Leaf anatomical traits of macadamia cultivars with potential links to drought tolerance

by

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DECLARATION

I, Khodani Maumela declare that the thesis/dissertation, which I hereby submit for the degree MSc Plant Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SIGNATURE: …………………………………

DATE: …………………………………

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TABLE OF CONTENTS

ACKNOWLEDGMENTS ii

LIST OF FIGURES v

LIST OF TABLES vii

ABSTRACT ix

CHAPTER 1: GENERAL INTRODUCTION 1

1.1 Hypothesis 5

1.2 Aims and Objectives 6

CHAPTER 2: LITERATURE REVIEW 8

2.1 Macadamia taxonomy 8

2.2 Climatic requirements for macadamia production 9

2.3 South African Macadamia Industry Statistics 9

2.4 Macadamia cultivars in South Africa 10

2.4.1 HAES 695 ‘Beaumont’ 11

2.4.2 ‘A4’ 12

2.4.3 ‘814’ 12

2.4.4 ‘816’ 12

2.4.5 ‘842’ 12

2.5 Leaf anatomical plasticity in response to environmental factors 13

2.5.1 Stomatal density 14

2.5.2 Palisade and spongy parenchyma 16

2.5.3 Huber value 18

2.5.4 Vein density 20

2.6 Summary 21

CHAPTER 3: MATERIALS AND METHODS 23

3.1 Study sites and weather variables 23

3.1.1 Levubu 25

3.1.2 Nelspruit 25

3.1.3 Komatipoort 26

3.1.4 KwaZulu-Natal South Coast 26

3.2 Leaf sample collection 27

3.3 Stomatal density measurements 27

3.4 Palisade parenchyma, spongy parenchyma, and petiole xylem cross-sectional area measurements 28

3.5 Vein density measurements 31

3.6 Statistical analysis 32

CHAPTER 4: RESULTS 35

4.1 Seasonal weather 35

4.2 Leaf anatomy 37

4.2.1 Stomatal density 37

4.2.2 Palisade parenchyma 43

4.2.3 Spongy parenchyma 45

4.2.4 Huber value 47

4.2.5 Vein density 50

4.3 Interactions between farms and macadamia cultivars 53

CHAPTER 5: DISCUSSION AND CONCLUSIONS 59

5.1 Stomatal density 59

5.2 Palisade and spongy parenchyma 60

5.3 Huber value 61

5.4 Vein density 62

5.5 Conclusions 62

5.6 Study limitations and future research 63

REFERENCES 65

APPENDICES 88

LIST OF FIGURES

Figure 2.1 Macadamia cultivars planted in 2021 (SAMAC, 2023). 11

Figure 3.1 Macadamia farm leaf collection locations (Kumari, 2021). 25

Figure 3.2 Procedure for stomatal peels from the macadamia abaxial leaf surface. 28

Figure 3.3 Method of holding the leaf blade A) and petiole B) specimen for free hand sectioning. 29

Figure 3.4 Example of the determination of the palisade and spongy parenchyma thickness (µm) in Macadamia leaves using ImageJ software. The red double-headed arrows on the image indicate the measurements for the palisade and spongy parenchyma length. 30

Figure 3.5 Example of petiole xylem cross-sectional area determination in Macadamia petioles (µm) using ImageJ software. The red freehand drawn lines outline the xylem cross-sectional area measured for the example petiole. 31

Figure 3.6 Veins on cleared macadamia leaves, A) original image and B) skeletonized image. 32

Figure 4.1 Stomata on leaves of macadamia cultivars, A- ‘814’ and B- ‘A4’. Scale bar = 50 µm. 42

Figure 4.2 Relationship between stomatal density (mm^{-2}) and stomata size (µm) for leaves of ‘695’, ‘A4’, ‘814’, and ‘816’ from Loerieroep, Sommerreg, Jacaranda, Valeen, and Outlook for the spring flush. The Regression equation, squared correlation coefficient (R^2), and P value are reported. 42

Figure 4.3 Cross-sections of the leaves of macadamia cultivars A- ‘814’ and B- ‘695’. pp= palisade parenchyma; sp= spongy parenchyma. Scale bar = 50 µm. 45

Figure 4.4 Cross-sections of petioles of macadamia cultivars A- ‘814’ and B- ‘A4’. Scale bar = 100 µm. 50

Figure 4.5 Veins on leaves of macadamia cultivars A- ‘695’ and B- ‘814’. Scale bar = 100 µm. 53

Figure 4.6 Macadamia leaf stomatal density on sun leaves across farms and macadamia cultivars during the spring flush. Box plots with the same letter are not significantly different at $P < 0.05$. 54

Figure 4.7 Macadamia palisade parenchyma thickness on sun leaves across farms and macadamia cultivars during the spring flush. Box plots with the same letter are not significantly different at $P < 0.05$. 55

Figure 4.8 Macadamia spongy parenchyma thickness on sun leaves across farms and macadamia cultivars during the spring flush. Box plots with the same letter are not significantly different at $P < 0.05$. 56

Figure 4.9 Macadamia Huber values on sun leaves across farms and macadamia cultivars during the spring flush. Box plots with the same letter are not significantly different at $P < 0.05$. 57

Figure 4.10 Macadamia vein density on sun leaves across farms and macadamia cultivars during the spring flush. Box plots with the same letter are not significantly different at $P < 0.05$. 58

LIST OF TABLES

Table 3.1 Geographical coordinates of farms and cultivars harvested from them.... 24

Table 4.1 Average seasonal minimum and maximum temperatures (˚C), vapour pressure deficit (VPD, kPa), and total reference evapotranspiration (ET$_o$, mm) during the spring (September- November 2021) and summer flush (January- March 2022). 36

Table 4.2 Best selected Linear mixed model effects model summary on leaf and petiole anatomical traits in macadamia cultivars '695', '814', and '842' across spring and summer vegetative flush at the Bridelia farm. '695' and spring flush are the intercepts.. 39

Table 4.3 Best selected Linear mixed model effects model summary on leaf and petiole anatomical traits in macadamia cultivar '842' across spring and summer vegetative flush at the Sommerreg and Jacaranda farms. Jacaranda farm, shade leaves, and spring flush are the intercepts. 40

Table 4.4 Best selected Linear mixed model effects model summary on stomatal density in macadamia cultivars '695', 'A4', '814', and '816' across spring and summer vegetative flush at the Loerieroep, Sommerreg, Jacaranda, Valeen, and Outlook farms. '695', shade leaves, spring flush, and Jacaranda farm are the intercepts. 41

Table 4.5 Best selected Linear mixed model effects model summary on palisade parenchyma in macadamia cultivars '695', 'A4', '814', and '816' across spring and summer vegetative flush at the Loerieroep, Sommerreg, Jacaranda, Valeen, and Outlook farms. '695', shade leaves, spring flush, and Jacaranda farm are the intercepts.. 44

Table 4.6 Best selected Linear mixed model effects model summary on spongy parenchyma in macadamia cultivars '695', 'A4', '814', and '816' across spring and summer vegetative flush at the Loerieroep, Sommerreg, Jacaranda, Valeen, and Outlook farms. ‘695’, shade leaves, spring flush, and Jacaranda farm are the intercepts. 47

Table 4.7 Best selected Linear mixed model effects model summary on Huber values in macadamia cultivars ‘695’, ‘A4’, ‘814’, and ‘816’ across spring and summer vegetative flush at the Loerieroep, Sommerreg, Jacaranda, Valeen, and Outlook farms. The intercepts are the ‘695’, shade leaves, spring flush, and Jacaranda farm. 49

Table 4.8 Best selected Linear mixed model effects model summary on vein density in macadamia cultivars ‘695’, ‘A4’, ‘814’, and ‘816’ across spring and summer vegetative flush at the Loerieroep, Sommerreg, Jacaranda, Valeen, and Outlook farms. ‘695’, shade leaves, spring flush, and Jacaranda farm are the intercepts. 52

ABSTRACT

In South Africa, *Macadamia integrifolia*, *Macadamia tetraphylla*, and their hybrids are commonly grown for their edible nuts and consequent nutritional benefits. To improve yields, irrigation is used to grow these trees in regions with inadequate rainfall. Selecting cultivars with desirable leaf anatomical traits, capable of withstanding drought, is one strategy to increase water use efficiency. In this study, we investigated differences in multiple traits related to water use efficiency in common South African macadamia cultivars, including stomatal density, palisade and spongy parenchyma thickness, Huber value, and vein density to determine if cultivars differed in their anatomical characteristics. Cultivar '814' consistently exhibited higher stomatal densities, thicker palisade parenchyma, thicker spongy parenchyma, and higher Huber values compared to cultivars '816; '695', '842', and 'A4'. In contrast, '695' showed greater vein density. The variability in leaf and petiole anatomy was influenced by factors such as the timing of vegetative flush, leaf type, location, and cultivar. Although climate may have played a role, no clear patterns emerged. Genetic factors likely contributed significantly to the observed differences among macadamia cultivars. These findings have implications for selecting drought-tolerant cultivars and improving water management practices in macadamia cultivation, especially in regions with inadequate rainfall.

CHAPTER 1: GENERAL INTRODUCTION

Macadamia (*Macadamia integrifolia* Maiden & Betche, *Macadamia tetraphylla* L. A. S Johnson, and their hybrids) originated in the subtropical coastal areas of eastern Australia (Bringhenti et al., 2023; Lin et al., 2022) , and is currently grown throughout the world including China, Australia, Kenya, Guatemala, United States, and Malawi, with the majority of macadamia nuts produced in South Africa, that has 76 348 hectares planted (SAMAC, 2023). However, South Africa's leading role in macadamia production comes with challenges. With an average annual rainfall of 495 mm, the country is considered one of the 30th driest in the world (De Villiers & De Wit, 2010; Schreiner et al., 2010). As a result, growers resort to supplementary irrigation to meet the needs of various crops, including macadamia, to improve yields (Stephenson & Searle, 2014).

The rapid expansion of macadamia cultivation in South Africa has implications for water management and necessitates a strategy to improve water use efficiency in the industry. However, little is known about the specific water requirements of macadamia trees, with existing research focused on the cultivar 'Beaumont' (Taylor et al., 2021). One potential way to address this knowledge gap and enable more effective water management practices without engaging in costly and time-consuming field experiments is to examine the internal structure of leaves (Farooq et al., 2019).

Leaves are the main organs of photosynthesis and transpiration, and consequently, they influence how plants respond to environmental conditions, in contrast to stems and roots (Guerfel et al., 2009). Macadamia, like other members of the family Proteaceae, exhibits a variety of leaf anatomical adaptations (Metcalfe & Chalk, 1950; Stephenson & Searle, 2014), such as sclerophylly and hypostomaty, which results in low carbon mesophyll conductance, low gaseous exchange, and lower transpiration rates (Lloyd et al., 1992; Stephenson, 2005). These adaptations have evolved in response to the arid conditions in other parts of their native regions, resulting in macadamia trees being recognized as drought-tolerant (Stephenson et al., 2003).

Drought tolerance in plants is a mechanism whereby plants can grow and thrive in environments with low water availability (Ennajeh et al., 2010). This tolerance involves various physiological processes, including photosynthesis (Chartzoulakis et al., 2002). In areas prone to drought, leaves play a major role in water management by regulating transpiration, and to survive in these areas they develop a variety of leaf adaptations (De Micco & Aronne, 2012; Farooq et al., 2012). These leaf adaptations exhibit variability between individuals of the same species, on the same leaf, and leaves of the same plant, emphasizing the essential role that leaf adaptation has for plant survival (Gratani, 2014).

Key leaf anatomical traits that enable a better understanding of how plants regulate water, especially in drought conditions, include stomatal density, mesophyll tissue thickness, vein density, and petiole xylem cross-sectional area (Bertolino et al., 2019; Caine et al., 2019; Guerfel et al., 2009; Li et al., 2021; Sack & Holbrook, 2006). The most important adaptation to drought is the presence, arrangement, and number of stomata on the abaxial and adaxial surfaces of the leaf (Bertolino et al., 2019). Upon their opening for gaseous exchange, water also exits the leaf, making stomatal density crucial for plant water use. Stomatal density is the number of stomata per leaf area, and a good indicator of potential transpiration rates (Bertolino et al., 2019; Stevens et al., 2021).

Another typical leaf adaptation under drought is the development of thick leaves, mainly due to increased palisade and spongy parenchyma thickness (Guerfel et al., 2009; Pompelli et al., 2019). Palisade parenchyma cells contain densely packed chloroplasts responsible for carbon fixation during photosynthesis and the spongy parenchyma contains loosely arranged cells that facilitate gaseous exchange and water transport within the leaf, regulating photosynthesis and transpiration (Nardini et al., 2010; Terashima & Saeki, 1983; Tian et al., 2016). Studies conducted by Bosabalidis and Kofidis (2002) and Ennajeh et al. (2010) have extensively investigated stomatal density, palisade parenchyma thickness, and spongy parenchyma thickness in Olive cultivars (*Olea europaea* L.) (Azadi, 2005). Their studies revealed that drought-tolerant cultivars exhibited higher stomatal densities, thicker palisade, and spongy parenchyma tissues.

The functioning of leaves under drought conditions is also linked to the hydraulic characteristics of the xylem in their petioles because efficient water flow into the leaf is required to compensate for water lost through transpiration. Thus, for transpiration to be sustained, the leaf must receive a constant supply of water through the xylem in the petiole, emphasizing how important petiole xylem characteristics are to plant capacity to withstand water stress (Sack & Holbrook, 2006; Schultz & Matthews, 1993). There is very limited literature on petiole xylem cross-sectional area, but given that the leaf petiole serves a similar function to the stem, it is reasonable to assume that conclusions regarding the xylem cross-sectional area of the stem to drought will also apply to the petiole. For instance, the Huber value, defined as the proportion of sapwood or stem xylem cross-sectional area to the leaf area has been observed to increase during periods of water stress (O’Grady et al., 2009; Tognetti et al., 1997).

The anatomical and architectural characteristics of leaf veins have an impact on plant productivity, and survival across all environments (Sack & Scoffoni, 2013a; Sack et al., 2012). Upon exiting the leaf petiole xylem, water moves through the major veins, and then exits into the minor veins (Sack & Holbrook, 2006). Given that the success of water transport through leaf veins is a major determinant of maximum rates of photosynthesis and transpiration (Brodribb et al., 2007), the venation traits of various plant species have been thoroughly investigated (Langer et al., 2021). These investigations into the total length of veins per unit leaf area under various water stress conditions have yielded valuable insights. For example, drought stress increased vein density in Hawaiian *Plantago* taxa (Dunbar Co et al., 2009; Wagner et al., 1990) and upland cotton (*Gossypium hirsutum*) (Campbell et al., 2009; Lei et al., 2018), allowing efficient water supply to the leaf tissues throughout the leaf lamina, boosting photosynthetic capacity and adaptability in various environmental settings (Ye et al., 2021).

The above-mentioned leaf anatomical structures are not only impacted by drought or water stress, but also show the effects of other environmental factors and their responses to these are very plastic (Brodribb & Jordan, 2011). These environmental factors may include light intensity, temperature, and vapour pressure deficit (VPD) (Jifon et al., 2005; Sack & Scoffoni, 2013a; Wang et al., 2007). This plasticity allows plants to adapt their transpiration rates and water use efficiency to the environment in which they are growing. Therefore, evaluating the plasticity of these leaf anatomical structures in response to environmental factors may help understand how different plants adapt to varying climatic conditions.

Consequently, for this study, the above-mentioned leaf anatomical traits will be investigated in macadamia leaves to determine if different cultivars exhibit differences in leaf anatomy. This will provide potential insights into transpiration rates among cultivars and may help improve water management for this economically important crop. If there are no differences amongst macadamia cultivars, this will eliminate the need for costly, time-consuming, and large-scale field experiments. However, if there are differences in leaf anatomy among cultivars, this will help with the selection of cultivars suited for water saving.

Therefore, the main research questions were:

- Does leaf and petiole anatomy remain constant among different cultivars of macadamia?
- Do these differences remain constant across different regions in South Africa?

1.1 Hypothesis

In South Africa, macadamia trees are widely grown in the Levubu, Nelspruit, Komatipoort, and KwaZulu-Natal South Coast regions (SAMAC, 2023), and for this study leaves were harvested in all these regions. These regions are suitable for macadamias as they are characterized by a subtropical climate with either hot-dry or humid climatic conditions, free from frost (Kriel, 2023; Zuza et al., 2021). The Levubu, Nelspruit, and Komatipoort regions are generally considered hot and dry, while the KwaZulu-Natal South Coast is recognized for its humid climate, likely due to its proximity to the ocean.

Taking into consideration that leaf anatomy is influenced by different environmental factors, leaves were harvested during the spring flush and summer flush, from sun-exposed and shaded parts of the trees. The leaf samples included cultivars of hybrids Beaumont (‘695’), ‘A4’, and *Macadamia integrifolia* cultivars ‘814’, ‘816’, and ‘842’, due to their significant contribution to the industry, bearing fruits at early ages, and for the high quality of their nuts (SAMAC, 2023).

The following hypotheses were formulated for the study:

- Leaf and petiole anatomy will vary significantly between *Macadamia integrifolia* and macadamia hybrid cultivars.

