Developing propagation methodology and determining seasonal variation and anti-bacterial activity for a cosmeceutical species, *Leucosidea sericea*

by

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DECLARATION

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DEDICATION

I dedicate this dissertation to my loving family, mother, daughter, fiancé, sisters, aunts, brothers and grandparents.

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ABBREVIATIONS

ANOVA:	analysis of variance
C:	control
DMRT:	Duncan's Multiple Range Test
CAGR:	compound annual growth rate
CTFA-SA:	Cosmetics, Toiletry, and Fragrance Association of South Africa
DMSO:	dimethyl sulfoxide
FDA:	food and drug administration
FTIR:	fourier transform infrared spectroscopy
GC-MS:	gas chromatography-mass spectrometry
GehA:	glycerol-ester hydrolase A
Hrs:	hours
^{1}H-NMR:	proton nuclear magnetic resonance
IBA:	Indole butyric acid
LC:	leaching
LC-MS:	liquid chromatography-mass spectrometry
LC-DADMS:	liquid chromatography-photodiode array detection-mass spectrometry
LSD:	least significance difference
M:	molar
MHz:	megahertz
MIC:	minimum inhibitory concentration
mg:	milligrams
mL:	millilitres
NAA:	1-naphthaleneacetic acid
nm:	nanometres
NMR:	nuclear magnetic resonance
OD:	optical density
OPLS-DA:	orthogonal partial least square discriminatory analysis
PCA:	principal component analysis
PPM:	parts per million
RCBD:	randomised complete block design
RPM:	revolutions per minute
SAS:	statistical analysis system
SC:	soaking cold
SH:	soaking hot

SM:	smoking
TSP:	trimethylsilylpropionic acid sodium salt
UNISA:	university of South Africa
µg:	micrograms
U.S.:	United states

ABSTRACT

The aim of this study was to determine propagation methodology of *L. sericea* taking into consideration the effect of season and storage. The response of tip and basal cuttings of *L. sericea* to three hormonal treatments and three growth media was investigated. Seed germination response to five constant temperatures and four pre-sowing treatments was also investigated. Using NMR spectroscopy, the effects of season and storage on metabolites in leaves of *L. sericea* were investigated. All treatments did not initiate or improve rooting of *L sericea* cuttings. Seed germination percentage was significantly affected by temperature and pre-sowing treatment compared to control. The optimum seed germination percentage was obtained at 15°C (53%) when treated with smoke water. Seasonal variation affected leaf metabolite profiles. Storage did not affect the antibacterial activity of *L. sericea* leaves and those harvested in winter showed an MIC value of ≤3.90µg/mL when tested on *Propionibacterium acnes*.

CHAPTER ONE

INTRODUCTION

1.1. GENERAL INTRODUCTION

The word “cosmeceutical” refers to a dermatological term that was introduced by Prof Albert Kligman at a society of cosmetic chemists’ meeting about 35 years ago, yet, to date there is no clear definition of the term “cosmeceutical” due to the absence of proper regulatory climate or framework regarding product claims associated with it (Vermeer *et al*., 1996; Gao *et al*., 2008; Draelos, 2009). In basic terms, cosmeceuticals are functional genomics meaning the ingredients of a cosmetic must come from safe raw material (Draelos, 2009), and these ingredients must not only act as a cosmetic but must also provide medicinal or drug like benefits (Lintner *et al*., 2009). A cosmetic is defined as “any article intended to be rubbed, poured, sprinkled or sprayed on, or introduced into, or applied to any part of the human body, for cleaning, beautifying, promoting attractiveness or altering the appearance and includes any article intended for use as a component of cosmetic” (Kuchekar, 2004). A drug is defined as “all medicines proposed for internal or external use of human beings or animals and all substances intended to be used for; or in the diagnosis, treatment, mitigation or prevention of any disease or disorder in human beings or animals” (Kuchekar, 2004). Cosmeceuticals are not legally recognised products in certain countries such as Thailand (controlled cosmetics), Hong Kong (cosmetic-type drugs) and Japan (Quasi-drug) (Dureja *et al*., 2005). However, in South Africa there is an appropriate regulatory system for cosmetics which mandates that cosmetic manufacturers must comply with the requirements of the Standards Act 29 of 1993 published by the South African Cosmetics, Toiletry, and Fragrance Association (CTFA-SA).

Although there is a lot of regulatory discrepancies about the fact that cosmeceuticals contain both health and beauty benefits (Newburger, 2009; Salvador and Chisvert, 2011), there is currently a very lucrative global cosmeceutical market that turned over US$37.9 billion in 2014 and is expected to reach US$57.3 billion by 2019, growing at a compound annual growth rate (CAGR) of 8.62% (Elsner and Maibach, 2005; Gao *et al*., 2008; Epstein, 2009).

Due to the fact that plant extracts are considered to be safe by the U.S. Food and Drug Administration (FDA) the plant kingdom provides the cosmeceutical industry with safe ingredients (Levin and Momin, 2010). Due to the ability of plants to survive in harsh environmental conditions which in turn makes them rich in endogenous anti-oxidants.

For this reason, plant parts such as roots, leaves, barks, fruits, stems, and flowers are widely collected for use as cosmeceutical ingredients.

In South Africa, there is a drive towards developing products from indigenous plants. According to Lall and Kishore (2014), there is a growing interest in the health benefits of plants grown in South Africa with regard to skin care. Therefore, there is great opportunity in the skin care industry to develop products such as cosmeceuticals, and by so doing improving the economy of the country. However, the main challenge of sustaining such industries is the lack of propagation methodologies for indigenous plant species especially those less studied in South Africa.

Plant propagation started some 10,000 years ago when ancient people who lived by hunting and gathering, began to cultivate plants and domesticate animals (Hartmann, 2011; Van Wyk, 2011). Plant propagation in its broad sense is defined as the multiplication and reproduction of plants by both sexual and asexual means (McMahon *et al*., 2002). Palgrave *et al*. (2002) added that plant propagation, preserves and at the same time, diversify the unique qualities of the mother plant. Sexual propagation is not a method aimed at producing more seed, but the primary goal rather, is to produce more plants, although more seeds are produced in the process (Hartmann, 2011). Compared to asexual propagation, sexual propagation is relatively inexpensive and large number of plants can be produced. However, plants grown from seeds tend to take longer to reach maturity as compared to plants produced using asexual or vegetative methods. The desirable factor about asexual or vegetative propagation methods is the reduced variability in new plants, because vegetative material used is the exact replica of the mother plant (Joffe, 2003).

Sharma *et al*., (2014) reported excellent anti-bacterial activity on crude *L. sericea* extract against *P. acnes* with MIC values of ≤15.62µg/Ml. Even better MIC values were obtained when alpha kosin was isolated from this plant as it was found to inhibit *P. acnes* at ≤1.95µg/mL. In light of this information, the main aim of this study was to determine optimum propagation methodology for this plant and to determine the effect of season and storage on the anti-bacterial activity of *L. sericea* leaves. Due to the fact that there are no previous scientific studies on the best or optimum propagation methodology for *L. sericea,* this study was therefore deemed necessary as large quantities of leaf material will be needed for development of cosmeceuticals. In this

study, a propagation methodology was developed to produce *L. sericea* plants on a large scale. Both the vegetative and sexual propagation methodologies were investigated by using seeds and cuttings of *L. sericea.* Both basal and apical cuttings were investigated and the nut-like fruit or seeds of *L. sericea* were used for sexual propagation.

