ANTIOXIDANTS COMPOSITION OF MORINGA (*MORINGA OLEIFERA LAM*) IN DIFFERENT PLANT ORGANS

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By

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DECLARATION

I, Fatima Abdelkarim Abdelrahman Mohammed, certify that this dissertation has not been submitted to any University and that it is my original work conducted under the supervision of Dr. S. Tesfay. All assistance towards the production of this work and all the references contained herein have been duly accredited.

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DEDICATION

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GENERAL ABSTRACT

Moringa oleifera Lam. is a versatile plant with various benefits; different parts of the plant have different pharmacological activity such as flavonoid, alkaloids, phenol, and carotenoids. The aim of the seed study was to investigate phytochemicals composition change and their utilization during seed and seedling germination. Seeds were germinated using three different temperature regimes (30/20 °C, 25/15 °C and 20/10 °C). Spectrophotometric determination of proline and phenols was carried out, while sugars were analyzed using HPLC-RID. The data were collected and analyzed using statistical software GenStat 14.1. Results of seed showed significant differences on speed of seed germination at temperature of 30/20°C followed by 25/15°C and 20/10°C respectively. Seed carbohydrates composition changes were observed with germination hours sucrose concentrations peaked within 24h (16 mg/g DW) and the same sugar showed sharp decrease for 96h (6.4 mg/g DW). Glucose and fructose concentrations also increased for 96h (2-6 mg/g DW). Also temperature had significantly influenced the biosynthesis and accumulation of biochemical compounds in the seeds especially 30/20°C. Temperature 30/20°C, increased seed phenols and proline concentration. Phenols and proline started to accumulate at 72h, after seed germination presumed within 48h. Results of leaves showed that Temperature also had significant effect on phenols the proline concentration particularly, 35/18°C, followed by 30/15°C, 25/12°C.

The antioxidant levels of post germination phase was investigated, the result showed significant differences in antioxidant concentrations and sugar distribution in various parts of Moringa seedling. The leaf was recorded the highest antioxidant concentration (1.7 mg g^{-1}). Moringa leaf also recorded the highest total phenols. The highest sugar concentration was found in root (258.9 mg g-1), and stem (245.72mg g-1) followed by root and seed. The highest concentration of total crude protein (110.4mg g-1) and vitamin E (28.57 µg g-1) was found in seed. The carotenoids concentration was the highest in the root (29 mg g-1). The result also showed different nutrients at various concentrations on different parts of Moringa tree. High phosphorous accumulated in leaf and seed; Potassium accumulation was found in root, stem, and seed coat.

It is concluded that temperature treatments of Moringa seed and leaves resulted in significant differences in the rate of germination and biochemical compounds. Furthermore, the present study showed that Temperature plays an important role in germination seed and development of *M. oleifera* and antioxidants, phenolic, proline and carbohydrates contents. Further research on the effect of temperature on germination of *M. oleifera* and biochemical compound is therefore recommended.

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CHAPTER ONE

Literature review

1.1 Introduction

Moringa oleifera Lam (*M. oleifera*) is the most extensively cultivated tree species of the genus Moringa that belongs to the family Moringaceae. *M. oleifera* is a highly valued plant, distributed in different parts of tropics and sub-tropics around the world (Anwar et al., 2007). Each part of this tree is edible and could be consumed by humans. The M. oleifera tree has numerous vernacular names such as Horseradish tree Marango, Kelor, Drumstick tree, Horseradish tree, Mlonge (Fahey, 2005). *M. oleifera* is considered as one of the most beneficial tree in the world, it has several traditional medicines, industrial and nutritional uses (Fuglie, 1999; Anwar et al., 2007; Wadhwa, 2013). This tree is a perennial softwood tree with timber of low quality, as an important crop in some countries in the world such as India, Ethiopia, and Sudan. Moringa tree has also been grown in African countries, Latin America, tropical Asia, and in Pacific (Meena et al., 2010). Various parts of *M. oleifera* are highly nutritious and contain important minerals, proteins, vitamins, antioxidant, β-carotene amino acids and various phenolic (Anwar et al., 2007). The leaves of *M. oleifera* are a good source of a natural antioxidant due to the presence of various compounds such as ascorbic acid, flavonoids, phenolic and carotenoids (Makkar and Becker, 1997). The antioxidants are capable of performing a number of functions including acting as free radical scavengers, enzyme inhibitors, reduce damage caused by free radical activity and oxidation (Larson, 1988), have significant role in preventing stress that might cause several degenerative diseases. The tree leaves have also been reported to become a rich source of β-carotene, protein, vitamin C, due to some different elements like calcium and potassium (Dillard and German, 2000). The tree roots and seeds extract have shown to have antimicrobial effects (Wealth, 1966; Eilert et al., 1981). *M. oleifera* has enormous medicinal value, and the different parts of the tree leaves, roots, seeds, bark, fruits, flowers and immature pods have been used for treatment of different human diseases in the indigenous medicine especially in South Asia (Anwar et al., 2007), and act as cardiac and circulatory stimulants, possess antitumor, antipyretic, antiepileptic, anti-inflammatory, antispasmodic, antioxidant, antibacterial and antifungal activities (Santos et al., 2011). Furthermore, *M. oleifera* is a great potential tree that

fast growing and cultivates best temperature ranging from 25 to 35ºC, but tolerates up to 48ºC in the shade and survives a light frost (Palada and Chang, 2003). The drought tolerant species grow healthy in areas with annual rainfall that ranges of 250 mm and 1500 mm for minimum and maximum, respectively. It favors a well-drained sandy loam or loam soil but tolerates clay (Palada and Chang, 2003; Coppin, 2008). However, temperature is important climatic factor affecting growth and geographical plant distribution (Grace, 1987; Sakai and Larcher, 1987). The Moringa trees are naturally found in tropical climates around the different zones in the world, the extent of their adaptability to cooler climates, adaptation to all these stresses is related with metabolic modifications that lead to the increase the several organic solutes such as sugars, proline polyols, and amino acids (Tesfay et al., 2011). The environmental stress increases the proline in plant and has a role of radical detoxification (Smirnoff and Cumbes, 1989). This review was to investigate various types of plant antioxidants distribution in different *M. oleifera* organs during plant growth and development in response to growing condition.

1.2 Specific objectives

- To determine the effect of temperature on Moringa seed phytochemicals' compositional changes and their utilization during seed germination
- To explored the levels of antioxidants concentrations in different parts of the *M. oleifera* seedling.
- To evaluate the effect on temperature of antioxidant activity, total phenolic, total flavonoid and proline concentration in Moringa seeds and leaves.

1.3 Botany and description

Moringa (*Moringa oleifera* Lam, Moringaceae) is one of the 14 species most extensively utilized of all the Moringa species (Ramachandran et al., 1980). *M. oleifera* is native to the Indian subcontinent, Pakistan, Afghanistan, Bangladesh, and different locations of African countries (Oliveira et al., 1999). Currently, it occurs and distributed widely in many tropics and sub-tropics and cultivated all over the world due to its multiple purposes. The tree is one of the world's most beneficial trees; every part of the tree has been used for food, medication and industrial purposes (Khalafalla et al., 2010). *M. oleifera* is perennial tree which has a maximum height of 5-12 m and a diameter of 20-40 cm at breast height, the branches grow in a disorganized manner and the

tree canopy is umbrella shape. The leaves are compound, pinnate double, and in oval shape (Morton, 1991). The fruits consist of three lobed pods that hang down from the tree branches as well as they open into three parts when they are dry. Each pod comprises between 12 -35 seeds. The seeds are round with a brownish semi-permeable seed hull. The hull has three white wings which run from top to bottom. The average weight per seed is 0.3 gram. The flowers are pleasantly fragrant; and have cream or white colored and yellow-dotted at the base (Meena et al., 2010).

1.4 Plant growth and cultivation

M. oleifera is a fast growing tree and able to tolerate wide range of environmental conditions, as it is drought tolerant and requires rainfall ranges between 250 to 1500 mm (Price, 2000). Temperatures ranging from 18.7 to 28.5 ºC per year to grow well but can survive up to 48 ºC and light frost (Palada and Chang, 2003). *M. oleifera* also tolerates a wide range of pH levels ranging from 5 to 9. The tree might be established in slightly alkaline soils up to pH 9 and acidic soils as low as pH 4.5 (Nautiyal and Venhataraman, 1987; Odee, 1998). It tolerates both sandy soils, heavier clay soils and water limited conditions and may survive in less fertile soils (Anwar et al., 2007). The Moringa trees yield a tuberous taproot that explains their tolerance to drought environments.

M. oleifera grows either by direct seeding or planting stem cuttings (Palada, 1996). Regarding the direct seeding, the *M. oleifera* seeds collect from dry pods and should be planted two centimeter (cm) deep that germinate about one to two weeks (Jahn et al., 1986), stated that the rate of germination is usually between 60%-90% for fresh seeds, for stem cuttings of about 1 m long, that is normally used for planting, development of plantlets in the greenhouse for 2-3 months and transplantation of mature stems between 1 to 1.5 m long to the main fields (Price, 2000; Bosch, 2004). Furthermore, the tree considered one of the trees of hot semi-arid zones, grows well in the humid or hot dry lands, tropical and subtropical zones.

1.5 Plant growing environmental condition

1.5.1 Temperature

Temperature is a major environmental factor plays a significant role in the plant growth process and natural geographical plant distribution temperature enzymes governing metabolic pathways within plants are affected by temperature variation; photosynthesis; growth and respiration are some of the plant processes controlled by metabolic pathways (Raghavendra, 1991). The temperature ranging between 20 and 30°C that is considered optimal for cultivation of *M. oleifera*, with no specific upper temperature limit known to date, however, trees have been observed to survive temperatures of up to 48°C (Palada and Chang, 2003).

1.5.2 Climate and soil requirements

M. oleifera is adapted to a wide range of environmental conditions. *M. oleifera* is a drought tolerant; it responds well to irrigation, fertilization as well as it grows perfect in various climate conditions (Radovich and Elevitch, 2011). The tree is tolerant to light frosts, however does not survive under freezing conditions as a perennial (Fuglie and Sreeja, 2001) *M. oleifera* grows more rapidly and has ability to reaching high level on the height. This tree grows in both humid tropics and hot dry lands. Moreover, it is also little affected by the drought (Anwar and Bhanger, 2003). *M. oleifera* can grow well in heavy clay soils, sandy soils and water limited conditions because it tolerates wide range of soil types and pH (Coppin, 2008). The tree can planted in slightly alkaline soils, acidic soils as low and is well suitable for a large range of contrary environments (Coppin, 2008).

1.5.3 Fertilization, irrigation, pest and control

M. oleifera leaves have various biological activities and are rich source in proteins, minerals and produce large quantities of leaves, that require receiving enough organic supplements (Ramachandran et al., 1980). It means that the soil needs to provide enough nitrogen and minerals to the plants (Ramachandran et al., 1980). Plant residue and chemical fertilizer can provide the necessary nutrients as well as improve the soil structure. Fertilization is very essential issue and need to be done before seeding and through land preparation (Fuglie and Sreeja, 2001). Moreover, the Moringa tree growth depends on rainfall (without irrigation). Its

tuberous root allows young plants to endure drought. However, for optimal growth, it is advisable to irrigate regularly during the first three months after seedling. Irrigation is also necessary to produce leaves in all seasons of the year, including during dry seasons (Mugal and Haq, 2010). Any suitable irrigation system can be used; to avoid and reduce evapotranspiration, the irrigation should be done in the early morning, night or evening. *M. oleifera* is resistant to most pests and diseases; its fast growth allows it to regenerate quickly from any disturbance. Insect and fungal diseases represent the highest risks for the tree plantations (Broin and de Saint Sauveur, 2010). The diplodia root may appear in waterlogged soils, affecting yellowing of leaves, severe wilting and death of plants, due to other insect and pests include termites, aphids, leaf miners, and whiteflies (Pandey et al., 2011). These pests can potentially affect the *M. oleifera* in different growing stages in different parts of the tree and led to tree death and mortality.

