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Assessing the effect of extreme temperature conditions on the morphology, anatomy and phytochemistry of *Moringa Oleifera* leaves

by

Luvo Ntsangani



UNIVERSITY OF THE
WITWATERSRAND,
JOHANNESBURG

School of Animal, Plant and Environmental Sciences

A thesis submitted to the Faculty of Science, in partial fulfilment of the requirements for the degree of Master of Science

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Declaration

I declare that this thesis is my own original work. It is being submitted for the Degree of Master of Science to the University of the Witwatersrand, Johannesburg. It has not been submitted for any other higher degree or examination in any other university.

The experimental work described in this thesis were carried out in the School of Animal, Plant and Environmental Sciences and School of Chemistry, University of the Witwatersrand, Johannesburg, South Africa, under the supervision of Doctor Ida Risenga (School of Animal, Plant and Environmental Sciences, University of the Witwatersrand) and Professor Luke Chimuka (School of Chemistry, University of the Witwatersrand).

Signature 

(Luvo Ntsangani)

Date 10 October 2018

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Abstract

Since its discovery in India many centuries ago, *Moringa oleifera* has been used largely in the tropics and subtropics where it is native for nutritional, medicinal and industrial benefits. These benefits are attributed to the antioxidant, antifungal and anti-inflammatory properties in the phytochemical compounds of *M. oleifera.* Agriculture of *M. oleifera* in South Africa is currently minimal despite its multiple benefits. Naturalisation of *M. oleifera* in the country is important in the aid to eliminate the shortage of nutritional food reserves, and utilise the plant as a source for realising and developing new medicinal compounds. Global warming and consequent climate change have caused extreme high and low temperature seasons in the past in South Africa. Studying the effects of extreme high and low temperatures on the growth, performance and phytochemistry of *M. oleifera* at controlled conditions is vital in understanding its responses and adaptability.

M. oleifera grown for three months in a greenhouse of the University of the Witwatersrand was subjected to two extreme temperature conditions of 15/10°C and 47/29°C for seven days (144 hours). The control was kept at ambient temperature (25/22°C). The morphology, histology, total phenolic contents, total flavonoid contents, quercetin and kaempferol concentrations were assessed using dissecting microscope, light electron microscope, UV-Vis spectroscopy and HPLC-UV respectively. The effect of high temperatures showed increased senescence. However, plants that were treated with low temperature appeared to be more tolerant by indicating a delay in leaf senescence. Yellowing, browning, curling and drying of the leaves worsened after 144 hours at high temperature treatment. The histological examination at high temperatures showed intact cellular structures such as upper epidermis, lower epidermis, spongy mesophyll, palisade mesophyll and intercellular spaces in green leaves after 24 hours. However, leaf size decreased over the seven days treatment. The cellular structures in leaves that became yellow had collapsed and showed evidence of necrosis. In the low temperature treatment, 1% of leaves showed signs of leaf curling after

48 hours while others remained green and intact. After 144 hours, 5% of leaves showed signs of senescence while others turned dark green and remained intact. Leaves cells such as palisade mesophyll had elongated and thus increased the thickness of the leaves. This was evidence of acclimation to cold temperatures.

The total phenolic content increased by 10.1% after 48 hours under high temperatures. However, a decrease of 3.5% and 3.1% was observed after 96 and 144 hours respectively. Quercetin increased by 8.5% after 48 hours then after 96 and 144 hours a decrease of 41.7% and 61.6% was observed respectively. A significant increase in kaempferol concentration of 203.5% after 48 hours was noted. This was followed by a decline after 96 and 144 hours of 43.0% and 24.8% respectively. Concentration of total flavonoid content increased by 11.6% and 33.8% after 48 and 96 hours, and after 144 hours, a decline of 0.4% was observed.

The combined evidence from the present study showed that the three months old *M. oleifera* plants are sensitive and cannot withstand temperatures as high as 47/29°C. However, the species has showed to be positively more tolerant to cold temperatures (15/10°C). This evidence may have meaningful contribution towards the industry, *Moringa oleifera* farming practices as well as advance the understanding of the effects of extreme temperatures on the species.

Symbols and abbreviations

M. oleifera	-	Moringa oleifera
A. D	-	after death
et. al	-	et alia
°C	-	degrees Celsius
mm	-	millimeter
cm	-	centimeter
%	-	percent
β	-	beta
α	-	alpha
ROS	-	reactive oxygen species
BHA	-	butylated hydroxy anisole
CO_2	-	carbon dioxide
cm^2	-	square centimeter
SA	-	salicylic acid
HPLC-UV	-	high pressure liquid chromatography – ultra violet
L	-	liter
mL	-	milliliter
µm	-	micrometer
TR	-	temperature regime
HT	-	high temperature
LT	-	low temperature
H_2O_2	-	hydrogen peroxide
DNA	-	deoxyribonucleic acid

O_2	-	oxygen
µmol	-	micromole
ET	-	extreme temperature
ppm	-	parts per million
mg/L	-	milligram per liter
mg	-	milligram
TPC	-	total phenolic content
TFC	-	total flavonoid content
v/v	-	volume per volume
$m^{-2}. s^{-1}$	-	per meter squared per second
µm	-	micrometer
rpm	-	revolutions per minute
g	-	gram
nm	-	nanometer
UV-Vis	-	ultra violet visible
C_{18}	-	carbon 18
PC	-	phenolic compounds
µL	-	microliter
CR	-	cellular response
CD	-	cellular damage/death
i.e.	-	that is

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CHAPTER 1: INTRODUCTION

1.1 Background on *Moringa oleifera* and its importance

Moringa oleifera is a plant species belonging to the family *Moringaceae* that has a major subdivision called *Moringa* which has a total of thirteen species including *M. oleifera* (Mahmood *et al*., 2010). It was discovered in the northwest region of India on the south side of the Himalayan mountains and its existence dates back as far as 150 A.D where it was used by royal families for healthy skin and mental alertness, and by soldiers for physical energy and wound healing (Gilani *et al*., 2007, Mahmood *et al*., 2010). *M. oleifera* is largely cultivated, harvested and distributed in Pakistan, India, Bangladesh, Nepal, Afghanistan, Sri Lanka, West Asia, the Arabian Peninsula, East and West African countries like Ethiopia and Nigeria as well as Central and South American countries like Brazil, Venezuela, Southern Florida, throughout the West Indies, and from Mexico to Peru and Paraguay (Anwar *et al*., 2006, Parrotta *et al*., 2009, Mahmood *et al*., 2010). This species grows best in tropical and subtropical climate conditions of 25-35 ˚C temperatures, on sandy or loamy soil that have slight acidic to alkaline pH. It is naturally fast-growing and can reach maximum height of 5-12 meters. It is a drought resistant plant and can withstand a variety of annual rainfalls that ranges from a minimum of 250 mm and a maximum of 3000 mm (Gilani *et al*., 2007, Mahmood *et al*., 2010, Kumar *et al*., 2016).

It has leaves that look like feathers and on a matured tree, the bark is thick and whitish, and has fragile branches that hang downwards (Gilani *et al.*, 2007, Parrotta *et al*., 2009). *M. oleifera* leaves' arrangement on the twigs is circling and alternate, the length and width of the leaflets varies from 1-2 and 0.6-1 centimeters depending on the plants maturity (Parrotta *et al*., 2009). The sideway leaflets take the form of an ellipse and the side-end leaflets are narrow at the base. *M. oleifera* leaves have few hairs on the abaxial side and completely hairless on the adaxial side and have red midveins, and the tips are rounded, and the base is

sharp (Parrotta *et al*., 2009) Figure 1.1. The tender leaves taste like watercress and are eaten raw, cooked or incorporated into food stuff as dry powder by human beings for nutritional and medicinal benefits (Anwar *et al*. 2006, Philominatal *et al*. 2016). It has been largely published that *M. oleifera* leaves are extremely nutritious because they contain more nutrients than the other parts of the plant and common food stuff. Industrially, a 30% aqueous extract of *M. oleifera* has shown to promote the plant growth, increased chlorophyll and phenolic content in maize seedlings (Basra *et al.*, 2011).

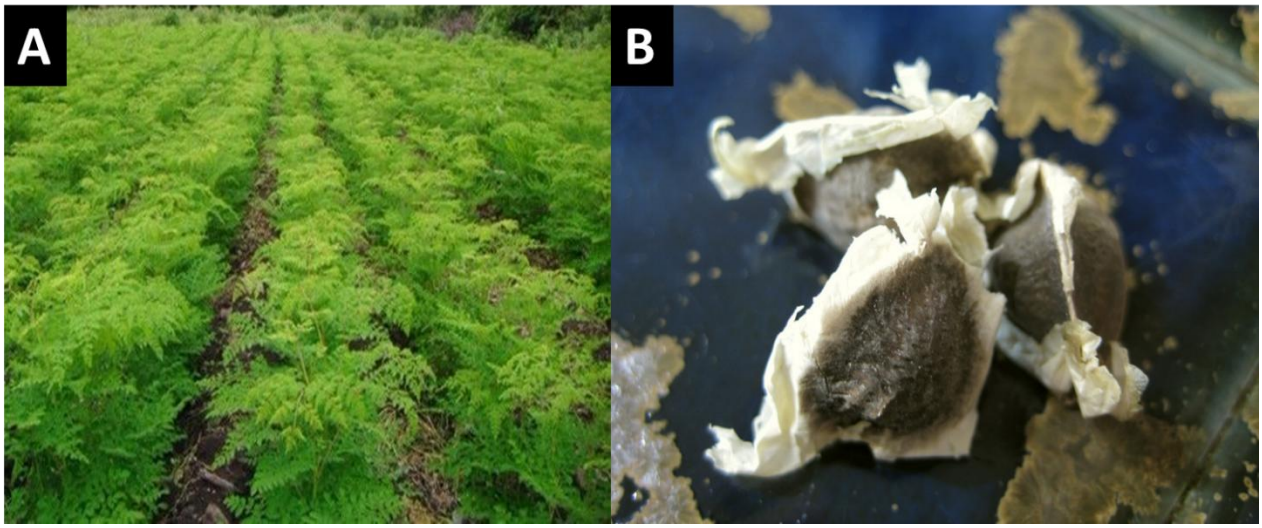


Figure 1.1 M. oleifera (A) Trees plantation. (B) Seeds. "Where in the World is Moringa Oleifera." (2017, July 24)

M. oleifera's nutritional, medicinal and industrial benefits to humans has multiplied drastically throughout the years. Although it has been used by humans for many centuries for multiple purposes, only in recent years the government of South Africa has supported the agriculture and harvest of this miracle tree in the country. Besides being used to combat malnutrition in impoverished communities in South Africa, it will be used to develop new

medicine and commercial products that will add value to citizens. *M. oleifera* is native to tropics and subtropics regions, South Africa does not have climate conditions conducive to the species. Naturalisation of *M. oleifera* in South Africa is critical to allow its large-scale agriculture and bioprospecting. Experimental research mimicking extreme South African climate temperatures is vital to understand the plants' response, performance and adaptation. Thus, the effect of high and low temperature on the morphology, anatomy and phytochemistry of *M. oleifera* is important for this study and are the objectives of this research project.

CHAPTER 2: LITERATURE REVIEW

2.1 Seed germination, plant growth and cultivation of *M. oleifera*

M. oleifera seeds are dark brown, large round seeds with white-greyish wings (Figure 1.1). Studies showed that they provide nutritional and medicinal benefits because they have antioxidant and anti-inflammatory properties due to their high contents in proteins, vitamins, minerals and metabolites (Copyright Moringasiam, 2018). Dimeric cationic proteins found in the seeds are the compounds responsible for its coagulation properties suitable for water purification (Anwar *et al*., 2007). To keep the species in existence, naturalisation and cultivation of the plant needs to be achieved in large quantities for sustainability. Cultivation starts with planting the matured ovule containing embryo and stored food supply protected by a seed coat in the soil to initiate seed germination (Jann *et al*., 1977). For the seed to develop into a seedling, the embryo needs to be active and able to undergo the germination process. Suitable environmental conditions such as water, temperature, oxygen and light need to be optimal to break dormancy (Bentsink *et al*., 2008). During germination, initially a rapid uptake of water by the seeds occurs and followed by a lag phase where less water is absorbed but vigorous metabolomic reactions occur such as maturation of the mitochondria, protein synthesis and production of substrates from stored macromolecules for energy to prepare for radicle protrusion (Schopfer *et al.*, 1984, Allen *et al*., 2000). The metabolomic

reactions result in cell elongation-division cycles while shoot and root meristematic cells form in the embryo which give rise to the seedling consisting of roots and shoots. Under favourable conditions, the seedling will grow into a matured tree (Muhl *et al*., 2009).

Another method of cultivation is by cutting the stem bark of matured trees and planting them in the soil. It has been reported that cultivating plants by cutting rather than planting the seeds is preferred because the plant cuttings root easily, the fruits become of high quality and the plant grow faster to sizeable trees in few months (Ramachandran *et al*., 1980). Plant tissue culture is another method of cultivation whereby plant cells, tissues or organs such as buds and apex from explants are planted in suitable growth medium under controlled conditions (Morales-Domínguez *et al*., 2017). Monitoring seed germination rate of *Eurotia lanata* at five different temperatures of 0-20˚C for four days imbibition period revealed that axial length and dried seed weight decreased as the imbibition temperature increased, so this means the seeds should be planted in the field late winter to take advantage of cold imbibition (Booth, 1992). Season also plays an important role on the initial growth of *M. oleifera*.

The effect of different plant densities (actual number of the same species in a specific area) of *M. oleifera* planted in autumn-winter season showed that plant height and stem diameter were not affected by these densities but instead they grew bigger when the plants were left to grow for a longer period. The initial growth rate of the plants at low temperatures were slow and rate of plant death was high (da Costa *et al.*, 2015). The seed germination rate increases with a decrease in temperatures of about 10˚C and the seedling, stem size, leaf size and plant height increases with increased temperatures of 25-30˚C (Muhl *et al.*, 2009). Commercial enhancers such as 2-(1-naphthyl) acetic acid and 4-indol-3-ylbutyric acid are sometimes used to promote healthy growth however they are often expensive and sometimes unavailable unlike *M. oleifera* aqueous extract.

2.2 Nutritional properties of *M. oleifera*

M. oleifera has been largely reported to contain high content of phytochemical compounds with high nutraceutical values because it plays a vital role in the growth, development and healing of mammals (Kumar *et al.*, 2016). Nutrients such as carbohydrates, proteins, fats, fiber, vitamins (A, B1, B2, B3, C and E), thiamin, niacin, riboflavin, folate and minerals (calcium, phosphorus, sodium, potassium, iron, zinc, magnesium and copper) are found in all the part of the species (Sanchez-Machado *et al.* 2010, Witt *et al*., 2011, Jayawardana *et al*., 2015, Kumar *et al*., 2016). The dried leaves contain seven times more vitamin C than the amount found in orange, ten times more vitamin A compared with carrots, more than seventeen times calcium in relation to milk, fifteen times more potassium found in banana, nine times more protein found in yogurt and twenty-five times more iron than the amount found in spinach (Mahmood *et al*., 2010, Mishra *et al*., 2012, Purohit *et al*., 2015). β-carotene, carbohydrates, fat, fiber, magnesium, phosphorus, copper, sulphur and natural antioxidants like phenolics, flavonoids and ascorbic acid are found in the leaves of *M. oleifera* and other selected plant parts such as the stem barks and flowers (Gilani *et al*., 2007, Purohit et al., 2015, Kumar *et al*., 2016).

This plant has also been incorporated in food eaten by animals to promote growth, carcass characteristics, digestibility and milk production (Sebola *et al*., 2015, Miron *et al.*, 2016). Due to its antioxidant, antifungal and antimicrobial activities, *M. oleifera* has been reported to prolong the shelf life, lowers the rate of spoilage, discolouration and foul smell of food products (Salam *et al*., 2004, Jayawardana *et al*. 2015). Its extracts are used as spray on plant crops to enhance plant growth and reduce disease and control pests (Mahmood *et al*., 2010). The leaf powder and seeds are also being used as biosorbent to adsorb metals and toxic compounds from aqueous and biological solutions (Seshaiah *et al*., 2010, Basheer *et al*., 2016). Rats were fed iron deficient diets for ten weeks and thereafter fed dietary iron from *M. oleifera* leaves and ferric citrate. Results revealed that *M. oleifera* diet was more superior in overcoming the effects of iron deficiency than ferric citrate diet. This means that the leaf powder can be used as dietary iron supplements (Giridhar *et al.* 2014). The concentration of

these compounds varies depending on the way the samples are preserved or processed. The content of vitamin E is at its highest in fresh leaf samples than in the freeze dried and hot air-dried samples while boiling the sample significantly reduces the concentration (Ningli *et al*., 2016). It has been reported that fresh leaves contain a high content of micronutrients such as β-carotene, α-tocopherol and lutein with very low bio-accessibility during digestion. A process of freezing, steaming and sterilizing the leaves preserve much of these micronutrients and makes them more bio-accessible as compared with sun-drying and frying but less bio-accessible to leaves that have been dried at 60˚C and finely grounded (Avallone *et al.*, 2017).

M. oleifera also contains phenolic and flavonoid compounds (Nadkarni *et al.*, 2005, Anwar *et al.*, 2006, Anwar *et al.*, 2007). Sholapur *et al*. (2012) found that the barks of this species contain triterpenoids, isothiocyanate glycosides, tannins, sulphoraphane and steroids using petroleum ether and ethanolic extracts of the bark and thin layer chromatography profiles. These findings were used to identify and show that the bark of the species have pharmacognostic and phytochemical characteristics. Furthermore, the study showed that the species contains all the essential nutrients which are vital for the functioning of the human and animal body (Nadkarni *et al.*, 2005, Anwar *et al*., 2006, Sholapur *et al*., 2012) as well as carotene and amino acids for better eyesight and tissue repair (Sholapur *et al*., 2012).

Amino acids such as lysine, methionine, cysteine, tryptophan and methionine, ascorbic acid, triterpenoids, isothiocyanate glycosides, tannins and sulphoraphane compounds are distributed in different parts of the species (Makkar *et al.* 1997, Nadkarni *et al.* 2005, Anwar *et al.*, 2006, Anwar *et al.* 2007, Moyo *et al.* 2012, Mishra *et al.*, 2012, Jayawardana *et al*., 2015, Zeren *et al*., 2016). To further demonstrate the potential of this *M. oleifera* as a vegetable, Pakade *et al*. (2012) compared the flavonoids and essential elements concentration on leaves to some selected vegetables such cabbage, spinach, cauliflower, broccoli and cow peas. Results showed that leaves contained higher concentrations of flavonoids identified as myricetin, quercetin and kaempferol as compared with the above-mentioned vegetables (Pakade *et al*. 2012).

2.3 Medicinal properties of *M. Oleifera*

The leaves also contain a good proportion of essential amino acids like arginine and histidine, which play a vital role in assisting infants to produce enough proteins so that they can grow properly (Mishra *et al*., 2012). It has been reported that not only the leaves of *M. oleifera* have nutritional and medicinal properties, but also the roots, stem barks, seeds, gums and flowers have these properties (Gilani *et al*., 2007). Nadkarni *et al*. (2005) found that the stem barks of *M. oleifera* are traditionally used to increase menstrual flow and as an antifungal agent, the leaves as intestinal deworming, antitubercular, antifertility agents and prevent the formation of kidney stones. All the different parts of the plant have been reported and scientifically proven to have anti-inflammatory, antidiabetic, antihypertensive, diuretic and cholesterol lowering activities (Chen *et al*., 2007, Sreelatha *et al*., 2009), antispasmodic, antiulcer and hepatoprotective activities (Purohit *et al*., 2015, Kumar *et al*., 2015), antibacterial and antifungal activities, antitumor and anticancer activities (Sholapur *et al*., 2012, Dubey *et al*., 2014). *M. oleifera* has also been reported to be a central nervous system depressant because it promotes calmness, induce sleep and protects against epilepsy (Anwar *et al*., 2006, Gilani et al., 2007).

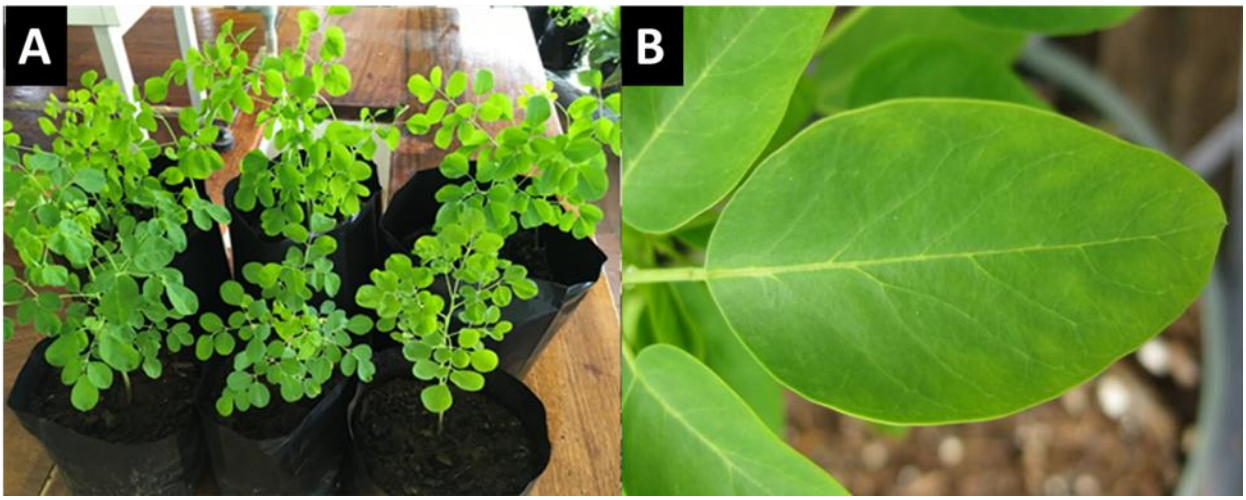


Figure 2.1 M. oleifera grown at the greenhouse, University of the Witwatersrand (A) Three months old plant. (B) Healthy green leaf

M. oleifera is also used to cure sicknesses such as tumours (Nadkarni *et al*. 2005), ulcers, wounds, scurvy and skin infections (Nadkarni *et al*. 2005 and Anwar *et al*. 2006). Nanoparticles therapy incorporating natural antioxidants has been reported to promote wound healing. Titanium oxide nanoparticles based on the leaf extract were synthesised to heal wounds of Albino rats by monitoring their closure for 12 days. This nanoparticle was compared with the healing rate of standard drug sulphadiazine and it was found that using *M. oleifera* titanium nanoparticles, healing rate was 92.36% as compared with 83.55% of sulphadiazine (Philominathan *et al.* 2016). Anxiety, asthma, fever, bronchitis and headaches (Anwar *et al.* 2007 and Gilani *et al*., 2007) are also reported to be cured by *M. oleifera*. Other ailments cured by this species are diarrhea, diabetes, semen deficiency, malaria, cardiovascular disorder and blood impurities (Chen *et al*., 2007, Mahmood *et al*., 2010). This 'miracle' tree possesses anti-inflammatory, antipyretic, antiepileptic, antispasmodic, antihypertensive, diuretic, hepatoprotective, antioxidant, antibacterial and antifungal activities and acts as cardiac and circulatory stimulant (Sholapur *et al.* 2012, Dubey *et al.*, 2014, Purohit *et al.*, 2015, Zeren *et al.* 2016). Anticancer activities were achieved by the silver

nanoparticles in the stem bark extracts. The nanoparticles inhibited cell division and multiplication of human cervical carcinoma cells (Dubey *et al*., 2014).

Aqueous extract and seed powder of *M. oleifera* has been found to cure type 1 (insulin deficient) and type 2 (insulin resistant/fails to sense glucose levels) diabetes by dropping fasting blood glucose and increasing antioxidant enzymes in serum which reduce reactive oxygen species that reduce insulin secretion, it is also used as an anticancer agent because it inhibits the growth of cancer cells by reducing the reactive oxygen species (ROS) in cancer cells (Kumar *et al*., 2016). Loss of the neurotransmitter acetylcholine in the brain results in the formation of Alzheimer's disease and the way to treat this disease is to use cholinesterase inhibitors to prevent the formation of acetylcholinesterase. *M. oleifera* showed 5% inhibition of acetylcholinesterase and 50% inhibition with *Withania somnifera* was observed (Prashanth *et al*., 2007).

2.3 Industrial applications of *M. oleifera*

M. oleifera is used in the agro-processing industry to convert the fresh leaves into leaf powder and extracting the oil from the seeds. Tea, tablets, capsules, soaps, powder, oil and beverages are some of the few bio-prospected products made from *M. oleifera* (Mishra *et al.*, 2012). In the baking industry, for example, studying the antioxidant and quality characteristics of the leaf extract on a Korean rice cake revealed that the addition of 0.1% of the extract on the ingredients increases the cake's antioxidant activities and gives better colour, moistness, texture and taste as compared with the cakes without the extract (Kim *et al.*, 2016). The replacement of wheat flour with 10% of dried leaf powder showed an increase in protein, β-carotene, dietary fiber, iron and calcium contents. Furthermore, the incorporation of the leaf dried powder in baking decreased the springiness, gumminess, adhesiveness and cohesion of the bakery products (Dachana *et al*., 2010).

