d}	1.76 ± 0.03^{cd}	0.35 ± 0.02^{cd}	1.55 ± 0.01^{e}	1.27 ± 0.02^{cd}	2.48 ± 0.02^{e}	1.68 ± 0.03^{e}
	HD	0.66 ± 0.01^{cd}	1.92 ± 0.01^{e}	1.35 ± 0.03^{cd}	1.55 ± 0.04^{f}	0.31 ± 0.01^{de}	1.62 ± 0.02^{d}	1.31 ± 0.02^{c}	2.57 ± 0.02^{d}	2.02 ± 0.04^{b}
	MD	0.67 ± 0.01^{cd}	1.99 ± 0.04^{de}	1.45 ± 0.02^{bc}	1.62 ± 0.04^{ef}	0.45 ± 0.02^{b}	1.69 ± 0.02^{c}	1.37 ± 0.02^{b}	2.71 ± 0.03^{c}	1.87 ± 0.01^{c}
	C	0.68 ± 0.01^{bc}	2.03 ± 0.03^{d}	1.47 ± 0.02^{b}	1.71 ± 0.03^{de}	0.51 ± 0.01^{a}	1.74 ± 0.03^{bc}	1.38 ± 0.02^{b}	2.81 ± 0.02^{b}	2.10 ± 0.05^{a}
HSD	Interaction (AxB)	0.04	0.09	0.10	0.12	0.05	0.06	0.06	0.05	0.06

Values are expressed as means ± standard deviations (n = 3). Within each column, different superscript letters indicate significant differences ($p < 0.05$). Abbreviations: Ultra-HDP= Ultra-high density planting; HD= High density; MD= Medium density; C= Conventional density; His= Histidine; Arg= Arginine; Thr= Threonine; Lys= Lysine; Met= Methionine; Val= Valine; ILe; Isoleucine; Leu= Leucine; Phe= Phenylalanine.

Table 4.2: Non-essentialamino acid composition of *Moringa oleifera* leaves grown under varying planting densities and leaf ages

		Ser	Gly	Asp	Glu	Ala	Pro	Tyr
Planting density (A)	Ultra-HDP	1.41 ± 0.01^{d}	1.39 ± 0.03d^{c}	3.14 ± 0.00^{c}	4.19 ± 0.01^{d}	21.98 ± 0.02^{c}	1.40 ± 0.03^{a}	1.16 ± 0.03^{c}
	HD	1.57 ± 0.02^{c}	1.45 ± 0.01d^{b}	3.22 ± 0.02^{b}	4.55 ± 0.04^{c}	2.00 ± 0.00^{c}	1.30 ± 0.00^{b}	1.36 ± 0.03^{b}
	MD	1.64 ± 0.00^{b}	1.56 ± 0.02a^{a}	3.33 ± 0.01^{a}	4.73 ± 0.03^{b}	2.06 ± 0.01^{b}	1.26 ± 0.00^{c}	1.39 ± 0.04^{b}
	C	1.80 ± 0.04^{a}	1.58 ± 0.03^{a}	3.37 ± 0.02^{a}	4.88 ± 0.04^{a}	2.23 ± 0.02^{a}	1.22 ± 0.01^{d}	1.52 ± 0.03^{a}
	HSD	0.04	0.04	0.04	0.15	0.05	0.02	0.05
Leaf age (B)	Young leaves	1.80 ± 0.02^{a}	1.51 ± 0.03^{a}	2.63 ± 0.02^{a}	4.93 ± 0.02^{a}	2.18 ± 0.03^{a}	1.33 ± 0.01^{a}	1.38 ± 0.00^{a}
	Mature leaves	1.41 ± 0.03^{b}	1.48 ± 0.03^{b}	2.91 ± 0.01^{b}	4.24 ± 0.02^{b}	1.96 ± 0.02^{b}	1.26 ± 0.00^{b}	1.34 ± 0.02^{b}
	HSD	0.02	0.02	0.02	0.08	0.03	0.01	0.02
Young leaves	Ultra-HDP	1.56 ± 0.00^{c}	1.41 ± 0.02d^{e}	3.59 ± 0.01^{b}	4.41 ± 0.04^{cd}	2.07 ± 0.01^{bc}	1.45 ± 0.01^{a}	1.15 ± 0.03^{d}
	HD	1.77 ± 0.04^{b}	1.43 ± 0.04d^{e}	3.60 ± 0.01^{b}	4.78 ± 0.00^{b}	2.09 ± 0.05^{bc}	1.34 ± 0.01^{b}	1.33 ± 0.03^{c}
	MD	1.81 ± 0.04^{b}	1.56 ± 0.03a^{b}	3.64 ± 0.02^{ab}	5.25 ± 0.02^{a}	2.12 ± 0.02^{b}	1.29 ± 0.03^{c}	1.45 ± 0.01^{b}
	C	2.07 ± 0.00^{a}	1.63 ± 0.01^{a}	3.67 ± 0.03^{a}	5.27 ± 0.01^{a}	2.44 ± 0.04^{a}	1.22 ± 0.02^{d}	1.59 ± 0.03^{a}
Mature leaves	Ultra-HDP	1.27 ± 0.01^{f}	1.36 ± 0.02^{e}	2.69 ± 0.02^{e}	3.96 ± 0.04^{e}	1.88 ± 0.02^{e}	1.35 ± 0.02^{b}	1.17 ± 0.01^{d}
	HD	1.36 ± 0.02^{e}	1.47 ± 0.01^{cd}	2.84 ± 0.02^{d}	4.33 ± 0.07^{cd}	1.92 ± 0.04^{de}	1.32 ± 0.01^{bc}	1.39 ± 0.04^{bc}
	MD	1.47 ± 0.01^{d}	1.55 ± 0.02^{b}	3.02 ± 0.01^{c}	4.20 ± 0.05^{de}	2.01 ± 0.03^{cd}	1.22 ± 0.00^{d}	1.34 ± 0.05^{c}
	C	1.53 ± 0.01^{cd}	1.54 ± 0.01^{bc}	3.07 ± 0.01^{c}	4.49 ± 0.05^{c}	2.02 ± 0.00^{c}	1.18 ± 0.01^{e}	1.45 ± 0.02^{b}
HSD	Interaction (A x B)	0.07	0.07	0.07	0.25	0.09	0.04	0.09

Values are expressed as means ± standard deviations (n = 3). Within each column, different superscript letters indicate significant differences ($p \leq 0.05$). Abbreviations: Ultra-HDP= Ultra-high density planting; HD= High density; MD= Medium density; C= Conventional density; Ser= Serine; Gly= Glycine; Asp= Asparagine; Glu= Glutamic; Ala= Alanine; Pro= Proline; Tyr; Tyrosine.

4.4.2 Discussion

4.4.2.1 Effect of planting density on nutritional composition

Previous research has indicated that planting density affects the nutritional composition of crops, normally as a result of competition for important resources such as light, water, and nutrients (Postma *et al*., 2021). Since competition of plants limits resource availability, high planting density systems, such ultra-HDP, are often linked to a decrease in nutrient composition. The results of this study corroborated this effect, which demonstrated that compared to the conventional planting density, ultra-HDP resulted in the lowest amounts of protein, carbohydrates, fat, ash, Mg, Ca, K and Fe. Zinc content, however, was significantly higher at ultra-HDP.

These results align with findings that plant spacing significantly affects the nutritional composition of *M. oleifera*, with higher densities leading to lower Ca, Mg, Mn, and Fe levels (Ponnuswami and Rani, 2019). Interestingly, an increase in Zn concentration at higher densities has also been reported, which supports the current findings. The increased Zn levels under ultra-HDP may be attributed to intensified root activity due to nutrient competition, which enhanced Zn uptake. Additionally, the competitive microenvironment may have triggered Zn's hormetic response, where low concentrations stimulate physiological processes that enhance nutrient absorption (Wei *et al*., 2022).

The results further indicated that planting density significantly influenced the moisture content of *M. oleifera* leaves. A reduction in moisture content at high planting densities has been observed in other crops, such as *Zea mays* L., where increased density led to higher water consumption and lower moisture retention (Okbagabir *et al*., 2016). Similarly, crops planted at lower densities tend to retain more moisture due to reduced competition for water (Zhang *et al*., 2021). This pattern may be explained by lower water availability per plant at higher densities, which results in water stress and a subsequent decline in yield (Carmi *et al*., 2006; Yarnia *et al*., 2011). However, higher planting densities have

also been shown to improve inter-plant water retention and increase relative humidity, which could mitigate some moisture losses (Wu *et al*., 2020).

Studies on soybean have demonstrated a quadratic response to planting density, with protein content decreasing and K concentration increasing as density increases (Rahman *et al*., 2011). In contrast, research on *Morus alba* L., reported only minor differences in nutritional composition due to planting density, suggesting that different crop species respond differently to variations in spacing (Boschini *et al*., 2000).

Moringa oleifera has also demonstrated varied responses to planting density across different studies. In some cases, nutrient composition remained largely unaffected, except for Fe and Mg concentrations (Mabapa *et al*., 2017). However, variations in crude protein and K content observed at different study sites suggest that environmental factors play a crucial role in nutrient accumulation. Similar results have been reported in *M. oleifera*, where nutrient composition was not significantly influenced by planting density, even under continuous harvesting conditions (Sánchez *et al*., 2006). When planting densities are not excessively high, the chemical composition of leaves appears to remain stable due to manageable competition for resources (Mendieta-Araica *et al*., 2013). This may explain why studies conducted under moderate densities fail to detect significant effects, whereas the extreme density conditions in this study revealed substantial variations.