Weed control is very important through soil cultivation before planting to suppress early weed growth (Palada and Chang, 2003). A weed free field should be preserved by repeatedly cultivating between the beds and the rows. The leaves and shoots can either be harvested manually or mechanically, rely on the scale of production. Harvesting of leaves from trees is best at a height of 30 cm to 1 m. The seed pods should be harvested as soon as they are mature, i.e. when they turn brown and dry (Muller and Rebelo, 2011). In addition, *M. oleifera* is usually used as a nutritional supplement for some communities and so it is important to know the nutritional benefits of the tree due to determine any detrimental effects of *M. oleifera* consumption (Foidl et al., 2001). Previous studies have reported that the tree is a valued component in human and animal feed due to its adequate amino acid profile and crude protein content, as it has own high level of vitamin (A and E), as well as low level of anti-nutritional compounds (Yang et al., 2006).

1.6 The nutrient content

The leaves of *M. oleifera* are highly nutritious and containing source of essential vitamins especially, vitamin A, C and E (Fahey, 2005). They are source of protein, amino acids, calcium, potassium and iron (Dahot, 1988). The leaves also are key source of natural antioxidants such as ascorbic acid, flavonoids, etc. (Coppin, 2008). In many countries, the leaves, fruits, flowers and

immature pods of this tree are used as a highly nutritious vegetable due to treating various types of illnesses (Patel et al., 2010). In addition, the *M. oleifera* seed powder is a perfect water purifier and contains polyelectrolytes, which constitute active ingredients in water treatment (Eilert et al., 1981; Jahn, 1989). The *M. oleifera* is an outstanding source of nutrition; and grows in a wide range of different climate condition such as semi-arid and tropical conditions (Fahey, 2005). All parts of the *M. oleifera* are edible as result it is used as source of nutritional component (Makkar and Becker, 1997). *M. oleifera* has been used to combat malnutrition, particularly among infants and nursing mothers in different developing countries in Africa (Fuglie, 1999; Elkhalifa et al., 2007). Generally, the tree provides especially promising as a food source for population and their animals in dry season because the tree is in full leaf at the end of that season when other foods obtained from other trees are typically scarce (Mugal and Haq, 2010).

1.6.1 Vitamin contents

The *M. oleifera* leaves, seeds, pods and roots provide a large range of necessary vitamins. These vitamins are; vitamin A, vitamin B, vitamin C, vitamin E, and β-carotene. They can ensure health and offer balanced nutrition (Khoo et al., 2008). The fresh leaves are rich in vitamin A. The tree have ability to cultivate in various climates conditions which makes the plant to be a perfect source of the vitamins in different zones in the world when people suffering from malnutrition (Dahot, 1988). These vitamins acts as antioxidant, defending the body from many deleterious influences of free radicals (Chambial et al., 2013), vitamins also play main roles in many physiological functions they can relate with enzymes in the metabolism to production energy and nutrients.

1.6.2 Mineral contents

Different parts of *M. oleifera* are excellent sources of minerals necessary to human health. Iron deficiencies cause anemia whereas calcium deficiencies can cause skeletal problems. The dried of Moringa leaves offer a great percentage of the daily requirements for minerals; calcium, iron, magnesium, potassium, zinc and other elements to avoid nutritional diseases (Dahot, 1988).

1.6.3 Amino acids contents

Amino acids are considered the building blocks of proteins, as they are important elements of a healthful diet (Balch, 2006). The different parts of *M. oleifera* are as source of essential amino acids; especially the leaves and seeds provide high amounts of amino acids (Anhwange et al., 2004). These essential amino acids are important for nervous function and provide high nutritional value for undernourished people in developing countries (Fuglie, 2005).

1.6.4 Antioxidants

Antioxidants are very important for human nutrition and play important role in the care of health and prevention some of diseases. Antioxidant activities play great an important role in preventing stress, inhibiting and scavenging free radicals that may cause several diseases such as cancer, heart disease, hypertension and stroke (Ames et al., 1993; Fang et al., 2002). Furthermore, antioxidants prevent or delay oxidation of food, initiated by free radicals formed during their exposure to environmental factors such as air, light and temperature when was added in foods (Ara and Nur, 2009). The plants are the potential source of natural antioxidants like carotenoids, flavonoids, ascorbic acid, etc. $^{\beta}$-carotene and ascorbic acid are the widely used antioxidants (McCall and Frei, 1999). *M. oleifera* is one of the plant species contains many natural antioxidants compounds such as flavonoids, vitamins and phenolic compounds (Siddhuraju and Becker, 2003). Although *M. oleifera* can tolerate a large range of rainfall, soil conditions and is drought resistant (Iqbal and Bhanger, 2006). These agro-climatic conditions can effect of the nutrient and flavonoid concentration in the Moringa tree (Siddhuraju and Becker, 2003).

1.6.5 Phenolic content

Phenolic compounds are secondary metabolites naturally present in plants, Secondary metabolites are chemicals produced by plants; and their functions in growth, photosynthesis. The most important groups of dietary phenolic are flavonoids, phenolic acids, and polyphenols. Phenolic compounds are produced as a response to environmental factors, defend injured plants (Kefeli et al., 2003), and beneficial in the defense function against the diseases. The bioactive compositions such as phenolic compounds, sugars and amino acids play an essential role in human nutrition and health as potential sources of foods and nutraceuticals (Ashfaq et al., 2012).

Phenolic compounds occur in plants, improves their ability to adapt to biotic and abiotic stress condition such as infection, water stress and cold stress (Shetty, 2004). Recently, phenolic compounds have attracted the attention of researchers due of their antioxidants capacity that can protect the human body from free superoxide radicals, and reduce the risk of many diseases (Halliwell, 1996). The anti-radical activity of flavonoids and phenolic is principally based on the structural relationship between different parts of their chemical structure (Rice-Evans et al., 1996). Phenolic phytochemicals constitute one of the most abundant groups of natural metabolites and form an important part of both human and animal diets. Phenols improve rally of antioxidant in the tissue, that promoting postharvest fruit quality subsequently improving the health benefit of fruit.

1.6.6 Flavonoids

Flavonoids are a group of polyphenolic compounds exist widely in many plant. They are particularly common in leaves, flowers and pollens (Larson, 1988). Plant flavonoids have vital role to the food because of their impact on human nutrition (Das and Pereira, 1990). Flavonoids have numerous properties, including anti-inflammatory, enzyme inhibition, and antimicrobial and antioxidant activity (Robak and Gryglewski, 1988). The antioxidant activity is the most important of flavonoids that responsible for biological activities in which the inhibition of oxidative stress. Anticancer activity of flavonoids is due to their capability to scavenge free radicals, so prevention the early stages of cancer (Cushnie and Lamb, 2005). *M. oleifera* is one of the plants which have high concentrations of flavonoids; these compounds distributed in various parts of plant especially in the leaves (Larson, 1988).

1.6.7 Proline

Proline is an important amino acid and has played a major role in plant salt tolerance and osmotic adjustment. Proline accumulates in several plant types under a wide range of stress conditions such as water shortage, salinity, and high temperatures (Büssis and Heineke, 1998). It defends folded protein structures against denaturation; stabilize cell membranes by interacting with phospholipids, act as an energy and nitrogen source in some plant species. Proline concentration has been reported to be commonly higher in stress tolerant plants Compared to

stress sensitive plants (Ashraf and Foolad, 2007). Proline and betaine are some of the most common compatible organic solutes found in these plants.

1.7 The uses

1.7.1 Food and nutritional

M. oleifera has a variety of potential purposes containing antioxidants like vitamin C, vitamin E, carotenes, polyphenols, and many other compounds reduce the diseases. Leaves, flowers and pods containing protein, vitamins (A, B and C), and some mineral like iron calcium and potassium (Verma et al., 1976). Moringa tree is considered as one of the important trees with reference to nutritional security of rural communities; therefore Moringa to improve human health used and assist in combating malnutrition especially for developing countries (Srikanth et al., 2014). Fresh or dried leaves and flowers of *M. oleifera* are used in different kinds of foods such as porridges and salads (Lockett et al., 2000). The flowers are cooked and used as a vegetable. The seeds can eat fresh as peas, roasted or powdered, and the seeds powder can be used for water treatment (Fahey, 2005). The young pods are eaten like green beans (Foidl et al., 2001). The root bark is used as a condiment (Villafuerte and Villafurte Abonal 2009).

1.7.2 Traditional uses

The *M. oleifera* has several traditional uses, almost all the parts of this tree (root, bark, leaves, fruits, flowers, seeds) and seed oil were utilized for many ailments in the indigenous medicine in South Asia (Anwar et al., 2007) . These parts have used for the treatment of inflammation and infectious human diseases like cardiac circulatory tonic and antiseptic (Wadhwa, 2013). For instance, pods were used as antipyretic, anthelmintic and diabetes. The flowers are cholagogue, stimulant, tonic and diuretic pods are antipyretic, anthelmintic, bark is used as antiviral, anti-inflammatory, and analgesic. The fresh root used as stimulant, diuretic and ant lithic (Singh and Kumar, 1999; Siddhuraju and Becker, 2003). Additionally, for human consumption, the young fresh pods, kernels and roots as pickles, sauces, juices and vegetables have been used due to traditional medicine purpose (Anwar et al., 2005). The seeds are used traditionally for the water purification in rural areas of Sudan and Malawi (Muyibi and Evison, 1995).

1.7.3 Medicinal application

M. oleifera has a several medicinal properties such as reducing blood pressure, tumour healing properties, antifertility activity, antibacterial activity (Eilert et al., 1981; Shukla et al., 1987). Flowers, leaves, bark, seeds, fruits, and roots are used for treatment different of ailments (Anwar et al., 2007) and chief medicinal properties of the plant include antitumor, cholesterol lowering antipyretic, anti-inflammatory antioxidant antidiabetic and fungicidal activities, etc. (Faizi et al., 1998; Ruckmani et al., 1998; Oliveira et al., 1999; Mehta et al., 2003). Previous studies of epidemiological have shown that leaves of *M. oleifera* are an important source of nutrition and exhibit antitumor, antiulcer, and anticonvulsant activities (Danmalam et al., 2001; Chumark et al., 2008).

1.7.4 Industrial uses

M. oleifera tree has many potential uses, as animal forage, green manure, foliar nutrient fertilizer, fungicide, biogas, and oil for cooking or lubrication (Fuglie and Sreeja, 2001). *M. oleifera* seeds contain ben oil (40%) that produced from this tree contains very nutritious and is non-drying resists rancidity, have been utilized for cooking, lubrication, perfume industry and cosmetic products (Ramachandran et al., 1980; Bosch, 2004). Moringa seed oil is a high anti-inflammation and antibacterial due to its high antioxidant potential (Roloff et al., 2009).

1.8 Conclusions

M. oleifera is multi-purpose tree with high nutritional value, the various plant parts have wide medicinal application for the prevention and treatment of diseases given the high nutritional value of *M. oleifera* and its implication as an important human food supplement, Therefore, the tree is an ideal crop to combat malnutrition and has been used to alleviate malnutrition. In view of, the Moringa tree needs to be largely cultivated in developing countries. By this way, the study can provide highly nutritious food at low cost, increasing incomes of smallholder farmers. Therefore, further research should be needed of the *M. oleifera* to develop strategy in terms of studying the concentrations of antioxidants in different parts of the tree to offer some benefits as well as warrant more attention for further investigation.

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CHAPTER TWO

The effect of temperature in *Moringa oleifera* seed phytochemical compounds and carbohydrate mobilization

2.1 Abstract

Temperature is one of the climatic factors that regulate seed and plant physiology, mainly carbohydrates and phytochemical compounds, resulted in regulating plant developmental stages. This study was designed to investigate effect of temperatures on Moringa seed phytochemicals' compositional changes and their utilization during germination. Spectrophotometric determination of prolines and phenols was carried out, while sugars were analysed using HPLC-RID. The data were collected and analysed. The 30/20°C significantly increased seed germination percentage, followed by 25/15°C and 20/10°C respectively. These seeds responded to above temperatures by instant accumulation various antioxidants. Sugars had significant effect in the plant developmental phases. In conclusion, plant antioxidants have vital role in seed germination and the synergy of these compounds can be regulated by growth conditions, mainly temperature.