- Leaves from hot and dry regions will exhibit higher stomatal densities, thicker mesophyll tissues, higher Huber values, and greater vein density, than leaves from humid regions.
- Leaves sampled in the summer flush will show higher stomatal densities, thicker mesophyll tissues, higher Huber values, and greater vein density than leaves sampled in the spring flush.
- Sun-exposed leaves will have higher stomatal densities, thicker mesophyll tissues, higher Huber values, and greater vein density than shaded leaves.

1.2 Aims and Objectives

The main aim of this study was to assess the variability in leaf and petiole anatomical traits among different cultivars of macadamia trees in South Africa, which may provide insights into potential transpiration rates and water use efficiency of macadamia trees, improving water management practices for macadamia in South Africa.

Objectives

- To assess leaf anatomical structures associated with drought tolerance in macadamia cultivars from selected production regions of South Africa.
- To compare leaf anatomical traits of macadamia leaves from South African macadamia-producing regions, including stomatal density, palisade and spongy parenchyma thickness, Huber value, and vein density.
- To assess implications for water use and drought tolerance in the cultivars Beaumont (‘695’),’ A4’, ‘816’, ‘814’, and ‘842’.

Outline of the dissertation

The dissertation includes an introductory chapter that describes the study background, research questions, hypothesis, aims, and objectives. Chapter 2 provides a literature review on macadamia species and their hybrids, their growth climate requirements, current South African macadamia production statistics, and the impact of various environmental factors on leaf and petiole anatomical traits related to plant water use. Chapter 3 details the study sites, leaf sampling procedure, stomatal density measurements, and the sectioning of and measurements of macadamia leaves and petiole cross sections. Chapter 4 examines and compares leaf and petiole anatomy between macadamia cultivars, across farms, between sun and shade leaves, and between spring and summer flush leaves. Discussions and conclusions are outlined in Chapter 5.

CHAPTER 2: LITERATURE REVIEW

2.1 Macadamia taxonomy

Macadamia is a genus within the family Proteaceae that consists of four species: *Macadamia integrifolia* Maiden & Betche, *Macadamia tetraphylla* L. A. S Johnson, *Macadamia ternifolia* F. Muell, and *Macadamia jansenii* C.L. Gross & P.H. Weston (Brežná et al., 2009; Trueman, 2013). They originated in northern New South Wales and southern Queensland on the east coast of Australia (Howlett et al., 2015; Peace et al., 2003). Of these, only *Macadamia integrifolia*, *Macadamia tetraphylla,* and their hybrids are cultivated commercially for their delicious kernels, whereas other species are unfit for ingestion due to high levels of bitter cyanide in the nuts, making them poisonous (Dahler et al., 1995).

Macadamia integrifolia is a large, tall tree with leaves arranged in whorls, each consisting of three leaves, 6 - 14 cm long by 3 - 6 cm wide, with rounded blade tips (Stanley & Ross, 1983). They have axillary, cream-white flowers arranged in 10 - 30 cm long racemes (Ranketse et al., 2022), and they produce smooth-shelled nuts (Yalemar et al., 2023). Other common names for *Macadamia integrifolia* include Queensland nut, bauple nut, Australian nut, and nut oak (Boyer & Cock, 2013). *Macadamia tetraphylla* is another large tree with leaves arranged in whorls, each consisting of four leaves with pointed blade tips, and serrated margins. Flowers have a pink to purple colour, arranged in 15 - 45 cm long racemes (Ranketse et al., 2022). *Macadamia tetraphylla* produces rough-shelled nuts (Yalemar et al., 2023); thus it is also known as bush nut and spiny-leaf macadamia (Wallace & Walton, 2011).

2.2 Climatic requirements for macadamia production

Although macadamia trees are from the subtropical rainforests of Australia, they can be cultivated across a range of different climates (Du Preez, 2015; Storey, 1976; Zuza et al., 2021). The tree prefers warm, humid sub-tropical climates (Kriel, 2023; Lim & Lim, 2013), with average monthly maximum temperatures of up to 29 ˚C and monthly minimum temperatures above 3 ˚C, with mean temperatures between 16 ˚C and 25 ˚C being ideal for their growth (Hardner et al., 2009; Zuza et al., 2021). Macadamia does not tolerate drought and frost, but mature trees can withstand mild frost for a short period, whereas frost damage in young trees can lead to death (Trochoulias & Lahav, 1983). Macadamia grows very well in areas with well-distributed rainfall at an average of 1500 mm per year but can be supplemented with irrigation to increase production (Zuza et al., 2021).

Macadamias can be grown in deep soil that drains properly, although in unfertile soils tree growth is not optimal and an ideal soil pH ranges from 4.5 to 6.5 (Hamilton & Fukunaga, 1959). South Africa is one of the countries suitable for macadamia production given these climatic and edaphic requirements, as some parts of this country are characterized by a subtropical climate similar to the indigenous range of the tree (Ranketse et al., 2022). These areas in South Africa include the frost-free coastal areas in the KwaZulu-Natal Province, the Lowveld areas in the Mpumalanga Province, and the Limpopo Province (Kriel, 2023; Taylor & Gush, 2014).

2.3 South African Macadamia Industry Statistics

According to the South African Macadamia Association (SAMAC), South Africa is currently the leading producer of macadamia nuts, followed by China, Australia, Kenya, Guatemala, the United States, and Malawi as of the year 2022, with South African production increasing by 1% as compared to the year 2021. The Macadamia industry is rapidly increasing in South Africa, with 9148 new hectares planted in 2022 compared to the 6235 new hectares planted in 2021. Farmers in KwaZulu-Natal planted 3705 new hectares in 2022, Mpumalanga planted 3403 new hectares, Limpopo planted 1547 new hectares, Western Cape planted 479 hectares, and other provinces planted 14 new hectares in the year 2022. Additionally, a tree census completed by the New England University in Australia revealed that 76,348 hectares are established in South Africa (SAMAC, 2023).

2.4 Macadamia cultivars in South Africa

A cultivar is a group of plants selected for desirable traits that are maintained during propagation (Dutfield, 2012). Macadamia cultivars are grouped based on their physical characteristics, and point of origin, although for some cultivars, the origins may not be clear due to limited information on the genetic variation of macadamia cultivars (Peace, 2002). Beaumont (‘695’) and ‘A4’ cultivars are the most planted in South Africa, followed by ‘Nelmak 2’ and ‘816’, while ‘A16’, ‘814’, ‘842’, and ‘788’ have the lowest percentages (Figure 2.1) (SAMAC, 2023).

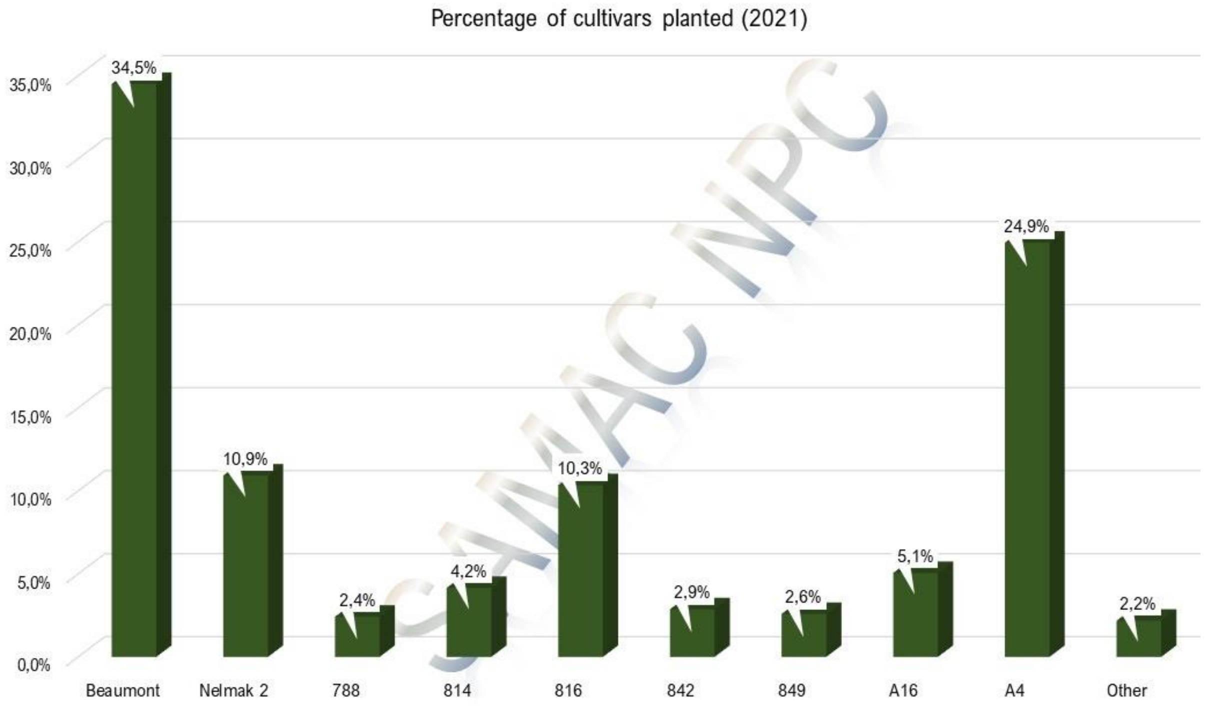

Figure 2.1 Macadamia cultivars planted in 2021 (SAMAC, 2023).

The present study focused on ‘695’, ‘814’, ‘816’, ‘842’, and ‘A4’, as they are beneficial to the industry by bearing fruits at early ages as compared to other cultivars, and for the quality of their nuts (SAMAC, 2023).

2.4.1 HAES 695 ‘Beaumont’

The most commonly planted hybrid of two macadamia species *(Macadamia integrifolia,* and *Macadamia tetraphylla*) in South Africa, Beaumont (‘695’), originated from Australia, and mature leaves are very spiny, with long racemes of pink flowers. An advantage of planting this cultivar is its precocity, bearing fruits at a young age of about three to four years (Vock et al., 1998). This is one of the most highly recommended cultivars in South Africa due to its ability to withstand South African weather conditions (Allan, 1993; Allan, 2007; Verma et al., n.d.).

2.4.2 ‘A4’

‘A4’ is an Australian hybrid of the two edible macadamia species (Peace et al., 2005). This medium-sized tree has pale, spiny, pointed leaves, and short to medium-sized petioles. ‘A4’ trees start bearing fruits from year three and are preferred for their nut quality (Allan, 2007; Vock et al., 1998).

2.4.3 ‘814’

‘814’ was derived solely from *Macadamia integrifolia* and originates from Hawaii (Peace et al., 2005). The trees are small compared to other macadamia cultivars. Mature leaves are mid-green, with undulating margins, few spines, and pointed tips. Compared to other cultivars, ‘814’ trees have more small branches, begin flowering later in the season, and are high-yielding even though they produce tiny nuts (Vock et al., 1998).

2.4.4 ‘816’

‘816’ was derived from *Macadamia integrifolia* and originates from a Hawaiian breeding program (Peace et al., 2005). These medium to large trees have pale lime green leaves with smooth margins and long petioles. They start bearing fruits between the ages of four and five years after planting, producing medium to large-sized nuts with a high kernel recovery rate (Vock et al., 1998).

2.4.5 ‘842’

Another cultivar derived from *Macadamia integrifolia*, which originates from the Hawaiian breeding program is ‘842’ (Peace et al., 2005). The tree is of medium to large size, with long leaves, multiple spines, and long petioles. After planting, they start producing small round nuts from year four (Allan, 2007; Vock et al., 1998).

2.5 Leaf anatomical plasticity in response to environmental factors

The world faces competition for water resources, particularly between agriculture and other industries, thus effective water management strategies are crucial to ensure sustainable food production (Ennajeh et al., 2010; Farooq et al., 2019). In regions like South Africa, where agriculture is the primary source of food for the population, large quantities of water are required for irrigation to increase crop productivity. Improving water use efficiency by selecting drought-tolerant cultivars is critical for future plantations. The leaf plays a major role in the hydraulic system of the plant and is the most responsive of all the organs to environmental conditions. Therefore, leaf internal tissues play an important role in plant water use and enable effective water management practices (Farooq et al., 2019).

Significant differences in leaf anatomical traits, such as stomatal density, mesophyll tissues, vein density, and petiole xylem cross-sectional area have been observed under varying environmental conditions, indicating the plasticity of these traits in response to various climates (Bacelar et al., 2004; Li et al., 2021; Sack et al., 2013). These changes are influenced by light availability, temperature, vapour pressure deficit (VPD), and soil water availability (Amitrano et al., 2019; He et al., 2018). Comparative studies have been crucial in demonstrating the relationship between environmental factors and leaf anatomical characteristics. While some studies produced unclear outcomes, others used these traits to determine transpiration rates and drought tolerance in various plant species (Guerfel et al., 2009; Luković et al., 2009; Xu et al., 2020). Such studies have emphasized the importance of analysing leaf anatomy in response to varying environmental factors leading to the selection of drought-tolerant and water-efficient cultivars (Ennajeh et al., 2010). The following section will describe how the above-mentioned leaf anatomical traits are influenced by various environmental factors, and how they have been successfully used to determine transpiration rates and drought tolerance in different plant species.

2.5.1 Stomatal density

Stomata are microscopic pores on the leaf surfaces, enabling gaseous exchange in plant leaves (Bertolino et al., 2019; Hill et al., 2014). Stomata may be located on the abaxial (hypostomatous) and adaxial (epistomatous) surfaces of the leaf (Driesen et al., 2023). Across plant species and individuals of the same species, stomata may vary in size and number, and these differences are influenced significantly by the environment and genetic factors of the plant species (Wakefield et al., 2021).

The carbon dioxide and transpiration demand is controlled by both stomatal size and density, thus changes in stomatal size and density may have a significant effect on gaseous exchange and water loss, making them play a major role in plant water use efficiency and the ability of plants to adapt to climate change (Hetherington & Woodward, 2003; Wakefield et al., 2021). Stomatal density (the number of stomata per leaf area), together with stomatal size, have been investigated under different environmental conditions in different plant species, and very plastic responses have been reported under varying light intensity, temperature, VPD, and water availability (Hill et al., 2015; Liu et al., 2018; Woodward, 1987).

Greatest stomatal densities have been reported on leaves exposed to continuous light or sun, and lowest on leaves in continuous dark or shade (Idris et al., 2019; Kardiman & Ræbild, 2018; Poole et al., 1996; Setiawati et al., 2018; Zacchini et al., 1997). Research on tomato plants grown in glasshouses revealed that high light intensity increased stomatal density (Gay & Hurd, 1975). Furthermore, higher stomatal density on macadamia cultivars ‘WJMAS-29’, ‘Beaumont’, and Daddow’ was recorded on northern crown position leaves exposed to high light intensity as compared to leaves on the southern crown position (Wakefield et al., 2021). The increase in stomatal density under high light intensity led to higher photosynthetic rates, transpiration rates, and allowed the leaf to cool down when water evaporated under the sun (Gay & Hurd, 1975; Setiawati et al., 2018), while low stomatal densities in shaded areas reduced the need for gaseous exchange because of reduced photosynthesis rate, thereby reducing yields (Jumrani & Bhatia, 2020; Wakefield et al., 2021).

In controlled growth experiments, stomatal density was observed to increase with increasing temperature (Ferris et al., 1996; Pandey et al., 2007). This increasing stomatal density under warm conditions increased the potential for leaf cooling. However, Beerling and Chaloner (1993) in their study on English oak (*Quercus robur)* presented contrasting findings, reporting that stomatal density under warm summer temperatures decreased, while leaves in cooler spring temperatures exhibited higher stomatal density. Additionally, Wu et al. (2018), also reported a decrease in stomatal density under warming temperatures, while other studies showed little or no response to the increase in temperatures in stomatal density (Zheng et al., 2013). While the findings of these studies differ, it is important to note that the relationship between temperature and stomatal density may not be consistent across all species or conditions. The decrease in the stomatal density observed on English oak leaves under warmer summer temperatures was described as an adaptive response to reduce transpiration (Beerling & Chaloner, 1993; Linnaeus, 1797).

An increase in temperatures leads to an increase in VPD (Yuan et al., 2019), which is the difference between saturation vapour pressure and actual vapour pressure (Carins Murphy et al., 2014; Will et al., 2013), making VPD a driving force for evaporative water loss through stomata (Grossiord et al., 2020; Hovenden et al., 2012). As expected, under high VPD, where ambient air is very dry, lower stomatal density is expected on leaves to reduce water loss through transpiration, and under low VPD, higher stomatal density is expected (Amitrano et al., 2022; Fanourakis et al., 2011). El-Sharkawy et al. (1985) reported that under high VPD, higher stomatal density together with smaller stomata was recorded on leaves of Red cedar (*Toona ciliata*) (Hossain et al., 2014).

Multiple studies have examined the effects of water stress on stomatal density revealing an increase in olives (Bosabalidis & Kofidis, 2002; Ennajeh et al., 2010; Guerfel et al., 2009), and in pistachio cultivars (Esmaeilpour et al., 2016). Generally, higher stomatal density under water stress is often associated with smaller stomata (Bertolino et al., 2019; Zhao et al., 2015). The combination of higher stomatal density and small stomata has been noted to be associated with improved water use efficiency in *Arabidopsis thaliana* (Meyerowitz, 1987), and stomatal conductance in rice varieties under drought conditions (Bertolino et al., 2019). Despite many studies that have been done, no studies have investigated the response of stomatal density to varying temperatures, VPD, and soil water stress on macadamia trees.