Butylated hydroxy anisole (BHA), butylated hydroxytoluene and tert-butyl hydroquinones are synthetic antioxidants that are mostly used to preserve food such as meat and chicken sausages during food processing (Jayawardana *et al*. 2015). These synthetic antioxidants have been found to contain trace amounts of toxic chemicals such as methyl alcohol and hydrogen sulfide (Salam *et al.*, 2004). The findings have made meat processors to seek alternative products that do not contain traces of toxic chemicals that might cause harm and risk to human and animal health. It has been reported that natural antioxidants and antimicrobial agents derived from plant sources such as *M. oleifera* do not possess any traces of toxic chemicals and thus they are preferred by meat processors and consumers (Salam *et al*., 2004). Juyawardana *et al*. (2015) showed that the shelf life of chicken sausage was extended when the dried leaf powder was incorporated inside the chicken sausages. Lower values of 2-thiobarbituric acid reactive substances were observed as compared with those incorporated with BHA. In addition, the chicken sausages incorporated with the leaf powder showed lower rate of spoilage, discolouration and foul smell (Jayawardana *et al*. 2015). The dried leaf powder is also used in animal feed industry. For example, when 18% of the leaf powder, wheat hay and sugar cane mixture was incorporated in solid additive foods of lactating cows, the food intake and protein milk content of the cows fed with the mixture was less than that of the cows fed with control additive. The milk yields, production efficiency, fat corrected milk and energy corrected milk was higher on the cows that were fed with the mixture that included *M. oleifera* (Cohen-Zinder *et al*., 2016). Sebola *et al.* (2015) showed that adding the dried leaf powder on broiler finisher diet for poultry improved the growth rate and overall carcass analysis (Sebola *et al*., 2015).

The oil in *M. oleifera* seeds have been largely extracted and used as a natural oil in combination with other natural and synthetic oils to produce healthy oil mixtures. There are many methods to produce oils that are trans fatty acid free, stable, high quality and good physicochemical properties, however oil blending method is the most efficient economically easy to use and control method that produces oil mixtures that are of high physicochemical quality, stable and highly nutritious (Torbati *et al*., 2016). Aqueous enzymatic extraction of oil

from seeds results in low oil yields as compared with the solvent extraction due to the formation of stable cream emulsion after extraction. Subjecting the seeds to high pressure processing rather than boiling them before extraction significantly reduces the thickness of the stable cream emulsion and improves oil yields (Yusoff *et al.*, 2017).

Natural coagulants such as *M. oleifera* seeds are thoroughly being studied as an alternative replacement to chemical-based coagulants. Cyanobacteria was successively removed from surface waters by the active bio-coagulate compounds in *M. oleifera* seed saline extracts. These seed extracts also removed aromatic organic compounds, chlorophyll *a*, initial turbidity of 5-10 NTU and 30-60 NTU from these surface waters (Yin, 2010, Camacho *et al*., 2017). These bio-coagulant properties of *M. oleifera* seeds are attributed to the thermoresistant homologous proteins present (Ghebremichael *et al.*, 2005). The use of M. oleifera to remove turbidity and microbiological contamination on surface and drinking water in poverty stricken areas would be ideal not only of the efficiency of this plant but because it is also cost effective (Lea, 2014). Heavy metal poisoning of manufacturing industry's effluent water is under high regulations by government agencies due to it high human and animal health risk when it poisons drinking waters. A chemically modified leaf powder has been used as a biosorbent to bio-absorb lead from effluent water and the results of thermodynamic properties using Langmuir method showed that the biosorption of lead onto the leaf powder biosorbent was spontaneous and this biosorbent was easily regenerated for further use when treated with dilute hydrochloric acid (Reddy *et al*., 2010).

The antioxidant properties of *M. oleifera* attributed to its phenolic compounds are also explored in the fuel industry. A leaf ethanol extract used as an antioxidative additive significantly inhibited oxidation in biodiesel, the biodiesel was stable during storage and had good quality physicochemical properties (França *et al*., 2017). The aquatic system is also vulnerable to contamination with toxic heavy metals from untreated wastewater discharges from mines. *M. oleifera* leaf powder was used as a biosorbent of cupper (II) ions from an untreated gold mine wastewater. It was observed that during the removal of copper (II) ions,

ion exchange of magnesium (II) and potassium (II) for copper (II) on the *M. oleifera* biosorbent occurred (Acheampong *et al*., 2011). In another similar experiment, leaf powder was chemically modified using an esterification process to make a cationic based biosorbent and the biosorbent successfully removed cadmium (II), copper (II) and nickel (II) ions from the aqueous solutions (Reddy *et al*., 2012). With all the literature readily available that confirms that this species contains all these phytochemicals that are essential for nutrition, medicinal and industrial benefits particularly when the leaves are consumed raw, cooked or incorporated into food products as powder by humans to counteract the effects of malnutrition, it is important to study whether environmental factors such as temperature can affect the concentration of these phytochemicals negatively or positively.

2.4 Environmental factors and effects on medicinal plants

Most often in scientific communications, the term climate change is not explained clearly, and this creates confusions in why it exists and its impact on global warming which is the increase in the Earth's surface temperature (Werndl *et al*., 2013). In simple terms, climate is the expected weather in a geographical area measured over a specific period. The measured weather conditions give average temperatures and predict the normally expected number of rainy days and droughts (Nation Research Council of the Academies). A change in these normally expected weather conditions gives rise to climate change. Technically, climate change is the distribution of climate variables such as surface air temperature, air pressure, sub-surface ocean temperature and ice sheets (Werndl *et al.*, 2013). It has been reported that human activities such as burning of fossil fuel, cement production and deforestation which results in the increase of carbon dioxide (CO_2) concentration in the atmosphere has resulted in climate change and in the past 100 years the earth's surface temperature has increased by 0.8 °C which is a significant change when this temperature is averaged over the entire globe (Bita *et al.*, 2013).

It has been predicted that the increase in temperature would be 0.2 °C per decade, and this will result in many droughts with less cold day and night cycles but more hot day and night cycles, extreme temperatures, Arctic and Antarctic sea ice to decrease and altered precipitation patterns (Aggarwal *et al*., 2008, United Nations Framework Convention on Climate Change, 2011). Extreme temperatures and droughts not only affects the production of food crops but also medicinal plants which play a vital role as an alternative to synthetic medicine in developing countries. Thus, understanding the effect of extreme temperatures on medicinal plants is vital so that adaptation strategies on plant growth can be implemented. In developing countries, it has been estimated that 80% of the population use medicinal plants for their primary health care (Gairola *et al*., 2010). Worldwide estimation of 50000 – 70000 medicinal plant species are used for medicine but there are still many species that are unidentified in the wild which are potential sources to medicines. Both the unidentified and already known species are most vulnerable to climate change and, therefore, studying the effect of climate change such as extreme temperatures on medicinal plants is of paramount importance.

2.5 High and low temperature stress on plant's morphology and anatomy

Due to climatic changes, distressing effects such as extreme temperatures stresses especially high and low temperatures on plant growth and metabolism have become a major concern because crop production is reduced by abiotic stresses (Hasanuzzaman *et al.*, 2013). High temperature stress causes irreversible damage to plant cells and the responses vary depending on the severity of the temperature, duration and plant type. Low temperature stresses also cause reduction in crop production and these are chilling stress that causes damage on plant cells without forming ice and freezing stress whereby ice is formed within plant tissues (Hasanuzzaman *et al.*, 2013). Retardation of growth evident through plant size, weight and height are observed when plants are exposed to high temperature stress due to the transformed cell elongation rate and cell division. Inhibition of metabolic reactions occurs in plants subjected to low temperature stress which results in injuries such as leaf yellowing,

leaf whitening, white specks, white bands, withering after transplanting. Furthermore, photosynthesis is also reduced, and this results in reduced plant growth and yield loss due to less carbohydrate formation (Hasanuzzaman *et al.*, 2013). Hatfield *et al*., (2015) reported that during controlled temperature experiments, the grain yield was reduced at warm temperature and the rate of pollination was higher than in cooler and ambient temperatures. A study conducted by Velasco *et al*., (2015) revealed that when *Brassica oleracea L* was exposed to low temperatures, stomatal conductance and leaf water content were reduced due to the leaves that were small and thick. At high temperatures, the leaves had reduced leaf weight and size due to the disturbed photosynthesis reactions (Velasco *et al*., 2015).

As much as the pharmacognostic and phytochemical properties of *M. oleifera* are important, microscopic studies are also important. The effect of extreme temperatures during growth and developmental stages adversely affect the fruit or grain production and flowering of all plant species. Muhl *et al*., (2011) studied the physiological adaptations of *M. oleifera* to lower and higher temperatures using different controlled temperature ranges from 10-30°C. Electron microscopy studies revealed that the thickness of the leaves was greater at lower temperatures and this was due to the spongier mesophyll and longer palisade cells in leaves on the 10°C temperature range as compared with leaves on the 30°C temperature range. They also found that the stomata and epidermal cells in the leaf area were higher in number at the lower 10°C temperature range than the 30°C temperature range, but the individual stomata were smaller in size for the 10˚C temperature range as compared with larger stomata size on the 30°C temperature range (Muhl *et al.*, 2011). Furthermore, it was shown that at 30°C temperature range (after 32 weeks) the leaves had higher average leaf area of 1980.34 cm^2 as compared with the 10°C temperature range with 1031.57 cm^2 average leaf area. The greater leaf area at higher temperatures resulted in a higher rate of photosynthesis (Muhl *et al.*, 2011 and 2012). Therefore, this demonstrated that higher temperatures favoured the plant growth and lower temperature range resulted in reduced plant growth. The plants were however still able to grow at low temperatures due to the physiological adaptation of the plant to lower temperatures (Muhl *et al*., 2012).

Abnormal growth in a plant causes destruction to its normal life processes and these are attributed to biotic diseases caused by viruses, bacteria and fungi or caused by non-living environmental conditions such as extreme temperatures, chemical toxicity, wind, frost, soil and water concentrations (Kennelly *et al.*, 2012, Small *et al.*, 2017). The morphology and histology of plant leaves is affected by factors such as the temperature of the environment, soil type with its pH, electrical conductivity and metal ion concentrations, water availability, oxygen, light and pathogen attack (Cramer *et al.*, 2004, Muhl *et al.*, 2011, Ter Heerdt *et al*., 2017). The physiology and metabolism of plants exposed to high temperature stress changes and the severity depends on the age of the plant, duration of exposure and these are attributed to physiological damage observed such as leaf senescence and abscission, leaf scorching, inhibition of shoot and root growth which results in the overall decreased plant growth (Bita *et al.*, 2013). Biochemical reactions within plants are sensitive to changes in temperatures and the inborn acclimation mechanism are triggered to allow the plant to adapt to these changes and off-set the stress-induced alterations. At extremely high temperatures, disastrous collapse of the cellular organization occurs due to cell damage or death (Fujita *et al.*, 2013).

Plants that are native to warm temperatures shows signs of damage when exposed to low non-freezing temperatures and crop yields are reduced. Plants reproductive development is negatively affected by cold stress and this is observed by the yellowing of leaves (chlorosis), wilting and reduced leaf expansion and possible tissue death (necrosis) (Yadav *et al.*, 2009). Also, when plants are exposed to high temperature and low soil moisture, scorching of the leaves, premature leaf drop, and overall plant death may occur. At temperatures above 35°, new leaves of *Pelargonium spp.* become bleached (Kennelly *et al.*, 2012). Although plants are sensitive to extreme temperature stresses, they contain heat shock proteins (HSP) that protect the plant against heat shock and maintain homeostasis (Yadav *et al.*, 2009 and Kennelly *et al.*, 2012). HSP prevent the multiplication of denatured proteins and promote renaturalisation of proteins (Iba *et al.*, 2002). The effect of high and low temperature on the

morphology and histology of *M. oleifera* leaves has not been studied. Therefore, this gives significance of this research project to be carried out.

2.6 High and low temperature stress on phytochemical compounds

Climate change not only changes the phenology of plants but also the phytochemicals such as secondary metabolites. In most cases, the concentration of secondary metabolites increases when plants are stressed because the biochemical reactions responsible for growth are inhibited more than photosynthesis. Furthermore, it has been shown that the composition of secondary metabolites varies depending on the type of species, stress and chemical type (Gairola *et al*., 2010, Harish *et al.*, 2013). Although these compounds do not play a vital role in the maintenance of plants life, they play a crucial role in making the plant interact with its environment for adaptation and defense (Ravishankar *et al.*, 2011). The effect of cold and hot seasons on the metabolites of *Thithonia diversifolia* were investigated. It was found that during the hot months, the concentration of secondary metabolites such as trans-cinammic acid ester derivatives were high while during the cold months, the concentration of sesquiterpene lactones were high.

During the transition period between the hot and cold months, intermediate concentrations of these secondary metabolites were obtained (Edrada-Ebel *et al*., 2016). As previously mentioned, plants respond differently to temperature stresses depending on the type of species. When *Arabidopsis* was subjected to hot and cold temperatures of 40°C and -4°C for 1 hour, the concentration of salicylic acid (SA) increased on both temperatures demonstrating that the species was in a defensive mode because an increase in SA meant there was an increased level of resistance to pathogens and mostly all other stresses within the plant. A similar increase in concentrations of amino acids, polyamine precursors and compatible solutes were observed on both heat and cold temperatures (Guy *et al.*, 2004). When *M. oleifera* leaves were harvested in different locations at different seasons, it was observed that

the leaves harvested in the cold season had a higher concentration of antioxidant compounds such as total phenolics, total flavonoids and ascorbic acid (Iqbal *et al*., 2005).

M. oleifera has been identified centuries ago as a medicinal plant and one of the plants used in traditional medicine. These types of plants play a crucial role in the development of novel drugs because they are a large source of natural antioxidants (Bag *et al*., 2015). Two types of metabolites are produced by plants and these are primary and secondary metabolites. Primary metabolites such as carbohydrates, proteins and lipids are responsible for the growth and metabolic reactions on the plant. Secondary metabolites such as alkaloids, phenolics, steroids and essential oils are not involved in metabolic pathways but give the plant its antioxidant properties (Croteau *et al*., 2000, Bourgaud *et al*., 2001). In a living animal, for energy to be produced during metabolism, O_2 must be present but because oxygen is highly reactive, highly unstable ROS chemicals called free radicals because of the unpaired electron in O_2 form. These chemicals play a vital role in the formation of many diseases because they react with most biological molecules such as protein and DNA and cause oxidative stress, hence a need of antioxidants to scavenge them in an aid of curing diseases caused by them. *M. oleifera* leaves have been largely reported to have antioxidant properties due to the presence of phenolic compounds which are reducing agent that can donate an electron or hydrogen to a free radical species as in Figure 2.1 below, can quench singlet O_2 and are able to chelate metals (Pandey *et al*., 2013, Matshediso *et al.*, 2014).

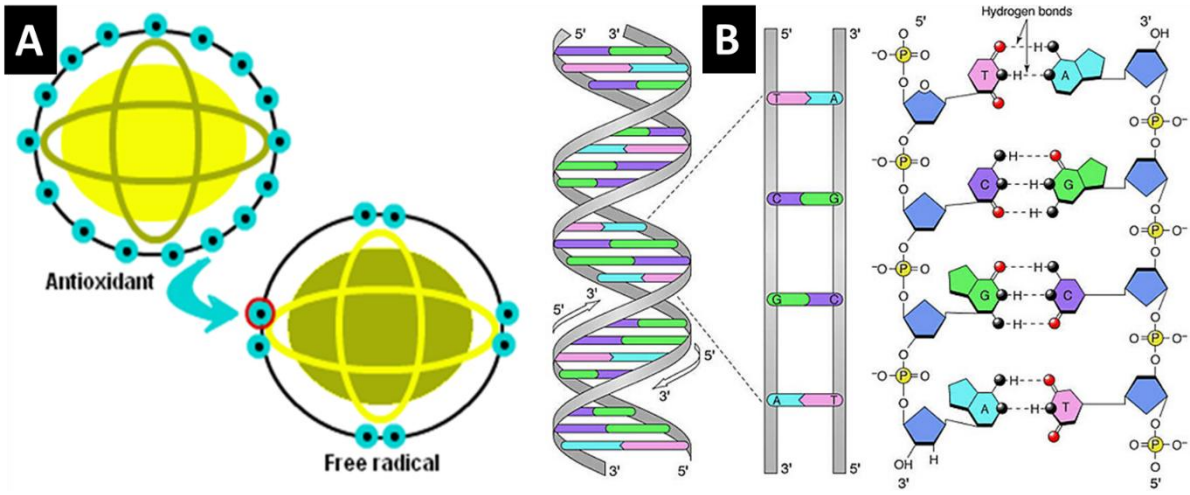


Figure 2.2 Photographs of (A) Antioxidant donating an electron to a free radical (Jockers, 2018). (B) Deoxyribonucleic acid strands showing hydrogen bonding (2014, The Gale Encyclopedia of Science).

Phenolic compounds (PCs) in plants exist as four main classes which are phenolic acids, flavonoids, stilbenes and lignans. Their basic structures and examples are shown in Figure 2.2 below. Due to the hydroxyl groups present in their structures, phenolic acids and flavonoids present in medicinal plants such as *M. oleifera* possess antioxidant properties needed to stabilise the free radicals by donating a hydrogen atom preventing tissue damage and sicknesses (Matshediso *et al*., 2014). These PCs especially phenolic acids and flavonoids have been extracted from *M. oleifera* using different solvents such as water, methanol, acidified methanol, ethanol and acidified ethanol just to name the few (Iqbal *et al*., 2006, Baba *et al*., 2015). Flavonoids such as flavones, flavonols and flavanones naturally occur as a 15-C skeleton with 2-benzene rings connected by a heterocyclic pyrane ring. Quercetin, kaempferol, myricetin, and fisetin are examples of flavonols that have been reported effect protective properties against infectious and degenerative diseases (Pandey *et al.*, 2013). It has been reported that the concentration of flavonols in different plants differs significantly. For example, onion leaves contain 1497.5 mg/kg of quercetin and 832.0 mg/kg kaempferol. Semambu leaves have 2041.0 mg/kg, bird chili 1663.0 mg/kg, black tea 1491.0 mg/kg, papaya

shoots 1264.0 mg/kg and guava 1128.5 mg/kg of total flavonols (Mohamed *et al.*, 2001). Analysing the content of total flavonols based on quercetin and kaempferol on different products of tomato (*Solanum lycopersicum* L.) revealed that tomato had concentrations of 3.1 to 10.0 mg/kg, tomato juice 19.8 mg/kg and tomato salsa 10.5 to 13.2 mg/kg (Tokusoglu *et al*. 2003).

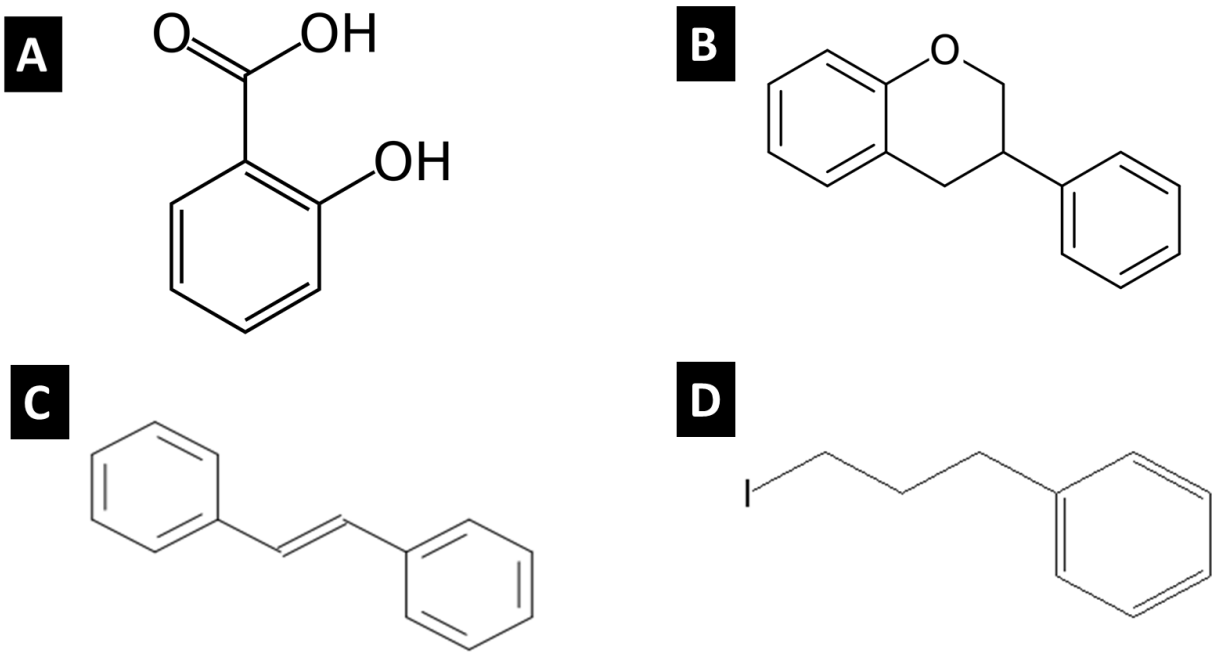


Figure 2.3 Phenolic compounds (A) Salicylic acid ["Phenols," (2017, November 11)]. (B) Isoflavan ["Flavonoids," (2017, November 28)]. (C) Trans-Stilbene ["E-stilbene," (2017, August 04)]. (D) 3-Iodopropyl benzene ["Lignan" (2015, Royal Society of Chemistry)].

CHAPTER 3: RESEARCH OBJECTIVES

3.1 Rationale

The nutritional, medicinal and industrial benefits that *M. oleifera* provides are attributed to the phytochemical compounds contained within the plant. The concentration of the phytochemical compounds in plants is influenced by geoclimatic conditions (Pakade *et al*. 2012). According to Pakade *et al*., (2012), *M. oleifera* grown at different locations and seasons would have different concentrations of total phenolics, total flavonoids and individual flavonoids such as kaempferol, quercetin and myricetin. The study showed that the total phenolics and flavonoid content was higher in winter than in summer season (Iqbal *et al*., 2005, Pakade *et al*., 2012). The morphology and histology of medicinal plants are also affected by environmental factors such as temperature. The steady increase in the cultivation of *M. oleifera* in South Africa is mostly in provinces where there is a high number of rural areas such as Limpopo, North West, Mpumalanga and Eastern Cape. Some of the provinces such as the Eastern Cape have been reported in 2015 to reach maximum temperature of 48.4°C (Ziervogel *et al*., 2014). This is an indication that climate change in South Africa is on the rise as it has been largely reported that extreme weather events are most likely to occur due to global warming (Harish *et al.*, 2013). The responses of medicinal plants such as *M. oleifera* are being studied at moderate high and cold temperatures. Environmental temperatures are not always moderate to growing plants due to global warming and climate change. Adaptation of *M. oleifera* to South African weather affected by global warming and climate change is critical for large scale agriculture. Thus, we are interested in assessing how extreme temperatures of 47°C and 10°C will affect the contents of phytochemical compounds along with anatomy of *M. oleifera*. Results obtained in this study provide an indication of what to expect from *M. oleifera* grown or exposed to extreme temperature stresses as well as create opportunities for future research to develop mitigation methods to counteract the effects of these stresses. This study is thus very important at both national and international levels.

3.2 Aim

The aim of this research project was to investigate the responses of *M. oleifera* leaves metabolome, morphology and anatomy on plants when exposed to extreme temperature conditions of 15/10 °C and 47/29°C for 7 days.

3.3 Objectives

- To examine the effects of low (15/10 °C) and high (47/29°C) temperatures on the *M. oleifera* leaf morphological and histological structures using dissecting and light electron microscopy. (15°C representing day and 10°C representing night) and (47°C representing day and 29°C representing night)
- Measure the micro thickness of leaves from *M. oleifera* plants exposed to 15/10 °C and 47/29°C treatments.
- To measure the total phenolic and total flavonoid contents in leaves of plants exposed to 15/10 °C and 47/29°C using methanol-based solvent extraction methods.
- To identify and quantify quercetin and kaempferol contents in leaves of plants exposed to 15/10 °C and 47/29°C using HPLC-UV
- To compare responses of the leaves of plants exposed to 15/10 °C and 47/29°C to the control (maintained under 25/22°C).

3.4 Statistical analysis

- Perform all the treatments, experiments and measurements in triplicate and analyse results using student's T test.
- Report the results in mean values and standard deviation at 95% confidence levels.
- Comparison using Post-hoc Tukey Honest significant difference test where $\alpha = 0.05$.

CHAPTER 4: A MORPHOLOGICAL AND HISTOLOGICAL EXAMINATION

4.1 MATERIALS AND METHODS

4.1.1 Plant cultivation and temperature treatments

Plant samples were grown from seeds obtained from Petece Wellness Centre Chuenespoort, Limpopo. Seeds were planted in 5L potting black bags containing potting soil grown at ambient temperatures in the greenhouse at the School of Animal, Plant and Environmental Sciences, University of the Witwatersrand. The plants were watered with approximately 100 ml of water daily and grown for three months. A total of 54 plants (3 months old) were used of which 18 were dedicated for the high temperature treatment of 47/29°C (47°C representing day and 29°C representing night) and 18 for the low temperature treatment of 15/10°C (15°C representing day and 10°C representing night). The remaining set of 18 plants was used as control and maintained at ambient temperature. Temperature controlled incubators were used to perform the treatments. The above-mentioned sets of plants were placed in separate incubators and treated for 144 hours (seven days). Control plants were kept at 25°C. Plants were watered daily during the treatments and leave samples were harvested after 48, 96 and 144 hours for assessment from both treatments as well as the control. The experiments were repeated three times.