Ultra-high density planting also led to a reduction in the energy content of *M. oleifera* leaves, which may be due to limited light penetration in densely planted systems. Restricted access to light reduces photosynthetic efficiency, ultimately lowering energy accumulation (Chapepa *et al*., 2020). A similar pattern has been observed in cotton, where high planting densities decreased light availability and reduced stored energy content (Chapepa *et al*., 2020). These findings suggest that ultra-HDP can negatively affect photosynthetic performance, resulting in lower energy production at the leaf level.

The effect of planting density on amino acid composition was also evident in this study. Ultra-high density planting resulted in higher proline concentrations compared to other

densities, while conventional density produced higher levels of other amino acids such as glutamic acid, arginine, threonine, lysine, and methionine. An increase in proline concentration under high planting densities has been reported in *Brassica napus* (Nasiri *et al*., 2017) and *Sorghum bicolour* (Alderfasi *et al*., 2016). However, in *Zea mays* L., proline levels were not significantly affected by planting density, highlighting species-specific responses (Rafiee, 2012). Since proline accumulation is commonly linked to environmental stress, the elevated levels observed under ultra-HDP suggest that this planting density may induce physiological stress in *M. oleifera* (Roychoudhury *et al*., 2015; Dikilitas *et al*., 2020).

4.4.2.2 Effect of leaf age on nutritional composition

Leaf age significantly influenced the nutritional composition of *M. oleifera*, with distinct differences observed between young and mature leaves as they progressed through developmental stages. Young leaves attained significantly higher protein content than mature leaves, suggesting that protein synthesis is more active at early leaf developmental stages (Agamou *et al*., 2015; Khalid *et al*., 2019). However, some studies have reported contrasting results, where mature leaves contained higher protein levels than young leaves, indicating potential variability due to environmental or genetic factors (Ojewuyi *et al*., 2014; Agamou *et al*., 2015; Qadir *et al*., 2022).

Consistent with the protein content, the carbohydrate levels in this study were also significantly higher in young leaves compared to mature leaves. This aligns with reports that young leaves exhibit greater carbohydrate accumulation due to higher photosynthetic activity (Zufferey, 2000; Yang *et al*., 2006; Bamishaiye *et al*., 2011; Agamou *et al*., 2015). Early leaf development is characterized by high metabolic demand, which could explain the elevated carbohydrate levels (Zufferey, 2000). However, carbohydrate differences between leaf ages may vary depending on species, as some studies found no significant variation in carbohydrate content (Ojewuyi *et al*., 2014). In contrast, fat content was higher in mature leaves, supporting the view that lipid accumulation occurs over time. However,

other research has found no significant differences in lipid content between young and mature *M. oleifera* leaves, although both leaf stages maintain a favourable lipid profile rich in unsaturated fatty acids (Sánchez-Machado *et al*., 2010).

Leaf age also significantly influenced the mineral composition of *M. oleifera*, with young leaves containing higher levels of Zn and Mg, suggesting greater nutrient uptake during early growth stages (Agamou *et al*., 2015). Conversely, mature leaves had higher Fe, Ca, and K levels, indicating potential nutrient accumulation as leaves age. The increased ash content in mature leaves further indicates that minerals accumulate over time, making older leaves a richer source of essential minerals (Ojewuyi *et al*., 2014). However, some studies have reported no significant differences in ash content between young and mature leaves, suggesting that overall mineral abundance remains relatively stable throughout leaf development (Agamou *et al*., 2015; Qadir *et al*., 2022).

Another key observation in this study was the significantly higher energy content in young leaves compared to mature leaves. The energy values recorded for young leaves were consistent with previously reported ranges for different *M. oleifera* cultivars, reinforcing their role as a reliable energy source (Ndhlala and Tshabalala, 2023). This suggests that young leaves may be more suitable for fresh consumption, where high energy availability is desirable.

Amino acid content also varied with leaf age, with young leaves exhibiting higher overall concentrations. This aligns with findings that young *M. oleifera* leaves tend to accumulate greater amino acid levels, except for certain amino acids such as arginine, tryptophan, tyrosine, aspartic acid, and serine, which have been reported in higher concentrations in mature leaves (Ndubuaku *et al*., 2014). Additionally, studies on *Arabidopsis thaliana* L., found that proline levels were higher in young leaves, suggesting that amino acid composition may be linked to developmental processes (Sperdouli and Moustakas, 2014). The elevated amino acid levels in young leaves may be attributed to active protein synthesis, which corresponds with the high protein content observed in this study.

Although mature leaves have lower amounts of certain nutrients, their capacity to store energy and minerals, as well as their ability to be dried with little loss of nutrients, make them generally more nutrient-dense (Yang, 2006; Palada *et al*., 2017). On the other hand, young leaves are better for the fresh market, and they contain more protein, carbohydrates, amino acids, energy, and minerals (Zn and Mg), making them more palatable. This age-related variation provides a unique opportunity for adapting harvest approaches based on desired nutritional benefits.

4.5 Conclusion

This study revealed that both planting density and leaf age significantly influenced the nutritional composition of *M. oleifera*. It was evident that ultra-HDP resulted in the lowest levels of most important nutrients such protein, carbohydrates, energy and some minerals. However, ultra-HDP managed to produce increased levels of Zn, demonstrating that even under stressful conditions this density could be able to maintain high levels of Zn, especially where Zn intake is prioritized. Young leaves, on the other hand, significantly increased the contents of important nutrients, demonstrating the importance of leaf age in determining the nutritional quality of *M. oleifera*.

This study further highlighted that planting density and leaf age significantly affected the amino acid concentrations of *M. oleifera*. Young leaves obtained the highest contents of most amino acids, especially under conventional planting density. However, ultra-HDP produced increased levels of proline content in young leaves, indicating stress responses. These results suggest that, although ultra-HDP might improve the number of plants per unit area, conventional planting densities must be taken into consideration for a better overall nutrient profile. It is, therefore, important to harvest at an appropriate time and further research is required to determine whether improved management practices can reduce nutrient losses at ultra-HDP.

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Chapter 5: Phytochemical response of *Moringa oleifera* to ultra-high density planting and leaf age

Abstract

Moringa oleifera is renowned for its diverse secondary metabolites, particularly in regions where combating disease is a priority. It is crucial to understand agronomic practices that promotes metabolite synthesis. This study assessed how the phytochemical composition of *M. oleifera* leaves was affected by the meter square ultra-high density planting (ultra-HDP) technology. It evaluated the effect of four planting densities (ultra-HDP, high, medium, and conventional densities) and leaf ages (young and mature) on phytochemical composition in the leaves. Following harvest, the leaves were analyzed for ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) polar untargeted metabolites, biochemical compounds (total phenols and total carotenoids), and antioxidant activities (total phenols and total carotenoids), and antioxidant activities, including 2,2-diphenyl-1-picrylhydrazyl (DPPH), Ferric Reducing Antioxidant Power (FRAP), and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)) which showed significant effects across all parameters ($p \leq 0.05$).

Key untargeted metabolites found included benzyl beta-primeveroside, gentisic acid 5-O-glucoside, cis-3-hexenyl beta-primeveroside, and glucomoringin. Heatmaps and variable importance in projection (VIP) scores both showed that ultra-HDP upregulated these metabolites. Ultra-high density planting also produced higher total phenolic content (1260.9 ± 3.12 mg GAE/100 g DW), and total carotenoid content (0.06 ± 0.01 mg β-carotene/100 g DW) compared to conventional density. Leaf age significantly influenced phytochemical content: young leaves resulted in higher total phenolic content (1035.5 ± 2.87 mg GAE/100 g DW) and carotenoid content (0.06 ± 0.0 mg mg β-carotene/100 g DW) than mature leaves. The combination of ultra-HDP with young leaves resulted in the highest phenolic content, while mature leaves from conventional density planting had the lowest phenolic content. Antioxidant activity, as determined by DPPH, ABTS, and FRAP assays, increased in young leaves of plants from ultra-HDP, though the DPPH activity

was significantly higher in mature leaves. These findings highlight the need of balancing planting density and leaf age in order to increase *M. oleifera* phytochemical production, providing useful insights for enhancing cultivation approaches.

5.1 Introduction

Phytochemicals refer to non-nutritious secondary metabolites which are indirectly essential for the plant's immediate survival (Yang *et al*., 2018). Different from primary metabolites, which are important for development and growth, secondary metabolites have roles in plant defense, signaling, and adaptability to environmental stress (Pang *et al*., 2021; Yang *et al*., 2018). These chemically varied substances, which come from primary metabolites or intermediates in primary metabolic pathways, contribute to the colour, taste, and scent of the plant (Piasecka *et al*., 2015; Pang *et al*., 2021). Moreover, they play a crucial role in how the plant reacts to biotic and abiotic stressors, serving as defenses against infections, herbivores, and even competing plants (Yang *et al*., 2018; Jan *et al*., 2021). Many of these secondary metabolites, such as polyphenols, and carotenoids are known to exert antioxidants and anti-inflammatory health benefits to plants and humans, highlighting the relevance of researching their production in plants (Fakhri *et al*., 2022). No single method fully captures antioxidant capacity, multiple assays, such as 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and Ferric Reducing Antioxidant Power (FRAP), are often employed to provide a comprehensive evaluation of antioxidant potential in plant matrices (Nobossé *et al*., 2018; González-Romero *et al*., 2020; Wang *et al*., 2015).