2.5.2 Palisade and spongy parenchyma

Mesophyll of dorsiventral plant leaves consists of palisade and spongy parenchyma tissues (Ivanova & P'yankov, 2002). These tissues differ in their location on the leaf, cell shape, and functions. Palisade parenchyma is located on the adaxial surface of the leaf, and it is thought to play a major role in photosynthesis, due to its high concentration of chloroplasts, as well as the fact that palisade cells are vertically oriented, allowing them to receive the most sunlight possible for photosynthesis (Esau, 1965; Ivanova & P'yankov, 2002; Vogelmann & Martin, 1993). Spongy parenchyma, located on the abaxial surface of the leaf, is mainly responsible for gaseous exchange during photosynthesis (Baillie & Fleming, 2020; Ivanova & P'yankov, 2002; Terashima & Saeki, 1983).

Light influences the palisade and spongy parenchyma thickness. Several studies (Cui et al., 1991; Fan et al., 2013; Fan et al., 2018; Feng et al., 2019; Oguchi et al., 2003; Rôças et al., 2001), have published results stating that leaves grown under high light intensity conditions typically have thicker palisade and spongy parenchyma tissues. For example, a study on sun and shade leaves of *Quercus coccinea* and *Quercus rubra* (Linnaeus, 1797), revealed that those grown in the sun had thicker palisade tissues than shade leaves. Thicker palisade parenchyma enables light penetration to the chloroplasts, whereas thicker spongy parenchyma improves light capture (Evans, 1989).

Furthermore, changes in temperatures have an impact on mesophyll tissue thickness. Studies have reported an increase in palisade and spongy parenchyma thickness under high temperatures while low temperatures result in thin palisade and spongy parenchyma (Djanaguiraman et al., 2011; Peet, 1976; Xu et al., 2020). To understand why plants use water differently and to identify cultivars with different levels of drought tolerance, researchers have also looked at how water stress affects palisade and spongy parenchyma thickness. Cultivars of olives, namely ‘Chemlali’ and ‘Meski’, were subjected to water stress and some were irrigated to determine the impacts of water stress (Ennajeh et al., 2010). Water stress increased the palisade and spongy parenchyma thickness on both cultivars, and this allowed more carbon dioxide intake and higher photosynthesis rates because more chloroplasts were closer to the cell surface (Kröber et al., 2015; Rivas et al., 2020).

Similarly, when other cultivars of olives were subjected to water stress, the findings were similar to those reported on ‘Chemlali’ and ‘Meski’ cultivars (Bosabalidis &

Kofidis, 2002; Chartzoulakis et al., 1999; Guerfel et al., 2009). Similar findings were also reported on avocado cultivars ('Hass' and 'Fuerte') by Kofidis et al. (2004). There is evidence to suggest that palisade and spongy parenchyma are affected by varying environmental factors, however, so far as we know, no investigation has been undertaken on different cultivars of macadamia.

2.5.3 Huber value

To compensate for the water lost because of transpiration through stomata, the leaves depend on the petiole xylem. Petiole xylem hydraulic properties therefore play an important role in the control of transpiration rates, which is related to photosynthesis and the general functioning of the leaf (Sack & Holbrook, 2006). The petiole's xylem cross-sectional area provides insights into how plants adapt to their environment because it is responsible for transporting water into the leaf blade (Blackman et al., 2023).

To determine the petiole xylem cross-sectional area supporting a particular leaf area, Huber values have been used (Tyree & Ewers, 1991). In tree stems, the Huber value is defined as the proportion of sapwood or stem xylem cross-sectional area to the leaf area that the sapwood or xylem feeds (Tyree & Ewers, 1991). Given that the leaf petiole serves the same function as the stem, the Huber value for the leaf can be defined as the ratio of the petiole's xylem cross-sectional area to the leaf evaporative surface (Roddy et al., 2016). Moreover, it may be reasonable to assume that conclusions on Huber values of stem and flower pedicles may also apply to the leaf petiole. Like other leaf anatomical traits that determine the leaf hydraulic conductance, the Huber value is also influenced by various environmental factors, although so far there is very little information on these responses.

Schultz and Matthews (1993) revealed that shoots of grapevines (*Vitis vinifera* L.) (Bombardelli & Morazzoni, 1996) exposed to the sun had higher Huber values in comparison to shoots in the shade. Similarly, Huber values on sun-exposed subtropical trees (*Cabralea canjerana*) (Pizo, 1997), were also six times higher than that of shade, minimizing the impact of high evaporative demand (Moretti et al., 2019). Furthermore, Huber values on Japanese oak (*Quercus crispula)* (Quang et al., 2008) shoots on the upper crowns exposed to more sunlight were larger, reducing hydraulic stress, a mechanism to deal with increased demand for water associated with sunlight exposure (Yoshimura, 2011).

Temperature also impacted Huber values in Scots pine (*Pinus sylvestris* L.) (Carlisle & Brown, 1968), with warmer conditions leading to higher Huber values as compared to those at cooler sites (Mencuccini & Grace, 1995). Macinnis-Ng et al. (2004) also found that high summer temperatures increased Huber values on branches of heathland, woodland, and mango groove trees in comparison to branches in low winter temperatures. Shoots under water stress conditions generally have higher Huber values to increase the capacity of the xylem to supply water to the leaves (Preston & Ackerly, 2003).

Huber values were higher for plant species in an arid biome as compared to those in humid biomes as an adaptation for drought tolerance to reduce stress due to high evaporative demand (Oyanoghafo et al., 2021). In response to elevated relative humidity, Sellin et al. (2013), reported an increase in Huber value in silver birch. Nolf et al. (2014), showed a decrease in Huber values with increasing soil humidity on *Solidago canadensis* (Mei et al., 2006). In contrast to the previous studies mentioned, some studies found no association between climate and water availability on Huber values (Choat et al., 2007; Edwards, 2006), indicating that other underlying factors may be influencing Huber values. Huber values are therefore influenced by varying environmental factors; however, no research has been conducted on different cultivars of macadamia.

2.5.4 Vein density

To efficiently replace water lost through stomata, water is transported through the petiole to the leaf midrib, which branches out to major and minor veins. Consequently, leaf veins play a role in how much water is distributed across the leaf, linking them to transpiration and photosynthesis rates (Sack & Holbrook, 2006; Zwieniecki et al., 2002). Across different plant species, vein systems vary in number, size, arrangement, density, and vascular bundles in veins (Roth-Nebelsick et al., 2001; Sack & Scoffoni, 2013b). Vein density is the total length of veins per unit area of a leaf (Sack et al., 2014). Of all the leaf venation traits, vein density has been most widely studied for different plant species under varying climatic conditions because it affects the leaf hydraulic conductance the most (Prado & Maurel, 2013; Zhu et al., 2012).

Vein density has been reported to be positively and negatively correlated with leaf area (Gupta, 1961; Schuster, 1908), whereas other reports state that the relationship between leaf area and vein density depends on plant species (Zhu et al., 2012). Leaves of plants growing under full sunlight are known to have higher leaf vein density, than those growing under shade conditions (Brodribb & Jordan, 2011; Esau, 1965; Martins et al., 2014).

Hu et al. (2014) reported an increase in vein density on tobacco leaves exposed to higher temperatures. However, Muller et al. (2014) reported that minor vein density was greater in leaves under cooler conditions than in warmer conditions on summer annual species, optimizing photosynthesis. In conditions of drought or water stress vein density tends to increase (Dunbar Co et al., 2009; Lei et al., 2018; Zhu et al., 2012). For instance, vein density was found to be higher on Hawaiian *Plantago* in dry sites, enabling greater leaf hydraulic conductance than in wet sites (Dunbar Co et al., 2009). The higher vein density enables greater leaf hydraulic conductance, increasing the capacity to transport water (Brodribb & Holbrook, 2005). While it is evident that varying environmental factors play a significant role in vein density, there is still limited data regarding this leaf anatomical trait, especially on macadamia cultivars.

2.6 Summary

The above-mentioned leaf anatomical traits have shown a high level of plasticity in response to varying environmental factors, offering valuable insights into transpiration, photosynthesis rates, and drought tolerance across different plant species and cultivars. However, very limited research has been conducted on the response of macadamia leaf anatomical traits to varying environmental factors.

Macadamia, as reported in existing literature, exhibits traits associated with drought tolerance. These features include sclerophyllous leaves, a common adaptation for plants adapted to dry environments. Additionally, internal self-shading (Shabalala et al., 2022), and reduced mesophyll conductance (Lloyd et al., 1992) contribute to the plant's ability to endure water stress. Sclerophyllous leaves, characterized by being hard with thick cuticles, exhibit reduced carbon uptake by photosynthesizing cells. Additionally, less sunlight reaches the lower leaves, consequently, minimizing water loss through transpiration (Edwards et al., 2000; Salleo & Nardini, 2000).

Macadamia are also hypostomatic, as they have stomata only on the abaxial side of the leaf (Wakefield et al., 2021). Stomata on the abaxial leaf surface receives less sunlight compared to stomata on the adaxial leaf surface, thus resulting in lower transpiration rates. This allows them to precisely control their water status to prevent desiccation (Taylor et al., 2021). Consequently, evaluating the leaf and petiole anatomy of different cultivars of macadamia from different regions, with varying climatic conditions may provide information on their plasticity as it relates to different environmental factors. It may also help deal with water resource challenges by selecting drought-tolerant cultivars.

CHAPTER 3: MATERIALS AND METHODS

3.1 Study sites and weather variables

Leaves were collected from four distinct macadamia production regions in South Africa, including Levubu, Komatipoort, Nelspruit, and the Kwa-Zulu Natal South Coast. The GPS coordinates for each farm are provided in Table 3.1, and the geographical locations of each farm are represented in Figure 3.1. These regions correspond to key centers of macadamia production in South Africa as a result of their subtropical climate, which is regarded as best for macadamia cultivation (Carr, 2013; Ranketse et al., 2022). The cultivars investigated were the hybrids HAES ‘695’ also known as ‘Beaumont’ and ‘A4’, as well as *Macadamia integrifolia* ‘814’, ‘816’, and ‘842’. Except for the Loerieroep farm in Nelspruit, which utilized both micro-sprinkler and drip irrigation, all farms utilized micro-sprinklers. The tree ages ranged from two to twenty years. Each cultivar's herbarium voucher specimen was collected and placed in the H.G.W.J. Schweickerdt Herbarium at the University of Pretoria, (PRU: accession numbers #128815 to #129026).

Climatic data was obtained from the Agricultural Research Council (ARC). Data for air temperature, solar radiation, wind speed, relative humidity, and rainfall were considered for this study, and saturation vapour pressure deficit (VPD) was calculated. Furthermore, reference evapotranspiration (ET_o) for a short grass reference surface was calculated according to Allen et al. (1998). ET_o is a climatic parameter or measurement used to estimate evapotranspiration from an extensive grassed area (Tabari et al., 2013). Each general region's climatic conditions are described separately.

Table 3.1 Geographical coordinates of farms and cultivars harvested from them.

Region	Farm	Cultivar collected	Geographical coordinates
Levubu	Bridelia	‘695’ ‘814’ ‘842’	23°06'00.20"S 30°07'34.19"E
Nelspruit	Loerieroep	‘695’ ‘814’ ‘816’ ‘A4’	25°25'57.96"S 30°54'09.45"E
Komatipoort	Sommerreg	‘695’ ‘814’ ‘816’ ‘842’ ‘A4’	25°32'15.87"S 31°50'30.88"E
KwaZulu-Natal South Coast	Jacaranda	‘695’ ‘814’ ‘816’ ‘842’ ‘A4’	30°43'29.65"S 30°17'13.15"E
KwaZulu-Natal South Coast	Valeen	‘695’ ‘814’ ‘816’ ‘A4’	30°52'36.12"S 30°19'38.38"E
KwaZulu-Natal South Coast	Outlook	‘695’ ‘814’ ‘816’ ‘A4’	30°53'7.37"S 30°19'10.90"E

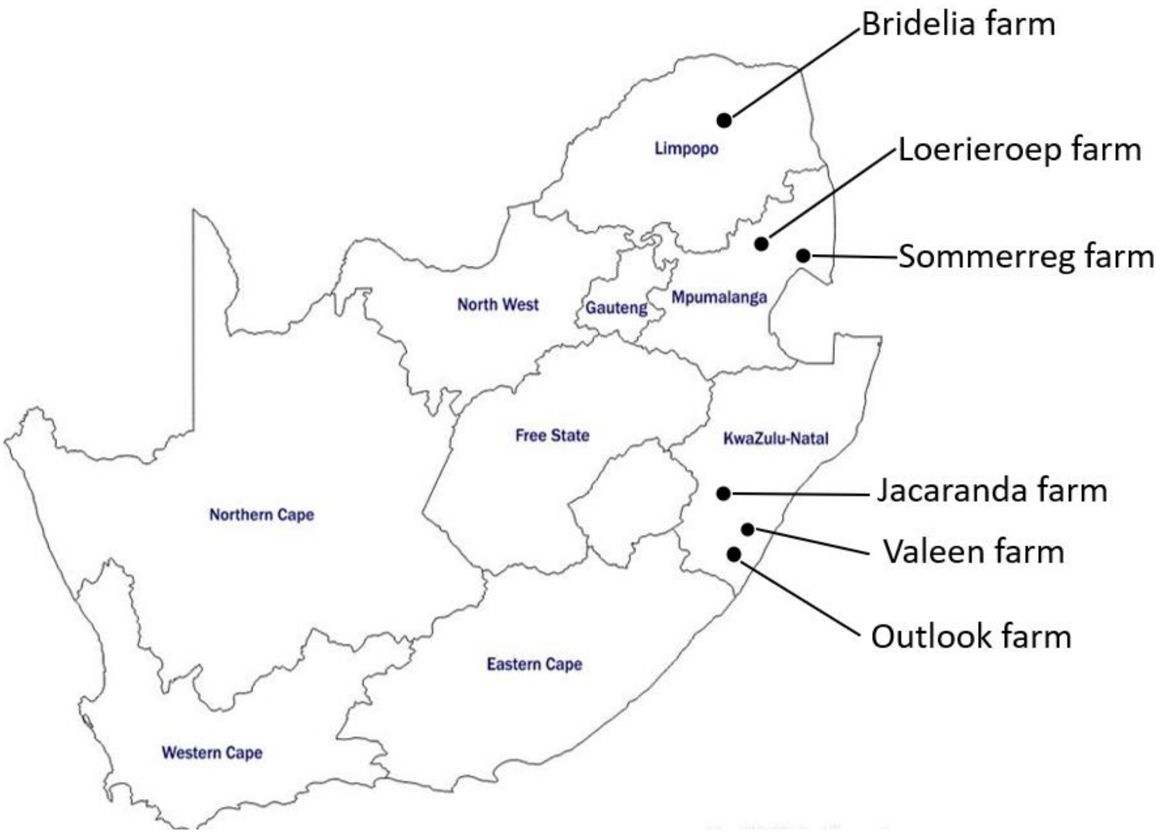

Figure 3.1 Macadamia farm leaf collection locations (Kumari, 2021).

3.1.1 Levubu

Levubu is in the Limpopo Province, within the Makhado local municipality. The region receives its main rainfall during the summer months between November and April, with an annual rainfall of approximately 1000 mm. Daily temperatures vary and can reach a maximum of 40 ˚C in summer, whilst winter months (June to August) are generally cool, with temperatures dropping below 20˚C (Kom et al., 2022; Lynch & Schulze, 2006).

3.1.2 Nelspruit

Nelspruit, also known as Mbombela, is in the Mpumalanga Province, within the Ehlanzeni district. The area normally receives 670 mm of rainfall per year, with most of the rainfall occurring from December to March. Daily temperatures range from 21˚C in June to 28˚C in December. The region is the coldest during July when the temperatures drop to 6˚C on average during the nights (Lynch & Schulze, 2006; Murovhi & Materechera, 2015).

3.1.3 Komatipoort

The Komatipoort sampling area is in the Mpumalanga Province, within the Ehlanzeni district. This region receives about 620 mm of rainfall per year with daily temperatures that vary between 38˚C- 40˚C in summer and the lowest temperature in the coldest months varies between 10˚C and 15˚C (Brotherton & Groenewald, 1982; Lynch & Schulze, 2006).

3.1.4 KwaZulu-Natal South Coast

Another major macadamia production centre is the KwaZulu-Natal Province's southern coast, within the Ugu district municipality. The region has a subtropical climate, whereby the winter months (April- August) are dry and cold, but summers (September to March) are humid and correspond to the main rainy season. Average daily summer temperatures rise above 25˚C and in winter drop to an average of 20˚C. The mean annual rainfall is approximately 1200mm per year (Lynch & Schulze, 2006; Ndlovu & Demlie, 2020; Nkosi et al., 2020).

Given the descriptions of each region's climatic conditions, they vary considerably. Levubu, Nelspruit, and Komatipoort experience extremely hot summers with higher temperatures and lower humidity than the KwaZulu-Natal South Coast. As a result of the hotter and drier conditions, it was hypothesized that macadamia cultivars from Levubu, Nelspruit, and Komatipoort would have greater stomatal densities, higher Huber values, greater vein densities, and thicker palisade and spongy parenchyma than macadamia trees grown in KwaZulu-Natal South Coast.