4.1.2 Morphological assessment

Images of plant samples were captured using a digital camera and a Nikon dissecting microscope to observe the possible changes in colour and the upper surfaces of the leaves at low (0.5X) and high (1X) total magnifications with emphasis on the exterior upper surface, thickness of the leaves and shape.

4.1.3 Histological assessment

Leaf samples were harvested at selected times as mentioned in 2.2.1 from both temperature treatments and control. The leaves were rinsed with deionised water for 1 minute to remove contaminants that might have accumulated on the plants' surface. Square leaf sample segments (3 mm x 5 mm) were cut from the harvested leaves by cutting in-between the midrib and the edges of the leaf. Segment samples were fixed with 2.5% glutaraldehyde in a phosphate buffer solution for 2 hours and then washed three times in a phosphate buffer solution. Osmium tetroxide (OsO_3) was further used to fix the leaf samples for 2 hours and then washed three times with deionised water. The dehydration procedure of the specimen was carried out using different concentrations of ethanol of 25, 50 and 75% respectively. The dehydrating solvent in the leaf specimen was embedded in Epoxy resin at 50 and 100% concentrations at ambient and 80°C temperatures each for 24 hours respectively. Samples were then left to cool, thereafter, semi-thin sections of the leaf samples were cut to 0.5 - 1.0 µm using a Reichert Ultramicrotome. The leaf samples were placed on the microscope slides and stained with Toluidine Blue pH 6.8 solution then gently evaporated for 1 minute on a hot plate. The slides were rinsed with deionised water and dried for further 1 minute. The samples on the slides were mounted with *p*-xylene-*bis*(*N*-pyridinium bromide) (DPX) and then glass cover slips were used to cover the mounted leaf samples (Spurr, 1969).

4.1.4 Light microscopy

After the leaves at different temperatures and times were harvested, they were rinsed with deionised water and air dried in a shade for 2 hours. The histology of the chemically fixed, stained, mounted and semi-thin cut leaf samples at different temperature treatments were examined with Olympus BX63 OFM light microscope attached with a Nikon DXM1200 digital camera with emphasis on the upper and lower epidermis, palisade parenchyma cells, spongy mesophyll cells, guard cells and stoma. The ultracellular thickness of the leaves was measured three times using an ocular grid software inside the light microscope.

4.2 RESULTS AND DISCUSSION

4.2.1 Effect of high and low temperature extremes on morphology and histology of *M. oleifera* leaves

Treatment of *M. oleifera* at high temperatures resulted in some of the leaves in individual plants turning yellow more rapidly than others while other leaves remained green throughout the entire seven-day treatment. At low temperature treatment, no prominent discolouration on most of the leaves was observed, this was due to the systematic responses of the plant leaves to overcome the impact of cold stress (Wang *et al.*, 2012).

4.2.1.1 Control samples at 25°C

The height of the plants and leaf sizes were almost equal and the effect of this temperature on *M. oleifera* is illustrated in **Figure 4.1**. The plants and leaf samples were healthy and green and the mid-rib and leaf veins clearly visible which showed that water and nutrients were easily transported onto the whole plant. Because *M. oleifera* is native to the tropical areas, these findings at controlled 25°C temperature confirms that this plant thrives best at this temperature (Gilani *et al*., 2007).

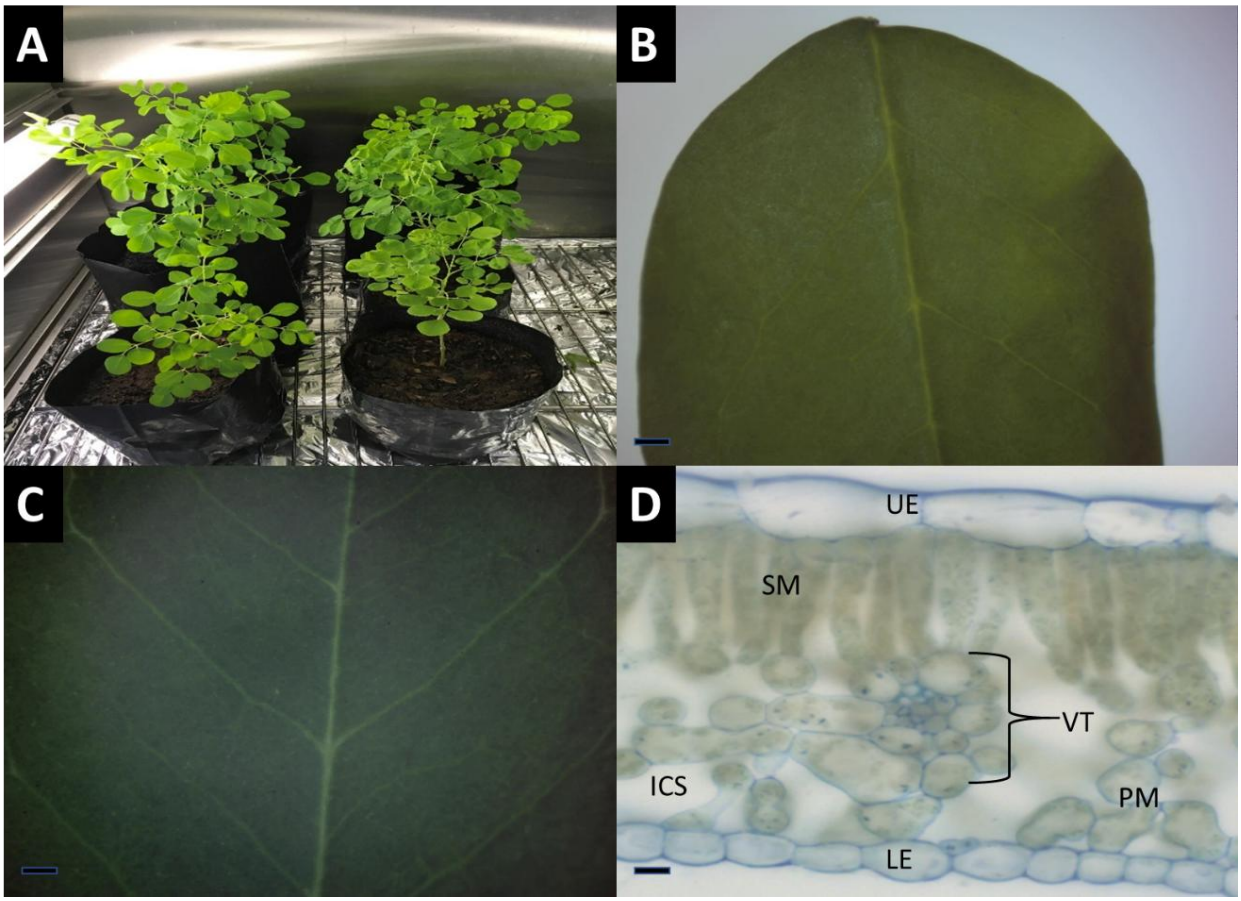


Figure 4.1 (A-C) Photographs of M. oleifera control samples at 25°C showing green healthy leaves. (D) Light micrograph of the leaf cross section of the control showing a clear and well defined cellular arrangement of a typical healthy and turgid leaf at 40x. UE=upper epidermis, LE=lower epidermis, SM=spongy mesophyll, VT=vascular tissue, ICS=intercellular spaces and PM=palisade mesophyll. Scale bar: 1cm represents 10µm (B and C), 1cm represents 20µm (D)

Morphologically, the leaves have a smooth shiny adaxial surface, while the abaxial surface is hairy and dull. Histologically, the leaves have a thin upper and lower cuticle layer, upper epidermal cells are bigger than the lower epidermal cells and both are oval **(Figure 4.1D)**. Evenly distributed intercellular spaces are well defined and look healthy. The spongy

mesophyll cells are rectangular-long and vertically aligned and the leaves have compact round vascular tissues. Furthermore, the palisade mesophyll cells are round shaped and evenly distributed below the spongy mesophyll cells **(Figure 4.1D)**. The abaxial region the leaves have stoma with guard cells that are oval and thick (not shown). The average leaf thickness of the control samples was 189.43 µm ± 0.55 µm.

4.2.1.2 Effect of high and low temperatures on M. oleifera after 48 hours

Immediately after the 48 hours treatment, morphological responses such as leaf rolling and wilting in *M. oleifera* leaves was observed as compared with the control. These responses have also been observed in plants such as maize and soybean under heat stress (Ashraf *et al*., 2004). These results, therefore, indicate that *M. oleifera* is more susceptible to heat stress. The changes in colour from green to yellow on some of the leaves of plants exposed to high temperatures (47/29°C) was observed. Normally, when green plants change leaf colour from green to yellow because of stress, it is an indication that the leaves are probably undergoing chlorosis, which is a deficiency of chlorophyll. Chlorophyll plays a major role in the development and growth of plants because it converts water and CO_2 in the presence of sunlight into carbohydrates. These are then used as energy for plant growth and development of new plant structures including new chlorophylls. Thus, deficiency in chlorophyll which is due to the destruction of the food manufacturing process within the plant, does not only cause leaves to turn yellow but it can result in low quality crop produce and eventually plant death (Wann *et al.*, 1930).

Extreme temperatures may affect the food manufacturing process and production of chlorophyll in plants negatively which eventually leads to chlorosis or plant death (Cramer *et al*., 2004, Wang *et al*., 2012, Ter Heerdt *et al*., 2017). When cucumber and wheat plants were exposed to low and high temperatures of 7°C and 42°C for varying amounts of time, chlorophyll biosynthesis inhibition was more severe at low temperatures than high

temperatures. This chlorophyll inhibition was due to the impairment of 5-aminolevulinic acid and protochlorophyllide biosynthesis which are the enzymes responsible to produce chlorophyll (Tripathy *et al.*, 1998).

Contrary to what was observed by Tripathy *et al.* (1998), at sub lethal and lethal temperatures of 47°C to 57°C, when matured leaves of Soybean and Elodea treated at these temperatures for four days, complete loss of chlorophyll and swollen chloroplasts were observed. 50% and 40% of cell death in Soybean and Elodea leaves was recorded at the extreme temperatures and this caused leaf cell plasmolysis, tonoplast membrane, plasmalemmal and tonoplast membrane disorganisation (Daniell *et al.*, 1969). These results relate to what was observed when *M. oleifera* was subjected to extreme temperatures which resulted in leaf curling and chlorosis.

Figure 4.2.A shows healthy green leaves after 48 hours at the temperature regime of 47/29°C. Approximately 80% of the plant leaves were still green while about 15% (represented by **Figure 4.2.B**) had turned yellow, and while the remaining 5% (represented by **Figure 4.2.C**) became yellow, dry and shrank. The percentage estimation was performed by randomly counting the leaves. **Figure (4.2.D)** shows the healthy green leaves at low magnification. Figure **(4.2.E)** shows that although the leaves had turned yellow, they still had the mid-ribs and veins intact which indicated that water and nutrients were able to reach the whole leaf, but chlorosis had occurred. Figure **(4.2.F)** show the leaves that were dry and shrank, containing dark mid-ribs and veins and this was the indication of sever high temperature stress damage. Cell death on the leaves occurred following the process of necrosis because the normal functioning of the cells on the leaves were disrupted by the extreme high temperature (Gunawardena *et al*., 2011, Risenga *et al*., 2014).

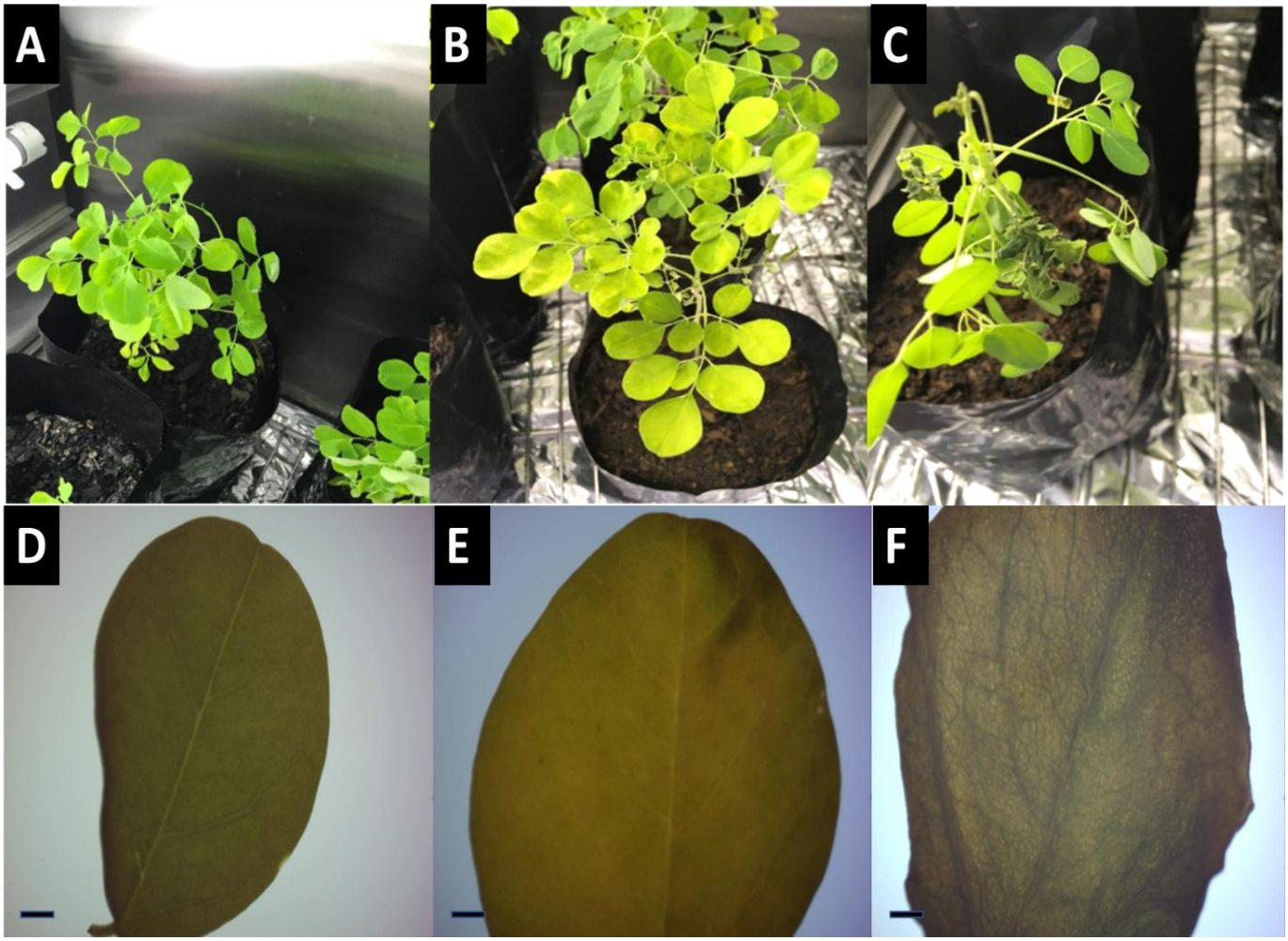


Figure 4.2 Photographs of M. oleifera showing variation in responses after 48 hours of high temperature (47/29°C). (A) Showing leaves that were still green. (B) Leaves that had turned yellow. (C) Leaves that had turned yellow and shrank. (D) Green leaf at low magnification, 0.5x. (E) Yellow leaf at low magnification, 0.5x. (F) Yellow shrank leaf at high magnification, 1x. Scale bar: 1cm represents 10µm (D, E and F).

In this high temperature regime (TR), the observed green and the yellow leaves were harvested after 48 hours and their anatomy was characterised. In both green and yellow leaves, the TR had an influence on the leaf thickness, shape of spongy mesophyll cells,

vascular tissue, intercellular spaces and palisade mesophyll cells as shown in **Figure 4.3 B and C** below. In the green leaves, the average leaf thickness was 267.52 µm ±0.35 µm. The average leaf thickness was thus increased by 41.1% in 48 hours of treatment in this high TR. The spongy mesophyll cells showed variation in size and shape, some were elongated, and others exhibited deformed shapes. The volume of intercellular spaces was increased, and the vascular tissue appeared to be dismantling. However, Palisade mesophyll cells seemed to remain intact as their shape remained the same. The observed changes in leaf colour i.e. from green to yellow, is indicative of possible chlorophyll degradation due to high temperature stress.

The severity of the stress in this TR on the yellow leaves was much greater as compared with the low temperature treatment. The average leaf thickness of the yellow leaves after 48 hours was 207.87 µm ±0.22 µm. This meant that the average leaf thickness was increased by 9.7%. Some of the spongy mesophyll cells were raptured and some had deformed shapes. The volume of the intercellular spaces increased dramatically, this was exacerbated by complete disintegration of some of the cells, leaving more spaces within the tissues. The number of chlorophyll containing cells decreased dramatically. This was observed as a sign of chlorosis which explains why the leaves turned yellow. Some vascular tissues were still intact and showed normal shapes.

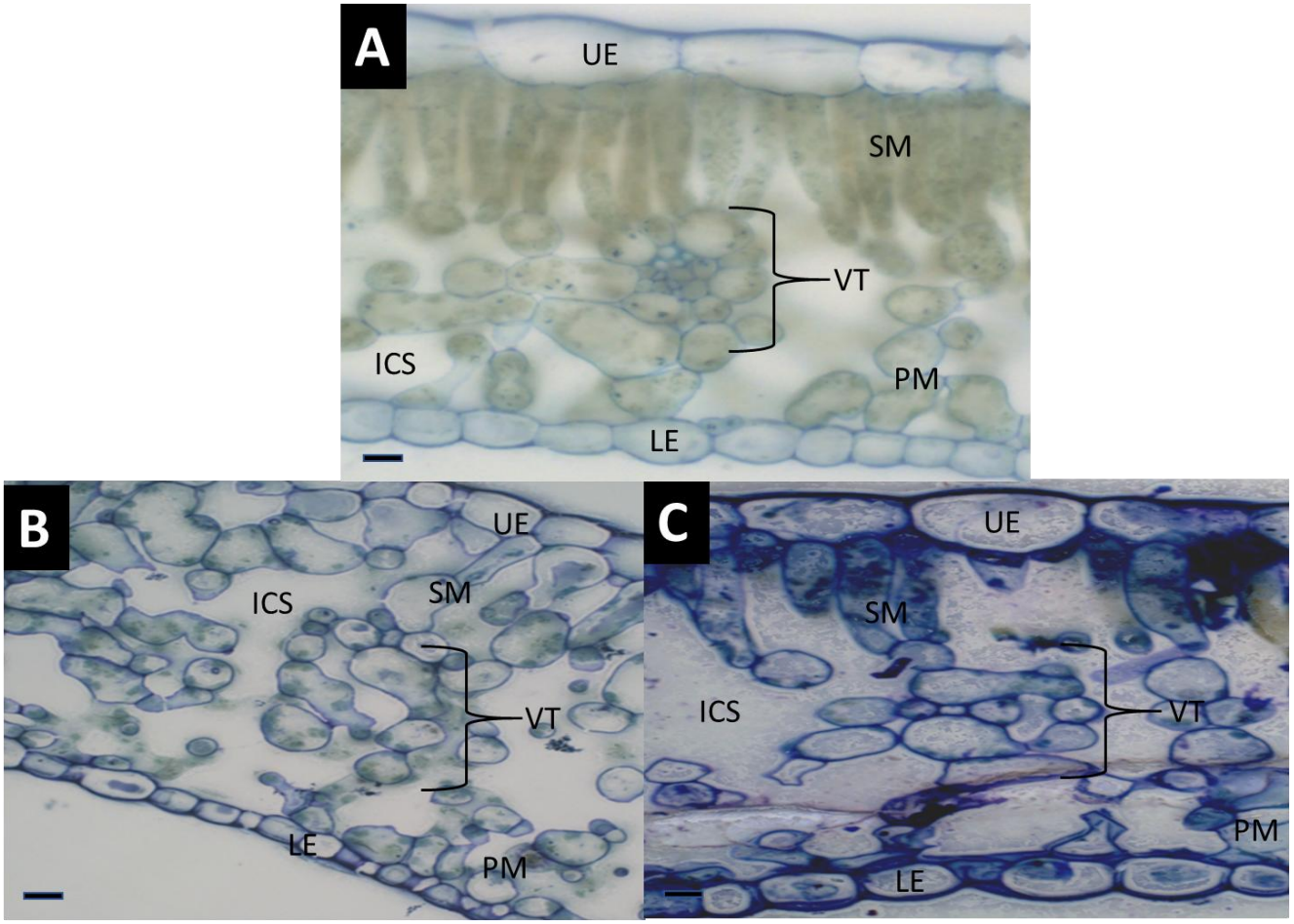


Figure 4.3 Light micrographs of M. oleifera control (A), green (B) and yellow (C) leaves for plants treated for 48 hours at 47/29°C ±1°C temperatures. (B) In the green leaves, the spongy mesophyll cells appeared to be swollen and deformed. The inner contents of the cells seemed to have decreased in quantity, and intercellular spaces had increased in size. (B) In the yellow leaves, more sever damaged was evident showing collapsed spongy and palisade mesophylls, disintegrated cells and abnormally wide intercellular spaces. UE=upper epidermis, LE=lower epidermis, SM=spongy mesophyll, VT=vascular tissue, ICS=intercellular spaces and PM=palisade mesophyll. Scale bar: 1cm represents 20µm.

When the three months old *M. oleifera* plants were treated with a low temperature regime of 15/10°C for 48 hours. The young leaves were shrinking and curling but remained green, this is shown in **Figure 4.4.D, E and F** below. **Figure 4.4 A, B and C** showed that plants remained green and no chlorosis had occurred at this stage of treatment. This may be indicative of cold stress tolerance as the damage was minimal after 48 hours at 15/10°C TR. **Figure 4.4.B and C** represent the green leaves at low and high magnifications whilst **Figure 4.4.E and F** shows the green shrunk leaves at low and high magnifications.

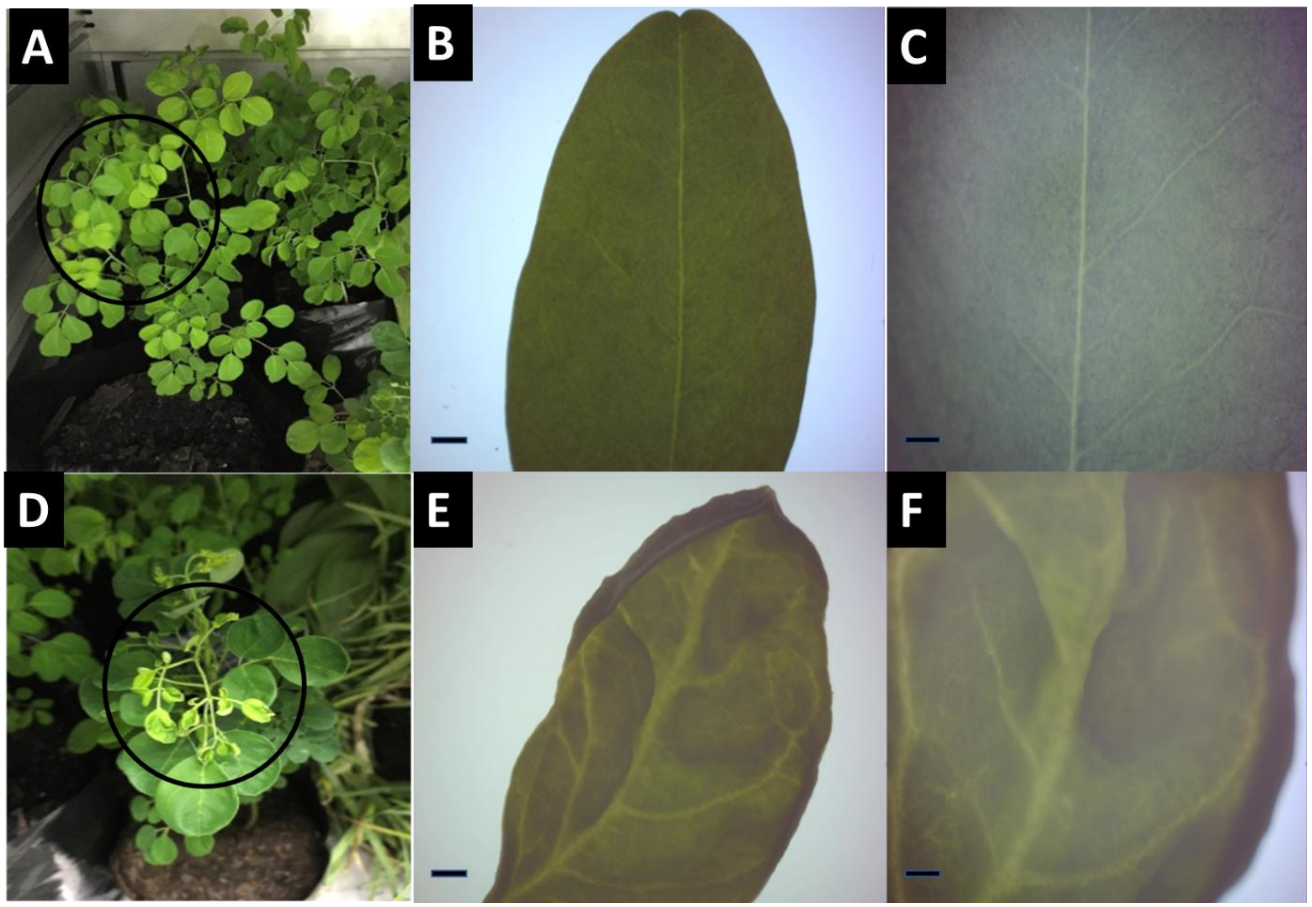


Figure 4.4 Photographs of M. oleifera under low temperature (15/10°C) for 48 hours. (A) Showing all green plant leaves. (B) Green leaf at low magnification. (C) Green leaf at high magnification. (D) Showing leaves that are green but have shrunk. Leaves at low (E) and high (F)

magnifications showing leaf shrinkage and curling. Scale bar: 1cm represents 10µm (B, C, E and F).