1.2. PROBLEM STATEMENT

Leucosidea sericea is widely used as an ornamental plant in urban settings whereas in the rural areas it is used mainly for its medicinal properties. In a previous study, *L. sericea* was found to have anti-bacterial properties against *P. acnes* (Sharma *et al*., 2014). For cosmeceutical product development, propagation methodology is required so that *L. sericea* can be harvested sustainably to avoid over harvesting and possible eradication or in extreme cases extinction from its natural habitat.

Currently there is no known methodology available to propagate *L. sericea* on a large scale and how this plant is affected by propagation and external cultivation. In this study, a methodology was developed to propagate *L. sericea* on a large scale with the focus on seed germination and cutting production, and to determine seasonal variation by employing metabolomics to develop a chemical profile to prevent compromising the efficacy of *L. sericea* extracts on *P. acnes*. Previous studies on *L. sericea* by Sharma *et al*. (2014) reported that this plant has anti-inflammatory, anti-bacterial and anti-oxidant activities. Four known and one new compound were isolated for the first time from *L. sericea* leaves (Sharma *et al*., 2014). Electron microscopy studies proved that *L. sericea* plant extract had lethal effects on the cells of *P. acnes.* In order to produce cosmeceutical products, this study was undertaken to ensure that the anti-bacterial activity is not lost with propagation and season. The current study investigated the best propagation methodology taking into consideration the effect of season and storage on the active compounds.

1.4. SIGNIFICANCE OF THE STUDY

For cosmeceutical product development, large quantities of plant material are needed, which cannot be sustainably harvested from nature. To determine different

propagation techniques on *L. sericea* and to identify the best propagation methodology will therefore ensure that the plant material can be produced sustainably on a large scale to satisfy the demand for product development. Determination of the optimum season for harvesting *L. sericea* leaves will ensure that the plant material is harvested when the active compounds are optimum in the leaves to ensure effective products against *P. acnes*. The storage time for powdered *L. sericea* leaves at room temperature was determined, by determining the anti-bacterial activity of stored leaves over different storage periods.

1.5. RESEARCH QUESTIONS

What is the best method for germinating *L. sericea* seeds?
Can *L. sericea* be propagated from cuttings?
How does season affect the growth and cosmeceutical properties of *L. sericea*?
How long can ground powdered *L. sericea* leaves be stored before anti-bacterial activity is affected?

1.6. RESEARCH OBJECTIVES

To determine the effect of different treatments such as smoking, leaching, soaking and temperature on the germination of *L. sericea* seeds.
To determine the effect of growth media and growth hormones such as Indole butyric acid (IBA), seradix no.1 and seradix no.2 on *L. sericea* cuttings.
To employ metabolomics to determine the effect of seasonal variation on the quality and the chemical composition of metabolites in *L. sericea*.
To perform biological assays to determine the effect of seasonal variation on the activity of the extracts.
To determine the length of time that ground *L. sericea* leaves can be stored using biological assays.

1.7. STUDY LAYOUT

This study is divided into seven chapters. Chapter one outlines the introduction including the objectives of the study, chapter two comprises of the literature review on

plant propagation methods (sexual and vegetative), seeds pre-sowing treatments, plant hormones, biological assays, metabolomics and factors that influence plant propagation. Chapter three and four presents the results on both vegetative and sexual propagation methods respectively. Chapter three presents result on the effect of growth media, cutting type and hormones on cuttings of *L. sericea* while chapter four presents effect of pre-sowing treatments and different temperatures on seed germination of *L. sericea.* Chapter five presents the anti-bacterial assay analysis and the effect of storage and season on the *L. sericea* leaves in relation to biological activity against *P acnes*. Chapter six presents the effect of season on the metabolomic profile of *L. sericea* leaves harvested in different seasons and chapter seven provides concluding remarks, a general discussion and recommendations for commercial production of *L. sericea*.

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CHAPTER TWO

LITERATURE REVIEW

2.1. INTRODUCTION

Plants have been used by rural communities as food, medicine, cosmetics and as building material for centuries (Van Wyk *et al*., 1997). There is an increased interest in the non-timber forest products specifically for use in medicine and cosmetics. Africa, especially Southern Africa, has rich plant diversity (Van Wyk *et al*., 1997). Many of these plants are of ethnobotanical significance are still used by local rural communities as medicine and food (Wang *et al*., 2004). Plants are a source of fuel, building material, craft material, dyes, food supplements and medicine all over the world (Van Wyk *et al*., 1997). For this reason plant propagation is a fundamental occupation to humankind (Hartmann, 2011) so that sufficient plant material can be harvested sustainably to meet all the needs. Hartmann (2011) and Van Wyk (2011) stated that agriculture began some 100,000 years ago and that the discovery of plant propagation is now referred to as civilization and that it initiated human domination over the earth. Ancient people, who lived by gathering and hunting, began to cultivate plants and domesticate animals around this period. These activities centred on stable communities and people began to select and propagate the kinds of plants that provided a greater and more convenient supply of food and perhaps other products for themselves and their animals (McMahon *et al*., 2002).

The relatively little research that has been conducted on plants indigenous to South Africa which has around 30 000 flowering plant species (Louw *et al*., 2002), has focused mainly on their medicinal properties (Venter and Venter, 1996) and not their use in their various applications such as skin care. However, in view of the rapidly growing size of the global and local cosmetic industry and their constant search for new products, there exists significant research opportunities to discover potential scientific use of this plant species for commercial development in the cosmeceutical industry. Ethnobotanical studies indicate that 117 selected plant species indigenous to South Africa are traditionally used for skin ailments (Lall and Kishore, 2014) and of these 117, 89 plants have been scientifically explored for pharmacological activity against various skin ailments (Mapunya *et al*., 2012). The rest are still to be researched and validated (Lall and Kishore, 2014). So far, little scientific research has been done on *L. sericea* for any skin ailment. Examples of plant families and species already investigated for cosmetic purposes are provided in Table 2.1.

Table 2. 1: Some South African plants scientifically explored for skin care

Family name	**Number of plants used traditionally**	**Examples of species used traditionally requiring further analysis**	**References**
Agapanthaceae	1	*Agapanthus oppositifolia* Lam.	Duncan, 1998
Aizoaceae	1	*Galenia africana L.*	Van der Lugt *et al*., 1992
Aloaceae	7	*Aloe aculeta* Pole-Evans *Aloe arborescens* Mill *Aloe ferox* Mill *Aloe greatheadii* Schonland *Aloe pretoriensis* Pole-Evans *Aloe sessiliflora* Pole-Evans *Aloe vera* L. Burn. f.	Mapunya *et al*., 2012 Mapunya *et al*., 2012 Kambize *et al*., 2007; Fawole *et al*., 2010 Boetes *et al*., 2008 Mapunya *et al*., 2012 Mapunya *et al*., 2012 Mapunya *et al*., 2012
Amaryllidaceae	3	*Scadoxus puniceus* L. Friis & Nordal *Crinum moorei* Hook. f.	Adewusi and Steenkamp, 2011 Fawole *et al*., 2010
Anacardiaceae	3	*Anacardium occidentale* L.	Okoye *et al*., 2009
Apiaceae	1	*Centella asiatica* L. Bolus	Rahman *et al.,* 2013