3.2 Leaf sample collection

One fully matured macadamia leaf from each tree out of 14 random trees of each cultivar per farm was harvested during the spring flush of 2021 and summer flush of 2022. Seven sun leaves were harvested from the sun-exposed part of the tree and another seven from the shade part of the tree, except in the Bridelia farm for the spring flush, where leaves were harvested without considering sun exposure. All the leaves were collected within a two-week period within each harvesting window.

3.3 Stomatal density measurements

The epidermal peel method was used to determine stomatal density (Liu et al., 2012; Zhao et al., 2016), see Figure 3.2. Each cultivar's fully expanded leaves were harvested and stored in zip-lock bags immediately after collection and processed the same day as collected. On the centre of the abaxial leaf surface, clear nail polish was applied and allowed to dry at room temperature. After the nail polish had dried, a strip of transparent tape was placed over it, peeled off the leaves, and then mounted on a microscope slide. The microscope slides were viewed using a Nikon Eclipse E200 (TOCHIGI Nikon Corporation) microscope with a 100x total magnification and images were captured using an Olympus EP50 camera (Olympus). The variation in stomatal density across the leaf was accounted for by selecting three zones at random across the centre of the leaf. Stomatal density (mm^{-2}) was calculated as the number of stomata on the entire field of view divided by the area of the field of view. All stomata within the field of view were tallied, including those that were partially visible.

1. Spread a thin layer of clear nail polish on the macadamia leaf's abaxial surface and allow it to dry.
2. Place a strip of clear tape over the nail polish; it should come off with it when you remove it.
3. Place the tape containing the imprint of the leaf on the microscope slides and observe under a microscope.

Figure 3.2 Procedure for stomatal peels from the macadamia abaxial leaf surface.

3.4 Palisade parenchyma, spongy parenchyma, and petiole xylem cross-sectional area measurements

To determine the thickness of the mesophyll tissues and petiole xylem cross-sectional area, the most recent fully mature leaves (including petioles) below the chosen apical meristems were harvested and immediately fixed in 70% ethanol. Due to the sclerification of the tissues in macadamia leaves (Syvertsen et al., 1995), classic microtomy approaches proved difficult to implement, as sections inevitably tore upon microtomy in wax media. Therefore, leaf blades and petioles were manually sectioned using the technique described by Yeung (1998), see Figure 3.3. Initial sections were placed in water-filled watch glasses. The selected sections were then placed in a drop of glycerol on a glass microscope slide and covered with a coverslip. The edges of the coverslip were sealed using clear nail polish to prevent desiccation. Under a Nikon Eclipse E200 microscope (TOCHIGI Nikon corporation) equipped with an Olympus

EP50 camera (Olympus), the petiole and leaf cross-sections were observed at 40x and 100x total magnification, respectively. Three petiole sections and five leaf blade sections per leaf were used. The measurements from these sections were then averaged to produce representative values for each leaf. Figure 3.4 and Figure 3.5 depict how measurements of palisade and spongy parenchyma length, and petiole xylem cross-sectional area, were done on ImageJ (Rasband, 2012), respectively. The Huber value was calculated using the petiole xylem cross-sectional area (calculated as the sum of xylem area across all vascular bundles in the petiole) relative to the leaf surface area. The leaf area for each leaf was measured by taking pictures of the leaves against a white sheet via a Nikon D3100 digital camera. Images were analyzed in ImageJ (version 1.53k) (Rasband, 2012). Leaf area was measured using the method of Ahmad et al. (2015).

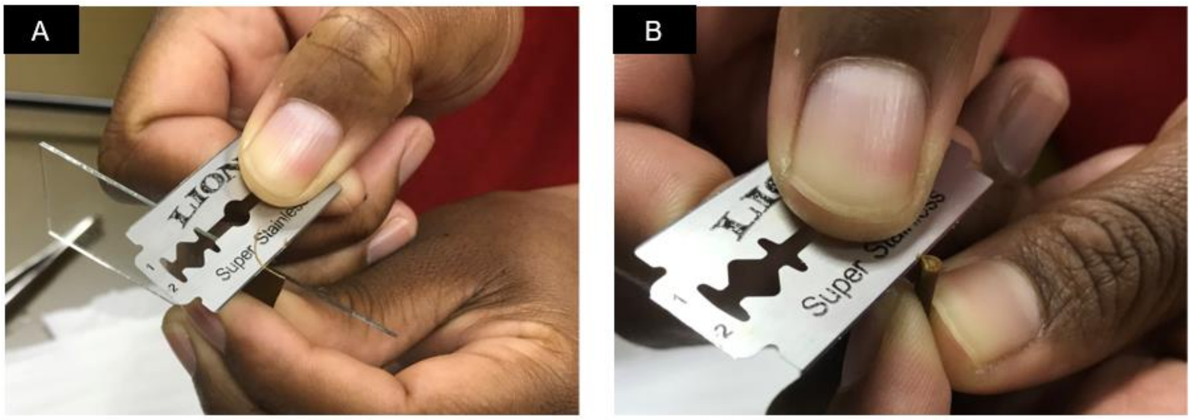

Figure 3.3 Method of holding the leaf blade A) and petiole B) specimen for free hand sectioning.

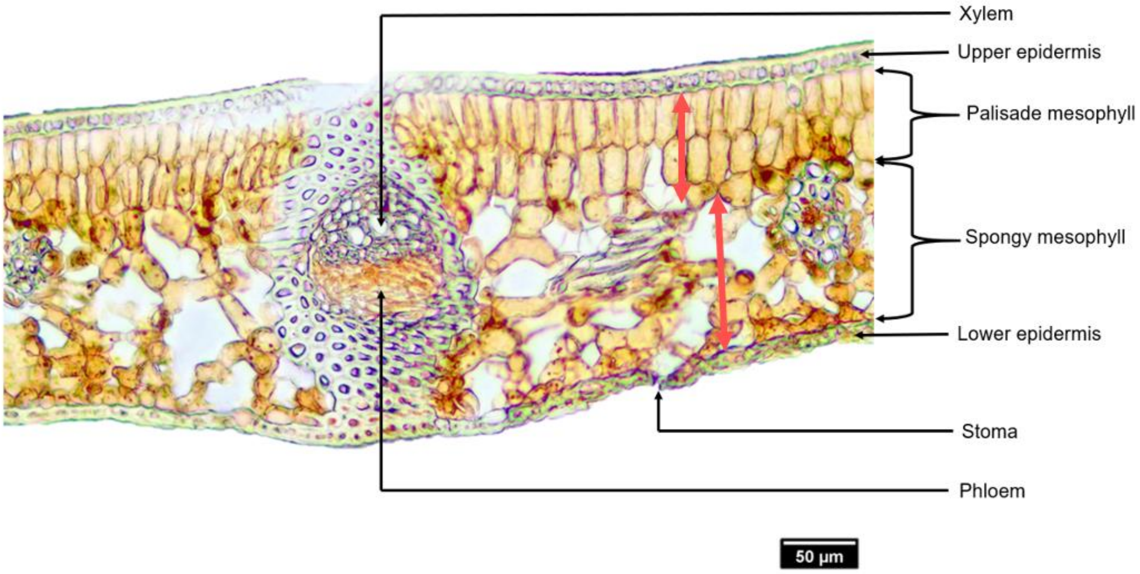

Figure 3.4 Example of the determination of the palisade and spongy parenchyma thickness (µm) in Macadamia leaves using ImageJ software. The red double-headed arrows on the image indicate the measurements for the palisade and spongy parenchyma length.

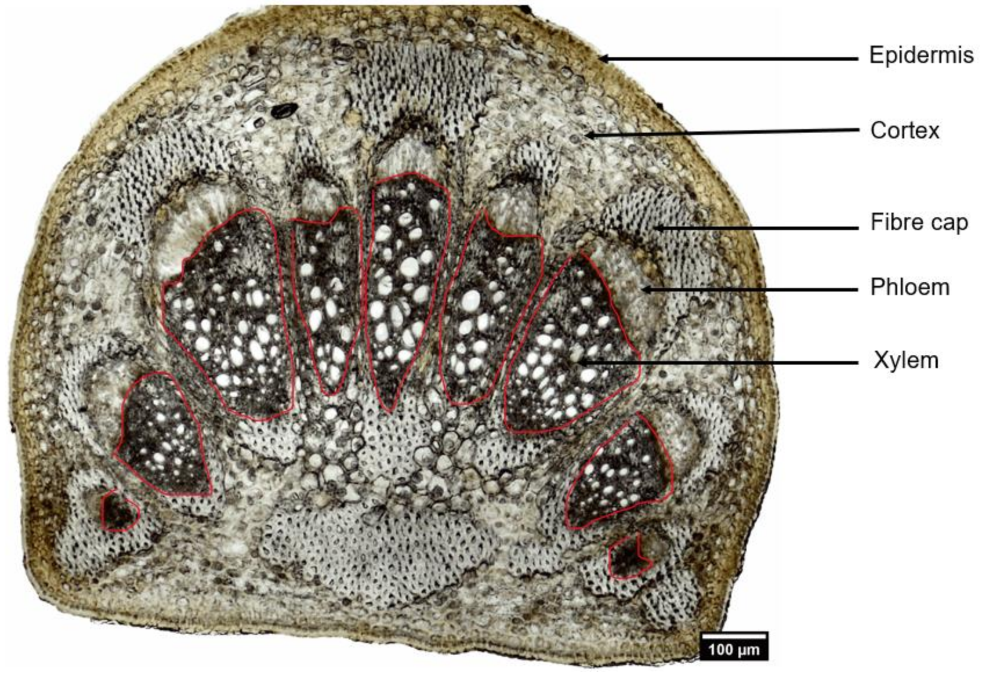

Figure 3.5 Example of petiole xylem cross-sectional area determination in Macadamia petioles (µm) using ImageJ software. The red freehand drawn lines outline the xylem cross-sectional area measured for the example petiole.

3.5 Vein density measurements

The vein orders were assigned based on Sack et al. (2014). To measure vein length, one cm^2 sample was taken from midway between the leaf midrib and margin, midway between the base and tip of the same leaves used for the measurements of mesophyll tissue thickness and petiole xylem cross-sectional area. The leaf samples were placed in 2 - 4% sodium hydroxide solution for clearing at room temperature (Roddy et al., 2013). The solution was replaced weekly until the leaf veins were visible. After soaking the samples in distilled water, they were transferred to a 3% bleach solution for a few minutes to complete the clearing procedure. The samples were then washed once more with distilled water before being placed in 95% ethanol (Roddy et al., 2013). After immersion in ethanol, samples were mounted on microscope glass slides and examined with a Nikon Eclipse E200 microscope (TOCHIGI Nikon corporation) equipped with an Olympus EP50 camera (Olympus). ImageJ (version 1.53k) (Rasband, 2012) was used to perform all image analyses in the following steps for each image: Open image; process; binary; create binary; image; adjust; threshold; triangle algorithm; light background; plugins; skeletonize image; and then analyze skeleton (Pagano et al., 2016). Vein information was manually extracted from skeletonized images, see Figure 3.6. Vein density was calculated as the total length of veins per unit area, and the values obtained from each leaf section were averaged to get an overall vein density value for each leaf. Similarly, the leaf area for each leaf was measured by taking pictures of the leaves against a white sheet via a Nikon D3100 digital camera. Images were analyzed in ImageJ (version 1.53k) (Rasband, 2012). Leaf area was measured using the method of Ahmad et al. (2015).

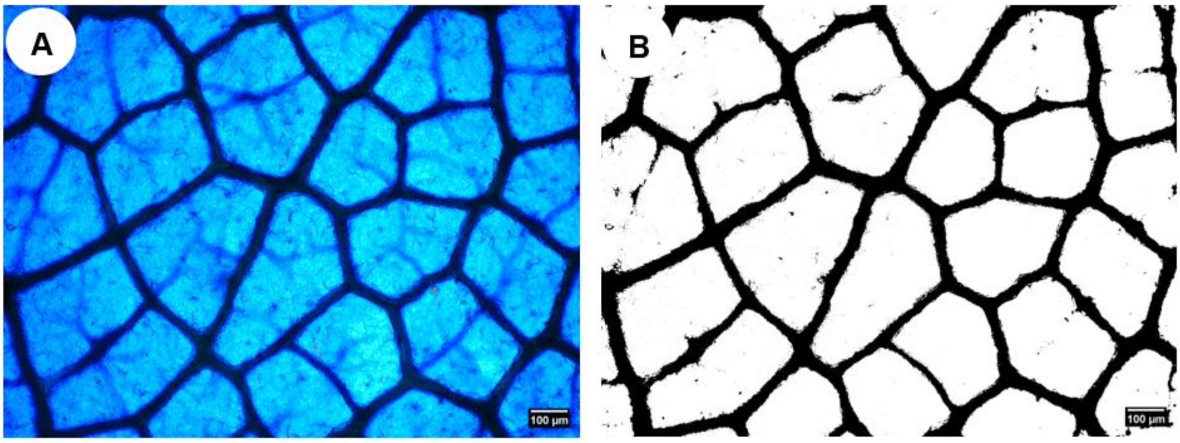

Figure 3.6 Veins on cleared macadamia leaves, A) original image and B) skeletonized image.

3.6 Statistical analysis

All analyses were run in R version 4.2.0 (Team, 2022) using the following packages: lme4 (Bates et al., 2014), ggplot2 3.3.6 (Wickham, 2009), MASS (Ripley et al., 2013), and multcompView (Graves et al., 2015). Before delving into the analysis, we performed a log transformation using the Box-cox method to make sure that the data met the normality and homoscedasticity assumptions. To investigate the influence of predictor variables such as season of leaf harvest, leaf type, farms, and cultivars on the response variables such as stomatal density, palisade parenchyma, spongy parenchyma, Huber value, and vein density, Linear Mixed Models (LLM) were fitted. These models were useful because they account for the fixed effects and their interactions, providing a robust framework for understanding the complex relationships between the variables involved. The best model was determined through backward selection, the findings presented in the tables have been back-transformed to the original scale. To examine potential interactions and patterns between farms and cultivars across the investigated leaf anatomical traits, a comprehensive statistical analysis was conducted. The relationship between stomatal density and stomatal size was examined by regression analysis.

During the spring flush, leaves from Bridelia farm were harvested without regard to shade or sun exposure. However, during summer, leaves were harvested from the sun-exposed and shaded parts of the trees. To facilitate a comparative analysis of leaf and petiole anatomical traits among different cultivars within the Bridelia farm, an average of sun and shade leaves was calculated. The intercepts were ‘695’ and spring flush. The ‘842’ was exclusively found on Sommerreg and Jacaranda farms. As a result, the analysis for this particular cultivar on these farms was conducted separately, and Jacaranda farm, shade leaves, and spring flush were the intercepts. The remaining cultivars (‘695’, ‘A4’, ‘814’, and ‘816’) commonly found in the multiple farms namely Loerieroep, Sommerreg, Jacaranda, Valeen, and Outlook were pooled together to facilitate a comparative analysis of leaf and petiole anatomical traits, and ‘695’, shade leaves, spring flush, and Jacaranda farm were the intercepts.

CHAPTER 4: RESULTS

4.1 Seasonal weather

Stephenson et al. (1989), outlined the vegetative flushing patterns of macadamia trees, with spring flush occurring from (September- November) and summer flush occurring from (January- March). The seasonal weather data for the Levubu, Nelspruit, Komatipoort, and KwaZulu-Natal South Coast regions is summarized in Table 4.1.

Temperatures during the spring and summer flush periods in the Levubu, Nelspruit, Komatipoort, and KwaZulu-Natal South Coast regions revealed seasonal variations. During the spring flush, maximum temperatures in all regions ranged from 23.5 ˚C to 29.7 ˚C. Minimum temperatures ranged from 14.3 ˚C to 16.0 ˚C. In contrast, during the summer flush, there was an increase in temperatures across all regions. Maximum temperatures increased drastically, ranging from 27.4 ˚C to 32.8 ˚C. Minimum temperatures also increased ranging from 18.5 ˚C to 20.7 ˚C. These findings suggest that during the summer flush, conditions were warmer as compared to milder conditions during the spring flush.

During the spring flush, the Komatipoort region exhibited the highest vapour pressure deficit (VPD) of 1.49 kPa. Nelspruit followed closely with a VPD of 1.47 kPa, while the Levubu and KwaZulu-Natal South Coast had lower VPD. During the summer flush, the Komatipoort region maintained the highest VPD, indicating elevated atmospheric dryness. The Nelspruit and Levubu regions also experienced an increase in VPD, recording values of 1.41 kPa and 1.13 kPa, respectively, and the KwaZulu-Natal South Coast region remained with a lower VPD. During the spring and summer flush, the Komatipoort region had a higher VPD than other regions, indicating drier conditions.

During the spring flush, the Komatipoort region had the highest total reference evapotranspiration (ET_o, 389 mm). Nelspruit closely followed with an ET_o of 338 mm, while the Levubu and KwaZulu-Natal South Coast had lower ET_o values. A similar pattern emerged during the summer flush, with the Komatipoort region exhibiting the highest ET_o of 406 mm. The Nelspruit region followed with an ET_o of 392 mm, while the Levubu and KwaZulu-Natal South Coast had lower ET_o values. The persistence of high VPD and ET_o in the Komatipoort region during both flush periods implies an increase in atmospheric water demand. Therefore, the Sommerreg farm in the Komatipoort region may have had drier atmospheric conditions than other farms.