Also at the low TR, responses were associated with the leaf thickness, spongy mesophyll cells, vascular tissue, intercellular spaces and palisade mesophyll cells. The leaf average thickness after 48 hours was 194.83 µm ±0.29 µm. This showed that leaf thickness increased by 2.7%. The upper and lower epidermis seemed to remain intact. The spongy mesophyll cells appeared to be swollen and more elongated as compared with the control. Vascular tissue remained intact with no obvious changes in shape and size. Intercellular spaces were much bigger and swollen than the control sample. Palisade mesophyll cells appeared smaller than the control sample as shown in **Figure 4.5** below.

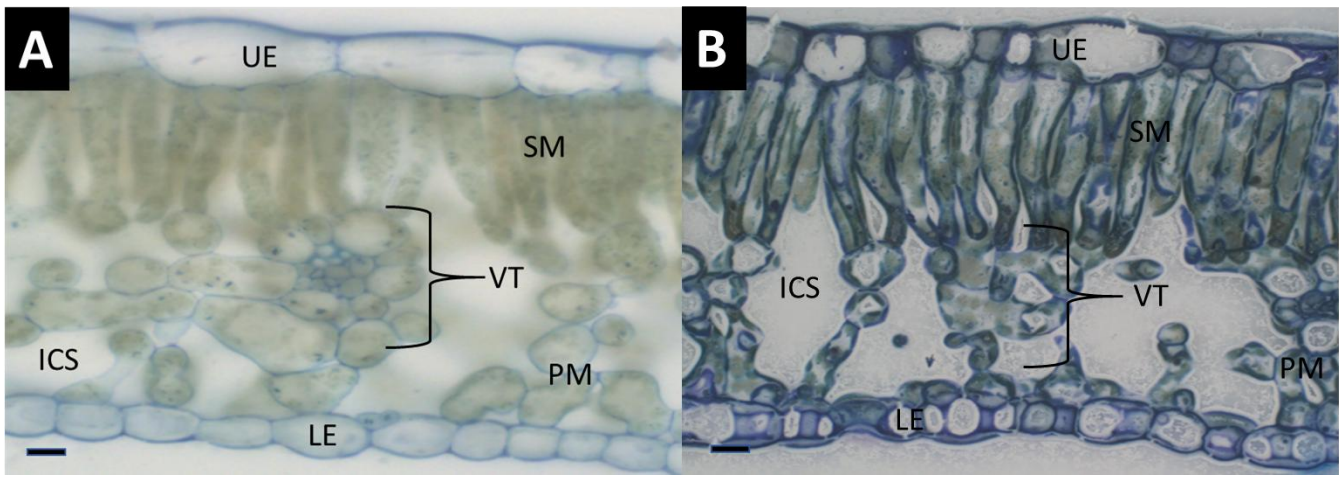


Figure 4.5 Light micrographs of M. oleifera cross sections. (A) Control and (B) Green leaves of plants treated for 48 hours at 15/10°C ±1°C temperatures. (B) The upper and lower epidermal cells seemed to remain intact while the spongy mesophyll cells appeared to be more elongated as compared with the control. The contents in the spongy mesophyll cells appeared to have been redistributed towards the more elongated side of the cells. Palisade mesophyll cells had decreased in size thus resulting

increased intercellular spaces. UE=upper epidermis, LE=lower epidermis, SM=spongy mesophyll, VT=vascular tissue, ICS=intercellular spaces and PM=palisade mesophyll. (A and B) Measured at 40x objective. Scale bar: 1cm represents 20µm.

Comparing the treatment results obtained at 47/29°C and 15/10°C temperature regimes after 48 hours, the temperature effect on *M. oleifera* at high temperatures is more than at low temperatures with respect to systematic response such as yellowing and shrinking of the leaves. The high temperature responses in this study mimic those that are observed in plants during heat and drought seasons were day/night temperatures reach maximum of 46/30°C (Jagadish *et al*., 2007). During the heat and drought season, plants that are still young would die faster than matured plants because the young ones are not matured enough to withstand the shock (Geyer *et al*., 2003). Similarly, this response was observed after 48 hours when three months old *M. oleifera* plants were treated with 47/29°C temperatures.

4.2.1.3 Effect of high and low temperatures on M. oleifera after 96 hours

After 96 hours at high temperature treatment, the percentage of green leaves had decreased from 100% to 60% **(Figure 4.6.A)**. A reduction of 40% in green leaves, 30% increase of yellow leaves and 10% increase on dry and brown leaves (dead leaves) was observed after this period. **Figure 4.6.B** represent all the leaves that were still green while **Figure 4.6. (C and D)** represents the dried-shrank brown and yellow leaves. Optimum temperature plays a role in the support of a plant's life, however high temperature may result in stresses that may cause fatal responses such as heat shock, leaf burn, physiological wilting and leaf roll disease (Wann *et al*., 1930, Breusegem *et al*., 2006). Furthermore, the photosynthetic process may be disturbed resulting in decline of carbohydrate reserves (Mondal *et al.*, 2015). During the consumption of carbohydrates reserves in plants, senescence occurs when the energy used during respiration is above optimal due to increased temperatures (Faust *et al.*, 2011).

Studying the formation of chloroplasts on the leaves of nine alpine plant species at 10 to 30°C exposed to dark and light conditions revealed that at dark conditions, an increase in chloroplasts formation was observed at temperatures above 20°C for seven species. In light conditions at temperatures above 20°C, no increase in chloroplast formation was observed; therefore, this meant that chloroplast formation is not involved in primary but secondary photosynthesis processes (Buchner *et al.*, 2007).

In the present study, it is therefore possible that *M. oleifera* plants turned yellow and eventually died because the production of chlorophyll may have seized due to the disruption of photosynthesis, blockage of mineral and water transportation to the leaves because of heat shock in the leaves. Grigorova *et al*., (2013) showed that when fourteen-day old wheat plants were exposed to high temperature of 40°C for 5 hours, chloroplasts and mitochondria in mesophyll cells of the leaves were swollen and had damaged cell membranes while the control sample had well defined cell membranes. At high temperature stress, the starch granules on the chloroplasts vanished. Furthermore, an increase in shape and size in mitochondria at high temperature was observed and leaf mesophyll cells were more damaged at high temperature stress than the control samples (Grigorova *et al.*, 2013). Similarly, in the present study the damage on the mesophyll cells and drastic reduction of chloroplasts was evident particularly after 96 hours of high temperature treatment **(Figure 4.7B)** below. Tomato (*Lycopersicon esculentum* MILL.) is normally grown during the winter season because during the summer season, due to high temperatures, yields are significantly reduced. Gruda *et al*., (2003) showed that exposure of tomato plants during the reproductive and vegetative stages to 37/27°C resulted in reduced plant height, leaf area, leaf weight and number of flowers as compared to the control (Gruda *et al.*, 2003).

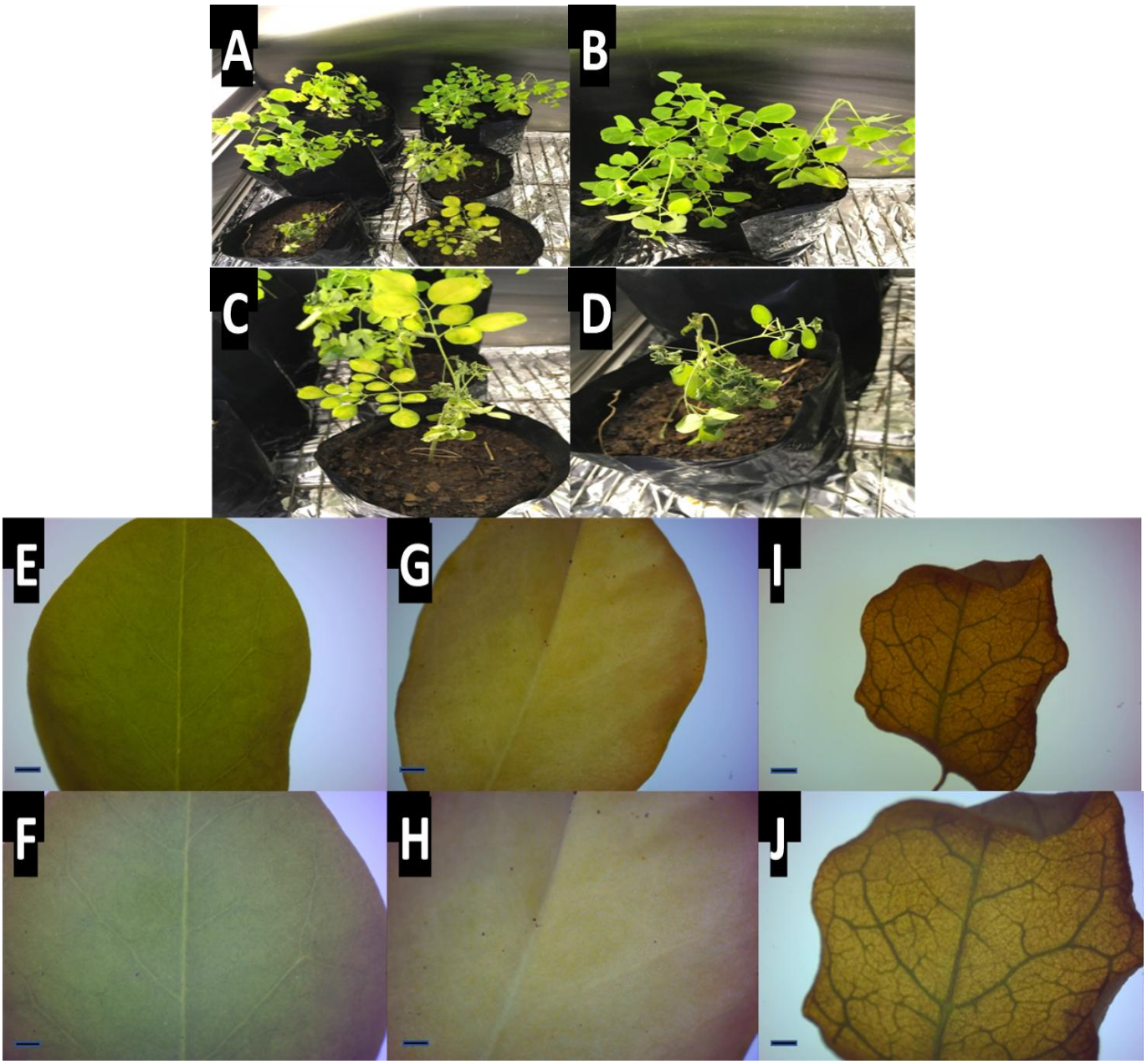


Figure 4.6 Photographs of M. oleifera under high temperature (47/29°C) after 96 hours. (A) Plants showing a range of responses. (B) Plant sample that still had green leaves. (C) Plant sample with dried shrunk brown leaves. (D) Plant sample with yellow leaves. (E and F) Represents green leaves viewed at low (E) and high (F) magnifications. (G and H) Yellow leaf samples at low and high magnifications. (I and J) Severely damaged

leaves that had shrunk, turned brown, dried and eventually died. Scale bar: 1cm represents 10µm (E, F, G, H, I and J).

In both green and yellow leaves, the high TR had an influence on the leaf thickness, spongy mesophyll cells, vascular tissue, intercellular spaces and palisade mesophyll cells. For the green leaves, the average leaf thickness was 191.66 µm ±0.28 µm. The average leaf thickness was thus increased by 1.1% after 96 hours of treatment in this TR. For the green leaves, the spongy mesophyll cells seemed to have remained intact, however slightly elongated **(Figure 4.7B)** as compared with the control cells **(Figure 4.7A)**. The palisade mesophyll cells also appeared to be intact, however, the chloroplasts in those cells appeared to be swollen and more pronounced **(Figure 4.7B)** as compared with the control **(Figure 4.7A)**. The volume of intercellular spaces was decreased, and the vascular tissue had dismantled. Smaller and bigger sizes and shapes of palisade mesophyll cells was observed. The amount of chlorophyll cells on the leaves decreased because the green colour on the cells decreased.

The average leaf thickness of the yellow leaves after 96 hours treatment was 113.81 µm ±0.19 µm resulting in the average leaf thickness decrease of 40.0% The spongy mesophyll cells collapsed and were of deformed shapes as shown in **Figure 4.7C** below. The volume of the intercellular spaces significantly decreased and became squashed. The green colour of chlorophyll cells disappeared due to chlorosis. The vascular tissues raptured and had abnormal shape. The swelling after 96 hours reduced and appeared normal. The palisade cells also followed the same trend as spongy mesophyll tissue at these temperatures and time periods. These findings obtained in these experiments agree with the experimental results published in the literature by another research group where they found that the average leaf thickness of *M. oleifera* grown at a TR of 30/20°C was smaller than the average leaf thickness of *M. oleifera* grown at a TR of 25/15°C and the spongy mesophyll tissue and palisade cells were fewer and shorter at TR of 30/20°C than the ones at 25/25°C (Muhl *et al.*, 2011).

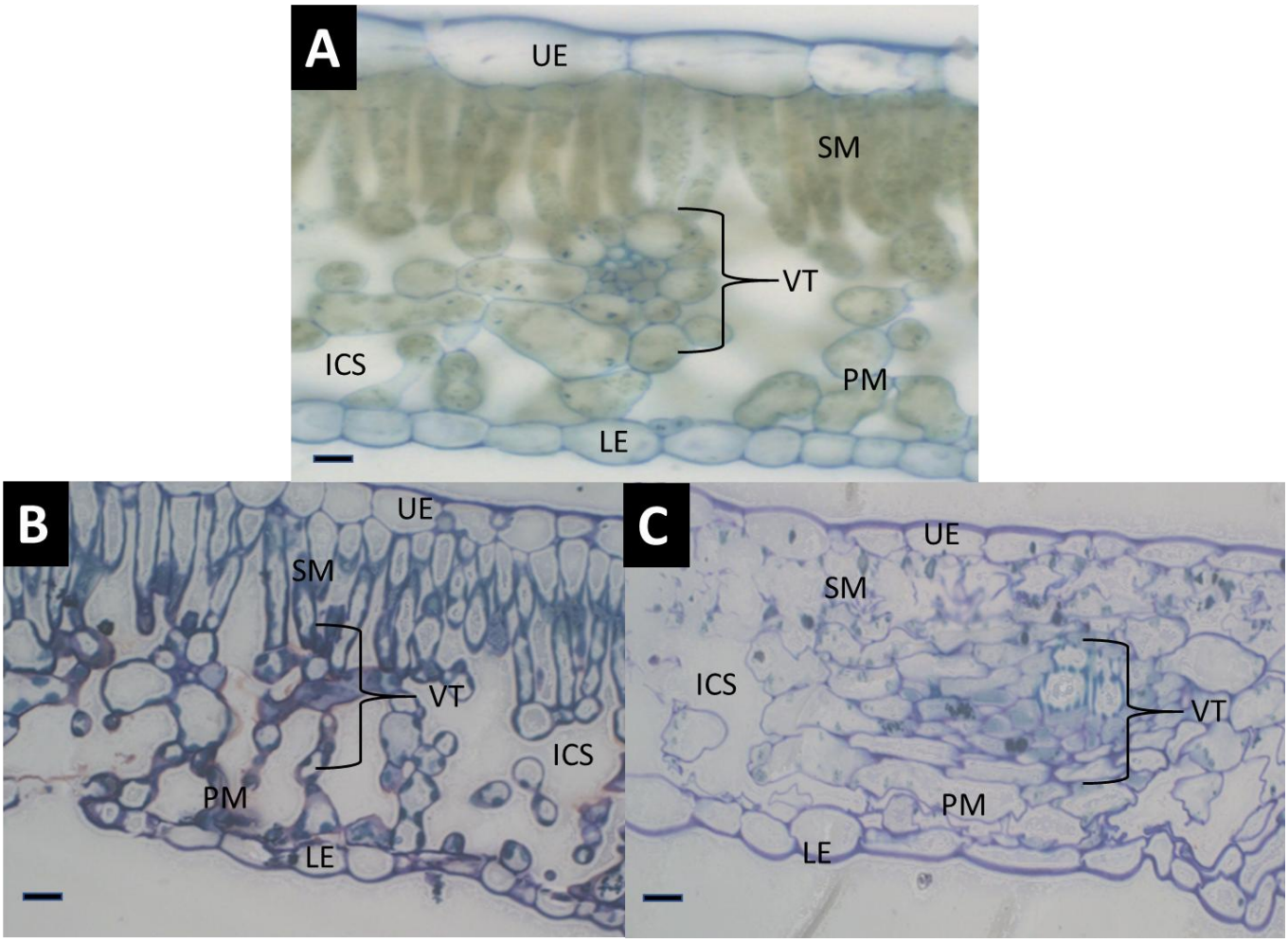


Figure 4.7 Light micrographs of M. oleifera control (A), green (B) and yellow (C) leaves of plants treated for 96 hours at 47/29°C ±1°C temperatures. (B) In the green leaves, the lower and upper epidermal cells appeared to have decreased in size. The mesophyll cells remained elongated and the cell contents had moved to one side of the cells. The palisade mesophylls were greatly reduced in size, thus increasing the intercellular spaces. The vascular tissues had disintegrated. C) In the yellow leaves, severe damage was evident showing disintegration of cells. UE=upper epidermis, LE=lower epidermis, SM=spongy mesophyll, VT=vascular tissue, ICS=intercellular spaces and PM=palisade mesophyll. Scale bar: 1cm represents 20 µm (A, B and C).

A similar response as in 48 hours low temperature treatment was observed after 96 hours of treatment at low temperatures of 15/10°C. A 98% of the leaves were still green and healthy. This was indicative of cold stress tolerance. Furthermore, on some of the older green leaves the green colour became darker as compared to the plants at 25°C **Figure 4.8**. This was the morphological response the plant showed to overcome the effect of cold temperature stress. Several previous studies have demonstrated similar responses. For example, Kimura *et al.*, (2016) showed that cold temperatures to freeze stress caused chlorosis in wheat leaves and the leaves' colour turned yellow. *Populus euphratica* has been found to naturally survive the effect of extremely low temperatures. This was observed when cells of this plant were subjected to varying temperatures of 5°C to 75°C for 20 minutes then cell viability and biomass contents were assessed. It was showed that cell viability and biomass were not altered at temperatures at lower temperatures. During recovery period, cell growth increased in cells exposed to lower temperatures, however at high temperatures a total decline in cells was observed which indicated that the high temperature stress tolerance of *Populus euphratica* is lower (Silva-Correia *et al.*, 2012). In the present study, the histological assessment similarly indicated a higher tolerance of lower temperatures as compared with the higher temperatures in *M. oleifera.*

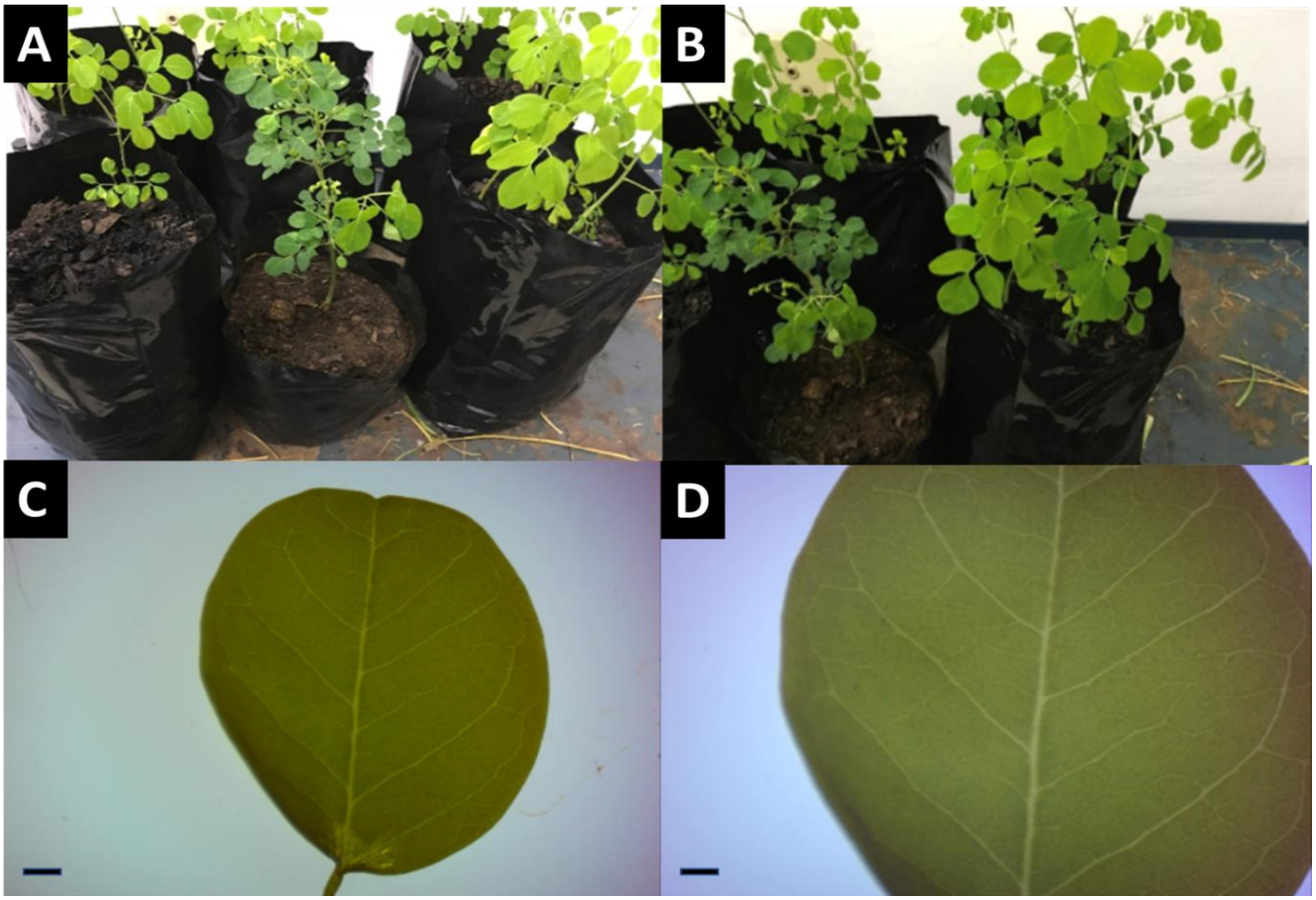


Figure 4.8 Photographs of M. oleifera low temperature (15/10°C) 96 hours samples. (A) Showing a range of responses in different plants. (B) Showing the predominantly green leaves. (C and D) Showing the green leaf samples at low and high magnifications. Scale bar: 1cm represents 10µm (C and D).

A continued influence on the leaf thickness, spongy mesophyll cells, vascular tissue, intercellular spaces and palisade mesophyll cells was observed at the low TR. The leaf average thickness after 96 hours was 222.06 µm ±0.24 µm. This showed that leaf thickness increased by 17.3%. There was no change in size and shape of upper and lower epidermis. The spongy mesophyll cells continued to swell and appeared longer with no change in shape. Vascular tissue was still intact with no obvious changes in shape and size. Intercellular spaces were

much bigger, and their volume continued to increase. Palisade mesophyll cells continued to get smaller and the chlorophyll cells were still visible. No cell death was observed at this low TR and these findings agree with literature where a research group revealed that the average leaf thickness of *M. oleifera* grown at 20/10°C TR was bigger than the plants grown at 25/15°C. Spongy mesophyll tissue and palisade cells on the leaves were many and longer at TR of 20/10°C than the leaves at 25/15°C (Muhl *et al*., 2009 and 2011) **Figure 4.9**.