Prior research on *Moringa oleifera* has revealed that it is rich in various secondary metabolites, including flavonoids, steroids, phenolic acids (gallic, vanillic and chlorogenic acids), alkaloids, anthraquinones, tannins, glucosinolates and terpenes (Goyal *et al*., 2007; Lin *et al*., 2019). Furthermore, studies show that *M. oleifera*'s phytochemical profile varies with environmental conditions, influencing both the type and levels of secondary

metabolites generated (Gopalakrishnan *et al*., 2016; Lin *et al*., 2019). This implies that certain conditions might be improved to increase the desired secondary metabolites in *M. oleifera*, which is valued for its therapeutic and nutritional qualities.

Biotic factors, including genetic characteristics and environmental factors such as genetic characteristics, humidity, soil nutritional availability, temperature, and light, all affect how these secondary metabolites are biosynthesized (Li *et al*., 2020; Selwal *et al*., 2023). Under stressful conditions, plant cell membrane receptors activate signaling pathways that cause secondary metabolite formation, a process adaptable through techniques like suspension culture, elicitation, and genetic engineering to improve metabolite production (Atanasov *et al*., 2015; Niazian *et al*., 2021; Fazili *et al*., 2022). For example, Shaaban and Maher (2016) discovered increased phenolic content in Moringa callus when subjected to drought-like conditions, indicating how stress can induce metabolite accumulation.

Planting density is one of the agricultural practices that has the potential to alter secondary metabolite levels. Research indicated that competition from high density planting might cause stress, which causes phytohormones, antioxidants, and stress-resistance chemicals to accumulate, as observed in *Panax notoginseng* (Liu *et al*., 2021). However, studies specifically examining *M. oleifera* plants grown at ultra-HDP conditions are still lacking. This study aims to address this research gap by investigating effect of the meter square ultra-HDP technology on the production of secondary metabolite of *M. oleifera*, with the goal of understanding whether density-induced stress could optimize synthesis of valuable compounds.

5.2 Materials and methods

5.2.1 Sample collection and analysis

Samples used for these analyses were collected as described in Section 3.2.3. The untargeted metabolites were analyzed at Stellenbosch University within the Central Analytical Facilities (CAF). Bioactive compounds were analyzed at the University of Limpopo Green Biotechnology Research Center of Excellence Laboratory.

5.2.2 Treatments, design, and sample preparation

The treatments, experimental design, and sample preparation followed the procedures detailed in Section 4.2.2.

5.2.3 Analysis of polar untargeted metabolites and biochemical compounds

5.2.3.1 Extraction of untargeted polar metabolites for UPLC analysis

To extract untargeted polar metabolites, 2 g of the *M. oleifera* leaf sample was placed in a 50 mL screw-cap centrifuge tube, and 15 mL of a 50% methanol and 1% formic acid solution was added. The mixture was vortexed for one minute after the tube was securely closed and then extracted using ultrasonic technology for an hour. At 14.000 rpm, a 2 mL aliquot of the extract was centrifuged for 5 minutes. The supernatant was then carefully poured into 1.5 mL glass vials for additional analysis.

5.2.3.1.1 Analysis of untargeted metabolites using UPLC

The method described by Mpai and Sivakumar (2020) was followed in the preparation of samples for the UPLC-QTOF-MS analysis of phenolic metabolites. A combination of Waters Synapt G2 QTOF mass spectrometer and a Waters Acquity UPLC system was utilized for untargeted metabolite analysis. A Photodiode Array (PDA) detector was positioned ahead of the mass spectrometer, enabling the concurrent acquisition of both ultraviolet (UV) and mass spectrometry (MS) data.. For sensitivity and resolution, the MS settings were optimized. Electrospray ionization was conducted in negative mode with the following settings: desolvation gas flow at 650 L/h, desolvation temperature set to 275°C, and a cone voltage of 15V. Data acquisition, covering a mass range of m/z 150–1500, was carried out in both resolution and MSE modes.. For fragmentation data, the MSE mode used two data channels: one with a collision energy ramp (40–100 V) and the other at low collision energy (4 V). Using leucine enkephalin as a lock mass and sodium formate for equipment calibration, an accurate mass determination was performed. A 2 µL injection volume and a Waters HSS T3 column (2.1 × 100 mm, 1.7 µm) were utilized for the separation. Formic acid in water (solvent A) and acetonitrile (solvent B) at concentrations of 0.1% each made up the mobile phase. The gradient that was used consisted of a 5-minute wash phase at 100% solvent B, a 12-minute linear transition to 28% solvent B over 22 minutes, reaching 40% B in 50 seconds, and a 4-minute re-equilibration to the initial conditions. With a fixed flow rate of 0.3 mL/min, the column was kept at 55°C. Relative quantification of the components was performed using quercetin standards (Li *et al*., 2023).

5.2.3.2 Extraction of biochemical compounds

A 100 mg quantity of *M. oleifera* leaf powder was carefully measured and placed in a 2 mL amber-colored microcentrifuge tube to prevent light exposure. After each tube received 1 mL of 80% aqueous grade methanol, the contents were vortexed to achieve a thorough mixing. Throughout the process, the tubes were sealed and wrapped in

aluminium foil to protect them from light. The samples were ultrasonically processed for 60 minutes in order to extract free, soluble phenolic compounds. After sonication, the samples were centrifuged for 5 minutes at 20,000 x g to separate the phenolic-rich supernatant from solid residues.

5.2.3.2.1 Total phenols content

The total phenols content of the extracts was quantified using a modified Folin-Ciocalteu assay, following the protocol outlined by Apea-Bah *et al*. (2020). Gallic standard was generated at values ranging from 0 to 800 mg/mL. The standards were made by weighing one mg of gallic acid into different one mL centrifuge tubes. These stock solutions were serially diluted to achieve a final volume of 500 µL for each concentration. A 96-well microplate was prepared by filling each well with 18.2 µL of either the extract or the standard solution. Each well was first treated with 36.4 µL of 10% (v/v) Folin-Ciocalteu reagent, followed by the addition of 145.4 µL of 700 mM sodium carbonate. The prepared mixtures were then incubated in the dark at room temperature (20–25°C) for 2 hours. After incubation, absorbance was recorded at 750 nm using a microplate reader. The total phenolic content of the extracts was expressed as gallic acid equivalents (mg GAE/g) per gram of dry milled sample.

5.2.3.2.2 Total carotenoid content

The total carotenoid content of the extracts was determined following the protocol described by Kaseke et al. (2020). Briefly, 0.2 g of M. oleifera leaf samples was dissolved in 2 mL of hexane, followed by the addition of 0.5 mL of 0.5% (w/v) sodium chloride (NaCl) solution. To facilitate phase separation, the mixture was vortexed for 30 seconds to ensure thorough mixing and then centrifuged at 1500 rpm for 10 minutes. The absorbance of the resulting clear supernatant was measured at 460 nm using a UV-visible spectrophotometer. The total carotenoid content was expressed as mg β-carotene per 100 g of the sample.

5.2.3.2.3 2,2-diphenyl-1-picrylhydrazyl

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of the samples was assessed utilizing the protocol outlined by Apea-Bah *et al*. (2020), which involved the preparation of a 60 µmol/L DPPH solution by dissolving 1.2 mg of DPPH in 30 mL of methanol. This assay assessed the capacity of the sample extracts to counteract the oxidative activity of DPPH-generated free radicals. Prior to the addition of 195 µL of 60 µmol/L DPPH solution, each microplate well received 5 µL of sample extract or Trolox standard solution. Following a 60-minute dark incubation period, absorbance measurements were obtained at 515 nm. The DPPH scavenging activity of the samples was given as micromoles of Trolox equivalents per gram (µmol TE/g) of the dry-weight sample.

5.2.3.2.4 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)

The antioxidant capacity of the samples was assessed using the Trolox Equivalent Antioxidant Capacity (TEAC) assay, following the method described by Apea-Bah et al. (2016). In separate sterile 2 mL amber centrifuge tubes, 1.62 mg of potassium persulfate and 8.23 mg of 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were weighed. Stock solutions were then prepared by dissolving potassium persulfate (6 mM) and ABTS (16 mM) in 1 mL of distilled water, followed by vortexing to ensure complete dissolution. For the creation of the ABTS stock solution, equal parts of these solutions were mixed, causing the solution to darken quickly. The mixture was then incubated for a period of 12 to 16 hours in the dark. For the working solution of ABTS to be obtained, the stock solution was incubated and then diluted with 200 mM PBS 30 times. A working solution containing concentrations of 0.1 mM potassium persulfate and 0.27 mM ABTS was prepared. The preparation included the dilution of 2 mL ABTS stock solution with 58 mL of PBS. A 96-well microplate was prepared for the assay by adding to each well, 190 µL of ABTS working solution, followed by the addition of 10 µL of diluted extract. A microplate reader was used to measure absorbance at 750 nm following a 60-minute

incubation period for the reaction solutions. The ABTS radical scavenging activity of the samples was shown as micromoles of Trolox equivalents per gram (μmol TE/g) of dry-weight sample.

5.2.3.2.5 Ferric Reducing Antioxidant Power

The Ferric Reducing Antioxidant Potential (FRAP) experiment was conducted using the method outlined by Llorach *et al*. (2004). The assay used a newly developed FRAP reagent. A solution was prepared by combining 25 mL of acetate buffer (0.3 M, pH 3.6) with 2.5 mL of ferric chloride ($FeCl_3 \cdot 6H_2O$) (20 mM) and 2.5 mL of TPTZ (10 mM) diluted in 40 mM hydrochloric acid. A 4:1 v/v methanol: water solution was used to homogenize a 5 g sample of fresh leaf pulp. Initiation of the reaction involved blending 50 μL of sample mixture with 950 μL of FRAP solution, previously heated to 37°C. The antioxidant capacity was measured and reported in μmol TEAC (Trolox Equivalent Antioxidant Capacity) per gram of dry weight (DW).