Apocynaceae	3	*Acokantera oppositifolia* (Lam) Codd *Rauvolfia caffra* Sond *Xysmalobium undulatum* L. Aiton f.	Addedapo *et al*., 2008 Erasto *et al*., 2011 Steenkamp *et al*., 2004
Aquifoliaceae	1	*Ilex mitis* (L.) Radlk	Mabona *et al*., 2013
Araceae	1	*Zantedeschia aethiopica* Spreng	Watt and Breyer-Brandwijk, 1962
Arecaeae	1	*Elais guineensis* Jacq	Neo *et al*., 2008 and Sasidharan *et al*., 2010
Asteraceae	11	*Artemisia afra* Jacq. Ex Willd *Athrixia phylicoides* DC. *Calendula officinalis* L *Dicoma anomala* Sond *Eriocephalus africanus* L *Eriocephalus punctulatus* L	Burits *et al*., 2001 and More *et al*., 2012 De Beer *et al*., 2011; McGaw *et al*., 2007 and Joubert *et al*., 2008 Muley *et al*., 2009 Steenkamp *et al*., 2004 Njenga and Viljoen, 2006 Sandasi *et al*., 2011

Asphodelaceae	1	*Bulbine frutescens* L. Willd	Abegaz *et al*., 2002; Pather *et al*., 2011
Bignonaceae	2	*Kigelia Africana*	Jackson *et al.,* 2000; Picerno *et al*., 2005 and Olalye and Rocha, 2007
Canellaceae	1	*Warburgia salutaris* Bertol. f. Chiov.	Kuglerova *et al*., 2013
Chenopodiaceae	1	*Chenopodium ambrosioides* L.	Grassi *et al*., 2013
Crombretaceae	1	*Terminalia sericea* Burch. Ex DC.	Nkobole *et al*., 2011
Cucurbitaceae	2	*Cucumis hirsutus* Sond.	Fawole *et al*., 2009
Cyperaceae	1	*Cyperus textilis* Thunb.	Nadkarni, 1976
Dryopteridaceae	1	*Polystichum pungens* Roth.	Jacobsen, 1983
Ebenaceae	2	*Diospyros lycioides* Desf. *Diospyros mespiliformis* Hochst. ex A.DC.	Fawole *et al*., 2009 Lamien-Meda *et al.*, 2008

2.2. BACKGROUND INFORMATION ON LEUCOSIDEA SERICEA (OLD WOOD)

Leucosidea sericea Eckl. & Zehy. is an evergreen dense shrub (although some leaves may turn yellow in autumn) which grows up to 7 m tall to 5 m wide in South Africa. *Leucosidea sericea* belongs to the family Rosaceae which includes the genus *Rosa*, the rose, one of the best known and loved plants in the world (Pooley, 1993; Grant and Thomas, 2011). *Leucosidea sericea* belongs to the poorly represented Rosaceae family in South Africa which has only eight native tree species and *L. sericea* is the sole representative of the genus *Leucosidea* (Van Wyk., 1997; Aremu *et al*., 2011; Nair *et al*., 2012). *Leucosidea sericea* is commonly known as “oldwood” (English), “ouhout” (Afrikaans) and “umTshitshi” in Zulu. The tree is traditionally used as a charm to protect the inhabitants of homesteads. Pooley (1993) reports that *L. sericea* occurs in places where the vegetation has been disturbed due to overgrazing, cultivation,

severe veld fires or flooding, felling or other similar causes. In such cases, *L. sericea* poses a serious problem as it grows in dense thickets which are difficult to eradicate. Grant and Thomas (2011) recorded that the tree also occurs in mountainous areas near water at high altitudes 1000 m in parts of Africa such as Zimbabwe, Lesotho, and South Africa (KwaZulu-Natal, Gauteng, Eastern Cape, Free State and Mpumalanga). In traditional medicine, *L. sericea* is an important plant used to not only treat various ailments such as opthalmia, intestinal worm infection but it is also used as a vermifuge and astrigent. The safety of using the stems and leaves of *L. sericea* in traditional medicine was evaluated by Aremu *et al*. (2011) as well as the anti-oxidant activity, acetylchlolinesterase inhibition, iridoid content and mutagenic activities. Nair *et al*. (2012) conducted a study and isolated cholestane triterpenoids, β-sitosterol and β-sitostenone which have anti-inflammatory effects. Despite the frequent use of this plant in traditional medicine, there is very little research on cosmeceutical application of *L. sericea.* Sharma *et al.* (2014) conducted a study where the potential use of *L. sericea* against *P. acnes* was determined. It was reported that four compounds namely phytol acetate, triacontanol, phytol and alpha kosin were present in this plant and an additional new compound (E)-3,7,11,15-tetramethylheptadec-2-ene-1,17-diol was isolated for the first time. In the same study, the ethanol extract of *L. sericea* inhibited the bacterial growth at MIC values of ≤15.62µg/mL and alpha kosin was found to be the most active compound against *P. acnes* with MIC value of ≤1.95µg/mL, which was recorded to be better than that of tetracycline (drug control) (Sharma *et al*., 2014).

Figure 2.1: A young *L. sericea* tree growing in Gauteng (A) and a mature *L. sericea* tree (B) (photo taken by Sehlakgwe P F 2014)

2.3. PROPAGATION METHODS

Plants have an ability to regenerate using two major techniques (McMahon *et al*., 2002). According to McMahon *et al*. (2002) and Basra *et al*. (2005) these two propagation methodologies are known as asexual or vegetative and sexual propagation. Each of the propagation method has sub techniques and each technique has its own advantages and disadvantages.

2.3.1. Vegetative or Asexual Propagation by Cuttings

Vegetative or asexual propagation is the reproduction of plants which results in offspring identical in genotype to the mother plant (Hartmann, 2011). A cutting can be defined as the detached vegetative plant part capable of regenerating the missing organs, which will eventually develop into a mature plant with all the characteristics of the parent plant. The main advantage of using vegetative propagation over sexual is the genetic uniformity, the fixing of populations and shorter time to reach maturity (Hartmann, 2011). The uniformity of individual plants is a desirable factor in commercial production where consistency in the product quality is required. Major methods used in vegetative propagation are budding, grafting, cuttings and layering (Reiley and Shry, 2002). Of these methods, cuttings are the most widely used because it is economical, rapid and easy to propagate (Reiley and Shry, 2002).

2.3.1.1 Types of Cuttings

Cutting types are as many and divergent as the parts of the stock plant and can be produced from leaf cuttings, leaf-bud cuttings, softwood cuttings, semi-hardwood to hardwood cuttings (Hansen, 1988). Cuttings from new growth are referred to as softwood or semi softwood or apical cuttings and a cutting taken from the previous season's growth is referred to as a hardwood or basal cutting (Hartmann, 2011). According to Hansen (1986) basal cuttings produces the highest rooting percentage in many researched vegetatively propagated plants. This is supported by Erez and Yablowitz (1981) where hardwood cuttings of *Prunus persica* (peach) rooted better when the cuttings were taken when the wood was mature. However, the success of basal cuttings is not only evident in the rooting percentage, it was also found that leaf area per cutting is greater and the root length and weight increased in cuttings taken from mature wood (Hartmann and Kester, 1983; Al-saqri and Alderson 1996). The

success of basal cuttings in providing better rooting may be attributed to the bigger size of most basal cuttings and the species as it is dependent on provision of space for food reserves such as total sugars, natural auxins and other root promoting factors which may not be present in thinner and newer apical cuttings (Hartmann, 2011). However the success of rooting basal cuttings is species dependent, since in some species apical, cuttings root better than basal cuttings (Hansen, 1988).