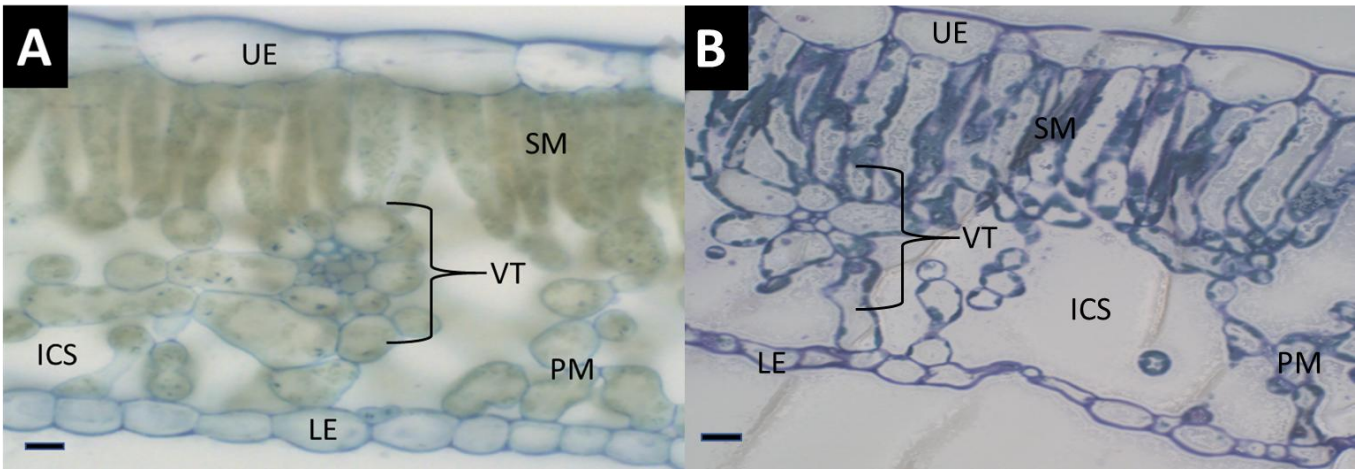


Figure 4.9 Light micrographs of M. oleifera cross sections. (A) Control and (B) Green leaves of plants treated for 96 hours at 15/10°C ±1°C temperatures. (B) The upper epidermal cells seemed to remain intact and lower epidermal cells seemed to have reduced in size while the spongy mesophyll cells appeared to be more elongated as compared with the control. The contents in the spongy mesophyll cells appeared to have been redistributed towards the more elongated side of the cells. Palisade mesophyll cells had decreased in size thus resulting increased intercellular spaces. UE=upper epidermis, LE=lower epidermis, SM=spongy mesophyll, VT=vascular tissue, ICS=intercellular spaces and PM=palisade mesophyll. (A and B) Measured at 40x objective. Scale bar: 1cm represents 20µm.

4.2.1.4 Effect of high and low temperatures on M. oleifera after 144 hours

The rate of leaf yellowing, browning, drying and death increased with time during temperature treatment of 47/29°C. These findings indicate the high level of sensitivity to high temperatures. The severity of the damage had increased in this temperature treatment after 144 hours. This was indicated by a further decrease in green leaves from 60% to 30% and an increase in yellow, brown and dry leaves to 70%. When the same plants were taken out of this temperature treatment after the 7 days period and placed at 25°C temperature and watered with 100 ml of water daily for two weeks, plants did not show signs of recovery from the treatment. Leaf browning in plants is driven by biochemical processes such as the total concentration of ascorbic acid and level of oxidants such as phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO), and peroxidases (PODs) (Luo *et al*., 2007). 12-day old pea (Pisum sativum) plants exposed to 7°C or 40°C for 48 hours showed that a decrease in chlorophyll a content was observed on both temperatures. This was due to the decline of protein Rubisco (pRSS) responsible for extreme temperature stress tolerance. A restoration of the chlorophyll a content was observed when the plants were relocated to room temperature (Dutta *et al.*, 2009).

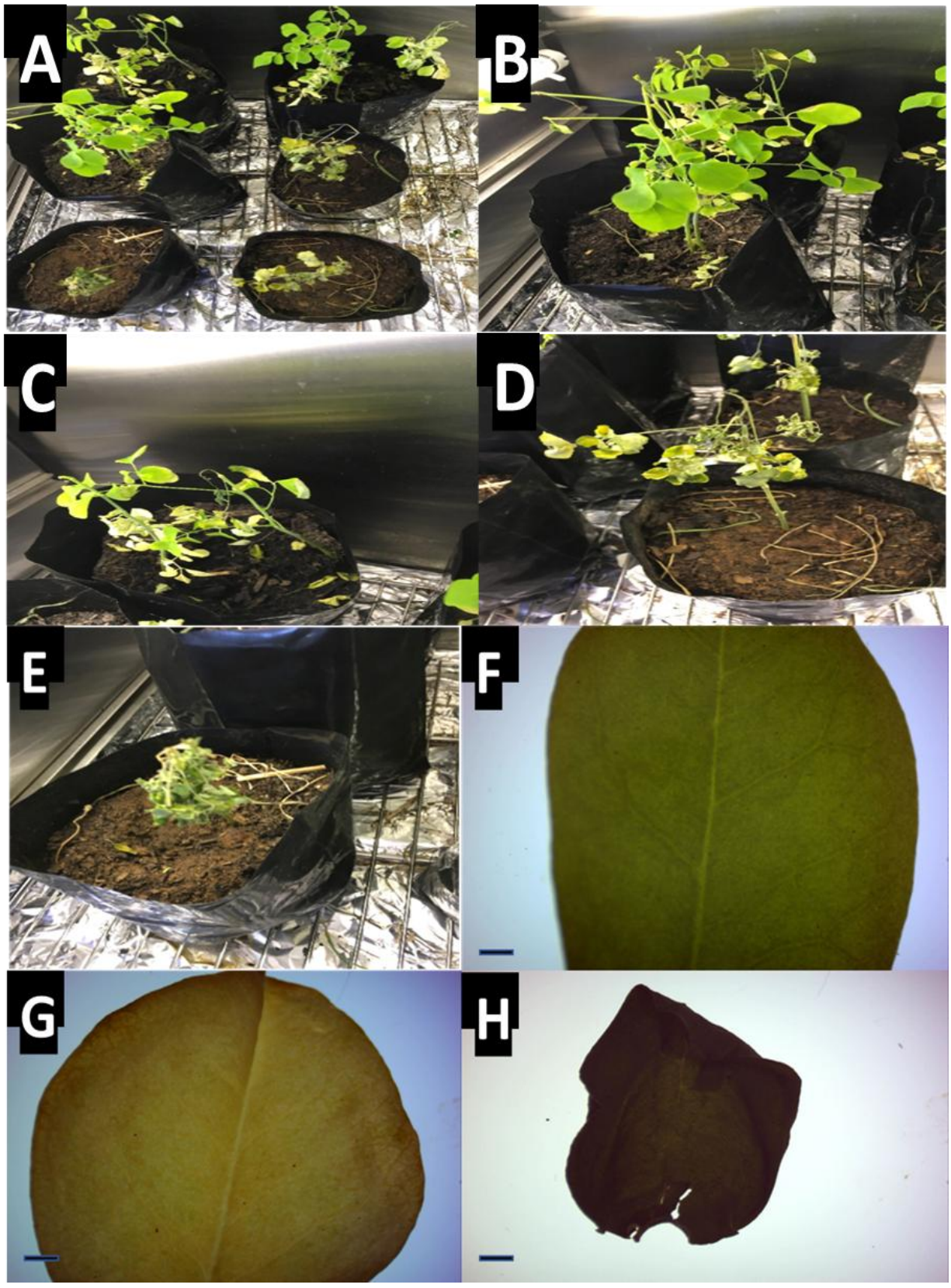

A
B
C
D
E
F
G
H

Figure 4.10 Photographs of M. oleifera high temperature (47/29°C) 144 hours samples. (A) Showing all the plant samples affected differently by the treatment. (B, C, D and E) Showing the individual plants representing leaves that were still green, ones that were yellow, ones that were yellow and have shrunk and ones that were green-yellow and shrunk. (F) represents the leaves that remained green. (G) Yellow sample leaves samples. (H) Dried-brown shrunk leaves. Scale bar: 1cm represents 10 µm (F, G and H).

In the green leaves, the average leaf thickness was 162.77 µm ±0.22 µm. The average leaf thickness was thus decreased by 14.0% after 144 hours of treatment. The spongy mesophyll cells became closely packed to each other and lengths appeared shorter. The volume of intercellular spaces was decreased significantly as compared to the control, indicating tissue collapse. The vascular tissue was intact but reduced in size. Bigger sizes and shapes of palisade mesophyll cells was observed. The amount of chlorophyll containing cells on the leaves decreased thus resulted in morphological changes in colour from green to yellow. There was no change in shapes and sizes of upper and lower epidermis. The average leaf thickness of the yellow leaves after 144 hours treatment was 105.40 µm ±0.16 µm resulting in the average leaf thickness decrease of 44.3%. The upper and lower epidermis collapsed and had abnormal shapes. The spongy mesophyll cells collapsed and were completely deformed in shape. There was no evidence of intercellular spaces because the tissues completely collapsed and became squashed. The vascular tissues raptured and was not visible. The complete destruction and collapse of the leaves was possibly due to necrosis.

During the seven days exposure of the three months old *M. oleifera* to the HT regime (47/29°C), the 47°C was the temperature that most likely caused cell death which was the temperature that represented day time when harvesting of the leaves occurred. The damage the 47°C temperature caused on the cells could not be reversed when the incubating machine

was switched to 29°C temperature which represented night time. The 29°C temperature could have not been the cause of necrosis because at this temperature, *M. oleifera* thrives and remain healthy. Therefore, the 47°C temperature was detrimental to the three months old plants.

Necrosis is accidental death of plant cells due to external factors such as intolerance to extreme temperatures. It results in permanent injuries of the plant's cellular membrane evident by swelling of the cytoplasm and damage of cellular constituents. Cell death in plants is triggered by reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), nitric oxide (NO) and superoxide ions. ROS are produced during photosynthesis and respiration. In plants, a constant balance in the production of ROS and its scavenging always must be maintained for the plants to function and grow normally. A disturbance in this balance by extreme temperatures results in high concentrations of ROS which reacts with all cellular components and causes destructive modification of proteins and deoxyribonucleic acid (DNA) strands breakage resulting in necrosis (Breusegem *et al.*, 2006, Ghorbani *et al*., 2011). The ROS content and DNA strands breakage were not determined nor measured in this study, however the extensive cellular damage and the complete disintegration that was observed in the tissues suggest that necrosis may have occurred due to the high temperature stress.

Adaptation of plants to HT stress is essential to achieve high crop yields. These adaptation mechanisms use plant inborn compounds such as ion transporters, proteins, osmoprotectants and antioxidants to effect stress tolerance (Fujita *et al.*, 2013). During reproductive stages, phenological development such as vegetative biomass was decreased at a temperature of 38°C and no change was observed at temperature of 25°C for maize and broccoli plants. This was due to the damaged cells and amyloplasts in the kernels which reduces gain sink and yield (Hatfield *et al.*, 2015).

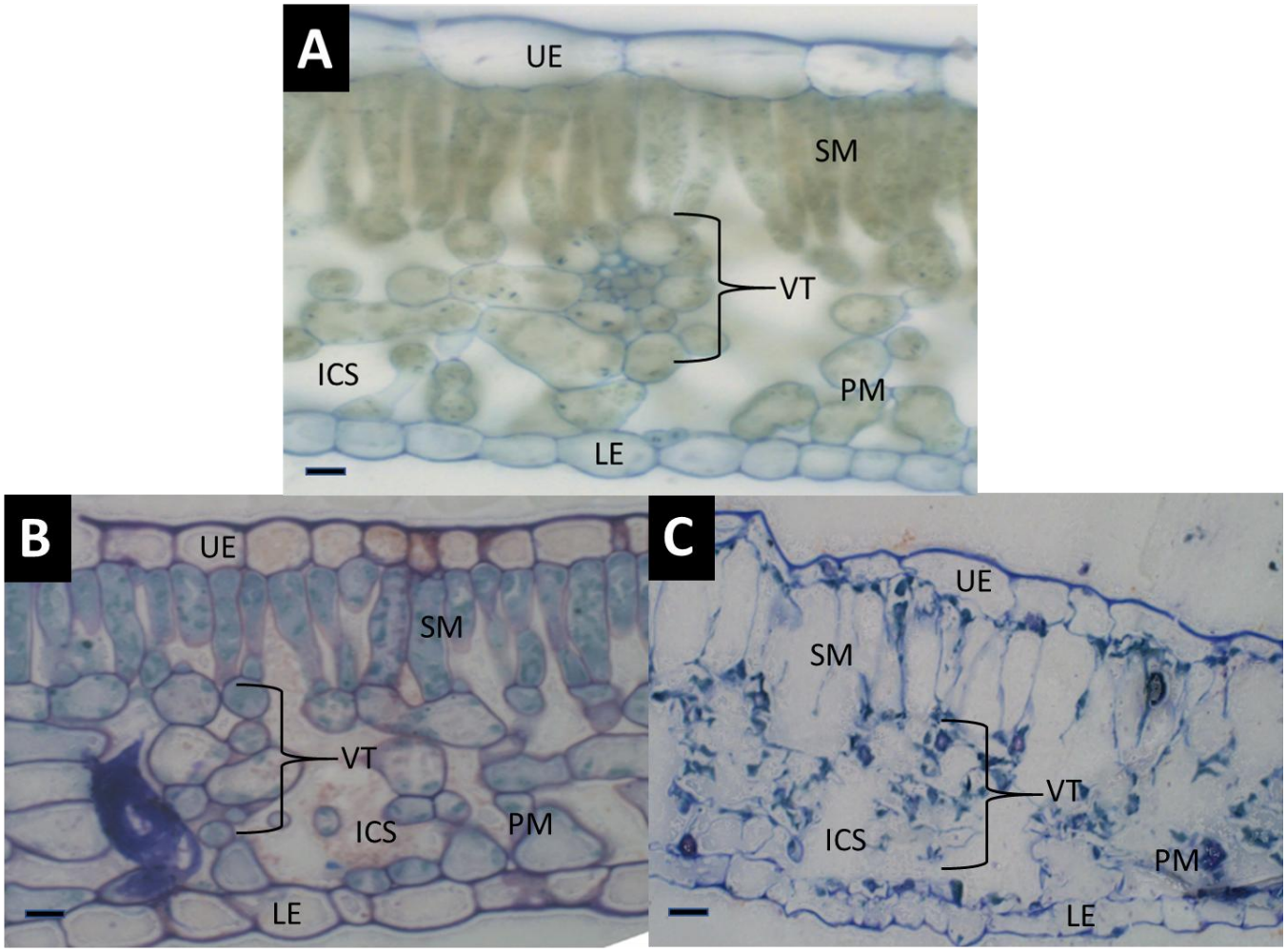


Figure 4.11 Light micrographs of M. oleifera control (A), green (B) and yellow (C) leaves of plants treated for 144 hours at 47/29°C ±1°C temperatures. (B) In the green leaves, the upper epidermal cells appeared to have decreased in size while lower epidermal cells have increased in size. The mesophyll cells remained elongated and the cell contents had moved to one side of the cells. The palisade mesophylls were greatly increased in size, thus decreasing the intercellular spaces. The vascular tissues had disintegrated. C) In the yellow leaves, severe damage was evident showing total disintegration of cells. UE=upper epidermis, LE=lower epidermis, SM=spongy mesophyll, VT=vascular

tissue, ICS=intercellular spaces and PM=palisade mesophyll. Scale bar: 1cm represents 20 µm (A, B and C).

Only after 144 hours at the temperature regime of 15/10°C a colour change of leaves from green to yellow was observed and this accounted for about 5% of the leaves when manually counted. More than 90% of the leaves after the seven days treatment at this temperature were still looking healthy and green and 5% (young and old leaves mixture) were green but shrank and were still looking healthy because they were not dry and did not show signs of severe stress damage.

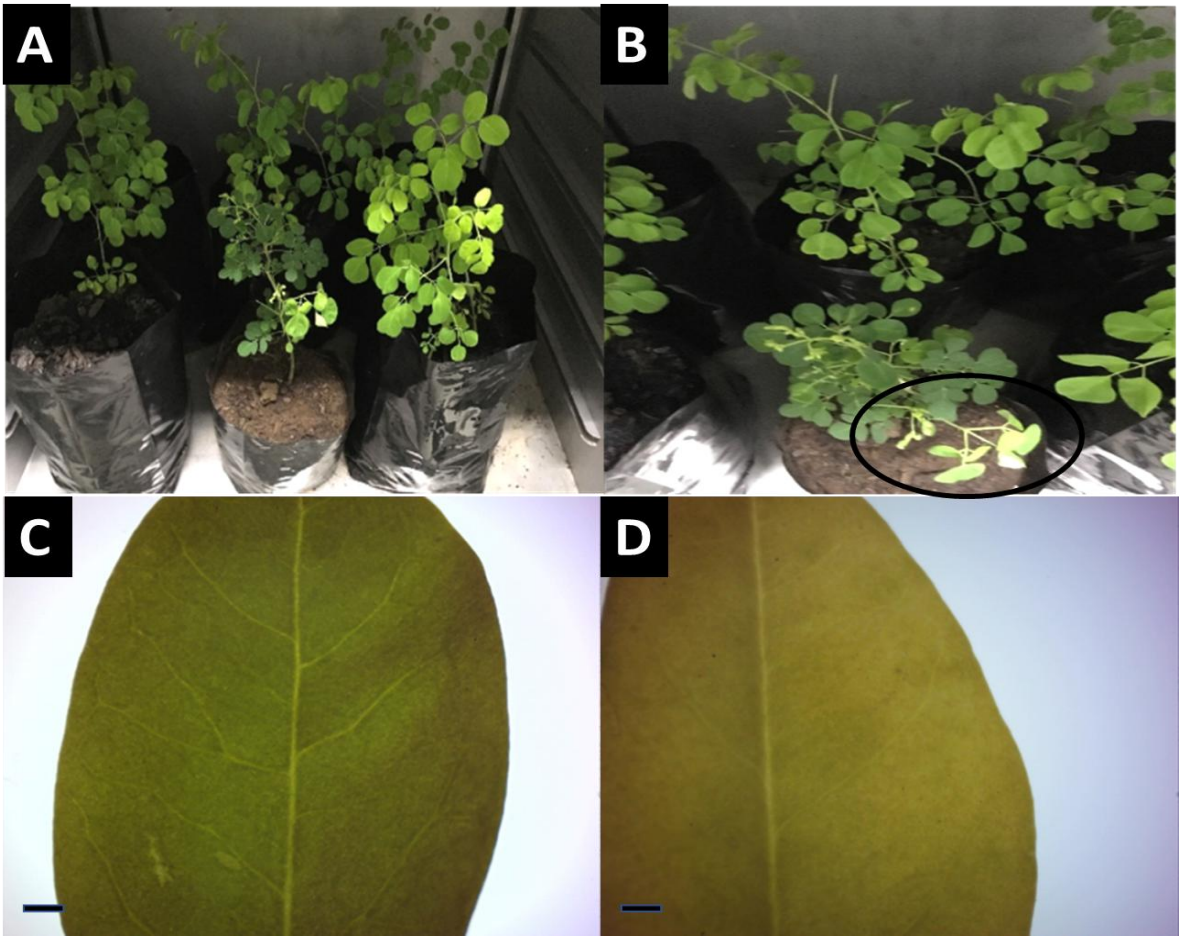


Figure 4.12 Photographs of M. oleifera under low temperature (15/10°C) treatment after 144 hours. (A) Showing that most of the plant leaves were still green. (B) Leaves starting to turn yellow in black circle. (C) Represent green leaves. (D) Yellow leaves. Scale bar: 1cm represents 10 µm (C and D).

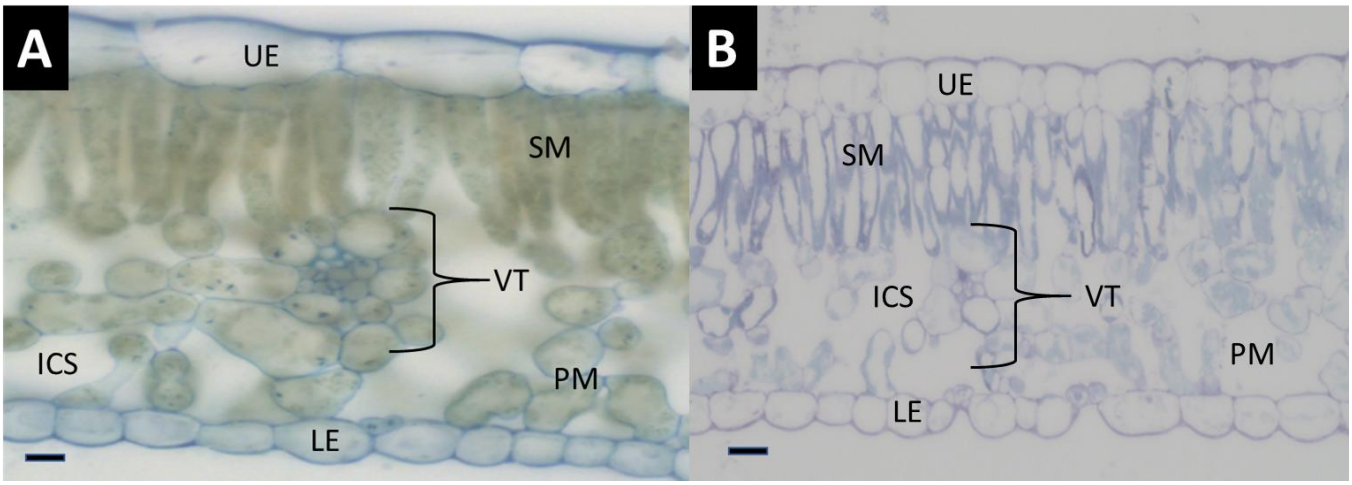


Figure 4.13. Micrographs of M. oleifera control (A), green (B) leaves of plants treated for 144 hours at 15/10°C ±1°C temperatures. (B) The upper and lower epidermal cells seemed to remain intact while the spongy mesophyll cells appeared to be more elongated as compared with the control. The contents in the spongy mesophyll cells appeared to have been redistributed towards the more elongated side of the cells. Palisade mesophyll cells remain intact and the contents are distributed at the center of the cells. Volume of intercellular spaces looked like those of the control. UE=upper epidermis, LE=lower epidermis, SM=spongy mesophyll, VT=vascular tissue, ICS=intercellular spaces and PM=palisade mesophyll. (A and B) Measured at 40x objective. Scale bar: 1cm represents 20µm.

A continued influence on the leaf thickness, spongy mesophyll cells, vascular tissue, intercellular spaces and palisade mesophyll cells was observed at the low TR. As shown above in **Figure 4.13**, the leaf average thickness after 144 hours was 319.19 µm ±0.27 µm. This showed that leaf thickness increased by 68.6%. There was no change in size and shape of upper epidermis, but the cells of the lower epidermis appeared bigger than in control samples. The spongy mesophyll cells continued to elongate and appeared longer. Vascular tissue was still intact with no changes in shape and size. Intercellular spaces remained in

proportion the elongated spongy mesophyll and intact palisade mesophylls. Palisade mesophyll cells remained intact and the chlorophyll containing cells were still visible due to the prominent green colour on the cells. Like chlorosis, leaf curling and browning especially at high temperature treatment increased with prolonged treatment time. At low temperature treatment, *M. oleifera* indicated tolerance to cold stress, however after 144 hours signs of chlorosis were observed. Upward leaf curling in some of the leaves (2%) occurred after 48 hours of treatment at low temperature stress. Besides causing chlorosis, leaf wilting and necrosis, prolonged low temperature stress results in plant's membrane damage caused by dehydration during the stress. Leaf curling is one of many responses to stress tolerance. For example, leaf angle change and curling occurred on *Rhododendron* species in the aid of protecting the plant from damage during winter. light intensity on leaf surface when in low temperatures results in leaf damage so when the leaves curl they prevent this effect to cause damage on the plant (Nilsen *et al.*, 2000). Possibly, this may have been the reason for the observed response in *M. oleifera.*

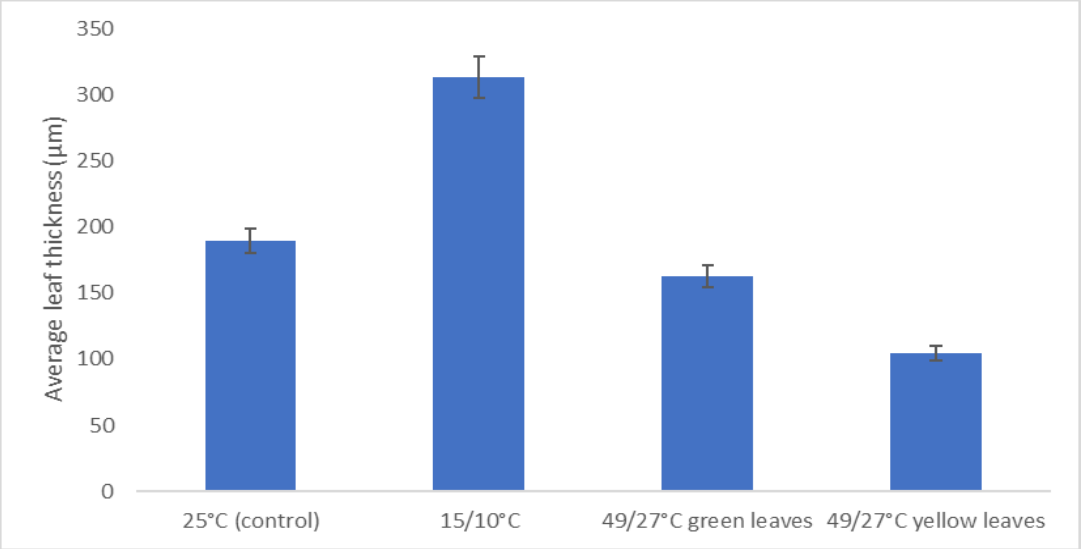


Figure 4.14 Average leaf thicknesses of M. oleifera after 144 hours of high (47/29°C) and low (15/10 °C) treatment, and the control (25°C). The average leaf thickness increased under low temperatures and decreased under high temperatures.