Table 4.1 Average seasonal minimum and maximum temperatures (˚C), vapour pressure deficit (VPD, kPa), and total reference evapotranspiration (ET_o, mm) during the spring (September- November 2021) and summer flush (January- March 2022).

Region	Farm	Spring (September- November)				Summer (January- March)			
		Max Temp (˚C)	Min Temp (˚C)	VPD (kPa)	ET_o (mm)	Max Temp (˚C)	Min Temp (˚C)	VPD (kPa)	ET_o (mm)
Levubu	Bridelia	26.9	15.1	1.32	234	28.5	18.5	1.13	263
Nelspruit	Loerieroep	28.2	14.3	1.47	338	30.4	18.8	1.41	392
Komatipoort	Sommerreg	29.7	16.0	1.49	389	32.8	20.7	1.57	406
KZN South Coast	Jacaranda Valeen Outlook	23.5	15.7	0.71	296	27.4	20.0	0.78	321

4.2 Leaf anatomy

This section presents the results of the Linear Mixed Model effects. At the Bridelia farm (Table 4.2), the intercepts were ‘695’ and spring flush. For the ‘842’ leaves from the Sommerreg and Jacaranda farms, detailed in (Table 4.3), Jacaranda farm, shade leaves, and spring flush were the intercepts. Furthermore, on analysis across multiple farms, presented in (Table 4.4, Table 4.6, and Table 4.8), ‘695’, shade leaves, spring flush, and Jacaranda farm were the intercepts.

4.2.1 Stomatal density

At the Bridelia farm, detailed in Table 4.2, ‘695’ exhibited significantly higher stomatal density than ‘814’ and ‘842’ ($P < 0.001$), while summer flush leaves had higher stomatal density than spring flush leaves ($P < 0.001$). Examining ‘842’ leaves from the Sommerreg and Jacaranda farms, detailed in (Table 4.3), Sommerreg farm leaves had significantly higher stomatal density ($P < 0.001$) than Jacaranda farm leaves. Furthermore, sun leaves had significantly higher stomatal density than shade leaves, and summer flush leaves had significantly higher stomatal density than spring flush leaves.

In the analysis across multiple farms, presented in Table 4.4, ‘814’ displayed significantly higher stomatal density than ‘695’, ‘816’, and ‘A4’ ($P < 0.001$). Sommerreg farm leaves exhibited significantly higher stomatal density ($P < 0.001$) compared to leaves from Loerieroep, Jacaranda, Valeen, and Outlook farms. Sun leaves had significantly higher stomatal density ($P < 0.001$) than shade leaves. Moreover, summer flush leaves exhibited significantly higher stomatal density than spring flush leaves. The significant interactions observed in (Table 4.4), demonstrate the impact of leaf type and flush season on stomatal density variability.

In summary, ‘814’ consistently displayed higher stomatal density than ‘695’,’842’, ‘816’, and ‘A4’, with notable differences when compared to ‘695’ (Figure 4.1). Sun leaves consistently exhibited higher stomatal density than shade leaves, and summer flush leaves also exhibited higher stomatal density than spring flush leaves. Sommerreg farm consistently exhibited higher stomatal density than leaves from the Bridelia, Loerieroep, Jacaranda, Valeen, and Outlook farms. Despite the observed patterns, a weak positive correlation between stomatal density and stomatal size was observed ($R^2 = 0.029$ and $P = 0.045$) (see Figure 4.2). This suggests that, while there is a relationship between stomatal density and stomatal size, it is relatively weak.

Table 4.2 Best selected Linear mixed model effects model summary on leaf and petiole anatomical traits in macadamia cultivars '695', '814', and '842' across spring and summer vegetative flush at the Bridelia farm. '695' and spring flush are the intercepts.

	Estimate	SE	*t*	*P* value
Stomatal density				
(Intercept)	184.357	7.692	23.967	**<0.001**
'814'	-31.299	9.421	-3.322	**0.001**
'842'	-10.868	9.421	-1.154	0.252
Summer flush	34.936	7.629	4.542	**<0.001**
Palisade parenchyma				
(Intercept)	88.274	4.306	20.502	**<0.001**
'814'	-17.328	6.089	-2.846	**0.005**
'842'	15.518	6.089	2.549	**0.013**
Summer flush	8.945	6.089	1.469	0.145
'814': Summer flush	9.937	8.611	1.154	0.252
'842': Summer flush	-21.437	8.611	-2.489	**0.015**
Spongy parenchyma				
(Intercept)	151.533	6.439	23.533	**<0.001**
814	-22.625	7.886	7.886	**0.005**
842	-5.309	7.886	7.886	0.503
Summer flush	-28.179	6.439	6.439	**<0.001**
Huber value				
(Intercept)	4.502e-06	5.122e-07	8.790	**<0.001**
814	-9.249e-07	7.244e-07	-1.277	0.205
842	1.672e-06	7.244e-07	2.308	**0.024**
Summer flush	-2.010e-06	7.244e-07	-2.774	**0.007**
814: Summer flush	3.153e-06	1.024e-06	3.078	**0.003**
842: Summer flush	1.620e-06	1.024e-06	1.582	0.118
Vein density				
(Intercept)	0.010499	0.026852	0.391	0.697
814	0.004703	0.037974	0.124	0.902
842	0.014210	0.037974	0.374	0.709
Summer flush	0.003112	0.037974	0.082	0.935
814: Summer flush	-0.006539	0.053703	-0.122	0.903

Bolded P values are statistically significant at $P < 0.05$. SE represents standard error and t represents t-value.

Table 4.3 Best selected Linear mixed model effects model summary on leaf and petiole anatomical traits in macadamia cultivar '842' across spring and summer vegetative flush at the Sommerreg and Jacaranda farms. Jacaranda farm, shade leaves, and spring flush are the intercepts.

	Estimate	SE	*t*	*P* value
Stomatal density				
(Intercept)	126.462	9.166	13.797	**<0.001**
Summer flush	32.353	11.594	2.790	**0.007**
Sun leaves	96.805	11.594	8.349	**<0.001**
Sommerreg	103.293	8.198	12.599	**<0.001**
Palisade parenchyma				
(Intercept)	60.037	3.630	16.539	**<0.001**
Sun leaves	30.539	4..753	6.425	**<0.001**
Sommerreg	48.656	4.753	10.237	**<0.001**
Summer flush	27.241	4.753	5.731	**<0.001**
Sun leaves: Sommerreg	-28.356	5.488	-5.167	**<0.001**
Sun leaves: Summer flush	-10.565	5.488	-1.925	0.06
Sommerreg: Summer flush	-30.752	5.488	-5.603	**<0.001**
Spongy parenchyma				
(Intercept)	128.959	5.381	23.964	**<0.001**
Sun leaves	5.527	4.813	1.148	0.256
Sommerreg	43.634	6.807	6.410	**<0.001**
Summer flush	8.693	6.807	1.277	0.207
Sommerreg: Summer flush	-41.709	9.626	-4.333	**<0.001**
Huber value				
(Intercept)	1.380e-06	6.653e-07	2.075	**0.043**
Sun leaves	1.871e-06	7.682e-07	2.436	**0.018**
Sommerreg	1.075e-05	9.409e-07	11.429	**<0.001**
Summer flush	2.902e-06	7.682e-07	3.778	**<0.001**
Sun leaves: Sommerreg	-3.302e-06	1.086e-06	-3.039	**0.004**
Sommerreg: Summer flush	-8.100e-06	1.086e-06	-7.456	**<0.001**
Vein density				
(Intercept)	0.00659	0.00138	4.769	**<0.001**
Sun leaves	0.00485	0.00274	2.778	**0.007**
Sommerreg	0.00897	0.00274	5.136	**<0.001**
Summer flush	0.00273	0.00273	2.213	**0.031**
Sun leaves: Sommerreg	-0.00944	-0.00944	-3.820	**<0.001**

Bolded P values are statistically significant at $P < 0.05$. SE represents standard error and t represents t-value.

Table 4.4 Best selected Linear mixed model effects model summary on stomatal density in macadamia cultivars ‘695’, ‘A4’, ‘814’, and ‘816’ across spring and summer vegetative flush at the Loerieroep, Sommerreg, Jacaranda, Valeen, and Outlook farms. ‘695’, shade leaves, spring flush, and Jacaranda farm are the intercepts.

	Estimate	SE	*t*	*P* value
(Intercept)	148.521	7.766	19.125	**<0.001**
Sun leaves	58.151	7.534	7.718	**<0.001**
Loerieroep	14.828	8.423	1.760	0.078
Outlook	19.041	8.423	2.261	0.241
Sommerreg	40.483	8.423	4.806	**<0.001**
Valeen	13.038	8.423	1.548	0.122
Summer flush	94.488	8.423	1.218	**<0.001**
‘814’	39.076	7.534	5.187	**<0.001**
‘816’	2.039	7.534	0.027	0.786
‘A4’	-20.439	7.534	-2.713	**0.006**
Sun leaves: ‘814’	-56.246	10.655	-5.279	**<0.001**
Sun leaves: ‘816’	-41.452	10.655	-3.890	**<0.001**
Sun leaves: ‘A4’	-18.754	10.655	-1.760	0.078
Loerieroep: Summer flush	74.773	11.912	6.277	**<0.001**
Outlook: Summer flush	-20.763	11.912	-1.743	0.081
Sommerreg: Summer flush	83.388	11.912	7.000	**<0.001**
Valeen: Summer flush	11.374	11.912	0.955	0.340

Bolded P values are statistically significant at $P < 0.05$. SE represents standard error and t represents t-value.

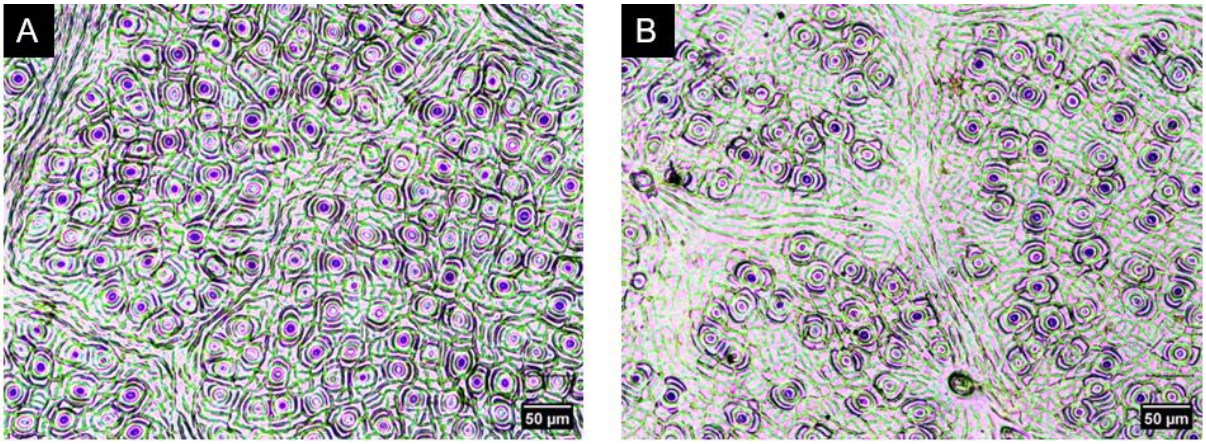

Figure 4.1 Stomata on leaves of macadamia cultivars, A- ‘814’ and B- ‘A4’. Scale bar = 50 µm.

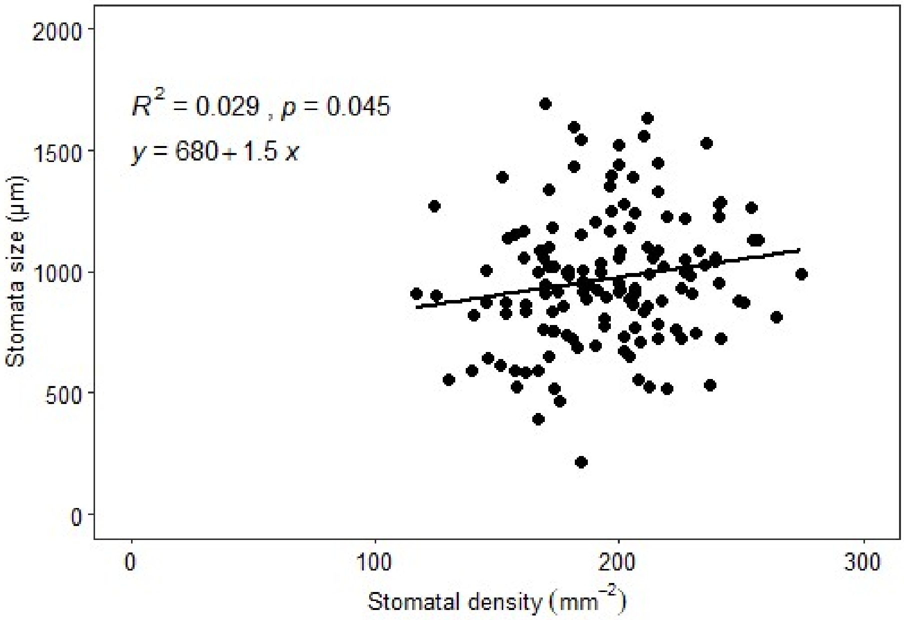

Figure 4.2 Relationship between stomatal density (mm^{-2}) and stomata size (µm) for leaves of ‘695’, ‘A4’, ‘814’, and ‘816’ from Loerieroep, Sommerreg, Jacaranda, Valeen, and Outlook for the spring flush. The Regression equation, squared correlation coefficient (R^2), and P value are reported.

4.2.2 Palisade parenchyma

At the Bridelia farm, detailed in (Table 4.2), ‘842’ had significantly thicker palisade parenchyma than ‘695’ and ‘814’ (*P= 0.013*). Although, not statistically significant (*P= 0.145*), summer flush leaves had thicker palisade parenchyma than spring flush leaves. In the case of ‘842’ leaves from the Sommerreg and Jacaranda farms (Table 4.3), sun leaves had significantly thicker palisade parenchyma (*P < 0.001*) than shade leaves. Additionally, summer flush leaves had significantly thicker palisade parenchyma than spring flush leaves, and leaves from Sommerreg also had significantly thicker palisade parenchyma than leaves from the Jacaranda farm.

Analyzing across multiple farms (Table 4.5), ‘814’ had significantly thicker palisade parenchyma than ‘695’, ‘816’, and ‘A4’ (*P < 0.001*). Moreover, leaves from the Sommerreg farm had significantly thicker palisade parenchyma than leaves from Loerieroep, Jacaranda, Valeen, and Outlook. Sun leaves also had significantly thicker palisade parenchyma compared to shade leaves, while summer flush leaves tended to have thinner palisade parenchyma, but were not statistically significant (*P= 0.655*). Significant interactions observed in Table 4.2, Table 4.3, and Table 4.5, demonstrate an influence of leaf type, cultivar, farm, and season of flush on the observed variability in palisade parenchyma.

In conclusion, ‘814’ consistently exhibited thicker palisade parenchyma than ‘695’, ‘842’, ‘816’, and ‘A4’, with notable differences when compared to ‘695’ (Figure 4.3). Sun leaves consistently exhibited thicker palisade parenchyma than shade leaves, and summer flush leaves also consistently had thicker palisade parenchyma than spring flush leaves. Furthermore, Sommerreg farm leaves consistently exhibited thicker palisade parenchyma than leaves from the Bridelia, Loerieroep, Jacaranda, Valeen, and Outlook farms.

Table 4.5 Best selected Linear mixed model effects model summary on palisade parenchyma in macadamia cultivars '695', 'A4', '814', and '816' across spring and summer vegetative flush at the Loerieroep, Sommerreg, Jacaranda, Valeen, and Outlook farms. '695', shade leaves, spring flush, and Jacaranda farm are the intercepts.

	Estimate	SE	*t*	*P* value
(Intercept)	76.639	2.831	27.071	**<0.001**
Sun leaves	18.412	2.762	6.665	**<0.001**
Loerieroep	8.676	3.383	2.564	**0.011**
Outlook	16.596	3.383	4.905	**<0.001**
Sommerreg	24.900	3.383	7.359	**<0.001**
Valeen	-2.586	3.383	-0.764	0.445
Summer flush	-1.559	3.494	-0.446	0.655
'814'	8.913	2.471	3.607	**<0.001**
'816'	2.308	2.471	0.934	0.350
'A4'	6.184	2.471	2.503	**0.012**
Sun leaves: Loerieroep	-9.603	3.907	-2.458	**0.014**
Sun leaves: Outlook	-13.230	3.907	-3.386	**<0.001**
Sun leaves: Sommerreg	-10.245	3.907	-2.622	**0.008**
Sun leaves: Valeen	-1.370	3.907	-0.351	0.725
Loerieroep: Summer flush	-25.033	3.907	-6.407	**<0.001**
Outlook: Summer flush	1.649	3.907	0.422	0.673
Sommerreg: Summer flush	-25.345	3.907	-6.487	**<0.001**
Valeen: Summer flush	9.280	3.907	2.375	**0.017**
Sun leaves: '814'	4.516	3.494	1.293	0.196
Sun leaves: '816'	9.508	3.494	2.721	**0.006**
Sun leaves: 'A4'	-0.008	3.494	-0.003	0.998

Bolded P values are statistically significant at $P < 0.05$. SE represents standard error and t represents t-value.