Apical cuttings originate from the upper part of stock plants and they mostly have young developing leaves and an apex. The presence of an apex and young leaves may have two fold effects on the cutting, they may contain rooting promoters or they may have inhibitors (Hansen, 1988; Hartmann, 2011). The study conducted by Amri *et al*. (2010) indicated that cutting position has an effect on the rooting percentage, the number of roots, root length and the percentage of callusing and that basal cuttings rooted better than apical and medial cuttings. In support of this statement, rooting percentage in four African mahogany species was higher from apical and medial cuttings when compared to basal cuttings (Owusu *et al*., 2014). However, a study conducted by Al-Salem and Karam (2001) indicated to the contrary that basal cuttings of *Arbutus andrachne* (greek strawberry tree) exhibited the highest rooting percentage when treated with IBA.

2.3.2. Sexual Propagation

Sexual propagation is a methodology whereby a seed or spore already separated from the mother plant is utilised. Sexual propagation is one of the major methods by which plants reproduce in nature (Hartman, 2011). Sexual propagation is one of the most efficient and widely used methods of propagating cultivated or domesticated crops and plants produced from seeds. Young crops produced from the recent germinated seeds are referred to as seedlings.

Watson and Himelick (1997) reported that a definition for seed germination differs from one specialist in the field of agriculture to the other. A plant physiologist defines seed germination as the emergence of the radical through the seed coat whereas a seed analyst defines seed germination as the emergence and development from the seed embryo of those essential structures, which for the kind of seed in question are indicative of the ability to produce a normal plant under favourable conditions. For the

purpose of this study the emergence and development of a seedling to a stage where the aspect of its essential structures (root system, shoot axis, cotyledons, terminal buds) indicates whether or not it is able to develop further into a satisfactory plant under favourable soil conditions was used (ISTA, 2006).

Glass and Parker (2009) stated that seed dormancy is the resting condition with reduced metabolic rate until the environmental conditions become favourable to trigger germination and they further highlighted that seed dormancy plays an important role in plant propagation. Numerous studies have been done to research the effect that seed dormancy has on seed germination by looking into factors such as scarification, smoking, temperature, leaching and soaking, but so far, no research has been conducted on the growth and propagation of *L. sericea* using these methods.

After maturation, seeds remain inactive until favourable environmental conditions are met which initiates growth and development of the seed embryo (Baskin and Baskin, 2014). The manner in which seeds respond to environmental conditions is species-specific. Some seeds may respond to favourable environmental conditions within a few days whereas others may still lie dormant even when favourable conditions are met for years (Basra *et al*., 2005;Hartmann, 2011).

2.4. PLANT PROPAGATION UNDER CONTROLLED ENVIRONMENTS

Plant propagation under controlled environments involves the manipulation of the propagation microclimate, edaphic factors and the biotic factors (Hartmann, 2011). Microclimate, which refers to the atmospheric characteristics prevailing within a small space (Schlegel, 2010) has the greatest effect on the success of plant propagation. Shry *et al*. (2010) states that the characteristics that influence plant propagation the most are water, nutrients, growth medium, temperature, light, and humidity.

Microclimatic factors that influence plant propagation the most are temperature, light, relative humidity and gases (Hartmann, 2011). It is further reported that plants may be propagated in the field, outdoors, or in protected culture environments such as greenhouses, shade nets and incubators. In all these instances plants are influenced by various environmental factors such temperature light, water and wind. Previous studies show that plant growth is very sensitive to temperature extremes and that each plant has a set of optimum temperature requirements.

A greenhouse is a structure with temperature, ventilation, light, humidity and moisture control where plants can be propagated throughout the year (Shry *et al*., 2010; Hartmann, 2011). Greenhouses have played a major role in agriculture by providing horticulturalists with an opportunity to manipulate and manage the propagating environment which results in a more rapid growth of plants. Greenhouse structures vary from uncomplicated home-structures to intricate commercial installations that are used by farmers in the agricultural sector (Shry *et al*., 2010). For this study, incubators, greenhouses and shade nets were used for manipulating the environmental factors to develop the best propagation methodology for *L. sericea*.

The advantages of growing plants under controlled environments are: optimal temperature or growth and development, protection against excess radiation, wind, rainfall and hail and effective insect and disease control (Zabeltitz, 2011). It also states that the disadvantages of this system in agriculture are: very expensive structures, high labour requirement, high irrigation cost, training, planting containers, growth medium, artificial ventilation and heating.

For successful plant propagation, regardless of the method used, a certain set of environmental conditions must be met (Shry *et al*., 2010). Jefferson *et al*. (2014) reported that climatic fluctuations, fires, volcanoes and other extreme forces have an effect on plant existence, diversity and propagation. Previous studies have documented the unpredictability of these environmental factors and how they dictate to farmers the planting dates or season, type of crops or plants to be planted, harvesting periods and which ultimately influence the price of the produce, hence farm profits. Mankind has developed various methods of mitigating and in other instances of bypassing these effects, and this has given the agricultural industry control over the environmental factors and to an extend control over what to plant, when and how (Hartmann, 2002). Currently there is a variety of specialised equipment and structures in place that allows the agricultural industry to produce crops and/or plants throughout the year such as growth chambers, incubators, plant tissue culture equipment, shade nets, green and/or glasshouses (Nelson, 2011).

2.5. CLIMATIC CONDITIONS AFFECTING PLANT PROPAGATION AND GROWTH

2.5.1. Temperature

Plant growth is greatly influenced by temperature in the form of heat and cold (Van Wyk, 2011). Air temperature, temperature of the propagation medium and temperature of the irrigation water affects plant propagation in many ways. Of all environmental factors, temperature is the most critical factor affecting seed germination. Temperature determines the length of the growing season, and there is also maximum and minimum temperature which designates the limits for plant growth and development. Drewes *et al*. (1995) also reported that 25°C was the optimum temperature for germinating lettuce seed. However, that being said, optimum maximum and minimum germination temperature requirements are species-specific. Zhou *et al*., (2012) found that 10°C was optimum, for two species (*Astragalus membranaceus and Panax notoginseng)* and yet for another species (*Magnolia officinalis*) a range of 15-25°C provided the highest germination percentages. Aflakpui *et al*. (1998) reports that temperature affects the growth season length of *Striga hermonthica* when seeds germination began at 10hrs for one temperature regime and at 48hrs at another temperature regime. These temperatures are known as cardinal temperatures and they have a direct effect on growth and development of plants. There has been extensive research done for most agricultural crops on the effect of temperature on seed germination where the critical minimum and maximum temperatures for germination, and the optimum temperature for the highest germination rate were documented (Aflakpui *et al.*, 1998; El-Keblawy and Al-Rawai, 2006; Pérez-García and González-Benito, 2006; Kettenring and Galatowitsch, 2007; Kumar *et al*., 2011). Aflakpui *et al*. (1998) conducted a study to determine the relationship between temperature and seed germination. Pérez-García and González-Benito (2006) studied the combined effects of temperature and pre-sowing treatments on germination. The study revealed that germination is dependent on temperature and in case of seed dormancy, pre-sowing treatments promoted germination in five *Helianthemum* species. Several studies have dealt with the effect of temperature on seed germination (Aflakpui *et al.*, 1998; Probert, 2000; Pérez-García and González-Benito, 2006; Zhou *et al.*, 2012) understanding the effect of environmental factors on seed germination. Studying the effects of temperature is important in plant propagation especially when developing a propagation strategy (Kumar *et al.*, 2011).