2.2 Introduction

Moringa (*Moringa oleifera Lam*., Moringaceae) is a perennial plant known for its high antioxidant contents. It is an important food commodity with all plant parts including leaves, flowers, fruits, and immature pods possessing nutritive value (Coppin, 2008). The seeds of *M. oleifera* are known to predominantly produce phenols and different fatty acids (Lalas and Tsaknis, 2002; Tesfay et al., 2011). In plants, the phenolic antioxidants are known to exist in the free phenolic form and usually stored in the vacuole. Furthermore, free phenolic are polymerized to lignans in the plant cell wall. The produced free phenolic can be polymerized on the cell walls of developing seedlings. Apart from the antioxidants potential evident in different organs of *M. oleifera* in response to growing conditions, its adaptations and tolerance to extreme environmental conditions is favored by their unique physio-chemical and physiological characteristics. Seeds of *M. oleifera* are reported to have multifunctional roles. The seeds in some instances are used as the best normal coagulants, which possess antimicrobial, antioxidant

properties and as a result the seeds are used for purification of water (Anwar et al., 2007).The seeds also are known to have valuable nutrients for human diet and contain oil range from 49.8% to 57.25% (Tsaknis et al., 1998; Osman and Abohassan, 2012). The oil extracted from Moringa seed is reported to be rich in high unsaturated fatty acid (70%) with oleic as the major component (up to 70. 52%). Also, it is found as the most stable oil since it has linolenic acid at undetectable level (Robiansyah et al., 2014). In addition, the seeds are known to possess approximately 18.9%–21.12% carbohydrate and 23.8%–33.25% protein (Al-Kahtani and Abou-Arab, 1993; Oliveira et al., 1999).

Plants activate several adaptive strategies in response to abiotic environmental stresses such as temperature fluctuations, dehydration, and osmotic pressure. These adaptive mechanisms include changes in physiological and biochemical processes. Adaptation to these aforementioned stresses is associated with metabolic adjustments that lead to the accumulation of several organic solutes such as sugars, polyols, phenols, and proline (Tesfay et al., 2011). The physiological and biochemical status of the seeds of *M. oleifera* including primary and secondary metabolites may also have huge roles for seed germination as well as post germination seedling establishment and plant development. Their adaptation to harsh conditions, which impact plant development, involves mobilization of different antioxidants. Previously, Muhl (2009) reported that 30/20 °C regime was the optimum temperature for seed germination and post germination seedling establishment when compared to 25/15 °C and 20/10 °C. However, in the physiological and biochemical insights underlying the seed germination, seedling establishment under the optimum temperature remained speculative. It is imperative to understand the seed germination process in relation to the antioxidant system and carbohydrate mobilization during germination at different temperature regimes (30/20 °C, 25/15 °C, and 20/10 °C). Thus, the current study aimed at evaluating the effect of these aforementioned temperatures on germination rate, antioxidant enzymes, phytochemical, and carbohydrate contents in the seeds of *M. oleifera*.

2.3 Materials and methods

2.3.1 Materials and source of seeds

All chemicals were obtained from Sigma-Aldrich®, Saarchem®, Fluka®, Separations®, or

Glycoteam GmbH. *M. oleifera Lam*. cultivar originally from Sudan; the seeds were generously donated by a commercial farmer and cultivated for leaf production at the Ukulinga experimental farm, Pietermaritzburg, KwaZulu-Natal. Species identification and authentication were done by a taxonomist in Bews Herbarium (NU), School of Life Sciences, University of KwaZulu-Natal, South Africa. Furthermore, a voucher of the specimen was prepared to be deposited in the Bews Herbarium, for future references.

2.3.2 Moringa seed germination

Moringa seeds were selected based on their size and color. Total number of 1350 seeds were sub-divided into three batches containing 450 seeds each and replicated into three (150 seeds per replication). Then for germination test, 100 seeds were arranged in moist germination paper towel and allowed to germinate in dark rooms under varying three temperature regimes (30/20 °C, 25/15 °C, and 20/10 °C). Seed samples (5 seeds) were collected every 24 h for 8 weeks until radicle emergency, and the seeds freeze-dried and stored in −75 °C for further biochemical analysis. The experiment was terminated after 8 weeks for statistical analysis to determine temperature treatment effects on mobilization of seed biochemical. Mean germination time (MGT) was also calculated according to the formulae by Ellis and Roberts (1981):

MGT =∑Dn \ ∑n **Equation 2. 1**

Where

MGT = mean germination time,

N = the number of seed which were germinated on day D, and

D = the number of days counted from the beginning of germination.

2.3.3 Determination of total antioxidant activity

Total antioxidant capacity (TAOC) was determined according to Benzie and Strain (1996), with slight modifications. These authors developed the FRAP assay which is based on the reduction of the ferric tripyridyltriazine (Fe(III)-TPTZ) complex to the ferrous tripyridyltriazine (Fe(II)-TPTZ) complex by a reductant, therefore determining the combined antioxidant capacity of antioxidant molecules present in the tissue under investigation. Aliquots of 0.1 g freeze-dried

plant material were extracted with 1 N per chloric acid, vortexed and centrifuged at 12,400g for 10 min at 4 °C. A fresh FRAP reagent solution (300 mM sodium acetate buffer pH 3.6, 10 mM Fe(II)-TPTZ prepared in 40 mM HCl, 20 mM $FeCl_3.6H_2O$ (10:1:1)) was prepared prior to measurement. Subsequently, an aliquot of the samples (30 µL) was mixed with 900 µl FRAP reagent solution, and the absorbance was measured at 593 nm after 10 min. The total antioxidant capacity was expressed as mg $FeSO_4 \cdot 7H_2O \times$ g DW^{-1} equivalent.

2.3.4 Determination of free soluble prolines

Free soluble prolines were extracted according to Bates et al. (1973), with slight modifications. Briefly, approximately 0.1 g of plant material was homogenized in 10 ml of 3% aqueous sulfosalicylic acid and the homogenate filtered through Whatman no. 2 filter paper. Two milliliters of filtrate was reacted with 2 ml acid-ninhdrin and 2 ml of glacial acetic acid in a test tube for 1 h at 100 °C, and the reaction was terminated in an ice bath. The reaction mixture was extracted with 4 ml toluene, mixed vigorously with a test tube stirrer for 15–20 s. The chromophore containing toluene was pippeted out from the aqueous phase into a glass cuvette and warmed to room temperature, and the absorbance read at 520 nm using toluene for a blank. Proline standards with different dilutions were used for calibration curve. Proline concentration was determined from a standard curve.

2.3.5 Determination of free-and membrane bound phenols

Phenols were determined according to Hertog et al. (1992), with slight modifications. Briefly, freeze-dried material (1 g each) was mixed with 10 ml 99.8% (v/v) methanol and vortexed for 30 s. Thereafter, the mixture was shaken overnight at room temperature to extract the free phenols. Subsequently, the mixture was centrifuged, and supernatant were filtered through Whatman no. 1 filter paper and the sample was again rinsed with 10 ml of solvent until color was no longer released. And acid hydrolysis was also used for the remaining plant residue (pellet) to efficiently release cell wall-bound phenols. Briefly, a 10 ml portion of acidified (2 M hydrochloric acid) 60% aqueous methanol was added to each sample and placed in an oven at 90 °C for 90 min exactly. Tubes were allowed to cool, and supernatants were filtered through a 0.45 µm filter and ready for analysis. The phenols concentration was determined spectrophotometric ally using

Folin-Ciocalteu reagent at 750 nm using gallic acid monohydrate as standard and the total phenolic concentration expressed as 'Gallic Acid Equivalents' (GAE).

2.3.6 Moringa carbohydrates (Non-structural carbohydrates)

Freeze-dried material (0.10 g) was mixed with 10 ml 80% (v/v) ethanol and homogenized for 1 min. Thereafter, the mixture was incubated in an 80 °C water bath for 60 min to extract the soluble sugars. Subsequently, the mixture was kept at 4 °C overnight. After centrifugation at 12000g for 15 min at 4 °C, the supernatant was filtered through glass wool and taken to dryness in a vacuum concentrator. Dried samples were suspended in 2 ml ultra-pure water, filtered through a 0.45 μm nylon filter, and sugars were analyzed according to Liu et al. (1999),using an isocratic HPLC system equipped with a refractive index detector on a Phenomenex® column (Rezex RCM–Monosaccharide). The concentration of individual sugars was determined by comparison with authentic sugar standards.

2.3.7 Total protein content

Total soluble proteins were extracted according to Kanellis and Kalaitzis (1992) from 1 g DW of frozen plant tissue. The extract was allowed to stand on ice for 15 min, was centrifuged at 20000g for 20 min at 4 °C, and the supernatant was used for enzyme assays after being passed through Miracloth®-quick filtration material for gelatinous grindates (20–25 μm pore size; Calbiochem, San Diego, CA, USA). The Bradford Micro assay was used to determine the protein content of the samples (Bradford, 1976). Bradford dye reagent was prepared by diluting the dye concentrate with distilled water 1:4. The dye (1 ml) was added to test tubes containing 20 μl sample extract, mixed, and incubated at room temperature for 5 min. Samples were then read spectrophotometrically at 595 nm and the protein concentration determined by comparing results with a standard curve constructed using bovine serum albumin.

2.3.8 Determination of alpha-amylase activity

The alpha-amylase (EC 3.2.1.1) activity in dry seed extract was assayed by quantifying the reducing sugars (glucose equivalent) liberated from soluble starch using HPLC-RID as described by Tesfay et al. (2012). Seeds samples frozen with liquid nitrogen and grounded to fine powder for further analysis. Samples were then homogenized with 2 ml ice-cold buffer of 50 mM Tris–

HCl (pH 7.5) containing 1 mM EDTA. The homogenate was centrifuged at 30000g for 45 min and the supernatant was heated with 3 mM $CaCl_2$ at 70 °C for 15 min to inactivate β–amylase, de-branching enzyme, and α-glucosidase (Sun and Henson, 1991). One unit of enzyme activity (U) was defined as the amount of enzyme required to release 1 μmol of glucose from soluble starch per minute under the assay conditions.

2.3.9 Determination of L-phenylalanine ammonia lyase (PAL) activity

Determination of PAL (EC 4.3.1.5) activity was done according to a modified procedure used by Schopfer and Mohr (1972). Samples of plant tissue (1.3 g) were homogenized at 4 °C in a glass homogenizer with 5 ml of 0.1 M sodium borate buffer, pH 8.8, containing 2 mm potassium meta-bisulfite. Following centrifugation at 20000g for 15 min, a 4.5 ml aliquot of the supernatant solution was passed through an 8 × 230 mm Sephadex G-25 (medium) column, which was previously equilibrated with the same borate buffer, to remove low mol. wt. substances. Before application of the supernatant solution, liquid in the void volume was removed from the column by suction. PAL activity was assayed using 2 ml of the sodium borate buffer, 0.5 ml of the enzyme extract, and 0.5 ml of 0.1 μM phenylalanine in 0.1 M borate buffer. The increase in absorption at 290 nm was measured after 2 h incubation at 37 °C.

2.3.10 Determination of catalase (CAT) activity

A method originally described by Beers and Sizer (1952) was used with slight modifications to determine CAT (E.C.1.11.1.6) activity. The reaction solution (3 ml) contained 0.05 M potassium phosphate (pH 7.0), 0.059 M hydrogen peroxide, 0.1 ml enzyme extract, and 1.9 ml distilled water. To start the reaction, the mixture, in absence of enzyme extract, was incubated for 4 to 5 min to achieve temperature equilibration and to establish a blank rate. To this mixture, 0.1 ml diluted enzyme extract was added, and the disappearance of H_2O_2 was followed spectrophotometric ally every 20 s for 3 min via the decrease in absorbance at 240 nm. The change in absorbance (Δ240 nm/min) from the initial (20 s) linear portion of the curve was calculated. One unit of CAT activity was defined as the amount that decomposes one μmol H_2O_2. Enzyme activity was reported as Units/ mg protein using the following equation: units/mg protein = (Δ240/ min × 1000) × (43.6 × mg enzyme/ml of reaction mixture) $^{-1}$.

2.3.11 Determination of superoxide dismutase (SOD) activity

SOD (E.C. 1.15.1.1) activity was determined by measuring the ability of the enzyme to inhibit the photochemical reduction of nitro blue tetrazolium chloride (NBT), as described by Giannopolitis and Ries (1977). The reaction solution (3 ml) contained 1.5 mM NBT, 0.12 mM riboflavin, 13 mM methionine, 0.1 M EDTA, 67 mM phosphate buffer (pH 7.8), and contained 10 to 100 µl enzyme extract. Riboflavin was added last, and tubes were shaken and placed under fluorescent lighting with an intensity 8.42 µmol m^{-2} s^{-1}. Blanks and controls were determined by with/ without illumination and without addition of enzyme, respectively. The absorbances of the illuminated and non-illuminated solutions were determined spectrophotometric ally at 560 nm. One unit of SOD activity was defined as the amount of enzyme inhibiting 50% NBT photo reduction. The results were expressed as units × (mg protein) $^{-1}$ = 1000 × (µg enzyme extract resulting in ½ max. inhibition) $^{-1}$.