Extreme temperatures negatively alter normal plant metabolism, cellular homeostasis, physiological and biochemical processes such as photosynthesis and photorespiration (Awasthi *et al*., 2015). Inborn redox components in plants regulate temperature and are always kept at equilibrium by biochemical processes. The extent of temperature stress determines if the plant will be able to maintain the redox components balance and cause the plant to adapt. Reactive Oxygen Species (ROS) are readily produced in small quantities in the organelles such as chloroplasts and mitochochdria in leaves. However, if the balance is disrupted, by the oxidative stress (due to extremely high and low temperatures) severe damage to proteins, lipids, carbohydrates ultimately results in cellular damage, and consequently chlorosis and necrosis (Awasthi *et al.*, 2015, Ribeiro *et al*., 2003).The data in the current study may suggest that the observed cellular damage in plants under high temperatures could have been linked with the oxidative stress, and ultimately caused necrosis in leaves that eventually turned yellow.

Plants have an adoptive mechanism due to the protein Rubisco which is responsible for changing the effects of chlorosis in plants. Increased levels of ROS above plants threshold resulted in the degradation of Rubisco protein resulting in plant cell death (Sedigheh *et al.*, 2011). It has been found that different membranes in a plant cell behave totally different to other membranes when exposed to the same temperature stress. Cells sense the changes in temperatures through cellular responses using a specialised histone protein called H2A.Z and heat stress inhibits the activation of Rubisco activase which ultimately reduces the rate of photosynthesis (Hemantaranjan *et al.*, 2014). When medicinal plants from Korea were selected and assayed for free radical scavenging and antioxidant activities. It was observed that root bark of *Morus alba* and the leaf of *Saururus chinensis* showed stronger scavenging of 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical due to the presence of flavonoids such as catechin, morin, naringenin, quercetin and rutin in the plants (Choi *et al.*, 2002).

CHAPTER 5: PHYTOCHEMICAL STUDY

5.1 Materials and methods

5.1.1 Plant material

The leaf samples of the *M. oleifera* grown at 25°C (control), 15/10°C and 47/29°C for 144 days were harvested every 48th hours (as mentioned in the previous chapter). After harvest, the leaves were cleaned with deionised water for 1 minute to remove contaminants that have accumulated on the plants surface. The leaves were then air dried in a shade for 30 days (to ensure proper drying) and after the drying process, the leaf samples were finely grounded into powder using pestle and mortar and then stored in a dark place that is low in temperature for future use (Pakade *et al*., 2012).

5.1.2 Chemicals and reagents

All the reagents and chemicals used in the experiments were of analytical grade. Methanol, acetonitrile and acetic acid (all HPLC grade). Quercetin, kaempferol, Folin - Ciocalteu reagent, catechin anhydride, gallic acid, sodium carbonate, sodium hydroxide, sodium nitrite, aluminum chloride, were purchased from Sigma Aldrich (Johannesburg, South Africa). The deionised water used in all the experiments was obtained from ultra-pure water purification system, Milli-Q. Millipore (MA. USA). Quercetin and kaempferol standards were prepared as stock solutions at 10 mg/L or 10 ppm in 80% methanol by weighing 10 mg of each standard and dissolving them with 80% methanol in a 1 L volumetric flask. The stock solution was stored in a refrigerator at -18°C when not in use. This stock solution containing a mixture of quercetin and kaempferol was used to prepare solutions of this mixture at lower concentrations of 1, 2, 3, 4 and 5 mg/L and these concentrations along with the concentration of the stock solution were used to construct a calibration curve during analysis.

5.1.3 Plant metabolomics

5.1.3.1 Extraction and acid hydrolysis of leaf powder with acidified methanol

In this procedure, 3.1 mL of 32% hydrochloric acid was added in a 100-mL volumetric flask containing 96.9 mL 80% methanol and this acidified methanol mixture was used to extract and acid hydrolyse the leaf powder. A 0.6 g of the dried leaf powder was weighed onto a 100-mL round bottom flask, 20 ml of acidified methanol was then poured onto the round bottom flask containing the powder and then connected to a condensation apparatus. The flask containing the mixture and sample was submerged in warm water (80°C) and allowed to extract for 3 hours. The flask was disconnected from the condensation apparatus and 10 ml of 80% methanol was added into the extract. The diluted extract was sonicated using Transsonic 460 ultrasonic bath (Elma. Sin-gen. Germany) for 10 minutes, transferred into 50 ml centrifuge tubes and then centrifuged for 10 minutes at 4000 rpm. The extract obtained in this procedure was characterised for individual flavonoids such as quercetin and

kaempferol. Before injection into the High-Pressure Liquid Chromatography – Ultra Violet (HPLC-UV) (Bischoff HPLC Metrohm. Johannesburg. RSA), the extracts were filtered with 0.45 µm filter paper. Also, total phenolic and flavonoid contents were characterised on this extract. When the extract was not in use it was stored in a 50-mL centrifuge tube in a refrigerator of -18°C (Anwar *et al.*, 2007).

5.1.3.2 Total phenolic content (TPC)

A Folin - Ciocalteu reagent assay reported by Folin *et al.* (1927) was followed in the aid to determine the TPC on the extracts. A 0.5 mL blank (80% methanol) and 0.5 mL samples of the extracts and standard (gallic acid) were placed in a 20-mL brown bottle, 2.5 mL of 10% Folin - Ciocalteu reagent and 2.5 mL 7.5% sodium carbonate were added into the brown bottle consecutively. The mixtures were incubated on a shaker in the dark at 45°C for 45 minutes. The absorbance of the TPC in the extracts were then measured with a spectrophotometer (Varian, Cary 50 Conc, Germany) at a wavelength of 765 nm. To obtain the concentration of the TPC, gallic acid working stock solution was prepared and a calibration curve plotted from six data points ranging from 2.5 to 100 mg/L was used to determine the concentration of the total phenolic content (Labal *et al.*, 2006, Matshediso *et al*., 2014).

5.1.3.3 Total flavonoid content (TFC)

A colorimetric assay published by Zhishen *et al.* (1999) and Kim *et al.* (2002) was followed for the determination of the TFC. The 1 mL samples of the black (80% methanol), extracts and standard (catechin anhydride) were diluted with 4 mL deionised water in a 20-mL brown bottle. At different time intervals, the following chemicals were added to the diluted sample. Initially, 0.3 mL 5% sodium nitrite and the mixture were hand shaken for 1 minute, at 5 minutes, 0.3 mL 10% aluminum chloride and then at 6 minutes, 2 mL of 1M sodium hydroxide and then 2.4 mL deionised water was added and the sample was shaken at medium speed

for 15 minutes. Spectrophotometer was used to measure the TFC on the samples at a wavelength of 510 nm. A calibration curve was constructed using catechin anhydride standards and the concentration of the total flavonoids were obtained from the calibration graph plotted from six data points ranging from 2.5 to 100 mg/L (Iqbal *et al*., 2006).

5.1.3.4 Individual flavonoid content using HPLC – UV

A high-pressure liquid chromatograph (Bischoff HPLC Metrohm. Johannesburg. RSA) consisting of Gemini Phenyl – C_{18} column (150 x 4.6 mL, 5 µm i.d) attached with a UV detector set at 368 nm was used for the quantification of quercetin and kaempferol from the extract. Samples were eluted with constant and uniform mobile phase of methanol, water and acetonitrile (40:39:20 v/v and 1% acetic acid). The flow rate was set at 0.5 mL/min and the injection volume of the sample was 20 µL. Samples were prepared in triplicates. A calibration curve plotted from six data points ranging from 1.0 to 10 mg/L using working stock solution of these individual flavonoids was constructed and their concentrations was obtained from the graph (Pakade *et al*., 2012, Matshediso *et al*., 2014).

5.1.4 Data and statistical analysis

All measurements were done in triplicates and data analysed using student's t test (95% confidence levels) and reported as mean ± SD, where SD is the standard deviation. All experiments were repeated three times and student's t analysis performed on the data at 95% confidence level.

5.2 Results and discussion

5.2.1 Total Phenolic and Flavonoid contents

5.2.1.1 Gallic acid and catechin anhydride calibration curves

In Appendix 2, **Figure 2.4 and 2.5** show the calibration curves used to quantify the total phenolic and flavonoid contents on the different extracts obtained from different leaf powders harvested at different TR at different times. The calibration curves were linear from 2.5 mg/L to 100 mg/L concentration range and the correlation factors were above 0.99. The figures also show the absorbances of UV-Vis light of different concentrations of gallic acid and catechin anhydride at wavelengths 768 nm and 512 nm.

5.2.1.2 Total phenolic and flavonoid concentrations at high and low temperature regimes

The effect of high TR on the TPC of *M. oleifera* at different times is shown in **Figure 5.1** below. It was observed that the effect of high temperature resulted in increased TPC after 48 hours by 10.1%. This was followed by a decline after 96 hours of 3.5%. An increase in concentration from the control sample to the 96 hours sample was observed to be 6.3% of TPC. TPC to continue to decline from 96 hours to 144 hours, a decrease in TPC was 3.1%. From the control to 144 hours, an increase in TPC was observed to be 3.0%. Although the leaves were randomly harvested, a similar trend was observed in all experiments. These finding suggested that, when a three-month-old *M. oleifera* is subjected to this TR for 7 days, an increase of TPC by 3.0% should be expected when the same conditions are followed. Abiotic stress such as extreme temperatures (ET) disturb metabolic homeostasis in plants and the production of compounds such as primary and secondary metabolites that are responsible in amending the effect of the stresses (Obata *et al*., 2012). At elevated temperatures, dramatic changes in primary and secondary metabolites occur due to destructions in membrane structure and function, protein and gene expression and tissue water content (Kralova *et al*., 2012).

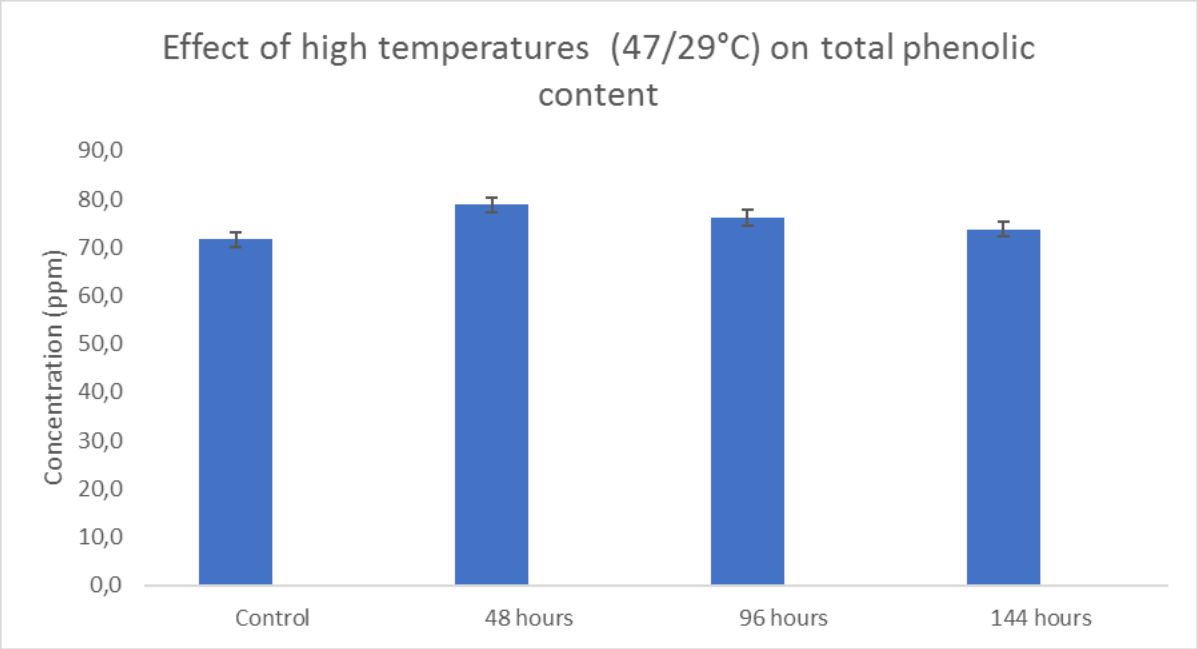


Figure 5.1 The effect of high temperature treatment on plant treated for 48, 96 and 144 hours. As compared with the control, total phenolics increased by 10.1% after 48 hours. Thereafter, a decrease of 3.5% after 96 hours and 3.1% after 144 hours was observed.

Plants that survive the high temperature stress have shown to undergo acclimation. The change in metabolic homeostasis may cause the plants to adjust to the abiotic stresses. Consequently, further change on the concentration of metabolomics in the plant such as high concentrations of phenolics may result. Jones *et al*., (2017) showed that when the Canadian and Australian wheat were grown to maturity at 20, 25 and 30°C, and changes on content of secondary metabolites was noteworthy. Results demonstrated that the concentration of secondary metabolites was directly proportional to temperature. The concentration of phenolic acids such as palmitic acid and oleic acid and total flavonoid contents increased with increasing temperature (Jones *et al*., 2017). Two cultivars of conifer, Scots pine (Pinus

sylvestris L.) and Norway spruce (Picea abies (L.) were grown for 50 days at 19/12°C or 23/16°C and their secondary metabolites were analysed. Terpenoids were higher in concentration at high temperatures (Holopainen *et al.*, 2003). Joshi, (2015) also demonstrated that secondary metabolites such as phenolics and flavonoids increased when the species was exposed to increasing temperatures of 13, 24 and 30°C for 15 days. Similarly, the current study demonstrated that high temperature stresses resulted in increased phenolic content. This result may be indicative of *M. oleifera's* possible adjustment to the high temperature stress.

Interestingly, the analysis of the results of the effect of low temperature treatment on the concentration of the TPC in *M oleifera* showed, as compared to the control, the concentration increased after 48 hours by 21.6% **Figure 5.2**. This is significant increase as compared to the response that was observed after 48 hours under high temperature treatment [10.1%, ($p<0.05$)]. Further increase 26.5% in TPC was observed after 96 hours. Those results have shown that TPC increased from 48 hours to 96 hours by 4.1%. This type of stress response is indicative of adaptation to the cold stress tolerance during that period of exposure. However, a sudden decline in TPC after 144 hours was observed. These analyses have shown that *M. oleifera* indicated to be more tolerant to the cold stress. This was characterised by a higher content of phenolic compounds of which indicates better radical-scavenging capacity together with stronger reducing power. Though the content of phenolics declined by 18.8%, it remained higher than the control by 2.7% **Figure 5.2**. Some researchers have shown that cold stress can increase the content of phenolics.

For example, Cook *et al.,* (2004) showed that *Arabidopsis thaliana* exposed to cold temperature stress, the metabolite concentration of compounds such as amino acids, glucose, fructose, inositol, galactinol, raffinose and sucrose increased due to cold acclimation. When subjected to low temperature of 4°C for 14 days, 75% of metabolites increased in concentration as compared with the control (Cook *et al*., 2004). Some plants survive cold temperature (CT) stress without injury due to the metabolome changes that occur within the

plant during cold acclimation. Many authors demonstrated that during acclimation to cold temperature stress, plants produce metabolites such as betaines, sugars (mannitol, sorbitol, and trehalose), polyols, polyamines, and amino acid (proline) to induce tolerance mechanism for normal cellular metabolism (Janmohammadi *et al.*, 2012, Kaplan *et al*., 2004). Chung et al. (2006) also demonstrated that cold stress resulted in an increase in the total concentration of phenolic compounds in *Rehmannia glutinosa*. Similar results were observed in seedlings of soybean subjected to low temperature stress (Posmyk *et al*., 2005). Similarly, the data in the current study suggest that low temperature stress results in significantly high phenolic content.

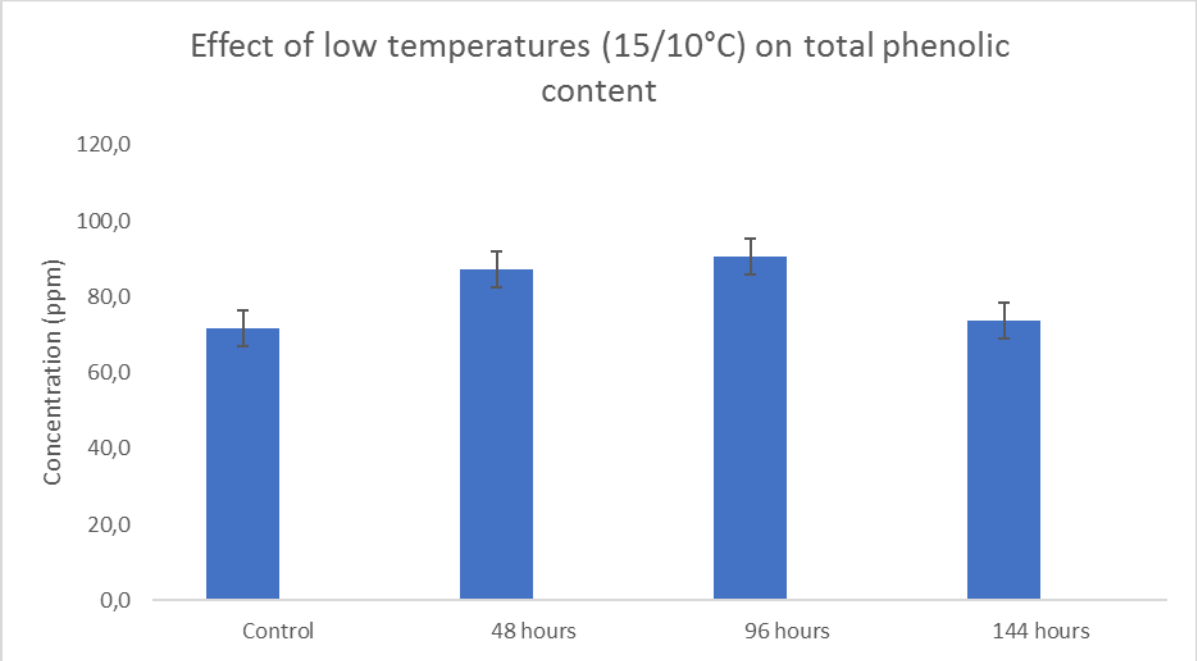


Figure 5.2 The effect of low temperatures treatment on M. oleifera total phenolic content treated for 48, 96 and 144 hours. An increasing trend in concentration occurred until 96 hours and a decline were observed after 144 hours. However, the phenolic content remained higher after 144 hours as compared with the control by 2.7%.

The effect of high temperature was observed to the increase the concentration of flavonoids after 48 hours of treatment. As compared with the control, TFC increased by 36.4% as shown in **Figure 5.3**. Conversely, TFC declined after 96 hours by 54%, and decreased further after by 17.8% after 144 hours. When plants adapt to abiotic stress metabolism reformation occurs and this results in different concentrations of primary and secondary metabolites. For instance, metabolite contents of Carrizo citrange and Cleopatra mandarin which are two citrus cultivars after being subjected to drought and high temperature conditions were assessed. Higher concentration of phenylpropanoid metabolites, scopolin, flavonols, flavones such as tangeritin were observed in Cleopatra. Higher concentrations of sinapic acid and sinapoyl aldehyde were observed in Carrizo. The metabolites formed in Cleopatra led to the formation of photoprotective and antioxidant secondary metabolites to fix and eliminate the damaging effects of these stresses (Zandalinas *et al*., 2017).

Panax quinquefolius plants were grown at 30°C and 25°C for one growing season. Analysis of secondary metabolites of these plants showed that the plants under high temperature reached senescence early and had decreased carbon, photosynthesis and biomass content than the ones at low temperature. Further, the concentration of ginsenosides was higher on the high temperature plants than the ones at low temperature (Jochum *et al.*, 2007). Seeds of four clones of *Eucommia ulmoides* were planted in different environmental locations such as altitude, annual sunshine duration, annual precipitation, and annual average temperature until maturity and their secondary metabolites were assessed. Results showed that secondary metabolite concentration was independent of the clone type but dependent on environmental factors. A combination of altitude and annual average temperature resulted in higher concentrations of chlorogenic acid and flavonoids while annual sunshine duration and annual average temperature gave higher concentrations of geniposidic acid. No change was observed in secondary metabolites on plants grown under the annul precipitation (Juane *et al.* 2011).

Metabolomic profiles of *Tithonia Diversifolia* grown during rainy and dry seasons of 21.3°C and 27.2°C were analysed using UHPLC-DAD-ESI-HRMS and NMR methods. Leaves that were

harvested during the dry seasons were rich in secondary metabolites such as sesquiterpene lactones and trans-cinammic acid ester derivatives. Secondary metabolites were of same concentration during the transition period between the rainy and dry seasons (Sampaio *et al.*, 2016). Toxic and antimicrobial secondary metabolites such as pyrrolizidine alkaloids (PAs) and naphthoquinones (NQs) were assessed on leaves of *Echium plantagineum* after being exposed to high temperatures for 3 days. It was found that concentration of NQs was significantly higher than that of PAs revealing that different types of metabolites will tend to respond differently to the same abiotic stress (Skoneczny *et al.*, 2017).

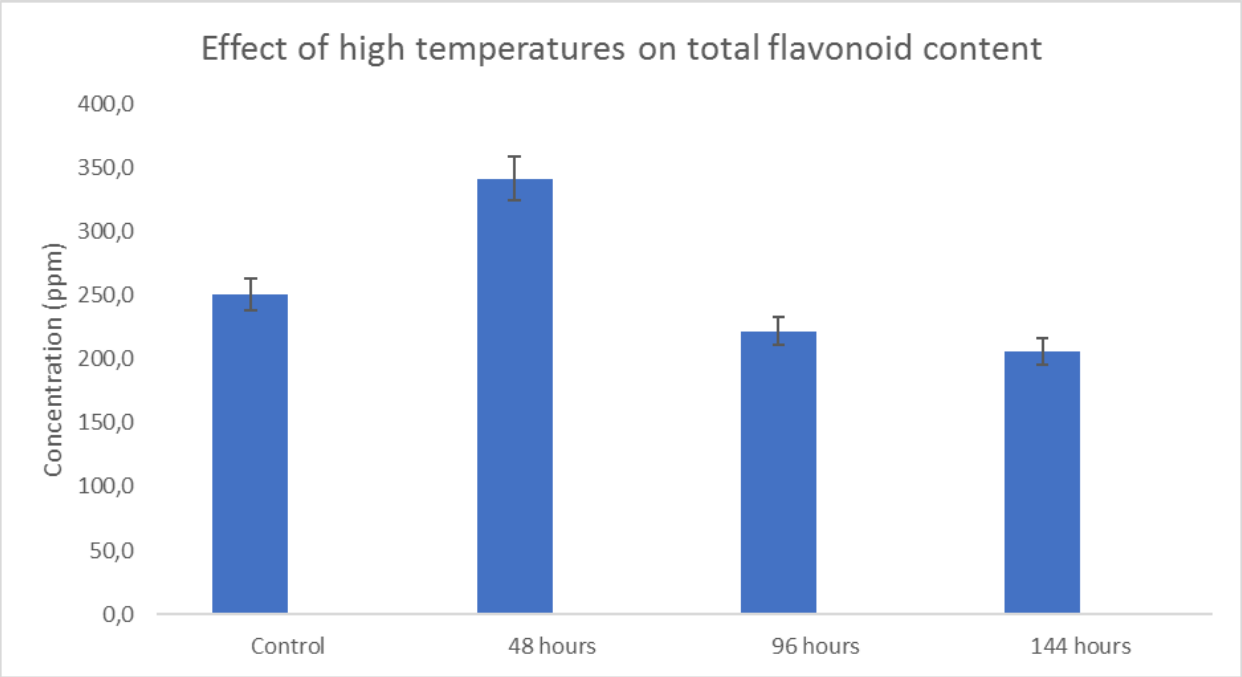


Figure 5.3 A significant 36.4% increase of total flavonoid concentration after 48 hours was observed. A decrease of 11.4% after 96 hours occurred. A continued decrease of 17.8% after 144 hours was observed.

Figure 5.4 below shows the trend of the concentration of total flavonoids when *M. oleifera* was subjected to a low TR. After 48 hours of treatment, an increase of 11.6%. in TFC was observed further increase in TFC was recorded after 96 hours. Therefore, the total increase after 48 and 96 hours was 33.8%. On the contrary, a 25.5% decrease in concentration after 144 hours was observed, however it was not significantly lower than the control. These findings confirm the higher tolerance of *M. oleifera* to cold stress. Furthermore, this is also supported by the results obtained from microscopic studies that showed that low TR does not severely affect the morphology of the leaves, the anatomy remained intact thus coursing the plants to adapt.

A high content of secondary metabolites is mostly produced in the plants when it is exposed to abiotic stresses to cause the plant to adapt and combat the stress. Concentration of cryo-protective compounds such as sugar alcohols (e.g. sorbitol, ribitol and inositol) soluble sugars (e.g. saccharose, raffinose, stachyose and trehalose), and low-molecular weight nitrogenous compounds such as proline, glycine and betaine in plants is increased during cold stress tolerance (Ravishankar *et al.*, 2011). Kovacs *et al.*, (2011) demonstrated that exposing wheat leaves to cold temperature resulted in increased concentration of putrescine, spermidine and, spermine. A similar pattern in response to low temperature stress was observed in the current study.