Temperature does not only affect plant growth and development but it also influences the plant's ability to resist a variety of diseases caused by fungi, insects, pathogenic

bacteria and viruses (Franklin and Wigge, 2014). However, understanding temperature thresholds in a changing environment is very scarce hence incubators are used in this study as they provide an accurate controlled environmental temperature in which to germinate seedlings. For the cuttings, greenhouse and a shade net were utilized to control the environmental factors especially temperature. Little or nothing is known about the effect of temperature on the germination of *L. sericea* seeds

2.5.2. Light

Light is important for rooting cuttings, germinating seeds, growing seedlings and in tissue culture propagation (Van Wyk, 2011). Light can be manipulated by propagating plants in structures like green or glasshouses, shade nets, growth chambers and incubators. Light inhibits or enhance plant growth depending on the intensity of light and photoperiod (day length) (McMahon *et al*., 2002).

2.5.3. Water

Water plays a major role in plant growth and development (McMahon *et al*., 2002) and it is one of the most crucial factors that affect germination (Baskin, 2001). Water is an important factor in rooting cuttings, germinating seeds, and growing young and mature plants (Beyl and Trigiano, 2014). Whether within the plant or outside the plant, water plays a major role in the wellbeing of the plant.

Water is vital to seed propagation especially but not limited to sexual propagation because of the reduced water content in seeds (Hodge, 2014). In the same study, it was also reported that moisture content of seeds decreased significantly with storage when the average moisture content of freshly collected, mature seeds of *Vallisneria americana* was 60.9 ± 3.9% and after storage the average seed moisture content ranged from 3.8 to 32.1% which reflects considerable decline in moisture content (Hodge, 2014). Moisture in the form of water determines the time at which seed germination in various species initiates and the success thereof (Finch-savage and Phelps, 1993). The presence of moisture arguably affects germination in seeds with epigeal germination (Baskin and Baskin, 2014).

The hypocotyl and roots grow downwards as an adaptation method to maintain and lengthen the contact with soil moisture during the initial growth stages when moisture is critical for success (Finch-savage and Phelps, 1993; Hartmann, 2002). Although water is critical for plant growth especially during seed germination, however, too much moisture may inhibit germination (Baskin, 2001). Water is involved in many activities surrounding seed germination such as enzyme activation, translocation, breakdown and usage of stored food reserves (McLaren and McDonald, 2003).

2.5.4. Fire

The first scientific literature linking smoke with its ability to promote seed germination was reported by Wicklow in 1977. However, the first real use of smoke as a germination cue occurred in 1990 when De Lange and Boucher promoted germination of *Audouninia capitata* (De Lange and Boucher, 1990).

De Lange and Boucher, (1990), Baldwin (1994) and Kulkarni *et al*. (2011) reported that wildfires and smoke help to maintain biodiversity in natural ecosystems and they were used for centuries in agricultural production. Fires stimulate and increase flowering which indirectly promotes seed production (Van Staden *et al*., 2004). Smoke produced from wildfires does not only affect the plant communities in the vicinity of the fire, but also affects plants far away from the fire as the volatile compounds produced from the smoke is released into the atmosphere (Van Staden *et al*., 2004). Other compounds that may be carried over vast distances includes but are not limited to aerosol smoke, aqueous media and water runoff into water reserves such as streams, lakes and impoundments (Van Staden *et al.*, 2004).

Though there is a lot of germination inducing factors that takes place after fires, such as scarification, increased nitrate levels in the soil, changed light levels and heat, it is reported that, the smoke itself induces germination (Powerful, 2002). In a study conducted by Downes *et al*. (2010), it was reported that seeds of *Tersonia cyathiflora* germinated due to other chemical(s) from smoke. However, the biologically active chemical constituents of smoke that stimulate germination have eluded scientists until recently (Jefferson *et al.*, 2014). Dixon *et al*. (1995) studied the role of cool gaseous smoke on germination of 94 plant species native to Western Australia where it was reported that 45 out of the 94 species germinated earlier when treated with smoke ex situ (glasshouse) and in situ (habitat).

Dixon *et al*. (1995) stated that smoke does not only aid with earlier uniform germination but seedlings from smoke treated seeds are more robust. Keeley and Fotheringham (1998) found smoke to be highly effective especially nitrogen dioxide in stimulating germination of deeply dormant seeds inducing germination up to 100% whereas in control of the same species would not germinate at all (zero percent germination). There are compounds that are released during wildfires from the smoke as the plant tissue is burning which were found to stimulate germination by breaking seed dormancy (Young and Evans, 1978). The most studied compounds produced during fires vary in their degree of influence on germination and these include gases generated by biomass smoke, nitrate oxide, ammonium and nitrate.

In a study by Zhou *et al*. (2012), the seed germination and seedling growth of *Astragalus membranaceus* was significantly improved by usage of smoke water. Van Staden *et al*. (2004) isolated a major germination cue from plant derived smoke named 3-methyl-2H-furo [2,3-C] pyran-2-one. The compound was found to stimulate seed germination and it is deemed to be highly active, long lasting and heat stable. Various researchers have studied the role of germination and this has led to a high interest in smoke as a germination inducing agent (Van Staden *et al*., 2004).

In a study conducted by Baskin and Baskin (2014) the effects of smoke water treatment was tested on forage legume species and it was reported that smoke solutions at varying concentrations influenced germination either positively or negatively. Highest germination rates were observed at 10 min of seed exposure to smoke water treatment (50%) whereas 45min exposure had little or no effect on germination (Baskin and Baskin, 2014). Kulkarni *et al*. (2011) highlighted that resource poor farmers in South Africa store their maize cobs over a fireplace where seeds are exposed to smoke and heat. Kulkarni *et al*. (2011) stated that it is believed that this traditional method of storage in maize cobs protects against insects, fungus and it also improves seed germination and seedling vigour. However, no study has so far attempted to investigate the effect of smoke water treatment on germination of *L. sericea* seeds.

2.6. HORMONES AND GROWTH MEDIA IN PLANT PROPAGATION

Plant hormones are naturally occurring chemicals responsible for growth regulation and development within the plant (McMahon *et al*., 2002). All types of growth

regulators affect root initiation and proliferation directly or indirectly in cuttings propagation (Pendleton and Meyer, 2004). Of all the classes of growth regulators auxins have the highest effect on root formation in cuttings (McMahon *et al*., 2002). For the purposes of this study a synthetic Indole-3-butyric acid (IBA) was used as a treatment for root initiation in cuttings propagation. Some synthetic auxins such as IBA do not disintegrate readily when applied to plant tissue, for this reason IBA and seradix were chosen as the auxins to be used in this study. Apart from its stability, IBA has the ability to remain non-toxic across a wide range of concentrations. Waziri *et al*. (2015) used 1000-4000 parts per million of IBA and positive significant differences were observed in all the measured parameters. In another study by Sebastinraj *et al*. (2014) high root formation was observed when cuttings were treated with 0.2 mg/L Indole-3-butyric acid (IBA). Cuttings are treated with auxins for a number of reasons; to increase rooting percentage, uniform rooting, to promote root formation and to increase the number of roots (Hartmann, 2011). IBA has widely been used in propagation studies to promote rooting of cuttings and other plant parts especially in tissue culture studies. In a study by Barbosa (2016) IBA promoted rooting of *Swietenia macrophylla* when compared to cuttings which were untreated. In another in vitro experiment conducted by Gupta and Sahu (2015), IBA was used with other hormones such as 1-naphthaleneacetic acid (NAA) to proliferate rooting. Together with NAA, IBA was found to increase and promote rooting of *Withania somnifera* (L.). Saraswathi *et al*. (2014) achieved rooting of *Musa* cv Udhayam (*Pisang Awak*, ABB) when IBA was applied at 0.5mg/L. IBA has helped researchers to propagate species that are challenging to reproduce. Harahap (2014) was able to propagate mangosteen which is challenging to growers due to limited seed set, slow rate of seedling growth, and difficulty with root formations. In vitro rooting of mangosteen shoots were obtained when 3mg/L IBA was used as treatment on the species. In certain species where seed propagation is successful but not acceptable due to high heterozygosis, IBA was used in tissue culture and 80% rooting were obtained with root length and root number averaging 4.0cm and 4.3cm, respectively. In a study by Mekonnen (2014) IBA was used in mass propagation of two commercial sugarcane clones. IBA when used with other auxins gave the highest rooting percentage (80%). IBA in conjunction with NAA was used to develop a propagation protocol for an important Indian medicinal plant and 83.3% rooting was obtained (Waheed *et al*., 2014). However, IBA has not only been used on cuttings but also on seedlings. Waheed *et al*. (2014) applied IBA on the seedlings of tomato and an increase in root number, root length, stem thickness and fresh weight