2.3.12 Determination of pyrroline-5-carboxylate synthetase (P5CS) activity

P5CS (EC 2.7.2.11) activity was assayed according to the method of Vogel and Kopac (1960) Modified as follows. Briefly, a 0.7 ml reaction medium containing 50 mM Tris (pH 7.5), 2 mM MgCl2, 10 mM ATP, 1.0 mM NADH, 50 mM glutamic acid, and 0.1 ml enzyme extract was incubated at 37 °C for 30 min. The reaction was then stopped by 0.3 ml of 10% (w/v) trichloroacetic acid. Color reaction developed by incubating with 0.1 ml of 0.5% (w/v) o-amino benzaldehyde for 1 h. After centrifugation at 12,000g for 10 min, the clear supernatant fraction was taken to measure the absorbance at 440 nm. Enzyme activity was calculated using the extinction coefficient of 2.68.

2.3.13 Data analysis

The data collected were analyzed using statistical software using GenStat 14.1 software. Standard error values were calculated where a significant standard deviation was found at P b 0.05 between individual values.

2.4 Results and discussion

The absorption of water by the seed (imbibition) activates metabolic processes that subsequently lead to expansion of the embryo and penetration of the radicle (or other organs) through the

surrounding tissues (Bewley and Black, 1994). Respiration to supply metabolic energy for these processes is activated immediately following imbibition.

Seed carbohydrates composition changes with germinations, as seed imbibes water. At temperature 30/20°C in germinating Moringa seeds, storage sugar, starch concentrations declined rapidly, even before radicle emergence (Figure 2.1), whereas sucrose concentrations peaked within 24h (16 mg/g DW) and the same sugar showed sharp decrease for 96h (6.4 mg/g DW). And glucose and fructose concentrations also increased for 96h (2-6 mg/g DW) and started to decrease as seeds starts to germinating, radicle emergence. Starch and glucose concentrations were negatively correlated (r = –0.61). Unlike 30/20°C, there was a shift on catalysis of starch, the sharp decline observed for 25/15°C, 20/10°C regimes after 48h and 72h respectively. Seemingly, this can be associated with radicle emergence, the seed requires more energy. This energy is provided via catalysis of storage compound, starch is then broken down to smaller units, producing sucrose, glucose, fructose and plant uses them for their growth and development. During the second phase of seed germination, water absorption and respiration are ongoing processes (Bewley and Black, 1994); simultaneously, starch, lipids, and proteins in the endosperm are hydrolyzed to sugars, fatty acids and amino acids, simple compounds that are soluble and mobile. Subsequently, these substances are mobilized to the growing points of the embryonic axis and are used in growth processes, which allow the growth of the radicle of the embryo (Sierra, 2005).

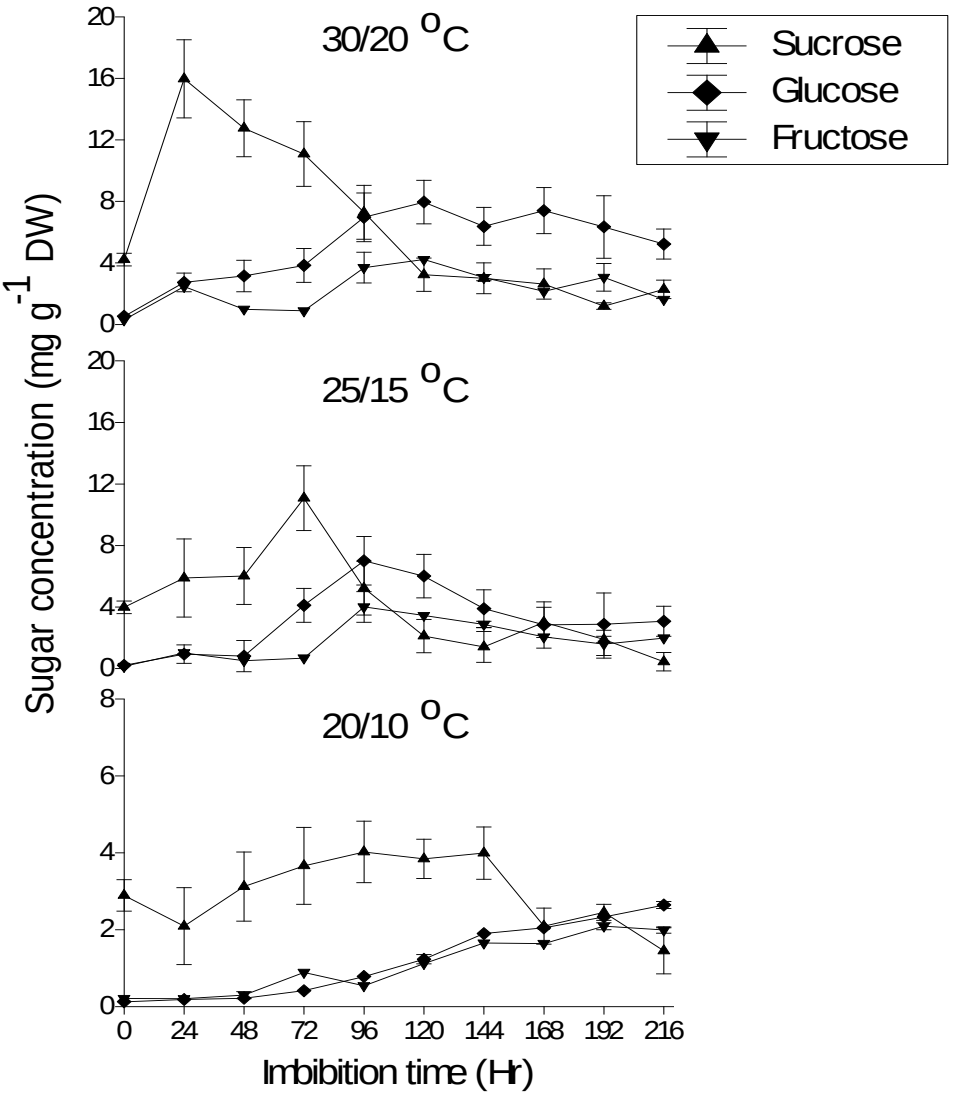

Figure 2.1: Moringa seed germination and seed carbohydrates germinated under three temperature regimes (30/20°C, 25/15°C, 20/10°C).

The temperature 30/20°C hastened radicle emergence, followed by 25/15°C and 20/10°C respectively (Figure 2.2). Temperature may have regulated some of the growth metabolites, such as carbohydrates, polyols and enzyme proteins. Seed germination at higher temperature might be due to rapid hydrolysis and mobilization of seed reserves through higher α-amylase activity. Similarly positive correlation between germination of wheat seed and α-amylase activity at various temperatures was also reported by Sultana et al. (2000). Our result is also found in agreement with Muhl (2009) they reported increased Moringa seed germination rate that are

grown under 30/20°C day/night temperatures. Starch rapid hydrolysis at 30/25°C resulted due to the amylase activity (Figure 2. 3), sucrose produced abundantly within 24h (16 mg/g DW), while glucose and fructose production fluctuating probably depending on the sucrose availability as affected by growth conditions, mainly water and temperature. Following the growth temperatures, the 30/20°C increased the amylase activity (6.4 U/mg/min) earlier, then followed by 25/15 °C (2.8 U/mg/min) and 20/10°C (1.6 U/mg/min) respectively. At 30/20°C temperature, the seed enzyme activity showed sharp increase immediately after 20h of seed imbibition (Figure 2.3). This could be associated with amylase catalytic effect; starch as storage compound is converted in to simple sugars, then can readily be used as energy source by the plant cell (Rahman et al., 2007) also reported amylase activity were tremendously increased 200-220 % at 24h of germination and decreased gradually from 48-96h of germination.

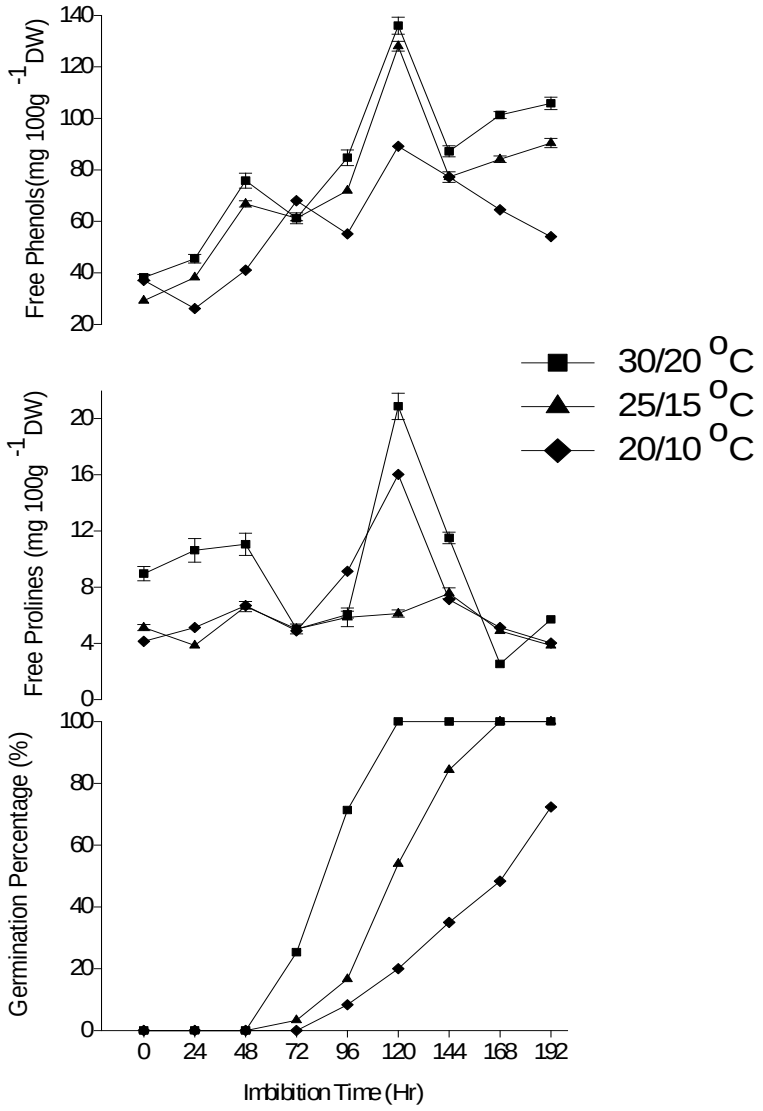

Figure 2.2: Moringa seed germination, concentration of seed prolines and phenols germinated under three temperature regimes (30/20°C, 25/15°C, 20/10°C).

Similarly, temperature 30/20^{0}C also significantly increased seed phenols and proline concentration (Figure 2.2). The result showed proline and phenols started to accumulate at 72h, after seed germination presumed within 48h. It was thought proline and phenol might have a role in seed germination. Proline accumulation in plant cell could have been stimulated in response to growing conditions. In some instances their accumulation linked to plant cell adjusting mechanism inter- and intracellular osmotic gradients that could be caused as result to high cell

metabolic activity which possibly symbolises a switch to next developmental phase, e.g. radicle emergence. Similarly, these temperatures had also significant effect in seed phenols accumulation during germination process. Seed might have started accumulating phenols before radicle emergence during germination. Temperature is one of the abiotic factors that can regulate the biosynthesis of prolines and phenols. The increase of these compounds could also be associated to a plant mechanism to adapt to temperature regimes, resulting in synergistic biochemical compositions. Similar results are also reported by Shetty et al. (2003), the increase of faba beans' prolines and phenols during seed priming, their production were occurred almost at the same time during germination process. In some instances other than seed quality assessment procedures, mung bean seed can be allowed to germinate and influence the phenolic compounds, seed bitterness and astringency changes (Rahman et al., 2007). In agreement to our findings, the same authors also reported the germination of mung bean for 72h would increase phenolic production and then influences bitterness and astringency. Seeds which are vulnerable to low temperatures during the early phase of imbibition result in decrease in percent germination, poor seedling growth and reduced plant productivity. Hare et al. (1998) observed that seed germination in *Arabidopsis thaliana* was enhanced by proline applied exogenously.