5.3 Statistical analysis

Data were analyzed using a two-way analysis of variance (ANOVA) with Statistix 10.0 to assess the effects of planting density and leaf age, as well as their interaction, on the studied variables. Mean comparisons were performed at a 5% significance level ($p \leq 0.05$) using Tukey's Honestly Significant Difference test. Principal Component Analysis (PCA), Partial Least Squares Discriminant Analysis (PLS-DA), Variable Importance in Projection (VIP) analysis, and heat map generation were performed using MetaboAnalyst version 6, released on July 22, 2024.

5.4 Results and discussion

5.4.1 Results

5.4.1.1 Effect of planting density and leaf age on the untargeted metabolites of *Moringa oleifera* young and mature leaves

The current study identified a wide range of untargeted metabolites present in the *M. oleifera* leaves, including flavonoids, phenolic acids, fatty acyl glycosides, amino acids, O-glycosyl compounds, terpenoids, and other specialized metabolites (Table 5.1). Among the phenolic acids, chlorogenic acid derivatives were prominent, with two isomers detected. The first eluted at retention time: 4.51 min, Pseudomolecular ion (*m/z*): 353.0882, while the second was detected at retention time: 5.53 min, with Pseudomolecular ion (*m/z*): 353.08844. The predominated phenolic acid (chlorogenic acid) was also the most identified in *M. oleifera* leaves. Another significant phenolic acid, 3-O-p-Coumaroylquinic acid, was identified at retention time: 5.24 min, with Pseudomolecular ion (*m/z*): 337.09354.

Flavonoids represented the largest group of metabolites, with distinct peaks for quercetin derivatives, such as quercetin 3-O-(6''-acetyl-glucoside), as well as quercetin 4'-glucoside. Kaempferol derivatives, which are also important for plant defense, were identified, including kaempferol 3-(6-acetylgalactoside), along with other kaempferol glucosides. Additionally, isorhamnetin 7-glucoside was detected as well as rutin was identified.

Fatty acyl glycosides, such as (S)-10,16-dihydroxyhexadecanoic acid and 13-L-hydroperoxylinoleic acid, were also detected. O-glycosyl compounds, including benzyl beta-primeveroside at retention time: 6.19 min,, were detected. Three isomers of Lucuminamide, a unique glycosyl compound, were identified: the first at retention time: 3.154 min, with Pseudomolecular ion (*m/z*): 444.15128 and fragment ion (m/z): 426.1405, the second at retention time: 3.251 min, with Pseudomolecular ion (*m/z*): 444.15152 and fragment ion (m/z): 440.18935, and the third at retention time: 3.367 min, with

Pseudomolecular ion (*m/z*): 444.15179 and fragment ion (m/z): 436.2604. The identified terpenoids included glucosyl 10,11-dihydroxyfarnesadienoate

These findings underline the biochemical diversity of *M. oleifera* leaves and highlight the presence of metabolites associated with plant defense, stress adaptation, and potential health benefits. Furthermore, this study represents the first report on the metabolite profile of *M. oleifera* leaves harvested from different planting densities and leaf ages, providing novel insights into the effects of ultra-HDP on secondary metabolite composition.

Table 5.1: Phytochemical composition of *Moringa oleifera* leaves identified through UPLC-MS untargeted metabolomics

Retention time (min)	Pseudomolecular ion (*m/z*)	Tentative identification	Formula	Fragment ion (*m/z*)
Phenolic compounds				
5.80	367.10403	3-O-Caffeoyl-4-O-methylquinic acid	$C_{17}H_{20}O_9$	134.03723:621
5.24	337.09354	3-O-p-Coumaroylquinic acid	$C_{16}H_{18}O_8$	163.03992:239
3.22	379.03455	4-hydroxybenzoic acid 4-(6-O-sulfo) glucopyranoside	$C_{13}H_{16}O_{11}S$	379.03375:962
4.52	353.0882	Chlorogenic acid	$C_{16}H_{18}O_9$	135.0452:3243
5.53	353.08844	Chlorogenic acid	$C_{16}H_{18}O_9$	191.0564:527
3.88	315.07208	Gentesic acid 5-O-glucoside	$C_{13}H_{16}O_9$	315.07196:713
3.76	390.11954	Glycoperine	$C_{19}H_{21}NO_8$	244.02843:446
7.50	563.22479	Methyl2-{6'-ethyl-5-[(6-hydroperoxy-trihydroxyoxan-2-yl) oxy]-oxo-spiro[indole-indolizin]}-methoxyprop-2-enoate	$C_{27}H_{36}N_2O_{11}$	165.06696:179
3.63	599.16223	Salicylic acid beta-D-glucoside	$C_{13}H_{16}O_8$	137.02388:103
Flavonoids				
8.30	505.09857	4''-O-Acetylmyricitrin	$C_{23}H_{22}O_{13}$	187.04086:907
8.39	607.13013	6''-(4-Carboxy-3-hydroxy-3-methylbutanoyl) hyperin	$C_{27}H_{28}O_{16}$	271.02527:702
8.47	593.15222	Astragalin 7-rhamnoside	$C_{27}H_{30}O_{15}$	284.03247:359
9.44	519.1131	Isorhamnetin 3-(2''-acetylglucoside)	$C_{24}H_{24}O_{13}$	199.03955:131
8.99	477.10251	Isorhamnetin 7-glucoside	$C_{22}H_{22}O_{12}$	243.03023:452
6.43	593.15118	Isovitexin 2''-O-glucoside	$C_{27}H_{30}O_{15}$	267.06598:530
9.13	489.1055	Kaempferol 3-(6-acetylgalactoside)	$C_{23}H_{22}O_{12}$	213.01926:351
9.74	489.10327	Kaempferol derivatives	$C_{23}H_{22}O_{12}$	284.0325:6908
8.70	447.09515	Orientin	$C_{21}H_{20}O_{11}$	183.04527:126
5.85	595.16663	Pinobanksin 5-[galactosyl-(1->4)-glucoside]	$C_{27}H_{32}O_{15}$	385.09344:790
8.55	505.09729	Quercetin 3-O-(6''-acetyl-glucoside)	$C_{23}H_{22}O_{13}$	243.02998:243
8.90	505.09946	Quercetin 3-O-(6''-acetyl-glucoside)	$C_{23}H_{22}O_{13}$	199.04005:490

7.98	463.08759	Quercetin 4'-glucoside	$C_{21}H_{20}O_{12}$	187.03993:786
7.77	609.14783	Rutin	$C_{27}H_{30}O_{16}$	227.0351:6732
Amino acids				
3.03	430.17206	2-amino-1-[2-hydroxy-3-(4-methoxyphenyl) propyl]-9-[4-hydroxy-5-(hydroxymethyl) oxolan-2-yl]-6,9-dihydro-1H-purin-6-one	$C_{20}H_{25}N_5O_6$	282.08411:366
4.03	397.1611	2-amino-4-{[2-({[2,3-dihydroxy-2-(1-hydroxyethyl) butanoyl] oxy} methyl) phenyl] carbamoyl} butanoic acid	$C_{18}H_{26}N_2O_8$	135.04585:732
3.80	202.07167	7a-Epialexaflorine	$C_8H_{13}NO_5$	202.07172:242
3.77	164.07153	L-Phenylalanine	$C_9H_{11}NO_2$	164.07202:455
3.53	267.06229	Portulacaxanthin III	$C_{11}H_{12}N_2O_6$	267.06161:124
5.27	441.15234	UNPD42237	$C_{20}H_{30}N_2O_5S_2$	337.0929:7614
Fatty acyl glycosides				
12.02	287.22299	(S)-10,16-Dihydroxyhexadecanoic acid	$C_{16}H_{32}O_4$	128.01036:892
13.23	311.22281	13-L-Hydroperoxylinoleic acid	$C_{18}H_{32}O_4$	128.00941:831
11.76	329.23227	9,12,13-TriHOME	$C_{18}H_{34}O_5$	128.00963:581
7.44	439.18158	cis-3-Hexenyl b-primeveroside	$C_{17}H_{30}O_{10}$	300.02939:411
11.37	327.21664	Corchorifatty acid F	$C_{18}H_{32}O_5$	271.02509:687
12.38	315.18127	D-Linalool 3-glucoside	$C_{16}H_{28}O_6$	128.01062:712
13.42	343.21286	Gallicynoic acid F;(-)-Gallicynoic acid F	$C_{18}H_{32}O_6$	128.0099:1024
O-glycosyl compounds				
6.19	401.14575	Benzyl beta-primeveroside	$C_{18}H_{26}O_{10}$	301.03482:810
3.73	399.15033	Isopropyl apiosylglucoside	$C_{14}H_{26}O_{10}$	389.1203:325
3.15	444.15128	Lucuminamide	$C_{19}H_{27}NO_{11}$	426.1405:435
3.25	444.15152	Lucuminamide	$C_{19}H_{27}NO_{11}$	444.15155:729
3.37	444.15128	Lucuminamide	$C_{19}H_{27}NO_{11}$	436.2604:521
2.17	290.08856	Pyroglutamic acid hexoside	$C_{11}H_{17}NO_8$	128.03529:112
4.90	382.11414	UNPD114682	$C_{17}H_{21}NO_9$	264.05063:406
Terpenoid compounds				