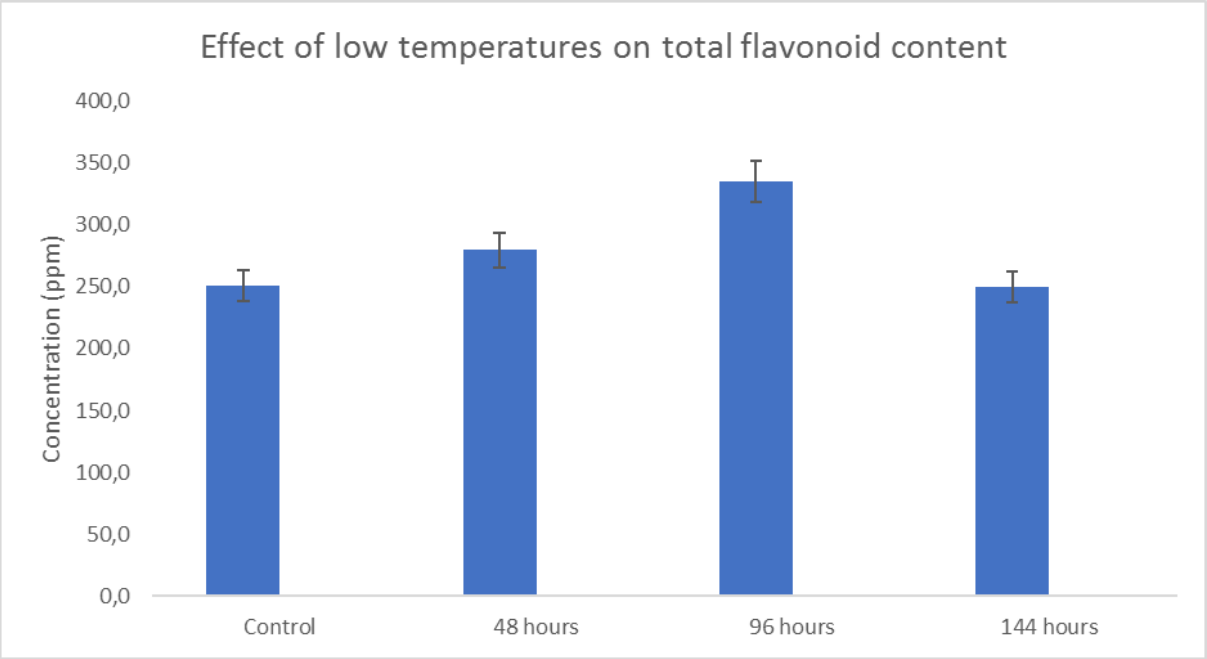


Figure 5.4 Increases in total flavonoid concentration in M. oleifera after 48 and 96 hours of 11.6 and 33.8% were observed and then a decrease of 0.4% after 144 hours was observed.

5.2.2 Linearity, LOD and LOQ of individual flavonoids

The calibration curve used to qualify and quantify quercetin and kaempferol is shown in Appendix 2. The calibration curves had acceptable correlation coefficients of 0.99 for both flavonoids and were linear from 1.0 mg/L to 10 mg/L concentration. A blank sample was run on the HPLC-UV to obtain the concentration of the background noise and from this concentration, limit of detection (LOD) and limit of quantification (LOQ) were determined by multiplying the background noise concentration by three and ten (Matshediso *et al.*, 2014). LOD and LOQ concentration are tabulated in Table 2.1 in Appendix 2.

5.2.3 Effect of high and low temperature on quercetin and kaempferol

The results obtained from the chromatograms for the standard mixture shows that quercetin gets eluted first from the C_{18} column at a retention time of 5-6 minutes and lastly kaempferol at 8-9 minutes. This showed that kaempferol is less polar than quercetin and, thus, it has more attraction to the stationary phase (Matshediso *et al.*, 2014). Quercetin and kaempferol are not readily available to be characterised in a plant material but rather exist as large groups polyphenols such as sugar conjugates or aglycones which when hydrolysed result in the breakage of the glycosidic bond forming small group aglycones that can be identified and quantified (Nuutila *et al.*, 2002). Quercetin has 5 hydroxyl groups and kaempferol has 4 hydroxyl groups as shown in Figure 5.5 and this confirms why quercetin elutes first from the C_{18} column as compared to kaempferol. This is because hydroxylation increases the polarity of the compound thus reducing its retention on the non-polar column (Matshediso *et al*., 2014).

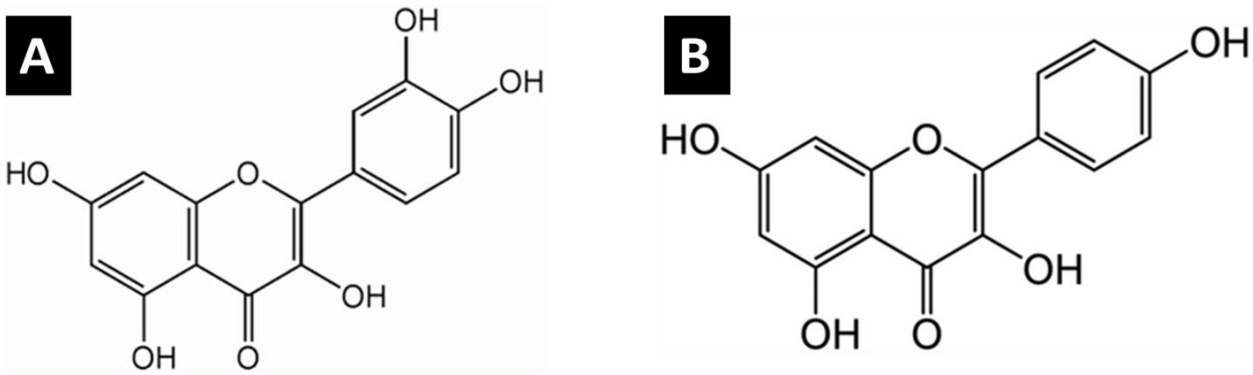


Figure 5.5: Chemical structures of (A) Quercetin and (B) Kaempferol ("Quercetin," 2017 December 12, "Kaempferol," 2017 December 24).

Both quercetin and kaempferol on the extracts showed a significant increase after 48 hours, thereafter a decreasing pattern was observed after 96 and 144 hours as seen below in **Figure 5.6**. In the control, the concentration of quercetin was relatively higher (15.39 ppm) than that of kaempferol (6.35 ppm). Although the concentration of quercetin and kaempferol compounds increased from 15.39 ppm to 16.70 ppm and 6.35 ppm to 19.27 ppm respectively, the concentration of kaempferol became higher than that of quercetin by 15.4% and three times higher than that of the control sample indicating an increase in concentration by 203.5%. These results indicate that HT stress has different effects on different types of flavonoids. Wang *et al.,* (2001) showed that when strawberry plants were exposed to four controlled temperature conditions of 18/12, 25/12, 25/22, and 30/22°C, their metabolomic contents increased in metabolites such as dihydroflavonol, quercetin 3-glucoside, quercetin 3-glucuronide, kaempferol 3-glucoside, kaempferol 3-glucuronide, cyanidin 3-glucoside and phenolic acids was observed at high temperature (30/22°C) than control. Also, at high temperatures, increased antioxidant capacity to scavenge peroxyl radicals, superoxide radicals and H_2O_2 was observed (Wang *et al.,* 2001).

After 96 hours, the concentration of both compounds decreased by 46.2% (quercetin) and 42.8% (kaempferol). In comparison to the control, quercetin's concentration reduced by 41.7% while that of kaempferol increased by 73.7%. After 144 hours, the concentration of both compounds decreased from the concentrations after 96 hours by 34.2% for quercetin and 24.8% for kaempferol. The overall effect of this temperature regime after 7 days treatment on the concentrations of these individual flavonoids was that the concentration of quercetin was reduced by 61.6% and that of kaempferol increased by 30.7%. It was noted that in the 7 days treatment at this temperature treatment, the concentration of quercetin is negatively affected after 96 hours because it becomes lesser than the control while that of kaempferol remained relatively higher than the control. These results may be correlated with anatomical responses that were observed. The cellular damage that was noted during the high temperature may be linked to the observed decline of flavonoids and quercetin. The

drastic increase in concentrations after 48 hours confirms the systemic responses to counteract possible oxidative stress.

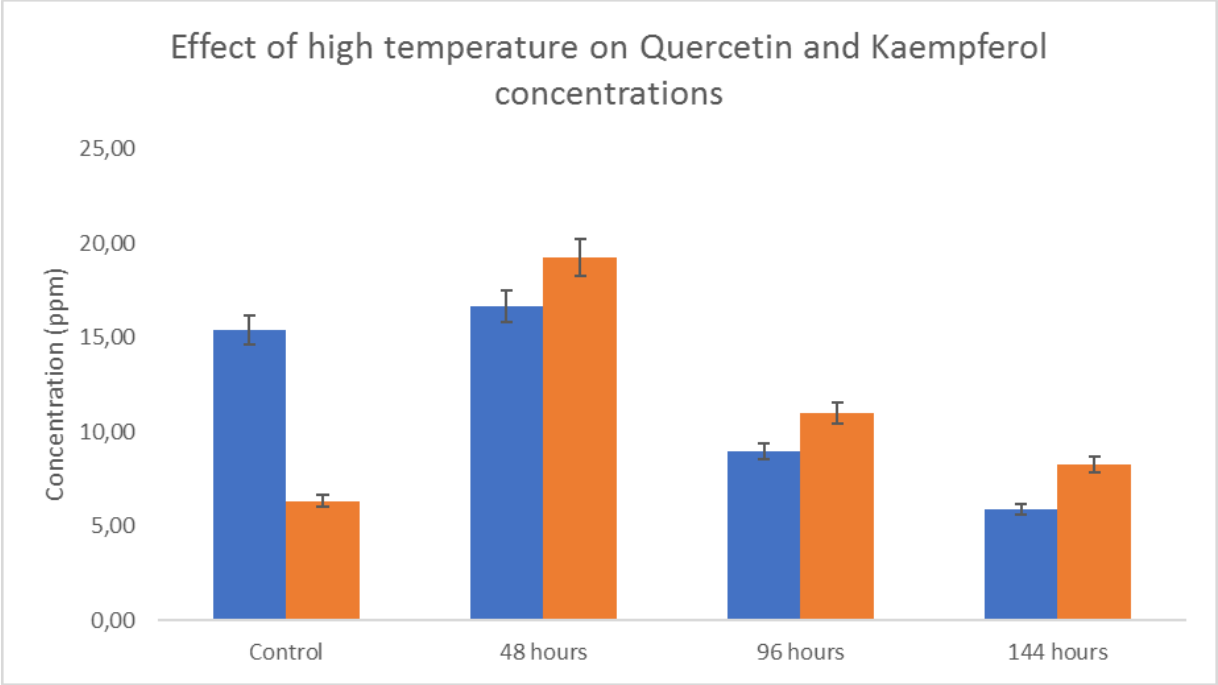


Figure 5.6 Concentrations of Quercetin (blue) and Kaempferol (orange) followed a similar trend when subjected to high temperatures. Quercetin increased by 8.5% after 48 hours, decreased by 41.7 and 61.6% after 96 and 144 hours. Kaempferol significantly increased by 203.5% after 48 hours. After 96 to 144 hours a decline of 43.0 and 24.8% was observed respectively.

Secondary metabolites are naturally produced by plants and assist in adaptation to its environmental stresses (Nuutila *et al.*, 2002). However, it has been shown that under one abiotic stress such as high temperature, different types of secondary metabolites react differently resulting in different levels of their concentrations (Kuokkanen *et al.*, 2001).

Figure 5.7 below shows that the effect of the low temperature regime on the concentration of quercetin was totally different from the concentration of kaempferol during the 7 days treatment. The concentration of quercetin significantly decreased throughout the 7 days treatment whilst that of kaempferol slightly increased after 96 hours and then decreased in 144 hours. The effect of the low TR reduced the concentration of quercetin by 58.3% after 48 hours while kaempferol increased by 12.6%. After 96 hours, the concentration of kaempferol continued to increase by 19.4% and that of quercetin continued to decline by 76.3%. After 144 hours both quercetin and kaempferol concentration decreased by 84.8% and 26.1% respectively.

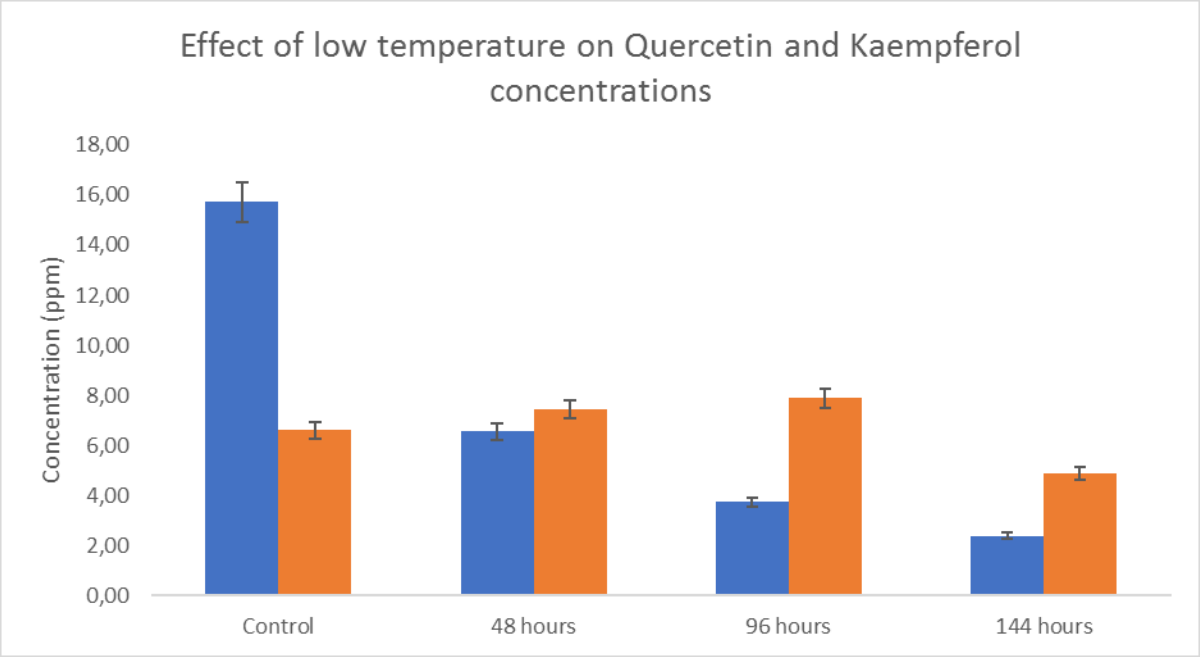


Figure 5.7 Concentrations of Quercetin (blue) and Kaempferol (orange) in M. oleifera leaves showed a different pattern when subjected to low temperature (15/10 °C). Quercetin significantly decreased by 56.2% after 48 hours, and further decreased by 74.2 and 82.8% after 96 and 144

hours respectively. Kaempferol increased by 16.4 and 23.0% after 48 and 96 hours. After 144 hours, it declined by 22.1%.

The responses of quercetin concentration in *M. oleifera* displayed a different pattern as compared with Kaempferol. A similar response was observed in three months old *Hypericum brasiliense* subjected to varying temperatures of 25/10 and 25/30°C along with a control for 15 days. Phenolic compounds such as quercetin, rutin, 1,5-dihydroxyxanthone, isouliginosin B were characterized, and an increase of phenolic compounds such as 1,5-dihydroxyxanthone, isouliginosin B under low temperature was observed while rutin and quercetin concentration decreased (Mazzafera *et al*. 2005).

CHAPTER 6: GENERAL DISCUSSION AND CONCLUSION

6.1 General discussion

M. oleifera is a commercially important plant species. for the species to be largely cultivated, harvested and used for nutritional, medicinal and industrial purposes, its adaptation and growth at different temperatures and environments that are not of the tropical nature needs to be studied and understood. Plant systematic responses are the key players in showing how the plant responses to different extreme temperatures. At high TR after 48 hours, the leaves displayed signs of senescence. This was demonstrated by the discolouration of the leaves from green to yellow colour. Wilting, curling and drying of the leaves was also noted as a sign of temperature stress. Furthermore, the rate of leaf damaged increased with increased exposure at this TR. Results from the histological studies of the plant's leaves at TR of 47/29°C correlated with the histological results. They showed that the rate of plant deterioration increases with time and changes in shapes and colours of the leaves were directly

proportional with a change in the cells of the leaves. When the leaves turned yellow, brown or shrank, a change in their cellular structures such as the epidermis, palisade mesophyll, spongy mesophyll and intercellular spaces was evident. Consequently, this resulted in necrosis after 144 hours of exposure. This has been shown to be a typical response in plants that are less tolerant to high temperature stress (Grigorova *et al.*, 2013; Cramer *et al*., 2004, Wang *et al*., 2012, Ter Heerdt *et al*., 2017). In the present study, the three months old *M. oleifera* certainly displayed high sensitivity and intolerance to high temperature stress. Despite the increase in the concentration of total phenolics, flavonoids, Quercetin and kaempferol after 48 and 96 hours to possibly combat oxidative stress, the data in present study suggest that the histological responses contributed to unsuccessful acclimatisation to high temperatures.

Under low temperature treatment (15/10°C), plants displayed more tolerance to cold stress as compared with the high temperature stress responses. After 48 hours results showed that only 1% of the leaves showed signs of leaf curling. After 96 hours, a similar response was observed. However, after 144 hours leaf senescence was observed in approximately 5% of the leaves in treated plants. Histological response further confirmed the species' tolerance to cold stress; tissue structures such as the epidermis, palisade mesophyll, spongy mesophyll and intercellular spaces remained intact and did not show severe damage. In addition, the significantly thickened leaf size, increased intercellular spaces and drastically elongated mesophyll palisade cells were indicative of cold acclimation (Tony *et al*., 2006). This has been shown to be a common response in plants that are cold tolerant and capable of cold acclimation (Pino, 2008; Janská *et al*.,2010; Zhu *et al*., 2007; Le Gall. 2015)

Thus, the current study demonstrated that three months old *M. oleifera* may survive cold temperatures for 7 days. The changes in the cellular structures of *M. oleifera* leaves under cold temperature stresses also correlated with the change in the concentrations of the phytochemical compounds within the plant. Previous studies have shown that total phenolic

and flavonoid concentration of *M. oleifera* harvested and characterised in winter were higher than those harvested and characterised in summer. Young *M. oleifera* plants grow best in the warmer season and mature in colder season, the concentration of the phytochemical compounds is higher in winter than in summer (Iqbal *et al*., 2006).

As shown in the previous chapter, at low temperatures, the concentration of total flavonoids increased from control to 48, 96 and 144 hours by 33.8, 25.5 and 0.4%. The concentration of total phenolics was increased after the 7 days treatment by 2.7%. Many researchers have shown that to avoid potential severe cellular damage caused by excess ROS as well as to maintain growth, metabolism and productivity, the balance between producing and eliminating ROS should be tightly regulated (Mittler, 2002; Mittler *et al.,* 2004). The capability of *M oleifera* to acclimatise at the cellular and morphological level together with the increased concentration of phenolic compounds, contributed to the adaptation to the cold temperatures. The changes in the phenolic activity to activate the antioxidant defense system in *M. oleifera* to control oxidative stress induced by cold temperature stresses may have occurred. Essentially, cellular structures remained intact throughout the treatments and thus provided structural support to ensure positive activation of ROS detoxification mechanisms.

6.2 Concluding remarks

In conclusion, the combined evidence from the present study showed that the three months old *M. oleifera* plants are sensitive and cannot withstand temperatures as high as 47/29°C. The increase in total phenolic and total flavonoid contents after 48 hours was an indication that the species was trying to combat the heat stress. The continued drop in these phytochemical compounds after 96 and 144 hours, meant that the species was losing the fight to counteract the heat stress. This is also indicative on the morphology and histology of the plants because senescence and necrosis increased with increased time of exposure. However, the species has showed to be positively more tolerant to cold temperatures (15/10°C). This positive tolerance to low temperatures was evident on the morphology and

histology of the leaves throughout the treatment process because no necrosis was observed. Also, most of the phytochemical compounds on the leaves were not negatively affected. This evidence may have meaningful contribution towards the industry, *Moringa oleifera* farming practices as well as advancing the understanding of the effects of extreme temperatures on the species. Further studies on the impact of extreme temperature on the ROS production and leaf ultrastructure on plants older that three months are recommended. Such studies will further improve the understanding of the responses of *M. oleifera* to environmental stresses.

APPENDIX 1

Light electron microscopy fixation and staining

1. Phosphate buffer

a) Solution A: 14.2 g/L disodium hydrogen orthophosphate anhydrous (Na2HPO4).

b) Solution B: 15.6 g/L Sodium dihydrogen orthophosphate dehydrate (Na2HPO4.2H2O)

A 100-mL buffer solution will be made by mixing 72ml of Solution A with 28ml of solution B.

2. Glutaraldehyde fixative (3% v/v)

Glutaraldehyde stock solution (2ml of 25% (v/v) will be mixed with 88ml of phosphate buffer.

3. OsO_4

Mix one vial of OsO_4 with an equal volume of phosphate buffer in the fume cupboard. Cover the specimen with OsO_4/buffer mixture and leave in the fume cupboard for 1h. 4.

Uranyl acetate (UA) (1% w/v)

Dissolve 0.25g of UA with 25ml of 75% (v/v) ethanol. Wrap the bottle with foil as solution is light sensitive. The solution must be made up fresh immediately prior to use.

5. Epoxy resin embedding medium (Spurr *et al.*, 1969)

Components:

- Resin: vinyl cyclohexene dioxide (CVD)
- Hardener: nonenyl succinic anhydride (NSA)
- Plasticiser: DER 736
- Accelerator: di-methylamino ethanol (DMAE)

Components must be at room temperature for use. Add components gravimetrically to a suitable container:

VCD 23g

NSA 62g

DER 14g

DMAE 1g (must be added after the other components have been added and mixed).

Standard polymerization time: 8 – 12h at 70°C.

Fixing and embedding procedure:

• Place samples in glutaraldehyde fixative for the appropriate amount of time.

• Change to buffer, making one double change followed by five further changes over 1h.

• Replace buffer with OsO_4 and keep in fume cupboard for 1h.

• Make up UA.

• Remove OsO_4 and change to buffer. Change the buffer twice more over ½h.

• Change to 10% (v/v) ethanol then to 25% and 50% (v/v) ethanol after 15min in each solution.

• Replace 50% (v/v) ethanol with UA solution, wrap samples with foil and keep refrigerated for 1h.

• Remove UA solution and follow with two double changes of 75% (v/v) ethanol (10min each).

• Remove 75% (v/v) ethanol and replace with absolute ethanol.

• Two changes of absolute ethanol (10min each)

• Remove absolute ethanol and follow with two changes of propylene oxide (10min each)

• Add equal amounts of resin and propylene oxide to samples after second change of propylene oxide. Leave for 5h.

• Remove propylene oxide/resin mixture and replace with whole resin. Leave for overnight.

• Remove whole resin, place samples in resin trays. Fill with fresh resin and incubate in an oven at 70°C for 8-12h.