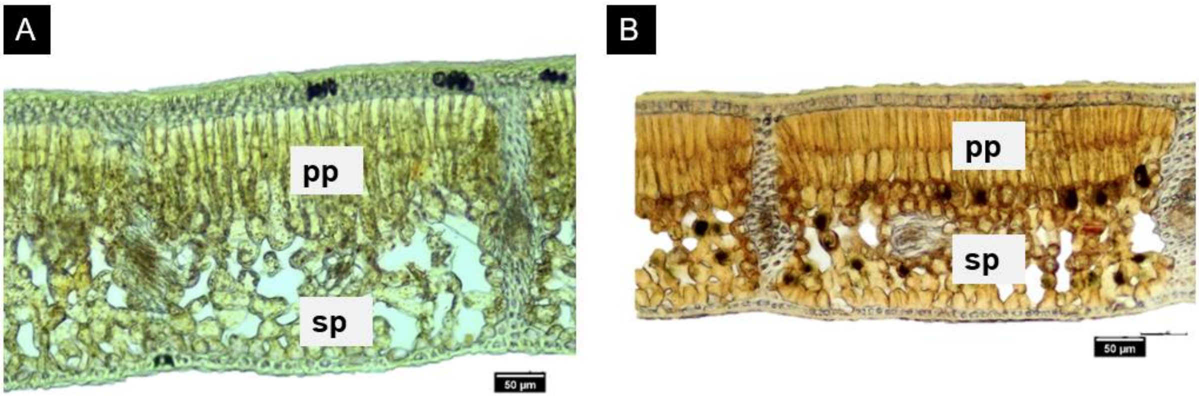

Figure 4.3 Cross-sections of the leaves of macadamia cultivars A- ‘814’ and B- ‘695’. pp= palisade parenchyma; sp= spongy parenchyma. Scale bar = 50 µm.

4.2.3 Spongy parenchyma

At the Bridelia farm (Table 4.2), ‘695’ exhibited significantly thicker spongy parenchyma than ‘814’ and ‘842’ ($P < 0.001$). Additionally, summer flush leaves had significantly thinner spongy parenchyma than spring flush leaves. For ‘842’ leaves from the Sommerreg and Jacaranda farms (Table 4.3), sun leaves had thicker spongy parenchyma than shade leaves, and summer flush leaves had thicker spongy parenchyma than spring flush leaves, though not statistically significant ($P = 0.256$ and $P = 0.207$, respectively). Additionally, leaves from the Sommerreg farm had significantly thicker spongy parenchyma than leaves from the Jacaranda farm.

Among multiple farms (Table 4.6), leaves of ‘814’ had significantly thicker spongy parenchyma than ‘695’, ‘816’, and ‘A4’ ($P < 0.001$). Sun leaves had significantly thicker spongy parenchyma than shade leaves, and summer flush leaves also had significantly thicker palisade parenchyma than spring flush leaves. Moreover, spring flush leaves had significantly thinner spongy parenchyma than summer flush leaves. Sommerreg farm leaves had significantly thicker spongy parenchyma than leaves from the Loerieroep, Jacaranda, Valeen, and Outlook farms. The observed significant interactions in Table 4.3 and Table 4.6 suggest that leaf type and season of leaf flush contributed to the observed variability in spongy parenchyma.

In conclusion, ‘814’ consistently stood out with significantly thicker spongy parenchyma compared to ‘695’, ‘842’, ‘816’, and ‘A4’, with notable differences when compared to ‘695’ (Figure 4.3). Sun leaves consistently exhibited significantly thicker spongy parenchyma than shade leaves. Spring flush leaves were also associated with thicker spongy parenchyma compared to summer flush leaves, and leaves from the Sommerreg farm consistently exhibited significantly thicker spongy parenchyma.

Table 4.6 Best selected Linear mixed model effects model summary on spongy parenchyma in macadamia cultivars '695', 'A4', '814', and '816' across spring and summer vegetative flush at the Loerieroep, Sommerreg, Jacaranda, Valeen, and Outlook farms. '695', shade leaves, spring flush, and Jacaranda farm are the intercepts.

	Estimate	SE	*t*	*P* value
(Intercept)	134.628	3.666	36.721	**<0.001**
Sun leaves	10.703	1.778	6.018	**<0.001**
Loerieroep	7.391	3.976	1.859	0.063
Outlook	9.523	3.976	2.395	**0.016**
Sommerreg	27.178	3.976	6.835	**<0.001**
Valeen	1.640	3.976	0.412	0.680
Summer flush	-13.603	5.030	-2.704	**0.007**
'814'	16.224	3.556	4.562	**<0.001**
'816'	7.888	3.556	2.218	**0.026**
'A4'	6.999	3.556	1.968	**0.049**
Loerieroep: Summer flush	-16.111	5.623	-2.865	**0.004**
Outlook: Summer flush	-25.327	5.623	-4.504	**<0.001**
Sommerreg: Summer flush	-19.060	5.623	-3.389	**<0.001**
Valeen: Summer flush	-16.139	5.623	-2.870	**0.004**
Sun leaves: '814'	0.416	5.030	0.083	0.934
Sun leaves: '816'	15.094	5.030	3.001	**0.003**
Sun leaves: 'A4'	-5.512	5.030	-1.096	0.273

Bolded P values are statistically significant at $P < 0.05$. SE represents standard error and t represents t-value.

4.2.4 Huber value

At the Bridelia farm (Table 4.2), '842' exhibited significantly higher Huber values (*P= 0.024*), than '814' and '695', while leaves from the summer flush had significantly lower Huber values (*P = 0.007*) compared to leaves from the spring flush. For '842' leaves from the Sommerreg and Jacaranda farms (Table 4.3) sun leaves had a significantly higher Huber value (*P = 0.018*) than shade leaves, and summer flush leaves had significantly higher Huber values than spring flush leaves. Moreover, Sommerreg farm leaves had significantly higher Huber values than Jacaranda farm leaves.

Analysis across multiple farms (Table 4.7), leaves of ‘814’ exhibited significantly higher Huber values than leaves of ‘695’, ‘816’, and ‘A4’. Sun leaves had higher Huber values than shade leaves, but not statistically significant ($P = 0.335$), and leaves from the summer flush had lower Huber values compared to leaves from the spring flush. Furthermore, leaves from the Sommerreg farm had significantly higher Huber values compared to leaves from the Loerieroep, Jacaranda, Valeen, and Outlook farms. There observed significant interactions in Table 4.2, Table 4.3, and Table 4.7, suggest the influence of leaf type, cultivar, farm, and season of flush on the observed variability in Huber values.

In conclusion, ‘814’ consistently stood out with significantly higher Huber values compared to ‘695’, ‘842’, ‘816’, and ‘A4’, with notable differences when compared to ‘A4’ (Figure 4.4). Sun leaves consistently exhibited significantly higher Huber values than shade leaves. Spring flush leaves consistently exhibited higher Huber values than summer flush leaves, and leaves from the Sommerreg farm consistently had significantly higher Huber values.

Table 4.7 Best selected Linear mixed model effects model summary on Huber values in macadamia cultivars '695', 'A4', '814', and '816' across spring and summer vegetative flush at the Loerieroep, Sommerreg, Jacaranda, Valeen, and Outlook farms. The intercepts are the '695', shade leaves, spring flush, and Jacaranda farm.

	Estimate	SE	*t*	*P* value
(Intercept)	4.785e-06	3.557e-07	13.451	**<0.001**
Sun leaves	3.209e-07	3.472e-07	0.924	0.355
Loerieroep	1.485e-07	3.472e-07	0.482	0.669
Outlook	1.105e-07	3.472e-07	0.318	0.750
Sommerreg	2.873e-06	3.472e-07	8.275	**<0.001**
Valeen	-3.395e-07	3.472e-07	-0.978	0.328
Summer flush	-2.829e-06	4.658e-07	-6.073	**<0.001**
'814'	1.930e-06	3.803e-07	5.074	**<0.001**
'816'	5.868e-07	3.803e-07	1.543	0.123
'A4'	-1.002e-06	3.803e-07	-2.636	**0.008**
Sun leaves: '814'	-8.871e-07	4.392e-07	-2.020	**0.043**
Sun leaves: '816'	1.883e-08	4.392e-07	0.043	0.965
Sun leaves: 'A4'	-4.433e-07	4.392e-07	-1.010	0.313
Loerieroep: Summer flush	3.043e-07	4.910e-07	0.620	0.535
Outlook: Summer flush	1.277e-06	4.910e-07	2.602	**0.009**
Sommerreg: Summer flush	-1.981e-06	4.910e-07	-4.034	**<0.001**
Valeen: Summer flush	1.531e-06	4.910e-07	3.119	**0.002**
Summer: '814'	6.176e-07	4.392e-07	1.406	0.160
Summer: '816'	2.029e-06	4.392e-07	4.621	**<0.001**
Summer: 'A4'	1.418e-06	4.392e-07	3.230	**0.001**

Bolded P values are statistically significant at $P < 0.05$. SE represents standard error and t represents t-value.

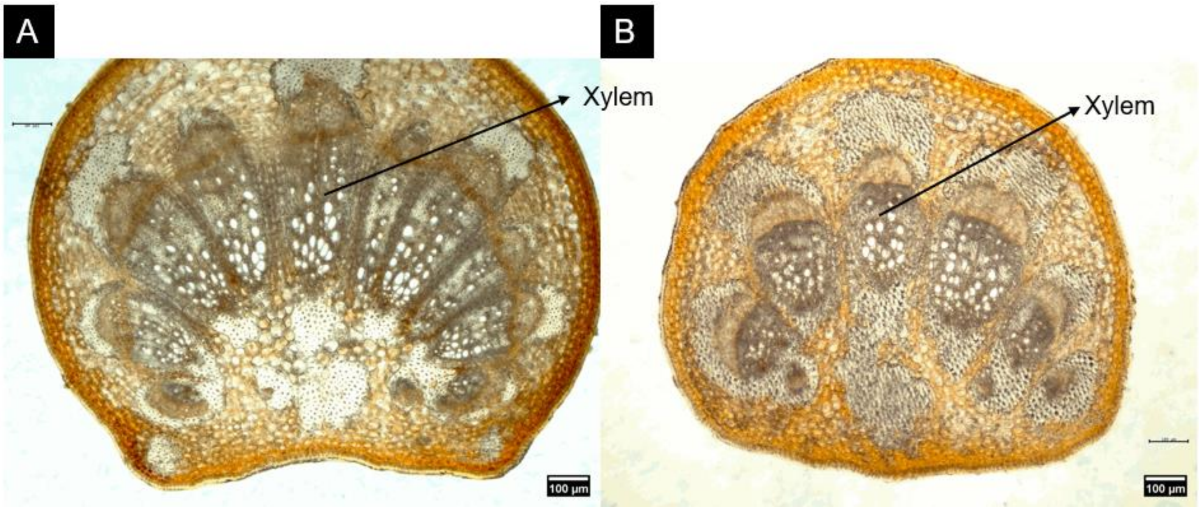

Figure 4.4 Cross-sections of petioles of macadamia cultivars A- ‘814’ and B- ‘A4’. Scale bar = 100 µm.

4.2.5 Vein density

At the Bridelia farm (Table 4.2), ‘842’ exhibited higher vein density than ‘814’ and ‘695’, but not statistically significant (*P= 0.709*). Moreover, summer flush leaves had higher vein density than spring flush, but not statistically significant (*P= 0.935*). For ‘842’ leaves from the Sommerreg and Jacaranda farms (Table 4.3), sun leaves had significantly higher vein density than shade leaves (*P = 0.007*), while summer flush leaves also had significantly higher vein density (*P = 0.031*). Furthermore, leaves from the Sommerreg farm had significantly higher vein density than leaves from the Jacaranda farm.

Among multiple farms (Table 4.8), leaves of ‘695’ had significantly higher vein density than ‘814’, ‘816’, and ‘A4’ (Figure 4.5). Sun leaves had significantly lower vein density compared to shade leaves. Furthermore, leaves from the Loerieroep had significantly higher vein density than leaves from the Sommerreg, Jacaranda, Valeen, and Outlook farms.

In conclusion, '695' consistently had higher vein density than '814', ‘842’, '816', and 'A4', with notable differences when compared to ‘814’ (Figure 4.5). Shade leaves consistently had higher vein density compared to sun leaves. Summer flush leaves consistently had higher vein density compared to spring flush leaves. Furthermore, the Loerieroep farm consistently exhibited higher vein density.

Table 4.8 Best selected Linear mixed model effects model summary on vein density in macadamia cultivars '695', 'A4', '814', and '816' across spring and summer vegetative flush at the Loerieroep, Sommerreg, Jacaranda, Valeen, and Outlook farms. '695', shade leaves, spring flush, and Jacaranda farm are the intercepts.

	Estimate	SE	*t*	*P* value
(Intercept)	0.01585	0.00129	12.236	**<0.001**
Sun leaves	-0.00201	0.00086	-2.336	**0.019**
Loerieroep	0.00814	0.00136	5.967	**<0.001**
Outlook	0.00633	0.00136	4.640	**<0.001**
Sommerreg	-0.00250	0.00136	-1.838	0.066
Valeen	0.00063	0.00136	0.466	0.641
Summer flush	-0.00325	0.00183	-1.777	0.076
'814'	-0.00079	0.00122	-0.648	0.517
'816'	-0.00151	0.00122	-1.240	0.215
'A4'	-0.00155	0.00122	-1.271	0.204
Sun leaves: Summer flush	0.00269	0.00122	2.202	**0.028**
Loerieroep: Summer flush	0.00493	0.00193	2.555	**0.010**
Outlook: Summer flush	-0.00646	0.00193	-3.349	**<0.001**
Sommerreg: Summer flush	0.00596	0.00193	3.091	**0.002**
Valeen: Summer flush	0.01083	0.00193	5.611	**<0.001**
Summer flush: '814'	-0.00317	0.00172	-1.835	0.067
Summer flush: '816'	-0.00584	0.00172	-3.381	**<0.001**
Summer flush: 'A4'	0.00158	0.00172	0.915	0.360

Bolded P values are statistically significant at $P < 0.05$. SE represents standard error and t represents t-value.

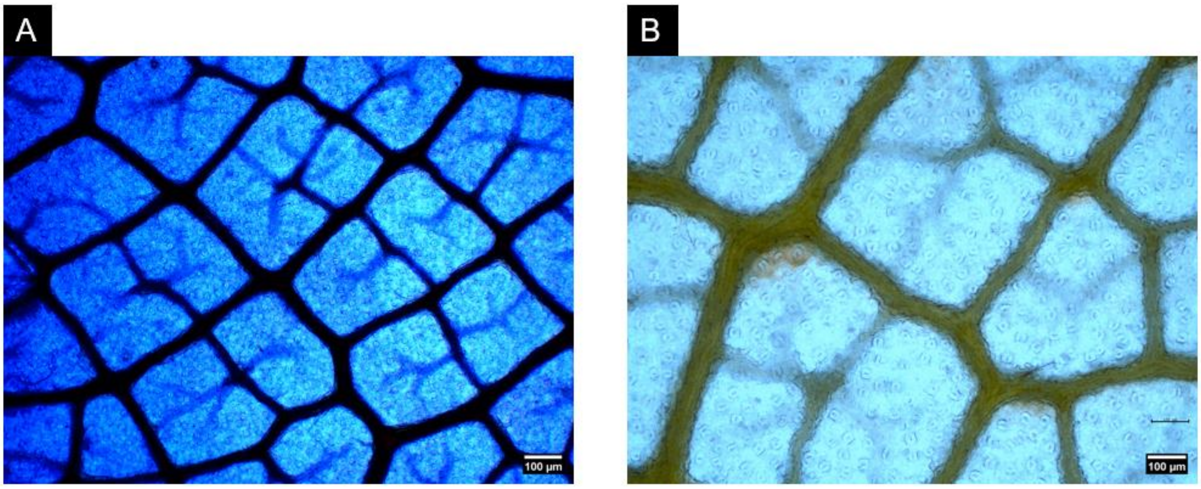

Figure 4.5 Veins on leaves of macadamia cultivars A- '695' and B- '814'. Scale bar = 100 µm.

4.3 Interactions between farms and macadamia cultivars

This section explored the interactions in stomatal density, palisade parenchyma, spongy parenchyma, Huber value, and vein density between multiple farms (Loerieroep, Sommerreg, Jacaranda, Valeen, and Outlook) and macadamia cultivars ('695', 'A4', '814', and '816'). This was done specifically on sun leaves because they are crucial for photosynthesis and transpiration due to sunlight exposure.

While non-significant interactions were observed in stomatal density (*P*= *0.785*, Figure 4.6) and spongy parenchyma (*P*= *0.11*, Figure 4.8), suggesting a limited impact of cultivar and farm location, significant interactions were observed in palisade parenchyma (*P*< *0.001*, Figure 4.7), Huber value (*P*< *0.001*, Figure 4.9), and vein density (*P*= *0.017*, Figure 4.10), indicating an influence of both cultivar and farm location on these leaf and petiole anatomical traits.