5.88	612.39246	6-{4,7-dihydroxy-5,10,10-trimethyl-11,21 dioxopentacyclodocos-6(22)-en-2-yl}-5-[(ethylamino)methyl]-4-hydroxy-2-methylhept-2-enoic acid	$C_{36}H_{55}NO_7$	195.03294:524
9.82	651.26813	Dicrocin	$C_{32}H_{44}O_{14}$	284.03183:472
11.83	431.22852	Glucosyl (2E,6E,10x)-10,11-dihydroxy-2,6-farnesadienoate	$C_{21}H_{36}O_9$	128.01035:586
9.29	489.10645	MINEs-366389	$C_{20}H_{26}O_{12}S$	146.96591:690
Tyrosols and derivatives				
4.62	342.11948	N-(1-Deoxy-1-fructosyl) tyrosine	$C_{15}H_{21}NO_8$	332.09088:541
4.36	238.07298	Phomonitroester	$C_{11}H_{13}NO_5$	238.07285:161
Tannins				
3.50	331.06647	Beta-Glucogallin	$C_{13}H_{16}O_{10}$	331.06702:212
Phenyl				
2.65	737.23651	7-[3,5-dihydroxy-12-(4-methoxyphenyl)-1,4,14-trimethyl-16-oxo-8-oxa-12,13-diazatetracyclohexadecane]-1-[2-(5-methoxyindolyl) ethyl]-pyrido[2,3-d] pyrimidine-2,4-dione	$C_{41}H_{34}N_6O_8$	545.17737:318
Glucosinolates				
3.49	570.09607	Glucomorigin	$C_{20}H_{29}NO_{14}S_2$	328.0856:1177
Heterocyclic compounds				
3.17	441.1261	CNP0449137	$C_{18}H_{26}N_4O_5S_2$	341.1084:606 4
Furofurans				
5.028	384.13016	6-[4-(methoxymethyl)-1H-1,2,3-triazol-1-yl] hexahydrofuro [3,2-b] furan-3-ylN-(3-cyanophenyl) carbamate	$C_{18}H_{19}N_5O_5$	359.11154:317
Vitamin B5				
4.022	218.10287	Pantothenic acid	$C_9H_{17}NO_5$	135.04478:133
O-glucuronides				
5.05	427.13605	Lacosamide-glucuronide	$C_{18}H_{24}N_2O_{10}$	427.13666:537
Others				
4.04	515.14044	Scopoletin 7-O-beta-D-sophoroside	$C_{22}H_{28}O_{14}$	179.03557:833

13.94	721.36493	(S)-Nerolidol3-O-[a-L-Rhamnopyranosyl-(1->4)-a-L-rhamnopyranosyl-(1->2)-b-D-glucopyranoside]	$C_{33}H_{56}O_{14}$	212.98749:514
13.95	675.35919	(S)-Nerolidol 3-O-[a-L-rhamnopyranosyl-(1->4)-a-L-rhamnopyranosyl-(1->6)-b-D-glucopyranoside]	$C_{33}H_{56}O_{14}$	212.9884:5613
5.48	325.09384	1-O-p-Coumaroyl-beta-D-glucose	$C_{15}H_{18}O_8$	119.05003:149
7.16	461.16644	Methyl 3-{2-[2-(2-ethoxy-2-oxoethyl)-3-oxopiperazin-1-yl] acetamido}-1H-indole-2-carboxylate	$C_{20}H_{24}N_4O_6$	300.02863:676
7.16	415.16107	Methyl 3-{2-[2-(2-ethoxy-2-oxoethyl)-3-oxopiperazin-1-yl] acetamido}-1H-indole-2-carboxylate	$C_{20}H_{24}N_4O_6$	300.02863:676
8.97	579.20074	UNPD168838	$C_{35}H_{32}O_8$	227.03461:841
2.85	476.17725	UNPD196040	$C_{20}H_{31}NO_{12}$	466.14966:338
7.60	769.24939	UNPD95029	$C_{34}H_{46}N_2O_{16}S$	393.07297:6
4.78	504.17276	UNPD203257	$C_{20}H_{29}NO_{11}$	150.05724:704

A principal component analysis (PCA) was utilized to characterize the diversity in the untargeted metabolites of *M. oleifera* leaves based on leaf age and planting density (Figure 5.1A). The score plot showed a clear distinction between young leaves (T1N, T2N, T3N, and T4N) and mature leaves (T1O, T2O, T3O, and T4O) along the horizontal axis (PC1), which accounted for 65.4% of the total variation. This suggests that leaf age was the primary factor influencing the metabolite profiles of the samples. Young leaves were grouped on the positive side of PC1, while mature leaves were grouped on the negative side.

Planting density also influenced variability, but to a lesser extent, observable along the vertical axis (PC2), accounting for 15.4% of the variation. The high density (T2O) was interestingly located nearer the center of the PCA plot, indicating a moderate influence of planting density. These samples shared characteristics with both ultra-HDP and conventional densities, implying that high density could provide a more balanced development environment of plants by reducing extremes in resource competition and environmental stress. In summary, the PCA analysis highlights the significance of both planting density and leaf age in determining the chemical and metabolic profiles of *M. oleifera*

To further explore the differences in the untargeted metabolite profiles of *M. oleifera*, Partial Least Squares Discriminant Analysis (PLS-DA) was performed. Based on planting density and leaf age, the PLS-DA analysis clearly shows metabolic differentiation across the samples (Figure 5.1B). The scores plot shows distinct clusters for young (N) and mature (O) leaves across all planting densities (T1 to T4), with the PC1 showing variance driven primarily by planting density. Secondary variation, captured by PC2, was likely influenced by leaf age. Samples from the ultra-HDP (T1O) are distinctly separated from those of the other densities, indicating significant metabolic differences driven by planting density. Furthermore, the separation of young and mature leaves within each density emphasizes the role of leaf age in metabolic variance, however to a smaller extent than planting density.

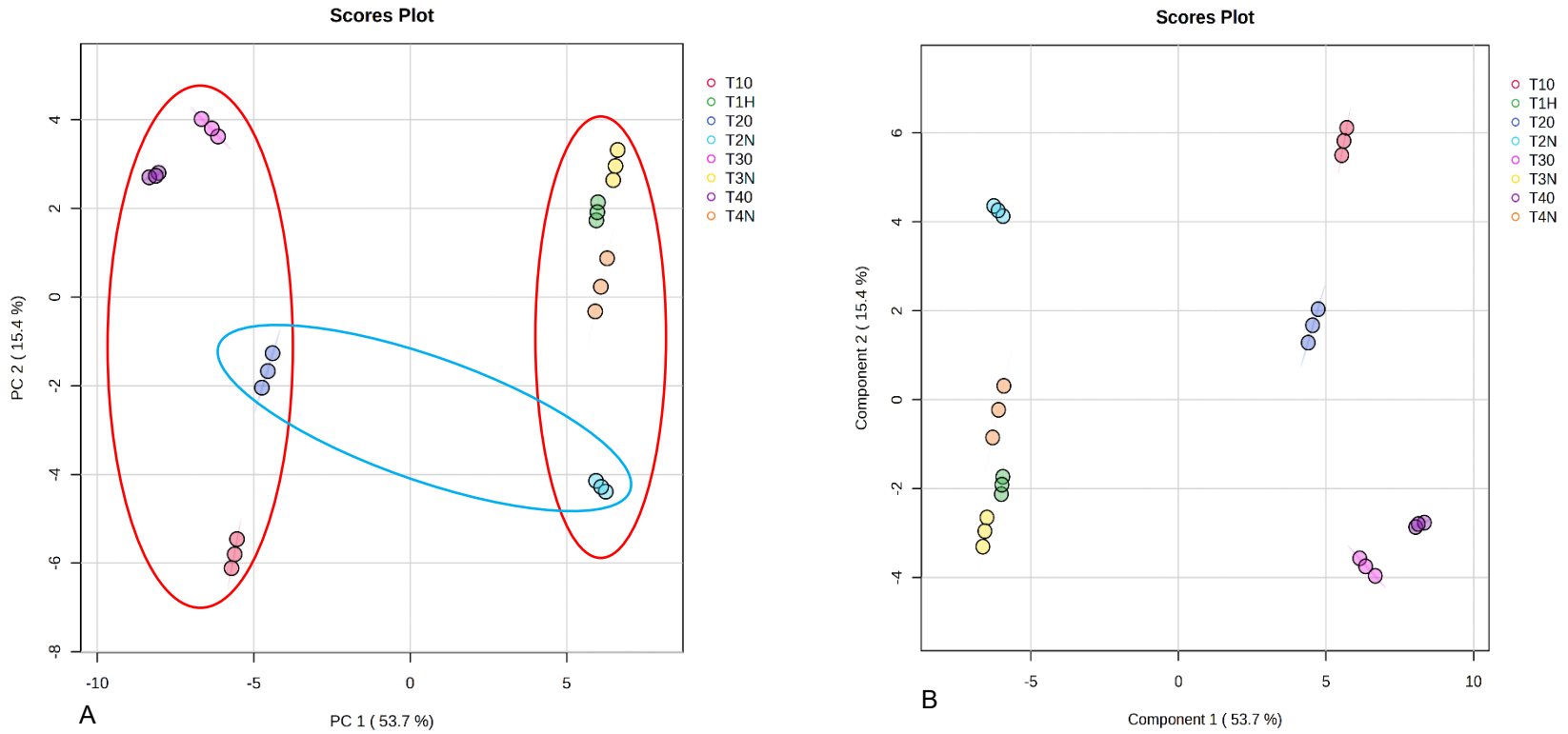

Figure 5.1: Principal Component Analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA) of *Moringa oleifera* leaves under varying planting densities and leaf ages. The data were mean-centered and Pareto-scaled, with ellipses in the PCA score plots representing Hotelling's T^2 at a 95% confidence interval. The models were computed using datasets comprising 96 metabolite features from *Moringa oleifera* leaves. A= PCA; B= PLS-DA; T1O= Ultra-HDP mature; T1N= Ultra-HDP young; T2O= High density mature; T2N= High density young; T3O= Medium density mature; T3N= Medum density young; T4O= Conventional density mature; T4N= Conventional density young.