Temperature also increased PAL activity (Figure 2.4). The 30/20°C increased the activity which reached to a maximum of 316 mmol/mg/m at 72h. The increment in enzyme activity in response to growth temperature a trend aligned in sequence of ascending temperature levels, most likely contributes to plant phenols accumulation. PAL activity which is highly sensitive to environmental condition, plays a major role in controlling the flux into total phenolic. The main function of phenolic is to maintain the stable concentration of free radical by producing and scavenging them and their physiological function may be shown by regulation of cell redox potential (Shetty, 2004). Furthermore, Hura et al. (2008) also reported a correlation between PAL activity and phenolic compounds in leaves of hybrid maize in drought stress and considered the accumulation of phenolic compounds as the indication of activated defence reaction in the drought resistance of that genotype.

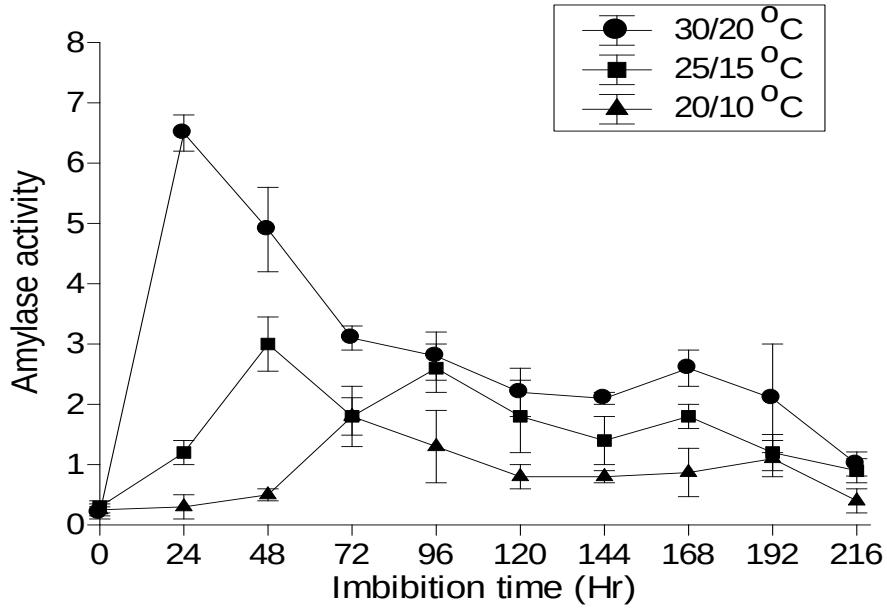

Figure 2.3: Moringa mean Amylase activity during seed imbibition time, germinated under three temperature regimes (30/20°C, 25/15°C, 20/10°C).

Temperature had significant effect in Pyroline-5-carboxylate synthase activity. Unlike PAL activity, the 20/10 °C had the highest P5CS activity, followed by 25/15 °C and 30/20°C respectively (Figure 2.4). The enzyme increased during the early hours and then declined towards the end of seed imbibition process. It is well known that higher plants accumulate free proline in response to a number of abiotic stresses such as drought, salinity, and freezing (Hare and Cress, 1997; Ashraf and Foolad, 2007). The accumulation of proline under stressed environments can result from enhanced biosynthesis and/or reduced degradation of proline. In plants, biosynthesis of proline is catalysed by pyrroline-5-carboxylate synthase (P5CS) and P5C reductase (Hare and Cress, 1997; Kishor et al., 2005). Manipulation of these *P5CS* genes has demonstrated that their overexpression increases proline production and confers salt tolerance in transgenic plants, including wheat (Vendruscolo et al., 2007). Temperature had a significant effect in CAT activity. At 30/20°C, CAT activity increased during seed imbibition, exhibited continuous increasing trend for 140h and declined afterwards (Figure 2.5). The enzyme activity could have increased in response to high accumulation of H_2O_2 due to high seed metabolic rate. The seed germination could therefore be effected in response to the cumulative effect of low molecular antioxidants produced over time during seed imbibition time.

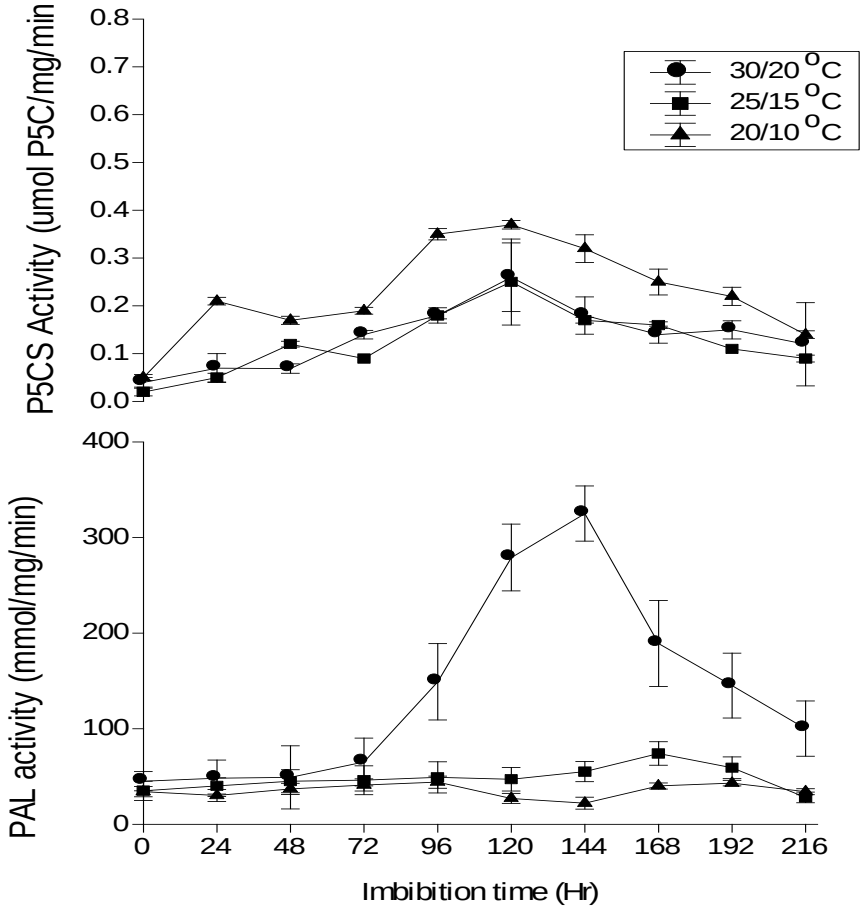

Figure 2.4: Moringa mean L-phenylalanine ammonia lyase (PAL) and pyrroline-5-carboxylate synthetase (P5CS) activities during seed imbibition time, germinated under three temperature regimes (30/20°C, 25/15°C, 20/10°C).

Temperature had significant effect in seed SOD activity. At 30/20°C, SOD was the highest, displayed increasing trend towards seed germination, the activity found increasing for 120h as it experiences high cell metabolic rise (Figure 2.5). While the other two temperatures 25/15°C and 20/10°C, although displayed increasing trend in SOD activity, recorded less activity compared to the former temperature. The SOD mainly dismutises the super oxides into H_2 O_2; the higher product of this ROS might be associated with increased SOD activity. This also confirms the holistic strength between SOD activity and CAT activity, the CAT activity depends on the H_2O_2 concentration which is the product of SOD. The control of steady-state ROS levels by SOD is an

important protective mechanism against cellular oxidative damage, since O_2^- acts as a precursor of more cytotoxic or highly relative ROS (Mittler et al., 2004). SOD has been established to work in collaboration with POD and CAT which act in tandem to remove 2 and H_2O_2, respectively (Blokhina et al., 2003). Early reports illustrate that increased SOD activities and cellular ROS levels were involved in the life of many plants including developmental course such as seed germination (Rogozhin et al., 2001). Enhanced SOD activity can be triggered by increased production of ROS or it might be a protective measure adopted by seeds against oxidative damage. Moreover, the changes of SOD activity in the degrading endosperms and developing cotyledons were correlated to those of POD and CAT activities. The findings, shown in (Figure 2.5) were also in line with Dučić et al. (2003) reported symptomatic of the participation of SOD in the defence mechanism during germination and early seedlings development.

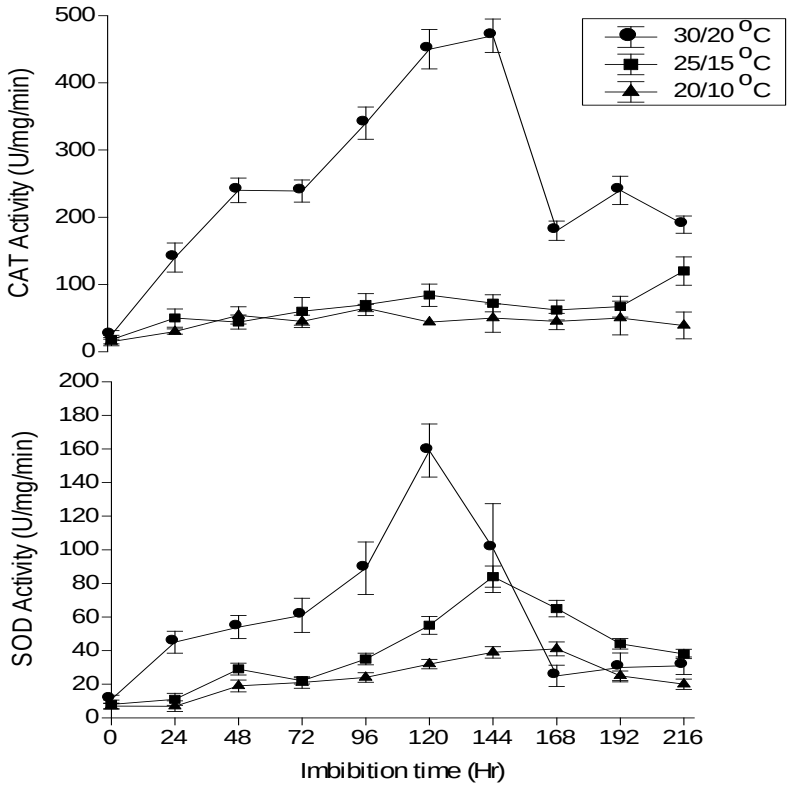

Figure 2.5: Moringa mean super oxide dismutase (SOD) and catalase (CAT) activities during seed imbibition time, germinated under three temperature regimes (30/20°C, 25/15°C, 20/10°C).

2.5 Conclusions

The temperature reported to have an effect in plant metabolites required for their growth and development. Moringa plants adapt to different temperature levels, it favours its adaptive strategy to this condition. Of which the 30/20°C was found the optimum temperature for most of the antioxidant metabolites which eventually impacted the seed germination process. During Moringa seed germination onset of metabolites, mainly energy storage reserves, regulated by optimum temperature to be used as energy source to stabilize seeds' high metabolic rate. During seed metabolic rise, cell biochemical process is accompanied by accumulation of antioxidants to maintain cellular redox balance, which enhances further plant development.

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CHAPTER THREE

Levels of antioxidants in different parts of Moringa (*Moringa oleifera*) seedling

3.1 Abstract

Different parts of *Moringa oleifera* comprising several bioactive compounds and used for various purposes. The present study explored the levels of antioxidants concentrations in different parts of the Moringa tree. Moringa seedlings were grown in pots under greenhouse condition, and then replicated into three. The samples of the different parts of the tree were collected and stored for further analysis used in this analysis. There were significant differences in antioxidant concentrations and sugar distribution in various parts of Moringa seedling. The leaf recorded the highest antioxidant concentration (1.7 mg g^{-1}) as well as leaf ascorbic acid (1.9 mg g^{-1}). Moringa leaf also recorded the highest total phenols (TP). The highest sugar concentration was found in root (258.9 mg g^{-1}), and stem (245.72 mg g^{-1}) followed by root and seed. The highest concentration of total crude protein (110.4 mg g^{-1}) and vitamin E (28.57 µg g^{-1}) was found in seed. The carotenoids concentration was the highest in the root (29 mg g^{-1}). Various parts of *M. oleifera* seedlings had different levels of antioxidants and sugars; this contributes potential of the plant to improve nutrition and health due to nutritional, medicinal and therapeutic properties.