was reported. Khan *et al*., (2010) conducted a study to observe the effects of seradix no.1 and IBA on cuttings of *Rosa bourboniana*. Seradix no.1 resulted in high root proliferation when compared to IBA.

Auxin is one of the most commonly recognized and naturally occurring plant hormones (Bonetta and Cutler, 2009). Auxin in plants plays a wide range of roles as it is involved in processes such as initiation of cell division, cell wall acidification, vascular differentiation, cell growth elongation, and organization of meristems (Bonetta and Cutler, 2009.) giving rise to either unorganized tissue (callus) or defined organs (generally roots).

Growth media is one of the most important factors that affect rooting in stem cuttings (Hartmann, 2011). The type of growth media used in vegetative propagation differs according to the type of species, the cultivar, water, temperature, the propagation method used, the cost and availability of the growth media and diseases (McMahon *et al*., 2002). Rooting media significantly increased the formation of adventitious roots of *Arbutus andrachne* (Al-Salem and Karam, 2001). However, even though there is no established ideal rooting media due to the reasons above, a growth media must meet certain requirements to ensure rooting. A growth media must provide nutrients, water (moisture or good water holding capacity) excellent aeration, and drainage and most importantly, it must be disease free (McMahon *et al*., 2002). It is important to use media that is compact enough to hold water, and still loose enough to allow drainage and aeration (Leakey, 1990; Hartmann, 2011). For instance in a study by Ofori *et al*. (1996), four growth media were utilised and a mixture of coarse sand and decomposed sawdust at a ratio of 1:1 was used to attain the balance. Kreen *et al*. (2002) reported that high primary roots were attained when a mixture of sand and perlite was used as substrate.

2.7. NUCLEAR MAGNETIC RESONANCE (NMR) AND PLANT METABOLOMICS

Metabolomics in its broad sense is a technology developed for the biochemical quantification, identification and analysis of complex structures under certain conditions (Barh *et al*., 2013) and in its simple terms, it is referred to as the study of the metabolome (Weckwerth and Kahl, 2013). In metabolomics, a wide range of analytical technologies can be used for non-biased, comprehensive and high

throughput analyses of complex metabolites from plant extracts such as gas-chromatography-mass spectrometry (GC-MS), liquid chromatography-photodiode array detection-mass spectrometry (LC-DADMS), nuclear magnetic resonance (NMR) and fourier transform infrared spectroscopy (FTIR). Nuclear Magnetic resonance (NMR) is a non-discriminatory spectroscopy technology used for determination of natural product structures in plant metabolomics (Ward *et al.,* 2007; Hall, 2011). NMR only identifies the most sensitive and commonly occurring magnetic nucleus. ^{1}H NMR spectroscopy provides a powerful complementary technique for the identification of plant metabolites (Krishnan *et al.,* 2005). This is supported by Neuhaus *et al.* (1985) when a more complete characterization of ^{1}H spin systems prior to the step of sequential resonance assignments was achieved by using two dimensional NMR techniques.

Hardy and Hall (2012) reports that there are two types of data that can be generated in a metabolomics experiment and these are fingerprints and profiles. Fingerprinting experiments are conducted at organ level and this type of data mainly deals or studies ^{1}H-NMR signatures as a whole in a non-targeted way before annotation (Rolin, 2012; Weckwerth and Kahl, 2013). NMR fingerprinting has a wide range of applications form medicinal plants, food all the way to functional genomics (Krishnan *et al.*, 2005; Kim *et al.*, 2011). NMR fingerprinting was proposed by Vogel *et al.* (1996) for use in the determination of orange juice authenticity and recently Spraul *et al.* (2009) used the proton NMR spectroscopy as a screening tool for the quality control of lemon, grape fruit, black currant, peach and pineapple juices. Estrada *et al.* (2009) conducted a study where fingerprint data was used on Hantavirus glycoprotein. The use of NMR fingerprinting in medicinal plants has been spreading widely over the past few years (Altman and Hasegawa, 2011; Rolin, 2012). In contrast to fingerprinting, NMR profiling simultaneously identifies and characterizes numerous metabolites from biological samples (Rolin, 2012). Moing *et al.* (2004) conducted a study where a 1-dimensional metabolic profiling was used to determine some secondary metabolites, amino acids, organic acids and soluble sugars in plant roots, fruits and leaves. NMR fingerprinting ignores the time consuming signal consignment and it thus compares and categorises samples in an unprejudiced way rapidly (Ward *et al.*, 2007). However, this type of data may also be performed using Fourier-transform spectrometers (FTIR) (Rolin, 2012). Similarly to NMR fingerprinting, ^{1}H-NMR profiling also has a wide range of application

such as food, plant breeding, functional genomics and for characterization of medicinal plants (Rolin, 2012; Barh, 2013).

Figure 2.2: 600MHz Nuclear Magnetic Resonance apparatus at the CSIR, Pretoria (photo taken by Sehlakgwe P F, 2015)

Plants are subjected to various environmental factors which they endure season in season out (McMahon *et al*., 2002) and the plants' response to this ever changing environmental factors occurs at multiple levels ranging from tissue, cellular, molecular, anatomical, whole plant physiological as well as at morphological level (Chaves *et al.,* 2003; Bartels and Sunkar, 2005). The impact of both biotic and abiotic factors has a tremendous effect on plant metabolism, which results in plants developing a complex metabolomic collection of compounds which differs with different environmental conditions. Altman and Hasegawa (2011) states that plants develop mechanisms that aids in enduring the stress from the abiotic and biotic factors. Thus, during the stress plants will go into an adaptive or protective mode (Orr and Raison, 1990). When exposed to stress, plants accumulate a wide range of osmolytes. The species-specific accumulated solute includes amino acids (proline), sugars (fructose, glucose, sucrose, trehalose etc), polyols and betaines (Altman and Hasegawa, 2011; Hardy and Hall, 2012; Barh *et al.*, 2013). Due to the fact that metabolic fluxes drops and rises under a wide range of environmental conditions, it is imperative that comparison is done to see variation of metabolites amongst seasons (Hardy and Hall, 2012). Environmental conditions vary from one season to the next, and this change not only affects plant growth but it also affects the formation of primary and secondary metabolites, especially the latter because they are mostly formed in young, actively growing tissues

(Sahoo *et al.,* 2012). A research study conducted by Sahoo *et al.* (2012) was to better understand variations in plant metabolites during different seasons in four important arid zone medicinal plants. The results revealed that the species had a high amount of total secondary metabolites in summer as compared to winter. So far, no study has investigated the effect of seasonal variation on the secondary metabolites of organs of the *L. sericea* plant including its stored seeds.