Blue denotes downregulated metabolites, while red denotes upregulated metabolites on the VIP scores (Liu *et al*., 2022). The variable importance in projection (VIP) score indicated that the mature leaves were more prevalent in untargeted metabolites compared to young leaves (Figure 5.2). These observations revealed significant differences in the metabolite profiles of the samples. The metabolites that were upregulated under the ultra-HDP include benzyl beta-primeveroside, Chlorogenic acid and gentesic acid 5-O-glucoside especially in mature leaves. Other planting densities also upregulated some metabolites including 4-hydroxybenzoic acid 4-(6-O-sulfo) glucopyranoside, chlorogenic acid and gentesic acid 5-O-glucoside.

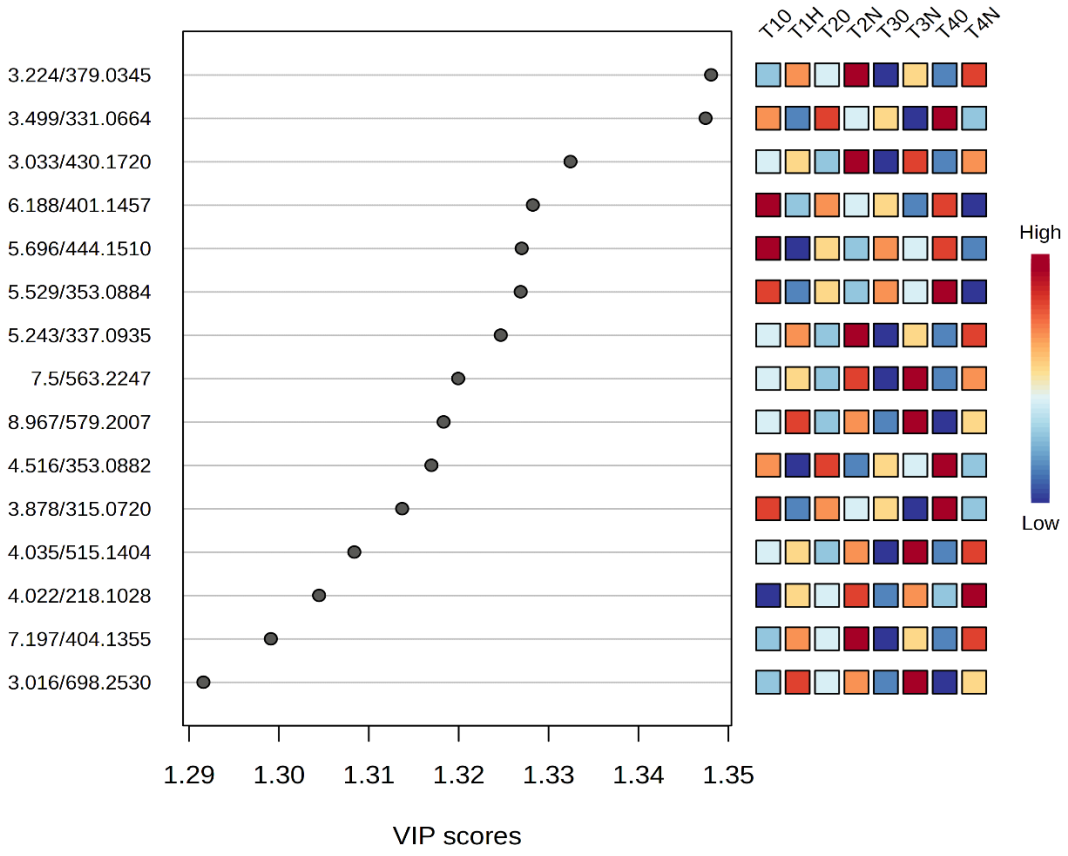

Figure 5.2: Variable importance in projection (VIP) of *Moringa oleifera* leaves showing effects of planting density and leaf age on untargeted metabolites. T1O= Ultra-HDP mature; T1N= Ultra-HDP young; T2O= High density mature; T2N= High density young;

T3O= Medium density mature; T3N= Medum density young; T4O= Conventional density mature; T4N= Conventional density young.

The heatmap shows the distribution of differentially expressed metabolites across samples, with rows representing samples and columns representing metabolites. Blue denotes downregulated metabolites, and red denotes upregulated metabolites (Liu *et al*., 2022). These findings reveal significant differences in the metabolite profiles of the samples. Notably, young leaves had higher concentrations of untargeted metabolites compared to mature leaves (Figure 5.3).

Under ultra-high density planting, mature leaves showed elevated levels of specific metabolites, including beta-glucogallin (a tannin), benzyl beta-primeveroside (a glycosyl compound), and a sulfanilide derivative (methyl 3-{2-[2-(2-ethoxy-2-oxoethyl)-3-oxopiperazin-1-yl] acetamido}-1H-indole-2-carboxylate) (Figure 5.3). For young leaves, medium-density planting yielded higher concentrations of untargeted metabolites such as Rutin (a flavonoid) and an amino acid derivative (N-[(1S,3R,4aS,9aR)-1-(hydroxymethyl)-3-[2-(4-morpholinyl)-2-oxoethyl]-3,4,4a,9a-tetrahydro-1H-pyrano[3,4-b]benzofuran-6-yl] methane sulfonamide). Conversely, under conventional planting density, mature leaves exhibited the highest levels of kaempferol derivatives, a class of flavonoids.

These findings highlight the significant impact of planting density and leaf age on the metabolite composition of *M. oleifera*. The results suggest that specific planting strategies can be optimized to enhance the accumulation of desired metabolites in either young or mature leaves.

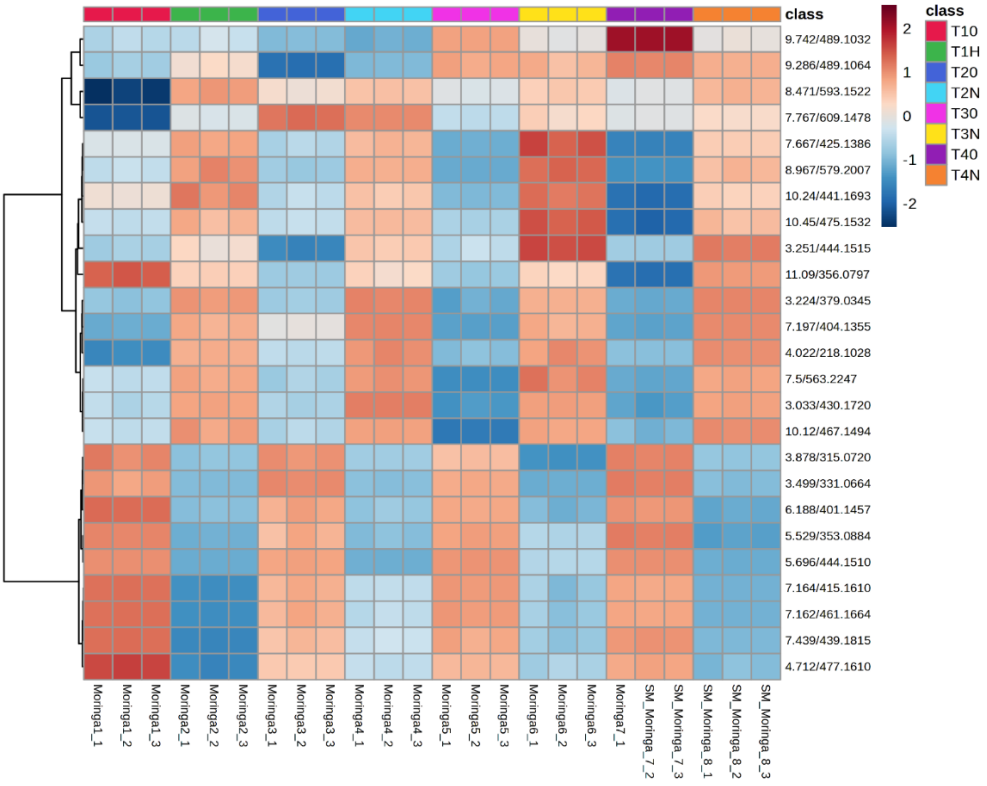

Figure 5.3: Heatmap illustrating the effects of planting density and leaf age on the abundance of untargeted metabolites in *Moringa oleifera* leaves. The data were derived from low-dimensional analysis based on PCA modeling, which highlights the sub-clusters present within the samples. T1O= Ultra-HDP mature; T1N= Ultra-HDP young; T2O= High density mature; T2N= High density young; T3O= Medium density mature; T3N= Medum density young; T4O= Conventional density mature; T4N= Conventional density young.

5.2.1.2 Effect of planting density, leaf age, and their interaction on biochemical compounds of *Moringa oleifera* leaves

5.2.1.2.1 Planting density effect on *Moringa oleifera* leaves

The total phenols results among the planting densities revealed that ultra-HDP (1260.9 ± 3.12 mg GAE/100 g DW) obtained the highest total phenols content in comparison to other studied densities such as high, medium and conventional densities. A similar trend

of the results was observed on carotenoid performance, whereby the ultra-HDP (0.06 ± 0.01 mg β-carotene/100 g DW) recorded higher total carotenoid content in comparison to the high, medium and conventional densities. The antioxidant capacity also followed this trend whereby the ultra-HDP (51.96 ± 0.4 µmol TE/g DW) obtained the highest under DPPH compared to high, medium and conventional densities. The similar results were observed under FRAP assay, whereby the ultra-HDP (3.85 ± 0.3 µmol TE/g DW) recorded the highest antioxidants compared to high, medium and conventional densities. Similar observations were made under ABTS assay whereby the ultra-HDP (0.29 ± 0.01 µmol TE/g DW) recorded the highest antioxidants compared to high, medium and conventional densities (Table 5.2).