3.2 Introduction

Moringa oleifera is most useful plants of the world which has some traditional medicine and industrial uses. *M. oleifera* tree cultivation is distributed in several countries of the tropics and sub-tropics in recent cultivated the entire world due to multiple benefits. *M. oleifera* tree known as excellent source of nutrition and different parts of this tree comprise essential amino acids, protein, vitamins A, B and C, mineral elements (Ca, Fe, P, and Cu) and protein (Makkar and Becker, 1997). These essential nutrients are necessary for improved health and nutrition, particularly in malnourished population, therefore the tree is an important food component in many countries in the world (Anwar and Bhanger, 2003; Anwar et al., 2005). *M. oleifera* also plays an important role to provide an important source of antioxidants, practically the leaf part that contains rich source of natural antioxidant such as ascorbic acid, flavonoids, phenolic and

carotenoids (Anwar et al., 2005; Lako et al., 2007). Antioxidants are vital substance that inhibits oxidative damage to molecules (Tiwari, 2001). They play main role in protecting the cell and tissue from damage caused by free radical induced oxidative stress (Paul et al., 2011; Hyldgaard et al., 2012). In addition, antioxidant compounds such as flavonoids polyphenol and phenolic acids, scavenge free radicals such as peroxide, hydro peroxide etc. Therefore, they prevent the oxidative stress that leads to degenerative (Helen et al., 2000). Free radical accumulation or oxidative stress adversely changes many essential biological molecules leading tissue damage and loss of plant function in a number of tissues and organs (Floyd and Carney, 1992). Oxidative stress is a disturbance in the imbalance between free radicals (FR), reactive oxygen species (ROS) and the antioxidant endogenous defense mechanisms (McCord, 2000). The human body requires both oxidant and antioxidant for metabolism and regulation of cellular functions. Thus, each cell maintains a condition of homeostasis between antioxidant and the oxidant (Halliwell, 1990; Halliwell, 1996). Oxidative stress has role in various pathological processes, including inflammatory condition, stroke heart diseases and cancers (Stefanis et al., 1997). Antioxidant defense systems in plant are present as enzymatic and non-enzymatic. The enzymatic antioxidant defense includes superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX); these antioxidants work effectively to mitigate cell damage against reactive oxygen species (ROS). The non-enzymatic antioxidant comprises vitamin C, vitamin E, carotenoids; flavonoids etc. (Jacob, 1995; Willcox et al., 2004; Stepien and Klobus, 2005). The present study reported the levels of antioxidants concentrations in different parts of the Moringa tree.

3.3 Materials and methods

M. oleifera seeds were germinated in paper-towel. The seedlings were transplanted into a plastic pot and were grown in a greenhouse for four months, the plant sample were collected; freeze dried and kept in cold storage at -21°C until further analysis.

3.3.1 Determination of bioactive compounds

3.3.2 Total antioxidant capacity (TAOC)

Total antioxidant capacity (TAOC) was determined using the ferric reducing ability of plasma (FRAP) according to Benzie and Strain (1996), with slight modifications. Freeze dried (0.1) g

plant sample were extracted with 1 N perchloric acid, vortexed and centrifuged at 12,400 g for 10 min at 4°C. A fresh FRAP reagent solution (300 mM sodium acetate buffer pH 3.6, 10 mM Fe(II)-TPTZ prepared in 40 mM HCl, 20 mM FeCl3 x 6H2O (10:1:1)) was prepared prior to measurement. Subsequently, an aliquot of the samples (30 µl) was mixed with 900 µl FRAP reagent solution and the absorbance was measured at 593 nm after 10 min. The total antioxidant capacity was expressed as mg $FeSO_4 \times 7H_2O \times$ g DW^{-1} equivalent.

3.3.3 Determination of ascorbic acid (AsA)

The Ascorbic acid (vitamin C) concentrations was determined according to Böhm et al. (2006) by comparing the absorbance of plant tissue extracts at 520 nm with values obtained using an L-ascorbic acid standard curve. The results were expressed in mg AsA g^{-1} DW.

3.3.4 Determination of total phenols

The total Phenols were determined according to Hertog et al. (1992)**,** using the Folin-Ciocalteu reagent. Freeze-dried tissue (1.0 g) was mixed with 10 ml 99.8% (v/v) methanol and vortexed for 30 second, and left overnight at room temperature to extract the free phenols (Hertog et al., 1992). Then , the mixture was centrifuged, the supernatant filtered through Whatman® no. 1 filter paper and the pellet repeatedly rinsed with 10 ml solvent until color was no longer released. Membrane-bound phenols were released from the remaining plant residue by acid hydrolysis. A 10 ml portion of acidified (2 M hydrochloric acid) 60% (v/v) aqueous methanol was added to each sample, which was then incubated at 90°C for 90 min. Samples were allowed to cool before the supernatant was filtered and analyzed filtered free phenol extract. Free and membrane-bound phenols concentrations were determined spectrophotometrically at 750 nm by adding Folin-Ciocalteu reagent to the extract and expressing the results in 'gallic acid equivalents'.

3.3.5 Determination of total carotenoids

The total carotenoids were determined according to Dere et al. (1998) by computing the absorbance values of the plant tissue extracts at wavelengths of 470, 646.8, and 663.2 nm**.**

3.3.6 Determination of vitamin E content

Freeze-dried sample (0.5 g) was immersed in 20 ml of ethanol for 30 min in a water bath at 85°C. The solution was allowed to cool and then filtered into a separating funnel. Heptane (10 ml) was added, and the solution was shaken for 5 min. Then, 20 ml of 1.25% sodium sulfate was added and the solution was shaken again for 2 min, and allowed to separate into layers. Total tocopherols were determined by a reaction with cupric ions and complexation with 2, 20-biquinoline (cuproine) according to Contreras-Guzman and Strong (1982). A volume of 0.5 ml of α-tocopherols in ethanol was processed in the same way as a sample and used as a standard.

3.3.7 Determination of sugar concentration

Sugar concentrations were determined according to Liu et al. (1999). Briefly, freeze-dried material (0.05 to 0.10 g) samples were mixed with 10 mL of 80% (v/v) ethanol and homogenized for 60 second. Thereafter, the mixture was incubated in an 80°C water bath for 60 min and kept at 4°C overnight to extract the soluble sugars. After centrifugation at 12000 g for 15 min at 4°C, the supernatant was filtered through glass wool and taken to dryness in a Savant vacuum concentrator. Dried samples were re-suspended in 2 ml ultra-pure water, filtered through a 0.45 μm nylon filter and analyzed using high performance liquid chromatography system equipped with a refractive index detector on a Phenomenex® column (Rezex RCM– Monosaccharide). The concentrations of individual sugar were determined by comparison with authentic sugar standards**.**

3.3.8 Determination of total soluble protein extraction

Total soluble proteins were extracted according to Kanellis and Kalaitzis (1992), frozen leaf tissue powder (1 g DW) was extracted in 5 ml 50 mM Tris-HCl buffer (pH 7.4). The mixture was allowed to stand on ice for 15 min and then centrifuged at 20000 ×g for 20 min. The supernatant was used for Bradford assay.

3.3.9 Total protein assay

The protein content was determined according to Bradford (1976). Bradford dye reagent was prepared by diluting the dye concentrate with distilled water 1:4. The dye (1 ml) was added to test tubes containing 20 μl sample extract, mixed and incubated at room temperature for 5 min.

Samples were read spectrophotometrically at 595 nm and the protein concentration determined by comparing results with a standard curve constructed using bovine serum albumin.

3.3.10 Data analysis

Data were analysed using GenStat version 17th edition. Standard deviation values were calculated and differences among treatments were separated by the least significant difference at $P \leq 0.05$ level.

3.4 Results

3.4.1 Total antioxidant capacity

Total antioxidant capacity (TAOC) differed significantly ($P \leq 0.05$) between seedling parts (Table 3.1). The (TAOC) was the highest concentration in the leaf (1.7 mg g^{-1}), followed by stem (1.3 mg g^{-1}) and seed coat (0.5 mg g^{-1}), respectively.

3.4.2 Ascorbic acid (AsA)

The ascorbic acid (AsA) concentration also differed significantly between plant parts (Table 3.1). Similar to the TAOC, the leaf of Moringa plant has the highest concentration of AsA (1.9 mg g^{-1}) followed by the root (1.6 mg g^{-1}).

Table 3.1: Distribution of different types of total antioxidant compounds in Moringa seedlings plant parts.

Plant parts	Vit. E (µg/g DW)	Total Carotenoids (mg/g DW)	Total crude protein (mg/g DW)	TAO (mg/g DW)	Asc. Acid (mg/g DW)
Leaf	3.5a	14.5a	76c	1.7c	1.9c
Stem	1.6a	16ab	51b	1.3c	0.7b
Root	0.6a	29b	29a	0.15a	1.6c
Seed	28b	12a	109d	0.09a	0.04a
Seed coat	0.04a	11.2a	39a	0.5b	0.31a
LSD 0.05	8.2	16.2	11.7	0.2	0.3

3.4.3 Total carotenoids

The root part had highest concentration of carotenoids (29 mg g-1) as compared to others (Table 3.1), while leaf, stem, and seed coat had relatively equally proportional concentration.

3.4.4 Vitamin E

The seed had highest concentration of vitamin E (28 µg g^{-1}) as compared to others (Table 3.1), while leaf and stem, had relatively equally proportional of vitamin E concentration.

3.4.5 Total crude protein

The Bradford reagent assay resulted in high concentration of crude protein in all tissues (Table 3.1). The total crude protein concentration was the highest in the Seed (109 mg g^{-1}), followed by leaf (76 mg g^{-1}).

3.4.6 Total phenols (TP)

The TP concentration was significantly the highest in leaf tissue, followed by root and seed (Table 3.2). Various Moringa parts showed different forms of phenols between free phenols and compared to membrane-bound ones. The membrane bound phenols concentration was the highest in the seed (45.2 µg g^{-1}) as compared to free ones.

Table 3.2: Free and membrane-bound phenols in different parts of *M. oleifera* seedling.

Plant parts	Free phenols ((µg/g DW)	Membrane bound phenols ((µg/g DW)	Total
Leaf	35b	28a	63
Stem	12a	18a	30
Root	28.4b	27.2a	55.6
Seed	4.3a	45.2b	49.5
Seed coat	6.6a	24.1a	30.1
LSD 0.05	10.8		

3.4.7 Non-structural soluble carbohydrates

There were significant differences on carbohydrate distribution on different parts of Moringa seedlings (Table 3.3). Root (258.9 mg g^{-1}) and stem (245.72 mg g^{-1}) recorded the highest sugar

concentration. Sucrose was found as the dominant sugar (245.16 mg g^{-1}), followed by glucose (180.19 mg g^{-1}). Raffinose was also detected only in series of stem, root and leaf, respectively.

Table 3.3: Concentration of carbohydrates of *M. oleifera* seedling on different plant organs.

Plant parts	Fructose	Glucose	Raffinose	Sucrose	Total
Leaf	12.99fg	14.51g	8.91fg	42.14d	78.55
Stem	19.85ef	74.81abc	66.32c	84.74ab	245.72
Root	70.69bc	90.04a	20.95ef	77.22abc	258.9
Seed	nd	nd	nd	33.74de	33.74
Seed coat	0.71g	0.83g	nd	7.32fg	8.86
Total	104.24	180.19	96.18	245.16	625.77

3.4.8 The environmental scanning electron microscope (ESEM) nutrient content

The ESEM result also showed different nutrients at various concentrations on different parts of Moringa tree (Table 3.4). Potassium predominantly accumulated in root, stem, seed coat; high accumulation of phosphorous was found in leaf and seed; sulfur was accumulated in leaf, seed, root and magnesium also distributed in similar concentrations over different organs of the seedling.

Table 3.4: ESEM nutrient analysis of *M. oleifera* seedling on different tissues.