2.8. ACNE

The history and use of cosmetics predates written history, however, in Africa especially in Egypt, it is documented that both men and women used cosmetics either in the form of scented oils, dyes, paint and ointment to clean their skins, mask body odour and to colour their skins and nails (Parish and Crissey 1988; Chaudhri and Jain, 2009). The importance and significance of the beauty industry and cosmetics exceeds the monetary value that the industry contributes towards the global Growth Domestic Products (GDP). The most striking factor about cosmetic products and the industry is its influence on social lives of humans worldwide (Kumar *et al.,* 2011).The use of plants in cosmetics is as old as mankind and recently there has been a spiked interest in producing cosmetic products containing natural plant extracts, oils and herbs.

Before methods of synthesizing substances with similar properties, plants were once the main ingredient in cosmeceutical products (Aburjai and Natesh, 2003). Due to the negative effects of cosmetic products made from animal-derived extracts, the use of plant extracts in cosmetic formulations is increasing. Plants such as peppermint, cedar, lily, aloe, olive, lavender, myrrh, thyme, chamomile, liquorice, walnut, gingko, bearberry, wheat and many more have been and are still used by companies in an effort to produce green cosmetic products (Winders, 2006; Vogels, 2009).

Acne vulgaris is a common skin condition in humans, which may be caused by several factors such as acne inducing bacteria (*Propionibacterium granalosum*, *Staphylococcus epidermis*, *Molassezia furfur* and gram positive *Propionibacterium acnes*) which affects sweat or oil glands and hair follicles (Aburjai and Natesh, 2003; Sharma *et al*., 2014), inflammation and keratinization. Hormonal imbalances in the body, which leads to excess production of sebum on the skin may also cause acne. The excess oil blocks the skin pores on the epidermis which leads to ideal conditions

for the bacteria to grow and multiply. Acne is one of the most commonly diagnosed conditions worldwide (Cordain *et al.,* 2002; Day, 2005) and it is often referred to as pimples, zits, lesions, clogged pores, black or whiteheads and rash.

Acne treatment as proposed by Strauss and Kligman (1960) involves inhibiting inflammation, excess sebum production, bacterial colonisation by *P. acnes* and correcting the altered pattern in which cells from underneath the human skin are transformed to hair and nails (keratinization) (Rahman *et al*., 2013; Williams *et al.,* 2013;). Though extensive clinical acne treatment studies have been undertaken (Becker, 1981; Stern, 1996; Pariser *et al.,* 2005; Leyden 2003), to this day, there is no cure for this chronic dermatological skin disorder (Coenye *et al.,* 2007). The most common forms of current acne treatment are oral and topical therapy (Kabongo, 1982, Kim *et al.,* 2008). Acne treatment is informed by the type of acne being treated and in severe cases both oral (tertracycline, doxycycline, erythromycin, minocycline, lime cycline etc) and topical (tazarotene, tretinoin and adapalene) treatments maybe used (Coenye *et al.,* 2012; Rahman *et al*., 2013). Hormonal (isotretinoin) therapy and laser treatments are also used for treatment (Rahman *et al*., 2013). Although antibiotics have been widely used for acne treatment for many years, there has been reported antibiotic resistance (Eady*,* 2003; Swanson, 2003), e.g. erythromycin has a widespread and most common resistance (Tan *et al.,* 2001; Rahman *et al*., 2013). Antibiotic resistance of *P. acnes* was not identified prior to the mid-seventies (Leyden, 2003). Since there is a current cumulative antibiotic resistance (Swanson, 2003), new and alternative treatments must be devised to treat acne as this disease affects human of all ages (11-30 years) majority of whom are adolescents (Swanson, 2003; Decker and Graber, 2012). The effects of acne are not only physical, but there are also emotional and psychological distresses associated with acne and therefore development of effective treatment is vital (Swason, 2003; Chomnawang *et al.,* 2005).

Extensive studies have been undertaken on medicinal plants as a possible alternative treatment for a wide variety of diseases (Mantle *et al.,* 2001; Williams *et al.,* 2013) including acne treatment (Chomnawang *et al.,* 2005; Decker and Graber, 2012; Hammer, 2015). Studies were conducted where plant extracts inhibited the development of bacterial biofilms even when applied at below minimal inhibitory concentrations which reduces development of antimicrobial resistance (Coenye *et al.,* 2012). Coenye *et al.* (2012) identified five traditional Chinese medicines which

exhibited a potent anti-biofilm activity against *P. acnes.* Weckesser *et al.* (2007) conducted a study where plant extracts and compounds were tested against 29 bacteria (aerobic and anaerobic) and several plant extracts were found to inhibit the growth of gram-positive bacteria such as *Usnea* CO_2-extract which inhibited the growth of *P. acnes*, *S. aureus* (including methicillin-resistant strains MRSA) and *Corynebacterium* species. It was therefore concluded that the extracts may be utilised for topical treatment of skin ailments such as acne vulgaris and sebhorrhoc eczema.

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CHAPTER THREE

INFLUENCE OF GROWTH REGULATING HORMONES ON TIP AND BASAL CUTTINGS OF *LEUCOSIDEA SERICEA*

3.1. INTRODUCTION

In recent years, seeds and cuttings propagation has seen an increase and this has led to less usage of soil as it is substituted by growth media especially in nurseries. Propagation by cuttings dates back to ancient history (Hartmann, 2011). One of the most important advantages of propagation by cuttings is the fact that a large number of plants can be produced from a single parent plant and it is therefore used in conservation of plants facing extinction and it also allows for rapid propagation of new cultivars (Hartmann and Kester, 1983; Lenton, 1997). Depending on the nature of wood used and the age and stage of stem growth in vegetative propagation, stem cuttings can be categorized into hardwood, softwood, semi hardwood and herbaceous (Mcmahon *et al*., 2002; Hartmann, 2002). Semi hardwood cuttings are obtained from the apical part of the plant and they mostly contain a developing apex with young leaves. Hardwood cuttings on the other hand are made from mature dormant hardwood. Rooting in cuttings is influenced by several factors such as the time of the year when the cutting was taken, stem structure and the chemical composition in the stem (Rice and Rice, 2000; Mcmahon *et al*., 2002 and Clarke and Toogood, 2004).

The role of auxins in root initiation is documented as one of the most important discoveries in plant propagation (Beyl and Trigiano, 2014). The usage of indole-3-acetic acid dates back to the early 1930's when it was first isolated and was shown to promote rooting (Beyl and Trigiano, 2014). In hard to root species, auxins are used to initiate, accelerate and to increase the rooting percentage, uniformity and quality of roots per cutting (Rice and Rice, 2000).

Leucosidea sericea belongs to the family Rosaceae which comprises of 120 genera with a total of 3000 species worldwide. Some of the most common fruit trees such as apple, peach, plum, prune, nectarine, pear, apricot, almond, cherry, and strawberry belong to this family. Plants in the Rosaceae family have been propagated through vegetative means in both *in vivo* and *in vitro* environments where growth promoting hormones have been used. In a study by Christensen *et al*. (1980) the effect of six auxins on root proliferation was investigated on cuttings of apple fruit trees. Rooting response of shoot cuttings of peach (*Prunus persica* (L.) was investigated by Tworkoski and Tadeka (2007) where up to 79% of rooting was observed when IBA was used. To date, there is no documented method for optimum propagation of *L.*

sericea and this study was therefore undertaken to determine the best methodology for propagating *L. sericea* using cuttings.