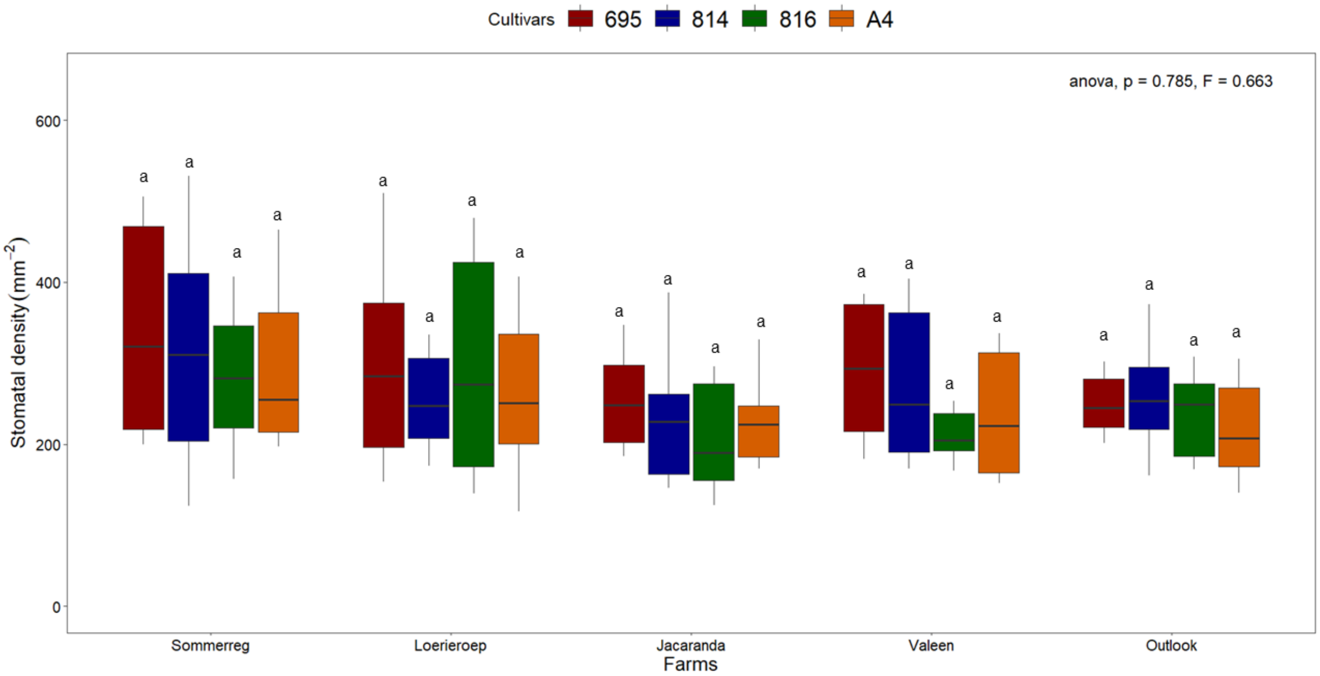

Figure 4.6 Macadamia leaf stomatal density on sun leaves across farms and macadamia cultivars during the spring flush. Box plots with the same letter are not significantly different at *$P < 0.05$*.

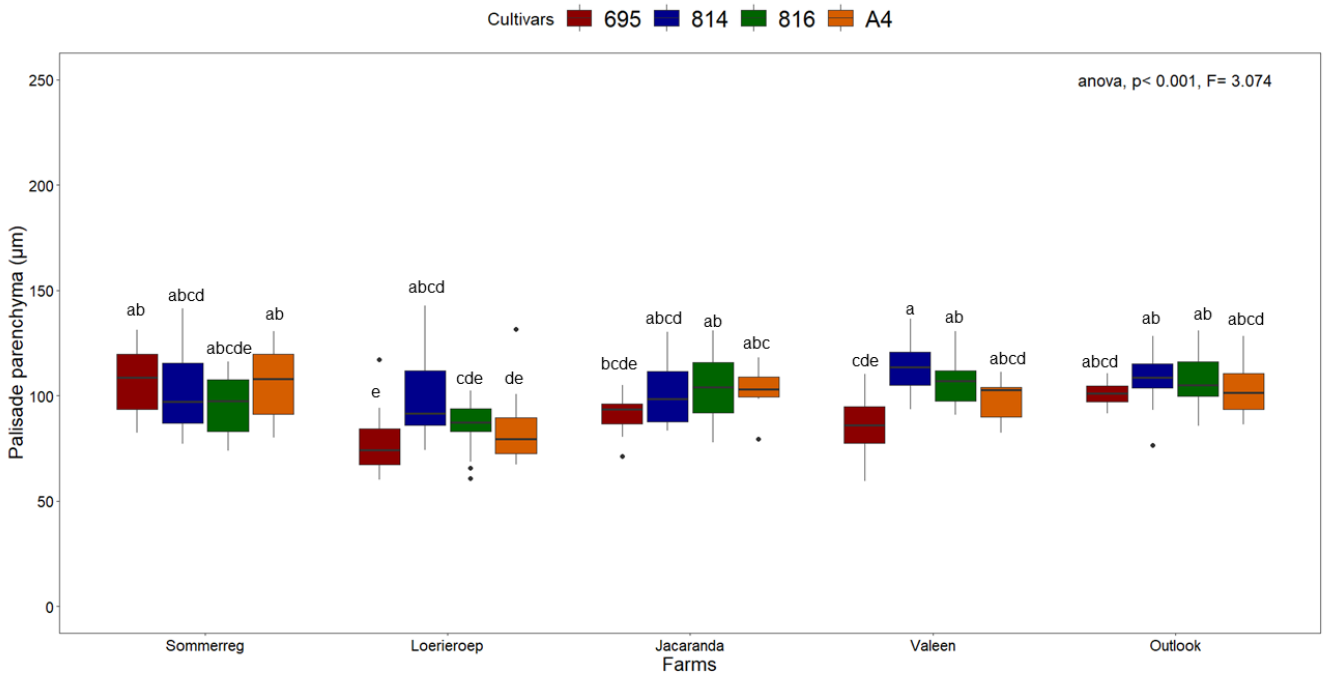

Figure 4.7 Macadamia palisade parenchyma thickness on sun leaves across farms and macadamia cultivars during the spring flush. Box plots with the same letter are not significantly different at *P < 0.05*.

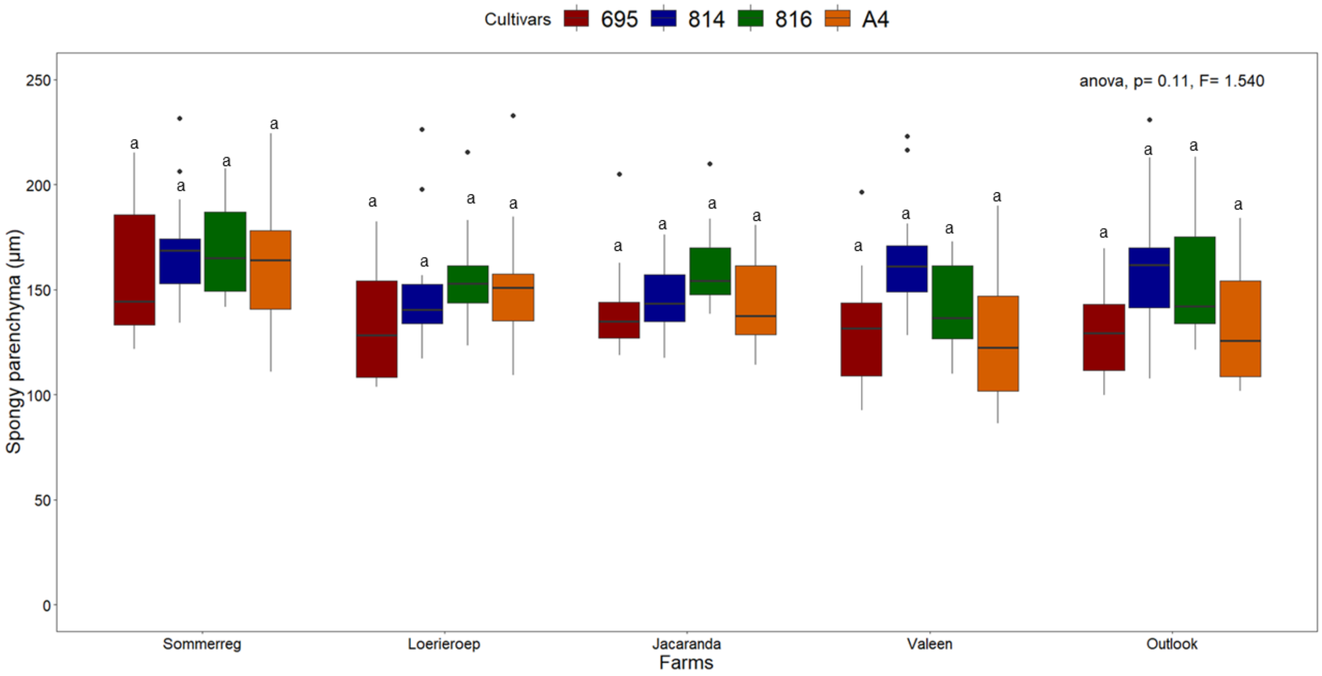

Figure 4.8 Macadamia spongy parenchyma thickness on sun leaves across farms and macadamia cultivars during the spring flush. Box plots with the same letter are not significantly different at *P < 0.05*.

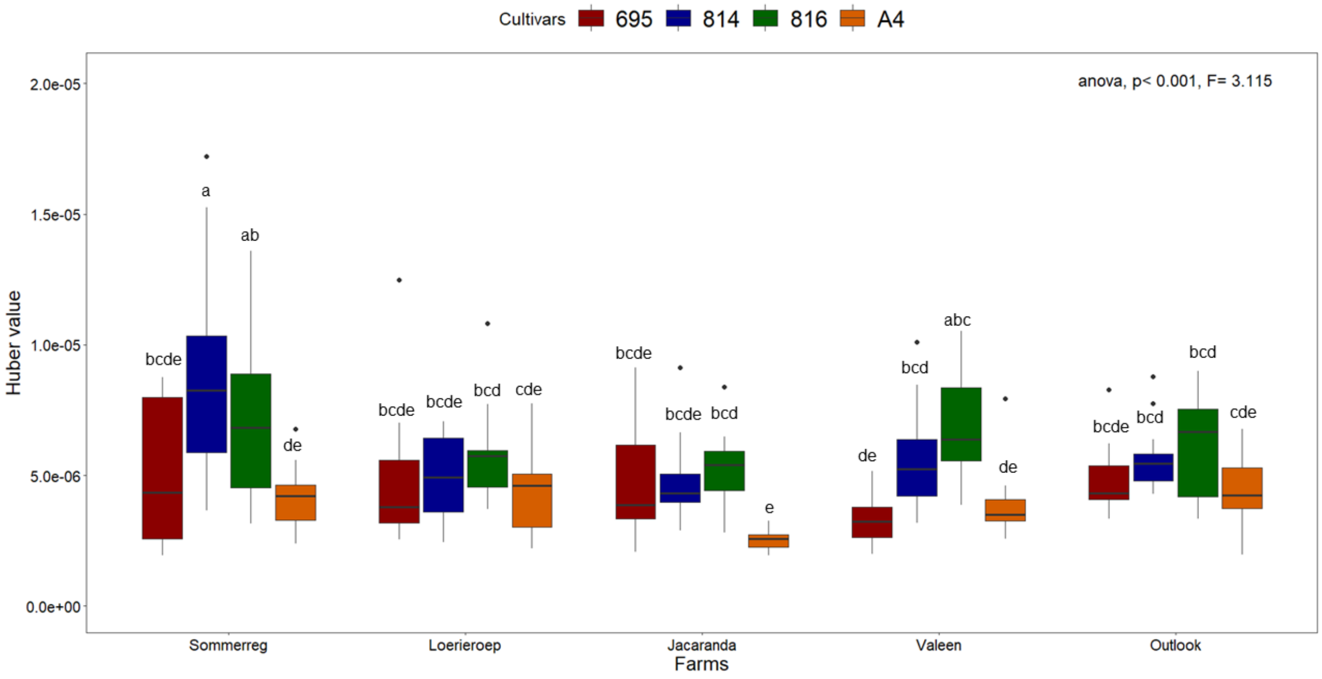

Figure 4.9 Macadamia Huber values on sun leaves across farms and macadamia cultivars during the spring flush. Box plots with the same letter are not significantly different at *P < 0.05*.

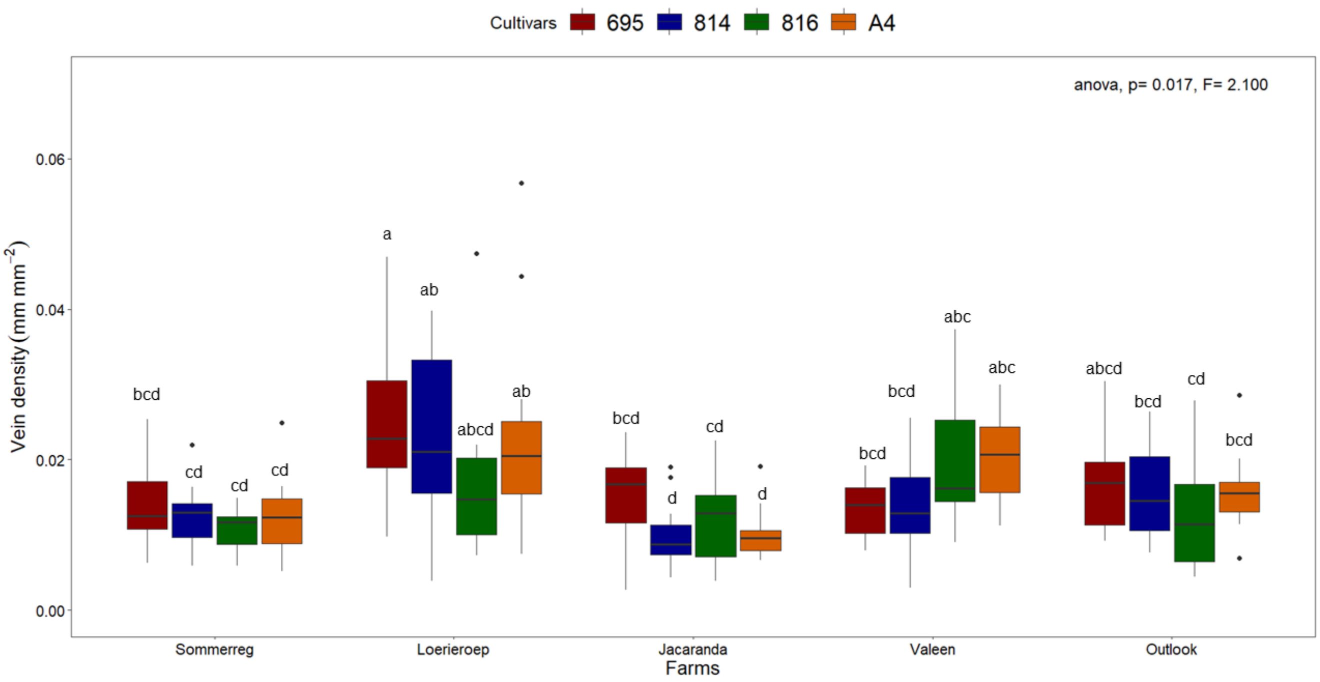

Figure 4.10 Macadamia vein density on sun leaves across farms and macadamia cultivars during the spring flush. Box plots with the same letter are not significantly different at *P < 0.05*.

CHAPTER 5: DISCUSSION AND CONCLUSIONS

In this study, we investigated the leaf and petiole anatomical traits of different macadamia cultivars across different regions in South Africa. The main aim of this study was to assess the variability in leaf and petiole anatomy among cultivars of macadamia trees in South Africa. The current findings suggest that leaf and petiole anatomy vary significantly between macadamia cultivars but that these differences are not consistent and are strongly influenced by the local environment. Furthermore, leaf and petiole anatomy also differed significantly between sun and shade leaves, as well as between leaves from the spring and summer flushes. These findings highlight the variation in macadamia tree physiology between cultivars and the environmental conditions.

5.1 Stomatal density

Notably, ‘814’ consistently displayed the highest stomatal density compared to ‘816’, ‘842’, ‘695’, and ‘A4’. This suggests that ‘814’ has the potential to be more drought tolerant than the other cultivars. The average stomatal density values obtained in our study align with those of other macadamia cultivars, such as ‘Keaau’, ‘Mauka’, and ‘Makai’ (Stephenson et al., 1989), as well as ‘WJMAS-29’, ‘Beaumont’, and ‘Daddow’ (Wakefield et al., 2021). The observed higher stomatal density in sun leaves corresponds to other situations for typical sun leaves in comparison to shade leaves, aiding in leaf cooling (Hill et al., 2014). Typically, sun leaves exhibit higher stomatal density and have smaller stomatal size to prevent excessive water loss during transpiration (Dickison, 2000; Dittberner et al., 2018). However, in our study, the relationship between stomatal density and stomatal size deviates from this pattern, suggesting that other factors, such as genetic factors, may have contributed to the variability in stomatal size.