APPENDIX 2

Phytochemical analysis calibration and experimental results

Table 2.1: LOD and LOQ concentrations for flavonoids

Flavonoid	**LOD (µg/mL)**	**LOQ (µg/mL)**
Quercetin	0.028	0.28
Kaempferol	0.038	0.38

Table 2.2: Concentrations of quercetin and kaempferol on the plants treated at HT of 47/29 ˚C for 48, 96 and 144 hours

Sample	**Quercetin (ppm)**	**Kaempferol (ppm)**
Control	15.39 ±0,709	6.35 ±0,806
HT48	16.70 ±0,689	19.27 ±0,699
HT96	8.98 ±0,733	11.03 ±1,084
HT144	5.91 ±0,721	8.30 ±0,627

2.3 Quercetin and Kaempferol

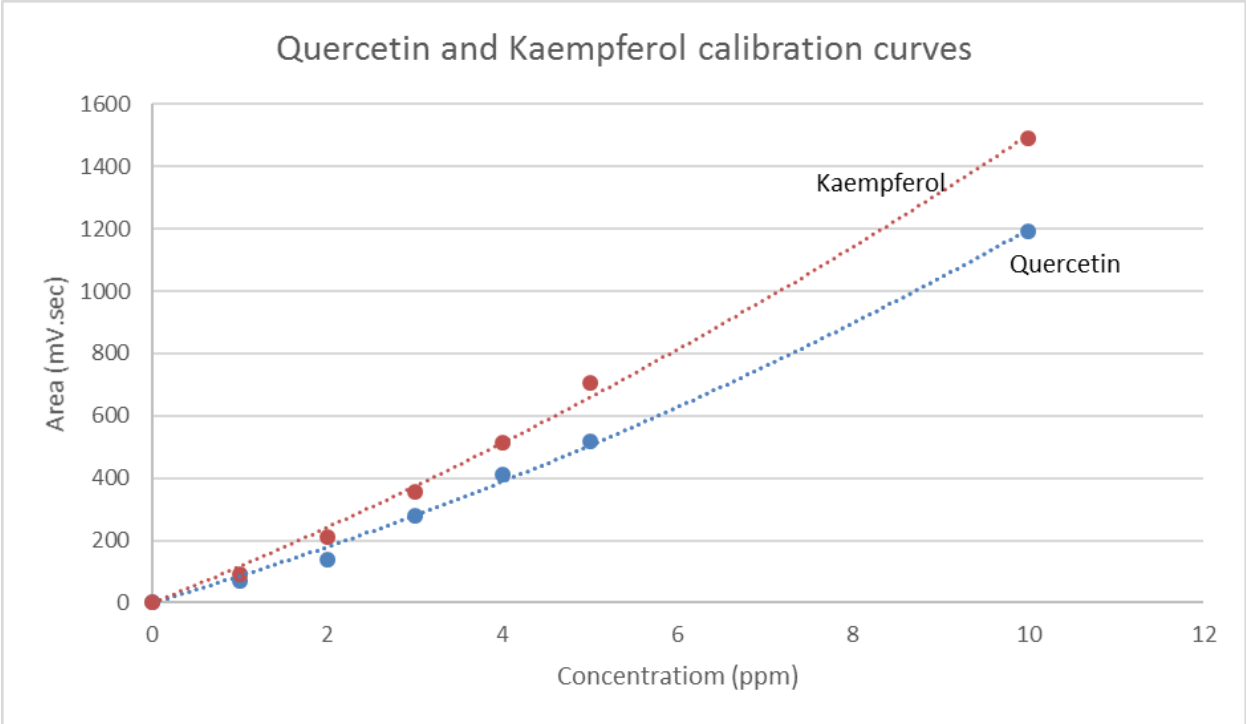


Figure 2.4 Quercetin and Kaempferol standards calibration curves

Table 2.5: Concentrations of quercetin and kaempferol on the plants treated at low temperature of 15/10 ˚C for 48, 96 and 144 hours

Sample	**Quercetin (ppm)**	**Kaempferol (ppm)**
Control	15.39 ±0,709	6.35 ±0,806
LT48	6,88 ±0,300	7,68 ±0,812
LT96	4,05 ±0,563	8,12 ±1,257
LT144	2,70 ±0,574	5,14 ±0,191

2.6 Gallic acid standard

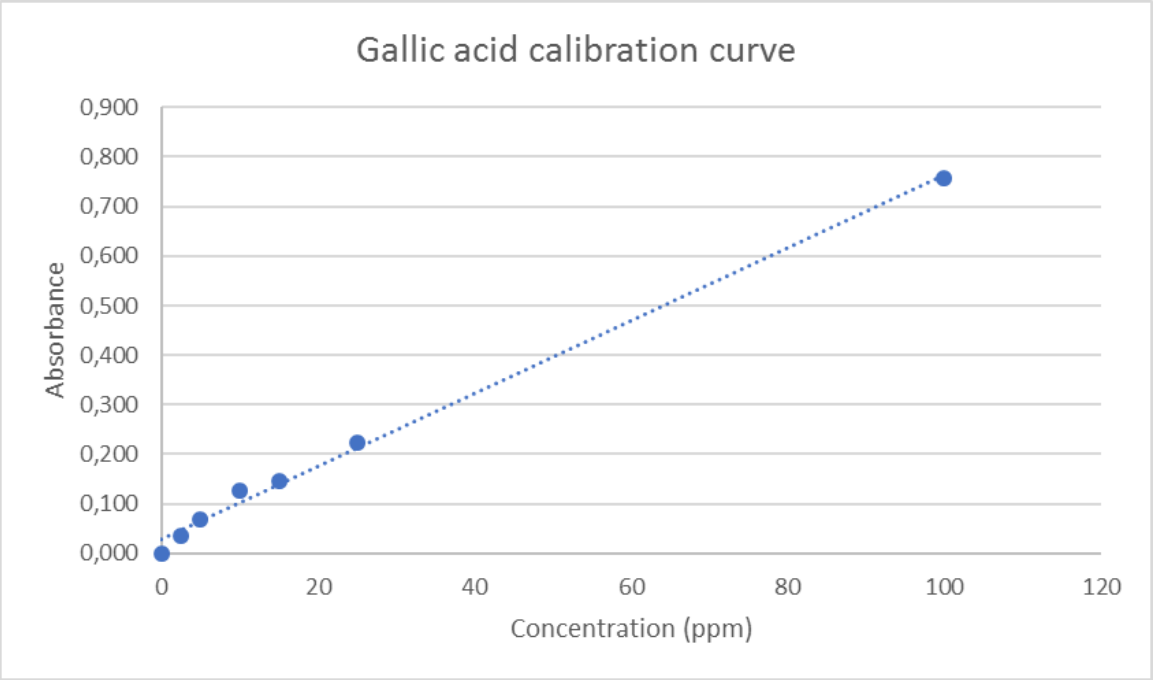


Figure 2.7 Gallic acid standard calibration curve

2.8 Catechin standard

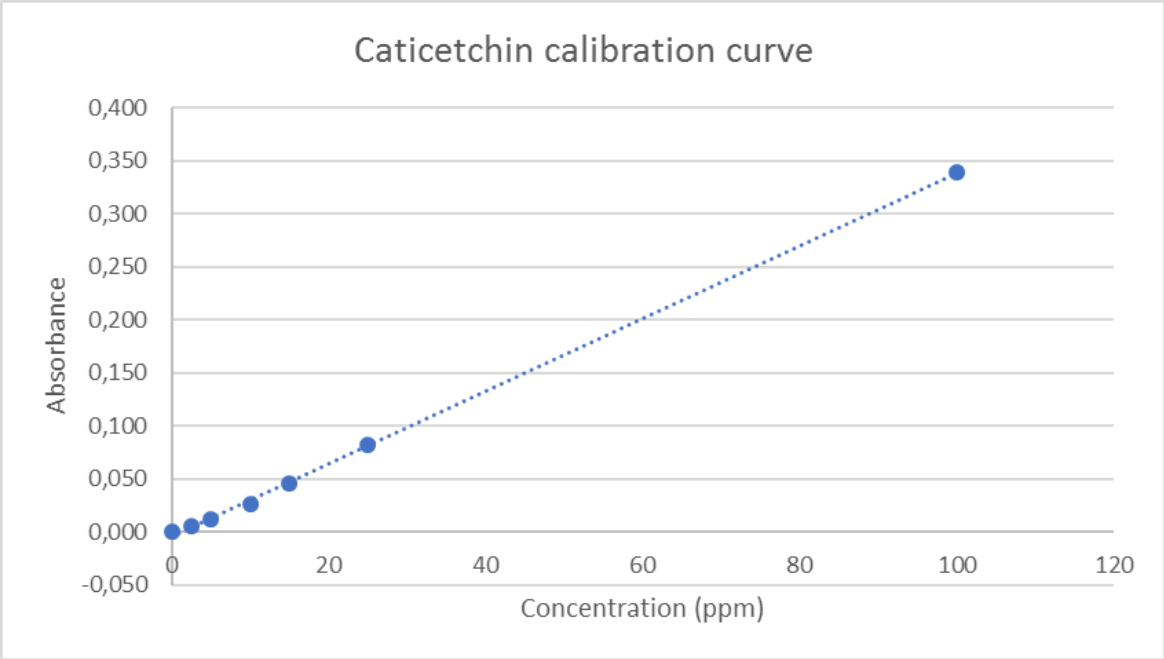


Figure 2.9 Catechin standard calibration curve

Table 2.10: Showing the concentrations TPC of high temperature treated plants

Extract	**Concentration (ppm)**
Control	71,636 ±0,191
HT48	78,873 ±0,089
HT96	76,142 ±0,137
HT144	73,796 ±0,140

Table 2.11: Showing the concentrations TPC of low temperature treated plants

Extract	**Concentration (ppm)**
Control	71,636 ±0,191
LT48	87,099 ±0,157
LT96	90,648 ±0,170
LT144	73,580 ±0,130

Table 2.12: Showing the concentrations TFC of high temperature treated plants

Extract	Concentration (ppm)
Control	250,386 ±0,069
HT48	341,529 ±0,234
HT96	221,922 ±0,158
HT144	205,941 ±0,194

Table 2.13: Showing the concentrations TFC of low temperature treated plants

Extract	**Concentration (ppm)**
Control	250,386 ±0,069
LT48	279,373 ±0,205
LT96	335,026 ±0,149
LT144	249,438 ±0,140

2.14 Raw data for quercetin and kaempferol from extracts

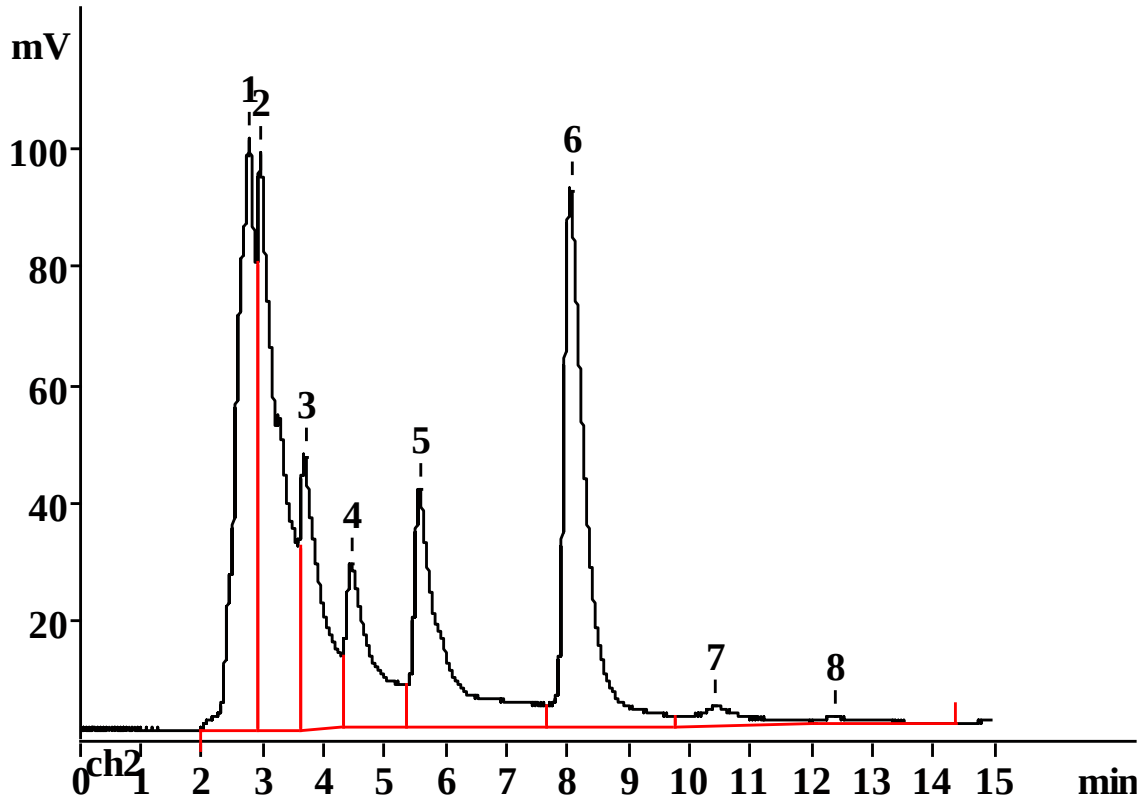


Figure 2.15 Concentrations of 5-quercetin and 6-kaempferol after 48 hours at HT

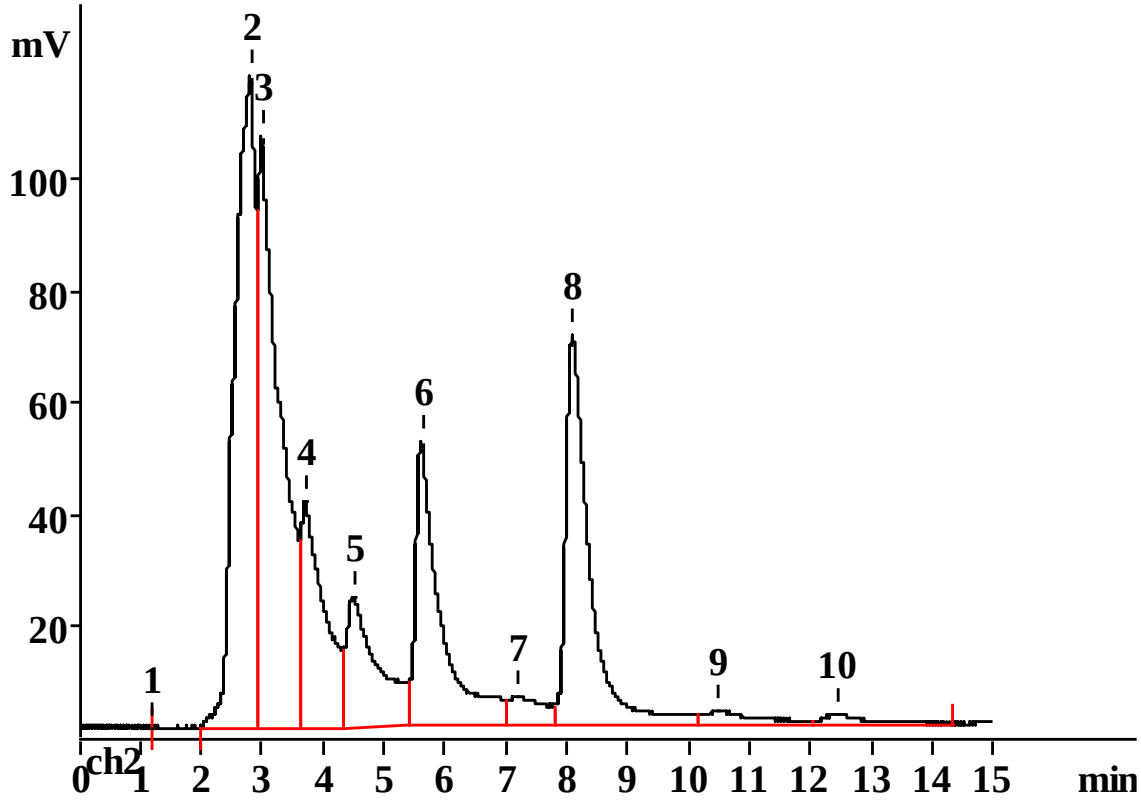


Figure 2.16 Concentrations of 6-quercetin and 8-kaempferol after 96 hours at HT

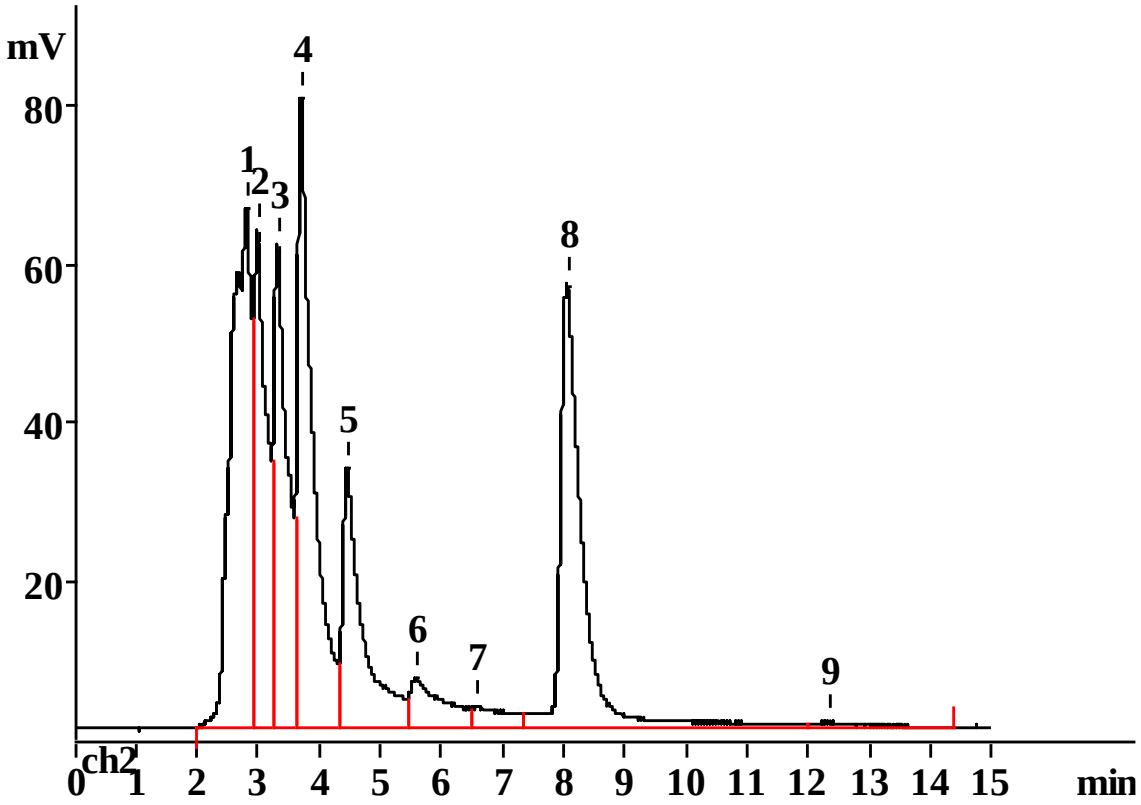


Figure 2.17 Concentrations of 6-quercetin and 8-kaempferol after 144 hours at HT

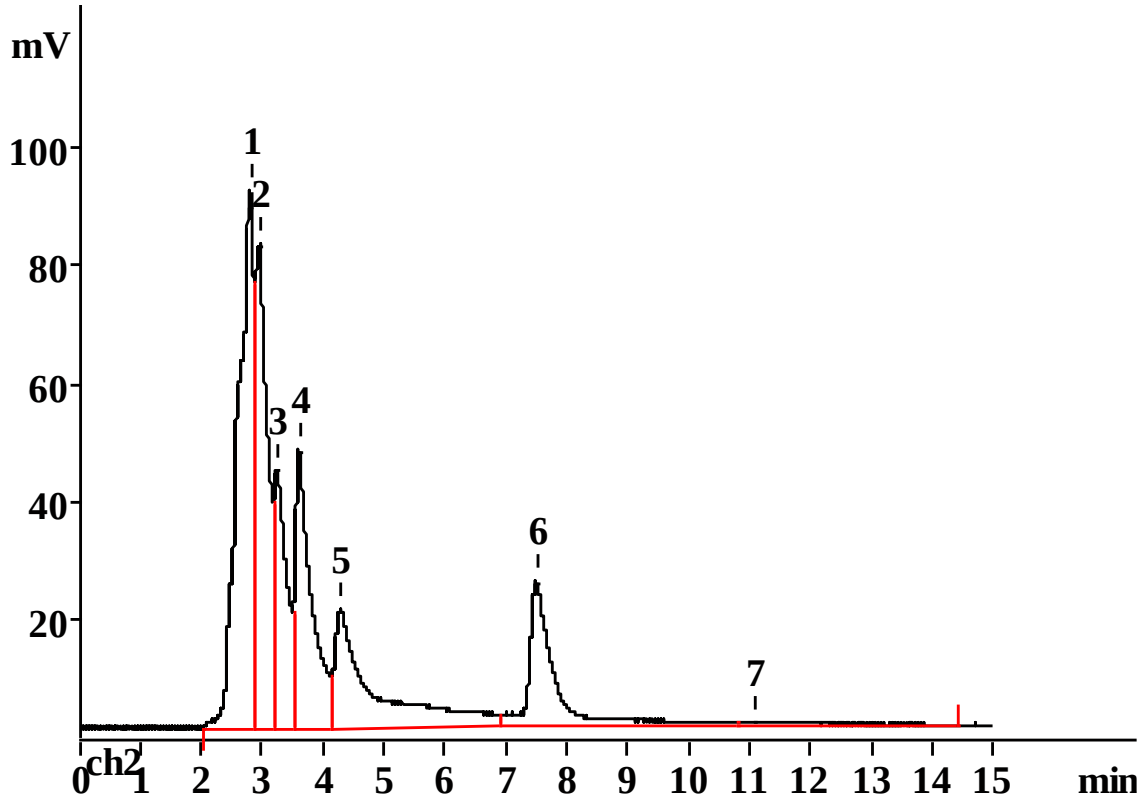


Figure 2.18 Concentrations of 5-quercetin and 6-kaempferol after 48 hours at LT

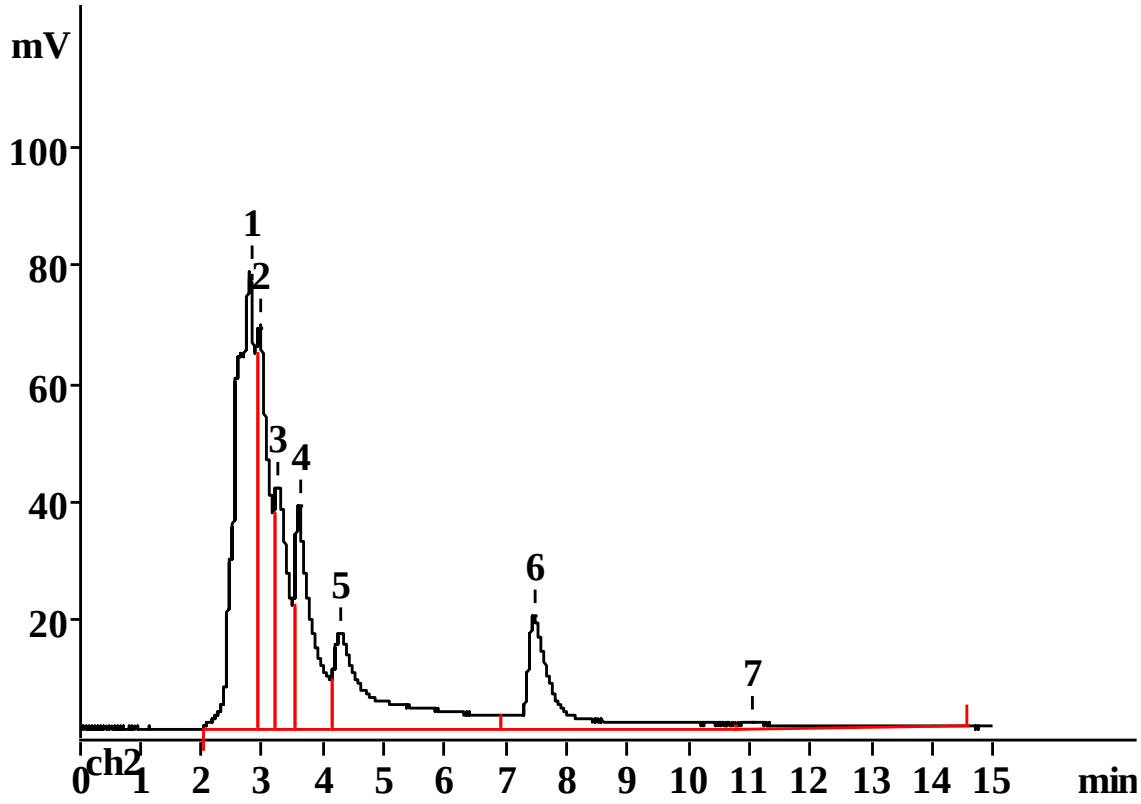


Figure 2.19 Concentrations of 5-quercetin and 6-kaempferol after 96 hours at LT

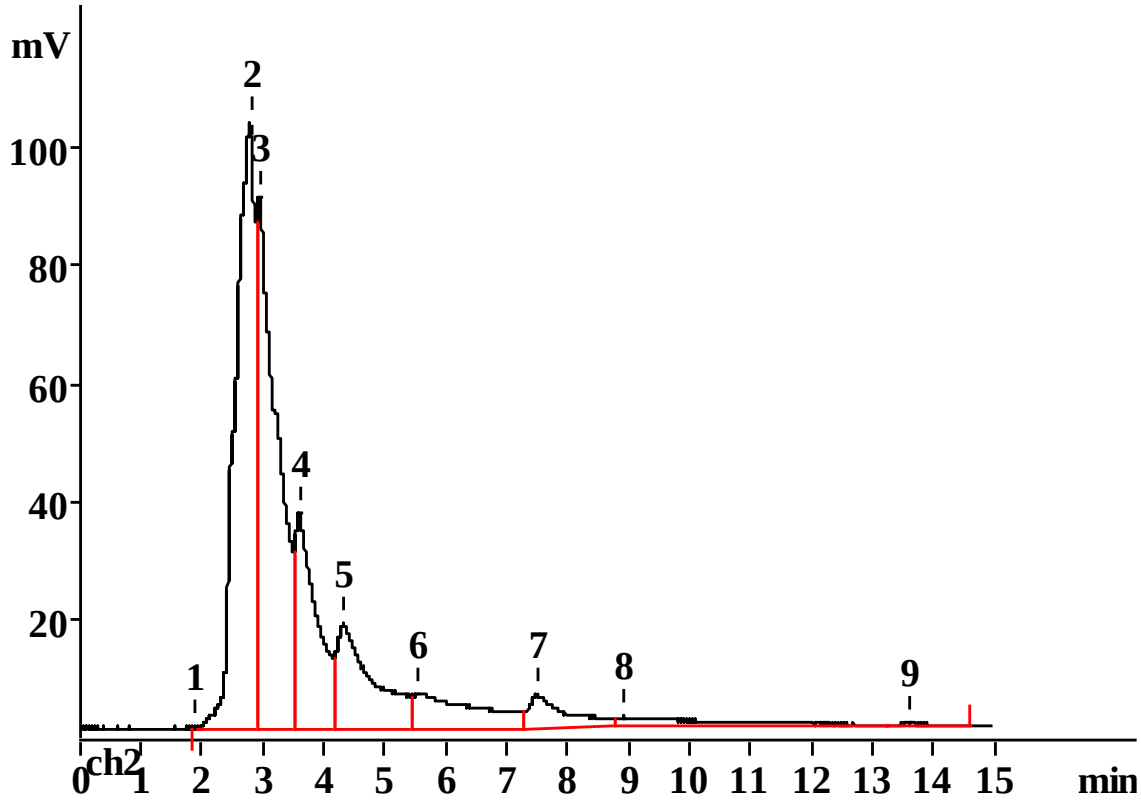


Figure 2.20 Concentrations of 6-quercetin and 7-kaempferol after 144 hours at LT

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