Plant parts	Magnesium (Mg)	Phosphorus (P)	Sulfur (S)	Potassium (K)	Calcium (Ca)
Leaf	5.65	20.63	47.4	21.62	4.67
Stem	7.11	nd	13.91	49.32	19.82
Root	4.62	6.54	36.4	54.38	14.12
Seed	5.95	20.47	46.43	22.38	4.04
Seed coat	10.1	7.3	16.73	40	19

3.5 Discussion

M. oleifera is a tree of multipurpose uses; it has been consumed for various applications such as nutritional, medicinal and industrial uses. Studies have shown Moringa species as being rich

sources of natural antioxidants compounds. The findings of the present study confirm that Moringa tree contains high antioxidants and nutrients that distributed in different part of tree. *Moringa peregrine* was found to also contain antioxidant however; ***M. oleifera*** contains the highest concentrations of nutritional values compared to other types of the plant (Reyes Sánchez et al., 2006). There is an increasing attention in antioxidants due to the ability to inhibit the deleterious effects of free radicals in the body. Moringa leaves have been reported as the major source of nutrition as it encompass many antioxidant compounds such as carotenoids, ascorbic acid, α-tocopherol and phenols (Perry et al., 1999).

In the present study, the Moringa leaf had high concentration of antioxidants and proteins. TAOC differed significantly ($P \leq 0.05$) among seedling parts. The Leaf showed the highest concentration of TAOC, AsA, as well as TP. In view of the high concentration of these useful nutrients, the tree can be used to combating malnutrition in the world. The leaves had been widely consumed by human for a long time in different ways. Fresh leaves can be eaten raw or cooked like spinach. The leaves also can be boiled and eaten like green beans. Moringa leaves powder can be stored for some time reaching about six months before it is consumed.

It has also been reported that Moringa leaves provide a huge concentration of essential nutrients including protein and fiber (Lockett et al., 2000; Fahey, 2005), for this reason, it is used as an alternative source for nutritional supplements to improving health in some countries (Anwar et al., 2007). In addition, Moringa leaves can prevent the risk of some diseases and these effects have been with the presence of antioxidant compounds (Ashok Kumar and Pari, 2003; Lipipun et al., 2003; Santos et al., 2012). The stem of the seedling was reported to contain higher concentration of TAOC that was found to be second following the leaf of plant. The Root and bark of Moringa are used for traditional medicine and treatment of various human diseases (Fahey, 2005). They are used as anti-inflammatory, antibacterial, antiviral and analgesic (Siddhuraju and Becker, 2003). These properties are related as they have strong antioxidant capacity. The roots comprise great levels of nutritional values e.g. protein, minerals, vitamins, and fiber (Fahey, 2005). The roots recorded high concentrations of carotenoids, AsA and TP following the leaf. The highest concentration of α- tocopherol was found in seed produced followed by TP. The phenols concentration was found no significant in seedling parts, excluding that the seed membrane-bound phenols concentration was predominantly produced. This result

supports the speculation that shows the vitamin E are dominantly produced in the seed as oil source and has additional position of cell defense. Moringa seed oil called (Ben oil) is high quality oil. It has been used for different purposes such as cooking oil in salads, for fine machine lubrication, it is also useful in manufacturing of cosmetic industry like soap, perfumes, and hair care products (Tsaknis et al., 1999; Price, 2000). The powdered seeds could be used for water purification, especially in rural communities in the African countries (Berger et al., 1984). The Moringa seeds are also eaten fresh, cooked, powdered and drenched for tea or use the powder in curries (Gassenschmidt et al., 1995). *M. oleifera* oil also can be used for the production of biodiesel.

3.6 Conclusions

The present study sought to explore the level of antioxidants compounds of different parts of Moringa tree. The results from our research showed that *M. oleifera* as a rich source antioxidant activity and numerous beneficial nutrients. Our findings also further support the use the Moringa leaves as a source of vitamins Particularly C and E; therefore *M. oleifera* is an excellent plant candidate to be used to improve the health and nutrition of communities in Sub-Saharan Africa.

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CHAPTER FOUR

Moringa oleifera phenols and prolines in leaves and plant development

4.1 Abstract

Moringa oleifera is tropical tree, as is drought tolerant plant that adapts various environmental conditions. Different parts of the tree have several pharmacological activity and used for various purposes. Temperature is a major climatic factor that impacts on growth plant and geographical distribution. The aim of the present study was to determine the effect of temperature on the phenols and proline of *M. oleifera* leaves. Moringa seeds were germinated in a tunnel approximately at 25°C. Seedlings transplanted after 45 days of germination into plastic bags and kept for two weeks. The seedlings transported into glass houses under three temperature regimes (35/18, 30/15, 25/12°C). Leaf samples were collected for six consecutive weeks, freeze-dried and stored at -74°C for further analysis. Temperature had significant effect on phenols and the membrane-bound phenols particularly, 35/18 °C. Temperature had also significant effect on prolines. The 35/18°C increased the proline concentration, followed by 30/15°C, 25/12°C. Overall, proline and carbohydrate content are increase with increasing environmental stress conditions and these results showed significant different of proline and carbohydrates in *M. oleifera* tolerance to wide range of environmental condition.

4.2 Introduction

M. oleifera is a plant with a great nutritional value due to high content of primary and secondary metabolites and pharmacological activities (Anwar et al., 2007). The leaves of *M. oleifera* are known to be a good source of natural antioxidant due to the presence of several types of antioxidant compounds such as ascorbic acid, flavonoids, phenolic and carotenoids (Makkar and Becker, 1997; Anwar et al., 2005). Antioxidants are substances that may protect cells from the damage caused by unstable molecules known as free radicals which are highly reactive molecules. Free radical accumulation or oxidative stress adversely changes many crucial biological molecules leading tissue damage and loss of function in a number of tissues and

organs (Floyd and Carney, 1992). Antioxidants are capable of deactivating free radicals, decreasing oxidative damage to lipids, proteins and nucleic acids induced by free radicals (Soler et al., 2000; Kaliora et al., 2006; Adesegun et al., 2008). Consequently, antioxidants reduce oxidative damage of a cell and tissue by enhancing natural defenses of cell; the antioxidants have defense mechanisms against free radical include preventative mechanisms, repair mechanisms, and physical defenses (Cadenas, 1989). In addition antioxidants are of great importance in preventing stress that may cause numerous degenerative diseases including cancer, atherosclerosis coronary heart diseases (Helen et al., 2000).

Moreover, *M. oleifera* leaves have been reported to have various flavonoids and phenolic compounds (Anwar et al., 2007; Elkhalifa et al., 2007). Phenolic compounds (Figure 4.1) are a major group of secondary metabolites that are produced by plants as response to biotic and abiotic stress conditions such as infection, water stress (Douglas, 1996; Shetty, 2004; Ignat et al., 2011). Flavonoids are a group of polyphenolic compounds, as they are synthesized in response to microbial infections (Kumar and Pandey, 2013). Flavonoids are capable of scavenging free superoxide radicals and protecting biological systems against the harmful effects of oxidative processes. Flavonoids also act as a secondary antioxidant defense system in plant tissues exposed to different abiotic and biotic stresses. They also regulate growth factors in plants (Agati et al., 2012). Furthermore, phenolic and flavonoid compounds from plants have been reported to have many biological activities, including free radical scavenging activity, activate antioxidant enzymes, and inhibit oxidases (Bors et al., 1996; Kahkonen et al., 1999; Heim et al., 2002; Cai et al., 2004).

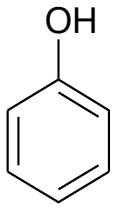

Figure 4.1: Structure of Phenolic

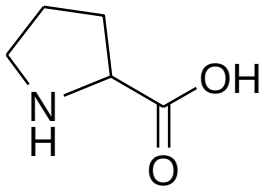

Figure 4.2: Structure of Proline

Proline (Figure 4.2) is one of the organic molecules that play important role ant oxidative defense molecule and a signaling molecule (Hayat et al., 2012). Conversely, proline accumulates in many plant species under a broad range of different environmental stresses conditions such as water shortage, salinity, drought, extreme temperatures (Yoshiba et al., 1995; Choudhary et al., 2005; Haudecoeur et al., 2009; Yang et al., 2009).

Temperature is a major environmental factor that plays a significant role in the plant growth process and natural geographical plant distribution. Temperature has huge influence on antioxidant activity (Pokorný, 1986). Temperature enzymes governing metabolic pathways within plants are affected by temperature variation, photosynthesis, growth and respiration (Raghavendra, 1991).

4.3 Materials and methods

All chemical were obtained from sigma-Aldrich®, Saarach®, Fluka®, Separations®, or Glycoteam GmbH. University of KwaZulu-Natal, Pietermaritzburg , South Africa.

4.3.1 Moringa seedling

Seeds were germinated in a tunnel in a controlled environment, approximately 25°C. Seedlings transplanted after 45 days of germination into plastic bags and kept for two weeks in the same tunnel to avoid transplanting shock. Hence, the seedlings transported into glass houses under three temperature regimes (35/18, 30/15, 25/12°C). Each room accommodated 20 pots. Leaf samples were collected for six consecutive weeks, freeze-dried and stored at -74°C for further analysis.

4.3.2 Determination of total phenols

Phenols were determined according to Hertog et al. (1992), with little modifications. Briefly, freeze-dried tissue (1.0 g) was mixed with 10 ml 99.8% (v/v) methanol and vortexed for 30 s. The mixture was then manually shaken using IKA® (ks 130, Staufen, Germany) and left overnight at room temperature to extract the free phenols. Subsequently, the mixture was centrifuged, the supernatant filtered through Whatman® no. 1 filter paper and the pellet repeatedly rinsed with 10 ml solvent until color was no longer released. Membrane-bound phenols were released from the remaining plant residue by acid hydrolysis. A 10 ml portion of acidified (2 M hydrochloric acid) 60% (v/v) aqueous methanol was added to each sample, which was then incubated at 90°C for 90 min. Samples were allowed to cool before the supernatant was filtered and analyzed filtered free phenol extract. Free and membrane-bound phenols concentrations were determined spectrophotometric ally at 750 nm by adding Folin-Ciocalteu reagent to the extract and expressing the results in 'Gallic acid equivalents' (GAE).

4.3.3 Determination of free soluble prolines

Free soluble prolines were extracted according to Bates et al. (1973) with slight modifications. Briefly, approximately 0.1 g of plant material was homogenized in 10 ml of 3% aqueous sulfosalicylic acid and the homogenate filtered through Whatman no. 2 filter paper. Two milliliters of filtrate was reacted with 2 ml acid-ninhdrin and 2 ml of glacial acetic acid in a test tube for 1 h at 100 °C, and the reaction was terminated in an ice bath. The reaction mixture was extracted with 4 ml toluene, mixed vigorously with a test tube stirrer for 15–20 s. The chromophore containing toluene was pippeted out from the aqueous phase into a glass cuvette and warmed to room temperature, and the absorbance read at 520 nm using toluene for a blank. Proline standards with different dilutions were used for calibration curve. Proline concentration was determined from a standard curve.

4.3.4 Non-structural carbohydrates

Freeze-dried material (0.05 to 0.10 g) was mixed with 10 mL 80% (v/v) ethanol and homogenized for 1 min. Thereafter, the mixture was incubated in an 80^{o}C water bath for 60 min to extract the soluble sugars. Subsequently, the mixture was kept at 4 ºC overnight. After centrifugation at 12000 g for 15 min at 4 ºC, the supernatant was filtered through glass wool and

taken to dryness in a vacuum concentrator. Dried samples were resuspended in 2 mL ultra-pure water, filtered through a 0.45 μm nylon filter and analyzed using an isocratic HPLC system equipped with a refractive index detector on a Phenomenex® column (Rezex RCM–Monosaccharide). The concentration of individual sugars was determined by comparison with authentic sugar standards.

4.3.5 Statistical analysis

Analyses of variance were performed using GenStat software 14.1. Standard deviation values were calculated and differences among treatments were separated by the least significant difference at $p \leq 0.05$ level.

4.4 Results and discussion

4.4.1 Total phenols (TP)

The leaf free and membrane-bound phenol concentrations showed a significant response towards temperature. The 35/18°C increased the free phenols, followed by 30/15°C and 25/12°C (Figure 4.3). And furtherly, the free phenols increased after third week and followed the same trend as the plant exposed to a temperature series from 35/18, 30/15 and 25/12°C (Figure 4.3). The results consistent with Wang and Zheng (2001), the highest day/night temperature (30/22°C) yielded fruit with the most phenolic content in strawberry.