Furthermore, our results revealed that summer flush leaves consistently had higher stomatal densities compared to spring flush leaves. This trend aligns with studies by Ferris et al. (1996) and Pandey et al. (2007), emphasizing the influence of elevated temperatures during the summer vegetative flush on stomatal density. The observed increase in stomatal density during warmer conditions suggests an adaptive response, aiding the regulation of leaf temperatures in macadamia trees. The consistent high stomatal density from the Sommerreg farm, characterized by higher vapour pressure deficit (VPD) and total reference evapotranspiration (ET_o), indicates a response to drier conditions. Higher stomatal density under drier atmospheric conditions may improve the external supply of carbon dioxide essential for photosynthesis and allow better control of transpiration in macadamia trees (Ennajeh et al., 2010).

5.2 Palisade and spongy parenchyma

The consistently thicker palisade and spongy parenchyma observed in ‘814’ compared to '695', ’842’, '816', and 'A4', suggests a potential that ‘814’ has the potential to be more drought tolerant than the other cultivars. Palisade parenchyma enables light penetration to the chloroplasts, while spongy parenchyma improves light capture and gaseous exchange (Evans, 1989), making them crucial photosynthesis tissues (Borsuk et al., 2022). Thicker palisade and spongy parenchyma in sun leaves, in comparison to shade leaves, indicate the potential for increased photosynthetic efficiency in leaves of macadamia leaves exposed to sunlight.

Variations in palisade and spongy parenchyma observed between spring and summer flush leaves suggest an adaptive response to the changing environmental conditions. The thickening of palisade parenchyma during the summer flush may enhance photosynthetic activity in macadamia trees under elevated temperatures (Xu et al., 2020). However, the thinner spongy parenchyma observed in summer flush leaves could be a strategic adaption by macadamia tree, to balance the trade-off between gaseous exchange and water conservation due to warmer conditions. Consistent thicker palisade and spongy parenchyma observed on leaves from the Sommerreg farm may also be an adaptive strategy employed by macadamia trees in response to potentially drier conditions in that region. This increases the chloroplasts available for carbon dioxide assimilation per unit leaf area and enhances the diffusion of carbon dioxide through the intracellular spaces, maintaining higher photosynthesis rates in macadamia trees, under water stress conditions (Ennajeh et al., 2010; Guerfel et al., 2009).

5.3 Huber value

The consistently higher Huber values observed in ‘814’ compared to '695', ‘842’, '816', and 'A4' indicate a potential that ‘814’ has the potential to be more drought tolerant than the other cultivars. Our findings resonate with previous studies (Moretti et al., 2019; Schultz & Matthews, 1993), with sun leaves consistently exhibiting higher Huber values. This adaptive response in macadamia trees suggests an adaptive mechanism for optimizing water use efficiency in response to the increased demand for water caused by sunlight exposure.

Our study revealed that leaves from the summer flush consistently had lower Huber values compared to leaves from the spring flush. This unexpected observation suggests a unique response of macadamia trees to seasonal changes. Lower Huber values in summer flush leaves could be a strategic response to the higher evaporative demand associated with warmer conditions during the summer flush, suggesting that macadamia trees may prioritize water-saving over increased water transport during the warmer periods. The higher Huber values at the Sommerreg farm align with the research findings by Oyanoghafo et al. (2021), suggesting that macadamia trees at the Sommerreg farm have developed an adaptive strategy in response to the increased evaporative demand due to high vapour pressure deficit and total reference evapotranspiration.

5.4 Vein density

The consistently higher vein density values observed in ‘695’ compared to '814’, ’842’, '816', and 'A4' indicate that ‘695’ has the potential to be more drought tolerant than the other cultivars. While previous literature report higher vein density in sun leaves compared to shade leaves (Martins et al., 2014), our results revealed the opposite trend, suggesting that a unique response of macadamia trees to light availability. Furthermore, higher vein density in summer flush leaves might be a response to elevated temperatures as suggested by Hu et al. (2014), enhancing water transport. The unexpectedly high vein density in the Loerieroep farm compared to the Sommerreg farm contradicts typical patterns observed in many studies where higher vein density was associated with arid or drier conditions (Dunbar Co et al., 2009; Lei et al., 2018; Zhu et al., 2012). This could have been influenced by other factors in the Loerieroep farm that were not investigated in our study.

5.5 Conclusions

Our results support our hypothesis that leaf and petiole anatomy will vary among different macadamia cultivars, different regions, between macadamia leaves from the sun and shade, and between spring flush leaves and summer flush leaves. However, the unexpected observations, for example, the lower Huber values in summer flush leaves and high vein density in Loerieroep require future research. Linear Mixed Models also revealed that the variability in leaf and petiole anatomy was greatly influenced by the leaf type and timing of vegetative flush. Additionally, the specific farms and cultivars also influenced the observed variability. Significant interactions were observed among different macadamia cultivars and farms, however, there were no consistent trends during the shift from arid regions to more humid regions. This implies that the diversity in leaf and petiole anatomy is due to a complex interplay between genetic factors and the unique characteristics of each farm rather than a uniform set of environmental conditions.

'814' stood out as the macadamia cultivar with leaf and petiole anatomical traits indicative of enhanced drought tolerance, as adaptations such as increased stomatal density, thicker palisade and spongy parenchyma, and higher Huber values indicate the ability to withstand water stress conditions. As a result, '814' could be a promising cultivar to grow in South Africa. The leaf and petiole anatomical traits investigated in this study could potentially be used as indicators to select drought-tolerant cultivars. Furthermore, these findings have shed light on differences in potential transpiration rates between macadamia cultivars and have implications for improving water management strategies in macadamia cultivation.

Beyond South Africa, the findings have implications for the global macadamia industry. The identification of cultivars with improved water used efficiency and drought tolerance can help macadamia growers in areas experiencing water scarcity. This study does not only add to academic knowledge, but also practical solution and opportunities for innovation in macadamia industry. Using the knowledge gained from this research, stakeholders can work towards a more efficient and sustainable future for macadamia cultivation, both locally and globally

5.6 Study limitations and future research

While our study provides valuable insights into the leaf and petiole anatomy of macadamia cultivars, some limitations may have impacted the findings. Our study limitations include not sampling sun and shade leaves during the spring flush at the

Bridelia farm, the absence of '842' at the Loerieroep, Valeen, and Outlook farms, and the sampling of leaves from random trees in the two sampling seasons. However, in-depth statistical analysis and the use of linear mixed models gave us rich and meaningful data. Recognizing these limitations allows us to improve future studies and increases the overall impact of our findings.

This study provides a solid foundation for future research, paving the way for future studies to investigate the molecular mechanisms underlying anatomical variations and their genetic basis. However, there is a need for a more direct focus on the complex relationship between genetic factors and environmental factors influencing leaf and petiole anatomy. There is a need for investigating the molecular mechanisms that control leaf and petiole anatomical trait development and gene expression patterns causing these anatomical variations. Conduct field trials in different regions in South Africa to evaluate the performance of the identified drought-tolerant cultivar ('814'), and track its growth, yield, and overall resilience under various environmental stresses. Furthermore, the study of leaf and petiole anatomy should be extended to other nuts trees, for a better understanding of comparative leaf and petiole anatomy.

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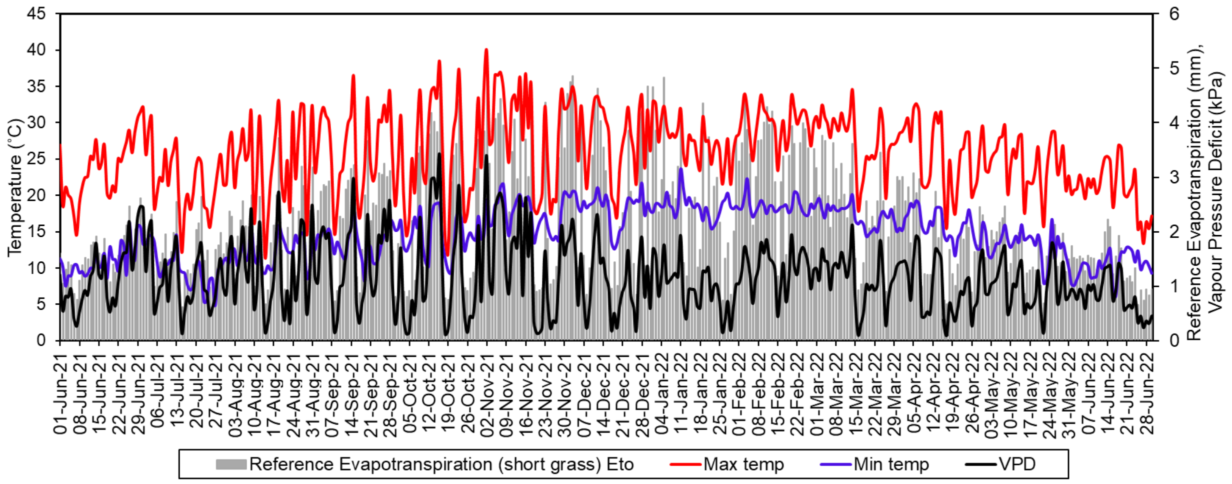
APPENDICES

Appendix A H.G.W.J. Schweickerdt Herbarium accession numbers for macadamia cultivars from the different farms.

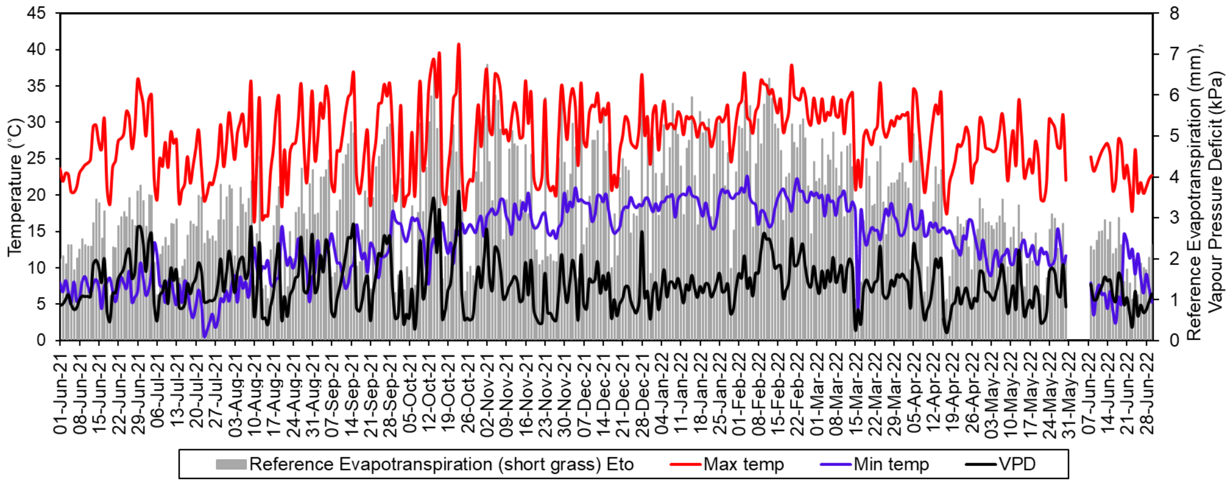
Region	Farm	Cultivar	Accession number
Levubu	Bridelia	‘695’	128815
		‘814’	128817
		‘842’	128816
Nelspruit	Loerieroep	‘695’	128821
		‘814’	128819
		‘816’	128818
		‘A4’	128820
Komatipoort	Sommerreg	‘695’	128824
		‘814’	128822
		‘816’	128826
		‘842’	128823
		‘A4’	128825
Kwa-Zulu Natal South Coast	Valeen	‘695’	129024
		‘814’	129025
		‘816’	129026
		‘A4’	129022
	Outlook	‘695’	129014
		‘814’	129016
		‘816’	129015
		‘A4’	129013
	Jacaranda	‘695’	129012
		‘814’	129018
		‘816’	129019
		‘842’	129020
		‘A4’	129017

Appendix B Tree ages for macadamia cultivars from the different farms.

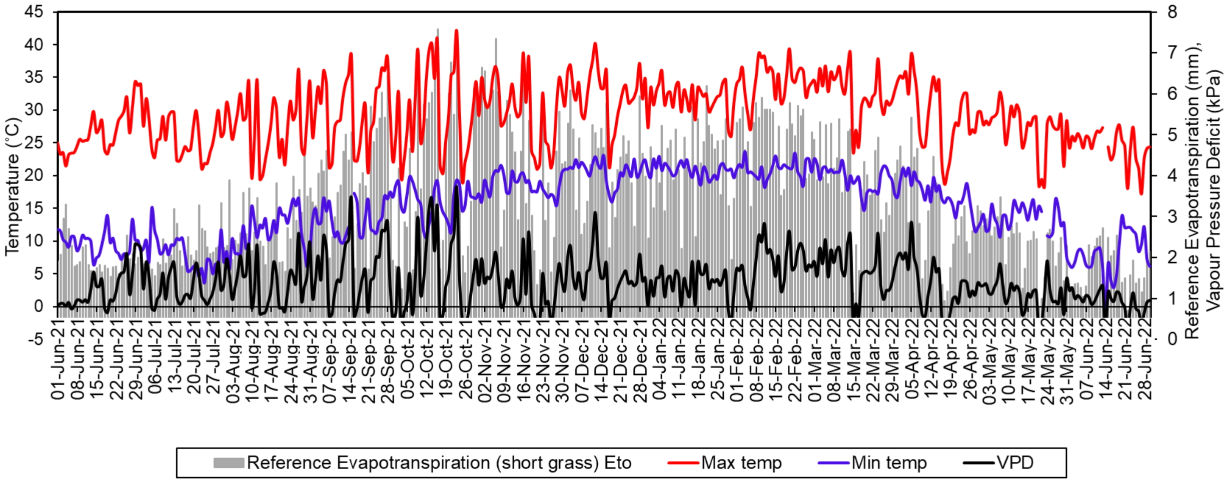
Region	Farm	Cultivar	Tree age
Levubu	Bridelia	‘695’	16 years
		‘814’	16 years
		‘842’	7 years
Nelspruit	Loerieroep	‘695’	15 years
		‘814’	8 years
		‘816’	8 years
		‘A4’	4 years
Komatipoort	Sommerreg	‘695’	6 years
		‘814’	2 years
		‘816’	2 years
		‘842’	2 years
		‘A4’	6 years
Kwa-Zulu Natal South Coast	Valeen	‘695’	20 years
		‘814’	6 years
		‘816’	8 years
		‘A4’	8 years
	Outlook	‘695’	12 years
		‘814’	5 years
		‘816’	7 years
		‘A4’	8 years
	Jacaranda	‘695’	15 years
		‘814’	13 years
		‘816’	13 years
		‘842’	8 years
		‘A4’	9 years



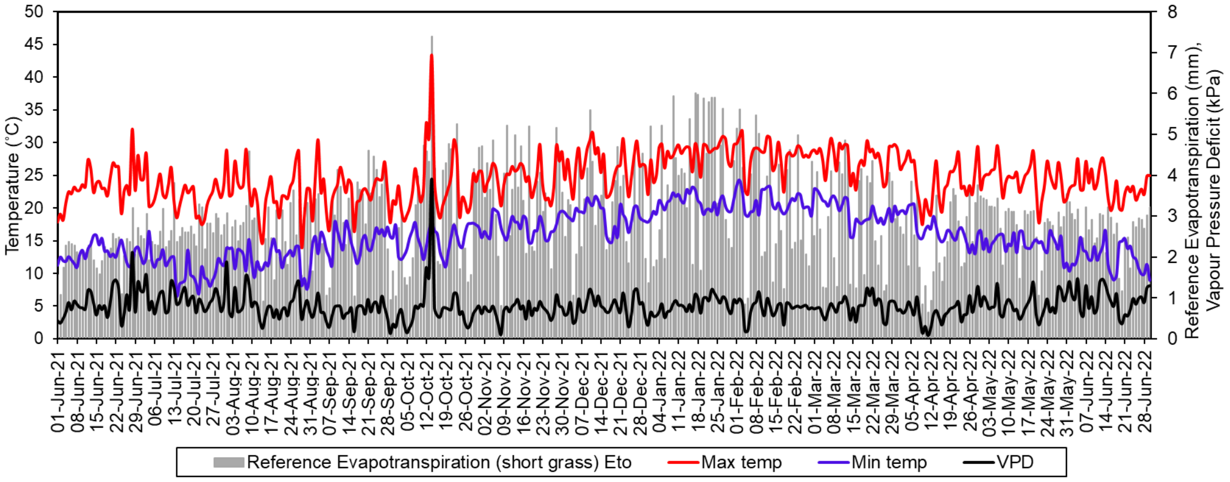
Appendix C Maximum and minimum daily air temperatures, vapour pressure deficit, and reference evapotranspiration (ET_o) from the variables measured by CITIMBA automatic weather station in the Levubu region from June 2021 to June 2022. Data source: ARC.



Appendix D Maximum and minimum daily air temperatures, vapour pressure deficit, and reference evapotranspiration (ET_o) from the variables measured by ITSG automatic weather station in the Nelspruit region from June 2021 to June 2022. Data source: ARC.



Appendix E Maximum and minimum daily air temperatures, vapour pressure deficit, and Reference evapotranspiration (ET$_o$) from the variables measured by AMANXALA automatic weather station in the Komatipoort region from June 2021 to 30 June 2022. Data source: ARC.



Appendix F Maximum and minimum daily air temperatures, vapour pressure deficit, and Reference evapotranspiration (ET$_o$) from the variables measured by SOUTHBROOM automatic weather station in the KwaZulu-Natal south coast region from June 2021 to June 2022. Data source: ARC.