5.2.1.2.2 Leaf age effect on *Moringa oleifera* leaves

The comparison between leaf ages also revealed a significant effect between mature and young leaves in terms of total phenols, total carotenoids, DPPH, FRAP, and ABTS antioxidant content. The total phenols content was higher in young leaves (1035.5 ± 2.87 GAE/100 g DW) than in mature leaves. Total carotenoid content also peaked in young leaves (0.06 mg β-carotene/100 g DW) compared to mature leaves. In contrast, DPPH indicated the highest antioxidant activity in mature leaves (49.86 ± 03 µmol TE/g DW) than in young leaves. Contrasting results were observed under FRAP assay whereby young leaves (3.10 ± 0.4 µmol TE/g DW) obtained the highest antioxidant activity than mature leaves. A similar trend was seen for ABTS content whereby young leaves obtained the highest antioxidant activity than mature leaves (Table 5.2).

5.2.1.2.3 Effect of planting density on the chemical composition of young and mature leaves of *M. oleifera*

The interaction between the planting density and leaf age was significant, as samples from the ultra-HDP and young leaves (1477.2 ± 3.31 GAE/100 g DW) contained the highest total phenols content, whereas the combination of conventional density and mature leaves produced lower total phenols content (Table 5.2). For total carotenoids, the combination of ultra-HDP and young leaves obtained the highest content, while the combination of conventional density and mature leaves produced lower carotenoid content. On the other hand, the combination of mature leaves and ultra-HDP had a greater DPPH antioxidant content. On the other hand, the combination of mature leaves and ultra-HDP had a greater DPPH antioxidant content than combination of conventional density and young leaves. A contrasting trend was observed in FRAP antioxidant activity whereby the combination of ultra-HDP and young leaves (4.70 ± 0.4 µmol TE/g DW) had greater antioxidant content than combination of conventional density and mature leaves. This trend was also observed in ABTS whereby the combination of ultra-HDP and young leaves (0.29 ± 0.01 µmol TE/g DW) obtained the higher content, while the combination of conventional density and mature leaves produced lower antioxidant content (Table 5.2).

Table 5.2: Effect of planting density and leaf age on the biochemical composition of *Moringa oleifera* Leaves

		TPC mg GAE/100 g DW	TCC mg β-carotene/100 g DW	DPPH µmol TE/g DW	FRAP µmol TE/g DW	ABTS µmol TE/g DW
Planting density (A)	Ultra-HDP	1260.9 ± 3.12^{a}	0.06 ± 0.01^{a}	51.96 ± 0.4^{a}	3.85 ± 0.3^{a}	0.29 ± 0.01^{a}
	HD	904.0 ± 3.01^{b}	0.04 ± 0.00^{b}	50.75 ± 0.2^{b}	3.09 ± 0.5^{b}	0.27 ± 0.01^{b}
	MD	814.4 ± 2.22^{c}	0.03 ± 0.00^{c}	46.65 ± 0.3^{c}	2.13 ± 0.2^{c}	0.24 ± 0.00^{c}
	C	804.6 ± 1.98^{d}	0.02 ± 0.00^{d}	46.04 ± 0.1^{c}	1.95 ± 0.3^{c}	0.22 ± 0.00^{d}
	HSD	3.94	3.83	0.69	0.60	3.85
Leaf age (B)	Young	1035.5 ± 2.87^{a}	0.06 ± 0.00^{a}	47.84 ± 0.3^{b}	3.10 ± 0.4^{a}	0.26 ± 0.01^{a}
	Mature	856.5 ± 2.54^{b}	0.02 ± 0.00^{b}	49.86 ± 0.3^{a}	2.40 ± 0.3^{b}	0.25 ± 0.01^{b}
	HSD	2.70	2.02	0.37	0.32	3.73
Young leaves	Ultra-HDP	1477.2 ± 3.31^{a}	0.08 ± 0.02^{a}	50.52 ± 0.3^{c}	4.70 ± 0.4^{a}	0.29 ± 0.01^{a}
	HD	964.8 ± 3.14^{c}	0.06 ± 0.01^{b}	49.56 ± 0.4^{c}	3.52 ± 0.3^{b}	0.27 ± 0.01^{b}
	MD	844.1 ± 2.78^{e}	0.04 ± 0.01^{c}	45.85 ± 0.4^{ef}	2.12 ± 0.3^{cd}	0.25 ± 0.00^{cd}
	C	843.1 ± 1.92^{e}	0.03 ± 0.00^{c}	45.43 ± 0.2^{f}	2.08 ± 0.4^{cd}	0.24 ± .0.1^{de}
Mature leaves	Ultra-HDP	1044.7 ± 3.15^{b}	0.03 ± 0.00^{d}	53.41 ± 0.3^{a}	2.99 ± 0.1^{bc}	0.28 ± 0.03^{ab}
	HD	855.8 ± 2.93^{d}	0.02 ± 0.00^{e}	51.91 ± 0.2^{b}	2.66 ± 0.2^{bcd}	0.26 ± 0.00^{c}
	MD	773.0 ± 2.01^{f}	0.01 ± 0.01^{f}	47.45 ± 0.1^{d}	2.13 ± 0.3^{cd}	0.23 ± 0.00^{e}
	C	765.1 ± 2.00^{f}	0.01 ± 0.00^{f}	46.65 ± 0.3^{de}	1.82 ± 0.4^{d}	0.21 ± 0.01^{f}
HSD	Interaction (A x B)	4.74	3.24	1.18	1.03	3.99

Values are expressed as means ± standard deviations (n = 3). Within each column, different superscript letters indicate significant differences ($p < 0.05$). Abbreviations: Ultra-HDP= Ultra-high density planting; HD= High density; MD= Medium density; C= Conventional density; TPC= Total phenols content; TCC= Total carotenoid content; GAE= Gallic acids equivalents; TE= Trolox equivalents.

5.4.2 Discussion

5.4.2.1 Identified untargeted metabolites of *Moringa oleifera* leaves

Moringa oleifera contains a significant amount of phytochemicals, including phenolic compounds and flavonoids, derived from phenylpropanoid compounds (Vogt, 2010). The synthesis of these compounds, including rutin, chlorogenic acid, and quercetin, is influenced by factors like planting density and leaf age. Studies have demonstrated that environmental factors, agronomic techniques, and genotype influence the bioactive component of vegetables (Biesiada *et al*., 2010). Increased planting density may cause competition for resources, triggering biosynthesis pathways that enhance antioxidant activity to mitigate stress (Naikoo *et al*., 2019).

This study identified rutin, which has been shown to increase antioxidant activity and photosynthetic rates of plants (Gorni *et al*., 2022). In line with these results, it has been observed that predominant flavonoid in *M. oleifera* leaf extracts was rutin, which demonstrates strong antityrosinase activity on par with kojic acid, a commercial inhibitor of melanin synthesis (El-Sharkawy *et al*., 2024). Chlorogenic acid, another important compound identified in this study and was found to have increased antioxidant activity of apples. It also possesses the ability to lower blood sugar levels in humans (Paikra and Gidwani, 2017; Mei *et al*., 2020).

The variable importance in projection (VIP) analysis found that ultra-HDP upregulated benzyl beta-primeveroside, a chemical implicated in plant defense and tea aroma quality necessary for human health (Ohgami *et al*., 2015). Ultra-high density planting also upregulated metabolites typically found in *Camellia sinensis*, key components of green tea (Ohgami *et al*., 2015). Furthermore, gentesic acid 5-O-glucoside's presence in ultra-HDP implies that biotic stress may initiate its synthesis, which is consistent with its established functions in plant defense, anticarcinogenic

activity, and fibroblast growth factor inhibition (Fernández *et al*., 2010; Sun *et al*., 2019; Nagai *et al*., 2020; Mechchate *et al*., 2021).

Heatmap analysis also revealed another metabolite associated with *Camellia sinensis*, cis-3-hexenyl beta-primeveroside (Jing *et al*., 2019). Other prominent compounds in ultra-HDP included benzyl beta-primeveroside, beta-glucogallin, glucomorigin, and gentesic acid 5-O-glucoside. Beta-glucogallin, a precursor to hydrolyzable tannins, has been associated with protective properties against infections, inflammation, hepatic damage, glaucoma, diabetes, and UV-induced skin damage (Khan *et al*., 2022; Rohilla *et al*., 2023). Similarly, glucomorigin has demonstrated potential in combating microbial infections, cancer, and tumors (Rajan *et al*., 2016; Jaafaru *et al*., 2018).

5.4.2.2 Effect of planting density on phytochemical composition of plant tissues

Planting density has a significant influence on plant population structure, increasing competition for the resources such as water, light, and nutrients (Wu *et al*., 2020). In this study, increased planting density resulted in higher contents of total phenols and carotenoids, along with enhanced antioxidant activity. Ultra-high density planting obtained the highest total phenolic content, consistent with the findings that high density planting increases phenolic concentrations in *Cynara cardunculus* L. and *Brassica oleracea* L. due to increased plant stress and resource competition (Lombardo *et al*., 2009; Kałużewicz *et al*., 2017). Similarly, increased planting densities were associated with high phenolic content (Danesi *et al*., 2014). This increase is probably the result of increased competition for resources, which triggers the synthesis of phenolic compounds as a defensive mechanism (Lombardo *et al*., 2009; Björkman *et al*., 2011).