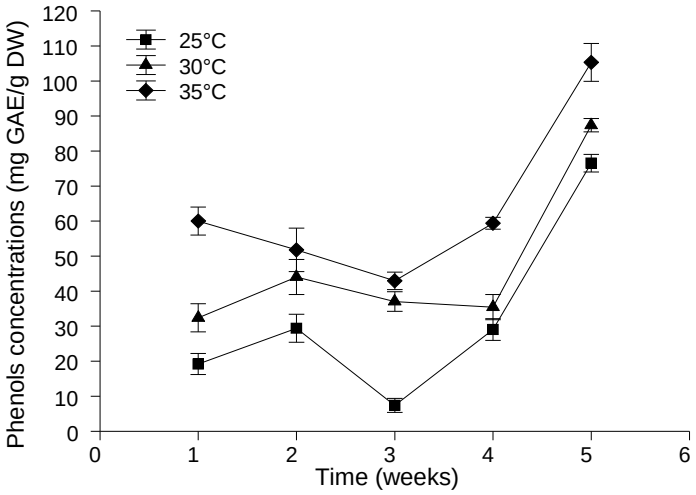

Figure 4.3: Free phenols concentrations of Moringa leaves grown in three different temperatures (25/12°C, 30/15°C, 35/18°C).

Interestingly, temperature regimes had also a significant effect to the membrane-bound phenols. The 35/18°C increased membrane-bound phenols, followed by 30/15°C, 25/12°C, respectively (Figure 4.4). Unlikely, membrane-bound phenols drastically decreased until second week, then produced almost in linear condition while maintaining the same trend towards temperature in series of highest to lowest temperature regimes (Figure 4.4).

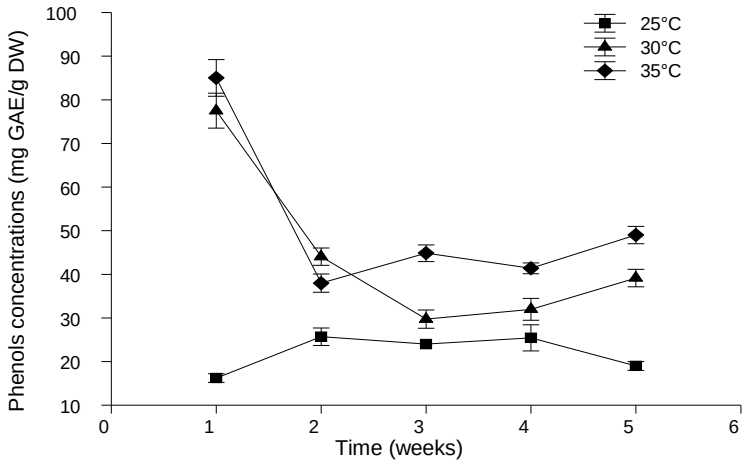

Figure 4.4: Membrane-bound phenols concentrations of Moringa leaf grown in three temperatures 25/12°C, 30/15°C, 35/18°C).

4.4.2 Total proline

The 35/18°C increased the proline concentration, followed by 30/15°C, 25/12°C. It was also observed proline started to accumulate over time (Figure 4.5), increasing in concentration until third week. A positive correlation was seen between proline levels and phenolic content during germination phase, except for day 8. This supports the hypothesis that with the proline stimulation, pentose-phosphate pathway was over expressed but not linked, which provides excess metabolic flux to shikimate pathway and phenylpopanoid pathway for secondary metabolite synthesis. However, increased proline and phenolic content have a negative correlation to G6PDH, the first committed step of the PPP. It is possible that since G6PDH was expressed in the early stages and for longer period (day 3) in gellan gum treatment, it makes all the desired precursors for anabolic reactions, including proline and phenolic synthesis. At later stage these precursors could be acting as feedback inhibitors for G6PDH enzyme. Proline levels may vary greatly according to the differing needs in various biosynthesis. The small decrease seen in proline content (Figure 4.5) against the highest level of phenolic content (Figure 4.3) on day 8 may be because the proline was oxidized at a faster rate than what could be measured. The result also indicates that phenolic acid and proline may accumulate in plant in response to oxidative stress.

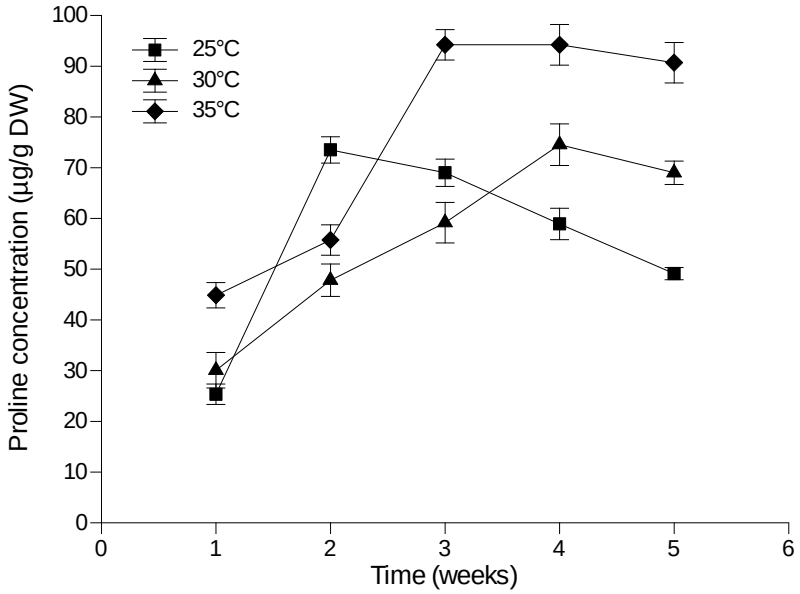

Figure 4.5: Proline concentrations of Moringa leaves grown in three different temperatures (25/12°C, 30/15°C, 35/18°C).

4.4.3 Non-structural carbohydrates

There were significant differences on carbohydrate of Moringa leaves. The 30/15°C recorded the highest sugar concentration, followed by 25/12°C, 35/18°C (Figure 4.6). Sucrose was the highest concentration in second week followed by glucose and fructose. Soluble sugar accumulation in plants as a defense mechanism is essential in osmoregulation.

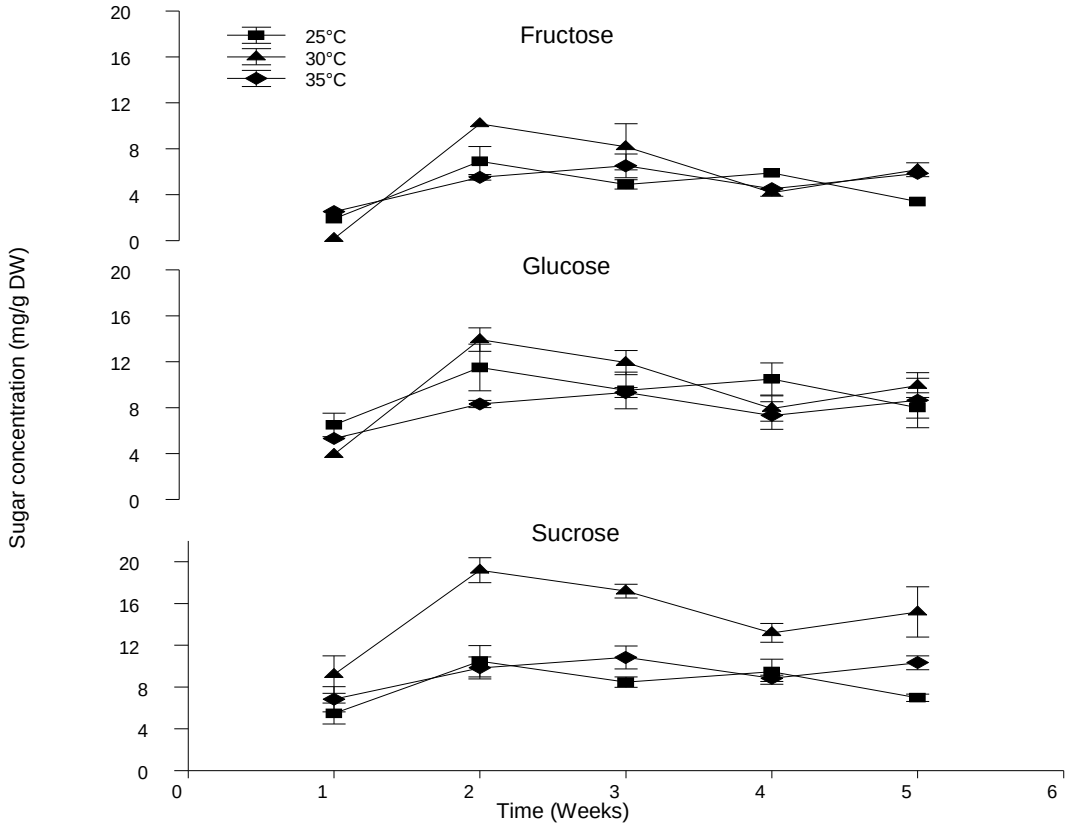

Figure 4.6: Non-structural soluble carbohydrates concentrations of Moringa leaves grown in three different temperatures (25/12°C, 30/15°C, 35/18°C).

4.5 Conclusions

The *M. oleifera* leaves are the most used part of the tree. They have several phytochemical compounds such as antioxidants, vitamins, carbohydrates, carotenoids, phenolic and flavonoids. Antioxidant systems are capable to protect the body against free radicals. The secondary metabolism is major for the plants; several secondary metabolites play main role as antioxidants defense compounds against free radical. Phenolic is one of the secondary metabolites produced by plants and it is has a significant role in human health which has various medicinal applications. Numerous plants respond to stress by an increase the proline and phenolic compounds. Proline may also act as an antioxidant through hydroxyl radical scavenging activity. In addition, Moringa tree adapted to a wide range of environmental conditions thus could improve the nutritional in Areas affected by malnutrition in developing countries.

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CHAPTER FIVE

General discussion and conclusions

The present study aims at investigate the effect of various temperatures on the growth of Moringa tree. Previous studies on *M. oleifera* have been focused on its nutritional and medicinal uses of the different parts of the tree (Lowell, 1999), and on the use of the seed in water clarification (Folkard et al., 1993); however, the best of our knowledge, few studies have been done on the effect of environmental factors on *M. oleifera*, one of such climate factors temperature is the most important role influencing on natural geographical plant distribution, physiology and productivity (Grace, 1987; Sakai and Larcher, 1987). Three temperature regimes were chosen to evaluate germination rate, antioxidant, phytochemical, and carbohydrate contents in the seeds and leaves of *M. oleifera*, the chosen temperature regimes were (30/20 °C, 25/15 °C, 20/10 °C,), 30/20 °C increased seed germination occurring within 48 h. Subsequently, germination was observed between 48 h and 72 h at 25/15 °C and after 72 h at 20/10 °C. Similarly, temperature especially 30/20 °C also increased total antioxidant, carbohydrates, phenolic and proline concentration. Proline and phenol might have a role in seed germination. Proline accumulation in plant cell could have been stimulated in response to growing conditions. Thus, temperature treatments of Moringa seeds and leaves effect in the rate of germination and biochemical changes, which are associated with various antioxidants and their mobilization**.** Previous research has shown that plant adaptations in response to their environmental conditions are often expressed through anatomical modifications (Shao et al., 2008).

The results of the present study also showed that M. *oleifera* parts such as leaf, stem, root, seed and seed coat contain high levels of antioxidants concentrations, vitamins, mineral, protein, and phenols. The leaf was recorded the highest antioxidant concentration (1.7 mg g^{-1}), total phenols (63 µg g^{-1}) as well as leaf ascorbic acid (1.9 mg g^{-1}). The highest concentration of total crude protein (110.4mg g^{-1}) and vitamin E (28.57 µg g^{-1}) was found in seed. Various parts of Moringa showed different nutrients such as Potassium accumulated in root, stem, and seed coat, phosphorous also accumulated in leaf and seed. Although Moringa is a tree that has received

interesting in many countries, due to the high nutritional value of its leaves, but this tree is unknown to many South African communities. It is thus recommended that:

- *M. oleifera* could be introduced and integrated into local food system to improve the local diets.

- *M. oleifera* is adapted to different temperature levels thus, it is recommended for cultivation in most of the areas where climatic conditions favor its optimum growth.

- The results showed that Moringa leaves contain different antioxidants compounds therefore it can be consumed as a sources of nutrition food in rural area.

- More research is needed in the physiology and growth of *M. oleifera* to follow its response to temperature.

- Further work to investigate performance and adaptation is required.

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