Phenolic compounds, characterized as major secondary metabolites, play a major role in plant defense mechanisms due to their free radical scavenging properties (Tlili *et al*., 2014). Their abundance in fruits and vegetables leads to enhanced antioxidant capacity

of plants and provides several health benefits to humans, including the reduction of oxidative stress (Tlili *et al*., 2014).

Carotenoid accumulation followed a similar trend, with ultra-HDP producing the highest content. These results are in line with those of Ren *et al*. (2017), who showed higher carotenoid concentrations with denser planting. The higher total carotenoid content observed in this study may be related to shade under ultra-HDP, which has been proven to stimulate carotenoid accumulation, as evidenced by leaves of *Camellia sinensis* L. grown in shade (Fu *et al*., 2022). However, contrasting findings have been reported, with decreased carotenoid levels under high density planting in *Cucurbita pepo* L. and no significant differences in carotenoid content of *Stevia rebaudiana* Lam. across different planting densities (Bhattacharjee et al., 2023; Śniegowska and Biesiada, 2024)

Multiple assays are used to offer a thorough evaluation because no single assay can fully capture the antioxidant capacity of a complex plant matrix (Wang *et al*., 2015). Out of the three assays used in this study, ultra-HDP attained the highest antioxidant activity, suggesting that antioxidant biosynthesis may have been triggered as a stress response to increased resource competition. Similar results have been observed in *Ipomoea batatas*, where drought stress enhanced antioxidant capacity, and in various plant species where environmental stress stimulated antioxidant activity (Lin *et al*., 2006; Zhao *et al*., 2016). These findings suggest that increased planting density may induce stress in *M. oleifera*, leading to the enhanced production of bioactive compounds with antioxidant properties.

5.4.2.3 Effect of leaf age on phytochemical composition

Secondary metabolite accumulation in leaves varies with leaf age, with young leaves synthesizing these compounds more actively due to their increased metabolic activity and greater requirement for protection against biotic and abiotic stress (Reifenrath and Müller, 2007; Qaderi *et al*., 2023). The findings of this study revealed significantly higher levels

of secondary metabolites and antioxidants in young leaves compared to mature leaves. This aligns with previous research indicating that young *M. oleifera* leaves contain higher phenolic content than mature leaves (Jahan *et al*., 2015; Qadir *et al*., 2022). Similarly, young leaves have been shown to accumulate more phenolic compounds, likely as a mechanism for photoprotection (Zhang *et al*., 2018). However, some studies have reported contrasting results, with mature leaves obtaining higher phenolic content, possibly due to a distinct regulatory mechanism governing phenolic production (Anwar *et al*., 2017). A similar pattern was seen in total carotenoid content, with young leaves showing higher levels, possibly because young leaves absorb more carbon during photosynthesis, which increases the production of carotenoid pigments (Dhami et al. 2018. These results are further supported by the possibility that mature leaves have a lower capacity for carotenoid storage. However, increased carotenoid accumulation in mature leaves has been noted, suggesting that these findings may be influenced by environmental variables or plant-specific reactions (Mibei *et al*., 2017 and Qadir *et al*., 2022).

Antioxidant activity also showed variation with leaf age, whereby young leaves attained greater FRAP and ABTS activity, indicating a higher capacity to scavenge free radicals. This aligns with findings that young *M. oleifera* leaves demonstrate strong antioxidant activity, likely due to their higher phenolic and carotenoid content, which enhances antioxidant defense mechanisms (Nobossé *et al*., 2018; Dhakad *et al*., 2024).

Interestingly, DPPH scavenging activity was higher in mature leaves, possibly due to their increased chlorophyll content, as reported in Chapter 3. A strong association between DPPH scavenging activity and chlorophyll levels has been observed, highlighting the role of chlorophyll in antioxidant capacity (Nobossé *et al*., 2018). Similar trends have been reported in other plants, where ABTS scavenging activity decreases as fruits ripen, though in some species, ABTS activity has been shown to increase with maturation (Adedayo *et al*., 2010; Haruenkit *et al*., 2010). These findings confirm that young *M. oleifera* leaves are a potent natural source of antioxidants due to their high phytochemical content and strong free radical scavenging ability. Their antioxidant potential suggests

potential applications in reducing oxidative stress and managing various human health conditions (Dhakad *et al*., 2024).

While planting density and leaf age have independent effects on antioxidant activity and secondary metabolite accumulation in a number of crops, little is known about how these factors directly affect *M. oleifera*. Furthermore, in any crop system, the relationship between planting density and leaf age is largely unknown. The results of this investigation indicate that both elements interact in complex ways to affect the nutritional composition and functionality of *M. oleifera* leaves.

5.5 Conclusion

Findings of the present study revealed that both planting density and leaf age significantly influenced the antioxidant activity and phytochemical composition of leaves of *M. oleifera*. Ultra-high density planting increased the levels of total phenols and total carotenoids, as well as antioxidant activity, as determined by the DPPH, ABTS, and FRAP assays. This suggests a link between density-induced stress and enhanced phytochemical production. The accumulation of phytochemicals was also significantly influenced by leaf age, with young leaves showing a higher phytochemical content than mature leaves. On the other hand, mature leaves showed higher DPPH antioxidant activity, most likely as a result of their higher chlorophyll SPAD index and stress-adaptation mechanisms. Moreover, the interaction between planting density and leaf age indicated that the phytochemical content was most significant when ultra-HDP was combined with young leaves.

Additionally, ultra-HDP upregulated important untargeted metabolites such as benzyl beta-primeveroside and gentisic acid 5-O-glucoside, emphasizing its potential to enhance phytochemical production. These results suggest that young leaves are a more plentiful source of these beneficial compounds than mature ones, and that ultra-HDP is an appropriate planting density for enhancing phytochemicals and antioxidants.

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Chapter 6: Summary of findings, significance, recommendations and conclusions

6.1 Summary of findings

This study revealed the significant effects of meter square ultra-high density planting (ultra-HDP) on the growth, yield, and phytonutritional composition of *Moringa oleifera*. Planting density and leaf age were critical factors influencing growth, yield, nutrient content, and phytochemical accumulation. Ultra-HDP significantly enhanced fresh and dry leaf yield per meter square compared to conventional density. However, individual plant height, stem diameter, and leaf yield were highest under conventional planting systems, which also promoted nutrient-dense leaves, including the highest levels of calcium (1.22 ± 0.02%) and potassium (2.38 ± 0.01%).

Leaf age strongly influenced nutrient composition: young leaves contained higher protein content (28.36 ± 0.1%) and essential amino acids such as leucine (2.64 ± 0.05%), valine (1.77%), and phenylalanine (1.95 ± 0.3%), particularly under lower planting densities. Conversely, mature leaves exhibited higher fat levels. Non-essential amino acids like glutamic acid were more abundant in conventional density leaves. Zinc concentration was also highest under ultra-HDP, while conventional density supported greater calcium and magnesium accumulation. Phytochemicals, including total phenolic and carotenoid contents were increased under ultra-HDP due to stress-induced responses, with phenolic content reaching 1477.2 ± 3.31 mg GAE/100 g DW compared to 765.1 ± 2.0 GAE/100 g DW in conventional density.

6.2 Significance

The findings of this study provide insights into the cultivation of *M. oleifera* under ultra-HDP, offering options for enhancing yield and bioactive compound production. This research elucidates the impact of planting density and induced stress on growth, yield, and phytonutritional composition, presenting potential practical applications for farming.

Ultra-HDP technology significantly increases leaf yield and bioactive compound content, crucial for addressing human health and nutritional challenges. In contrast, conventional density promotes higher concentrations of essential nutrients, which may offer flexibility based on specific agricultural goals. This suggests that *M. oleifera* is adaptable to varying cultivation systems, making it an important crop for improving food security and addressing malnutrition, in resource-constrained environments. These findings pave the way for the development of agricultural techniques that optimize yield and quality while addressing global food and nutrition security challenges.

6.3 Recommendations

It is recommended to adopt ultra-HDP technology in areas where maximizing yield per unit area and phytochemical production are priorities. This technology is particularly advantageous in space-limited environments and can support small-scale farmers in resource-scarce regions to achieve higher production without compromising crop quality. Further research should explore the application of ultra-HDP to other nutrient-dense crops with potential to address health related issues and food insecurity. Investigating its effects under diverse environmental conditions, including water-scarce regions, could optimize its application. Additionally, long-term studies are needed to assess the effects of ultra-HDP on soil health and water management to ensure sustainability. This knowledge will enable the broader implementation of ultra-HDP for improving agricultural productivity and crop quality in various contexts.

6.4 Conclusion

This study demonstrated that planting density significantly influences its growth, yield, and phytonutritional attributes. Ultra-HDP increased leaf yield per meter square and phytochemical composition due to stress-induced responses, while conventional density favoured individual plant biomass and nutrient accumulation.

Leaf age also played a vital role, with young leaves containing higher protein, amino acids, and carbohydrates, while mature leaves obtained higher fat levels and other nutrient concentrations. These findings emphasize the importance of optimizing planting density and harvesting strategies to achieve desired outcomes. The adaptability of *M. oleifera* to diverse planting systems, coupled with its high nutritional and phytochemical composition, underscores its potential as a valuable crop contributing to addressing global food security and nutritional challenges. This study provides a foundation for optimizing *M. oleifera* cultivation to meet agricultural and dietary needs.