3.2. MATERIALS AND METHODS

3.2.1. Collection and Cutting Preparation

To determine the effect of cutting position both the basal (hardwood severed at the base of the stem) and tip (semi-hardwood severed at the tip of the stem) cuttings of *L. sericea* were harvested in November 2013. The cuttings were collected early in the morning from 07:30 to 8:30 for all the trials. *Leucosidea sericea* cuttings were collected from uniform, healthy vigorous plants from Clearwater Florida, South Africa (26°9'26"S 27°54'10°E) using sharp sterile secateurs. The cuttings were kept moist using water in a cooler bag during transportation to the Horticulture Centre at the UNISA Florida campus. At the Horticulture Centre, the cuttings were then transferred to the potting shed where the cuttings were planted into black plastic bags (2.5L) containing various growth media. Four-node cuttings were prepared by making an angled cut between buds on actively growing shoots. All leaves were removed and the cuttings were then trimmed to about six (tip) to ten (basal) cm in length. The bottom parts of the cuttings were treated with growth hormones.

3.2.2. Growth Hormones and Application

To promote rooting of the cuttings, three synthetic IBA growth hormones namely Indole butyric acid (IBA) in liquid form, (three levels of IBA: 1%, 3% and 8%), seradix no.1 and seradix no.2 were used for treating the cuttings. Seradix no.1 and seradix no.2 were applied using the dry quick dip method and the long soak method was used to apply different levels of the growth hormone IBA (in liquid form) (Aminah *et al*, 1995).

3.2.3. Media

Initially, the aim was not to determine the best growth media for *L. sericea* cuttings, but rather to determine whether *L. sericea* can be propagated from cuttings. However, due to lack of positive results from the initial experiment, a decision was taken to change growth media as well as the environments where the cuttings were being

propagated to improve rooting. The experimental lay out is presented in Table 3.1 with each treatment replicated three times with three different growth media and growth media combinations. The following growth media and growth media combinations were used; compost, sand, plug mix extra plus and a 50:50 combination of these growth media, in four trial carried out in the following sequence: In trial one, compost (Garden World, South Africa) was used in a greenhouse at the University of South Africa (UNISA) (26.1750^{o}S, 27.9230^{o}E). The compost had high water holding capacity which led to waterlogging and growth of algae during week five of the experiment and no rooting.

Hence sand (Garden World, South Africa) was used in trial two in a greenhouse to increase porosity. However, rooting was not improved and a 50:50 combination of sand and compost was used in trial three and the temperature was altered as described in section 3.2.5 below. Although, the rooting was not improved by a large percentage, the overall health and response of the cutting was observed to be better when compared to the cuttings used with the previous two growth media. Hence in trial four, a 50:50 combination of sand and a specialised cutting rooting mix called plug mix extra plus (Greenhouse Technologies PTY, South Africa) and a 50:50 combination of sand and compost was used in a shade net at Mothong, Mamelodi in Pretoria (Latitude:-25.703876, Longitude:28.34592). The growth media was well watered before planting. As described by Hartmann (2002), a hole was made in the growth media with a planting stick in each planting bag to avoid brushing off of the rooting growth hormones during planting. In all the trials, cuttings were planted 2cm deep in black 2.5L planting bags at the same time ensuring that two nodes are above and two more are below ground at the UNISA Florida Science campus. At Mothong, cuttings were planted in the same manner however, raised seed beds were used where a net was used to hold a 50:50 combination of sand and compost and another 50:50 combination of plugmix and sand in a shade net.

3.2.4. Data Recorded

Data was collected for all the trials in terms of the following parameters: number of sprouted buds, number of roots, root length, rooting percentage, number of days after planting and the effect of cutting type. Evaluation of rooting parameters was done at

the end of the experiment and sand was removed from the rooted cuttings before counting and measuring.

3.2.5. Trial Layout at Florida (Johannesburg)

The first, second and third trials were carried out at UNISA (26.1750^{o}S, 27.9230^{o}E), Florida Science campus (horticulture centre) on a mesh bench (3 x 1.6m) in a greenhouse. Throughout the experimental period, the temperature in the greenhouse was monitored on a supra computer (Fertigo 3000, Spain). Throughout the experimental period the cuttings were irrigated through overhead sprinklers three times a day. A randomised complete block design with 12 treatment combinations and three replicates as shown in Table 3.1 was set up to determine the possibility of propagating *L. sericea* by cuttings. Each treatment combination comprised of 10 cuttings. Compost, sand and a 50:50 mixture of sand and compost were used in these trials.

Table 3.1: Cuttings trial layout

Block 1	**Block 2**	**Block 3**
IBA_2Tip	ControlBasal	IBA_3Tip
IBA_3Tip	Seradix no.1Basal	Seradix no.1Tip
Seradix no.2Tip	IBA_1Basal	IBA_3Basal
IBA_1Tip	IBA_1Tip	IBA_1Tip
ControlTip	IBA_3Basal	Seradix no.2Tip
IBA_2Basal	Seradix no.2Tip	IBA_2Tip
IBA_1Basal	Seradix no.2 Basal	Seradix no.1Basal
IBA_3Basal	IBA_2Tip	ControlTip
Seradix no.1Basal	ControlTip	IBA_1Basal
Seradix no.1Tip	Seradix no.1Tip	IBA_2Basal
ControlBasal	IBA_2Basal	ControlBasal
Seradix no.2Basal	IBA_3Tip	Seradix no.2Basal

IBA_1= 1%

IBA_2=3%

IBA_3=8%

Seradix no.1

Seradix no.2

3.2.6. Trial Layout at Mothong

The fourth experiment was carried out at Mamelodi Mothong heritage site, northeast of Pretoria, Gauteng in South Africa (Latitude:-25.703876, Longitude:28.34592). The cuttings were prepared as stipulated in 3.2.5 above and planted in raised seed beds where a net was used to hold a 50:50 combination of sand and compost and another 50:50 combination of plugmix and sand in a shade net. The cuttings were irrigated with a hose pipe three times a day for the duration of the experiment. A randomised complete block design with 12 treatment combinations and three replicates as shown in Table 3.1 was set up in a shade net.

3.2.7. Statistical Analysis

Data were analysed using Statistica program (Statistical analysis system institute Inc, 2010). The collected data were subjected to ANOVA and the means were separated using the Duncan's multiple regression analysis. The means were tested at 5% confidence interval.

3.3. RESULTS AND DISCUSSION

The results of the application of different growth hormones and growth hormone levels in combination with different growth media, two cutting types and growth environment (shade net and greenhouse) did not yield positive results to improve cutting propagation of *L. sericea* cuttings. The interaction of these treatments did not yield positive results either.

To establish the best cutting type and interpret better the relationship between the rooting ability and the cutting material's initial physiological characteristics, two collection points (basal or tip) were used. *Leucosidea sericea* basal and tip cuttings produced no results and comparison between the two cutting types showed no significant differences in the rooting percentage, rooting length and all the other measured parameters. There were no significant differences for rooting percentage, number of sprouted buds, and number of leaves of both basal and tip cuttings for the duration of the trial in all the tested growth media and growth hormone concentrations of IBA at all the three levels (1, 3 and 8%), seradix no.1 and seradix no.2. Untreated

cuttings (control) did not produce positive results (Figure 3.1). The rooting number, rooting length and the overall rooting percentage did not vary with the different growth hormone treatments and different growth hormone levels (Figures 3.1 and 3.2).

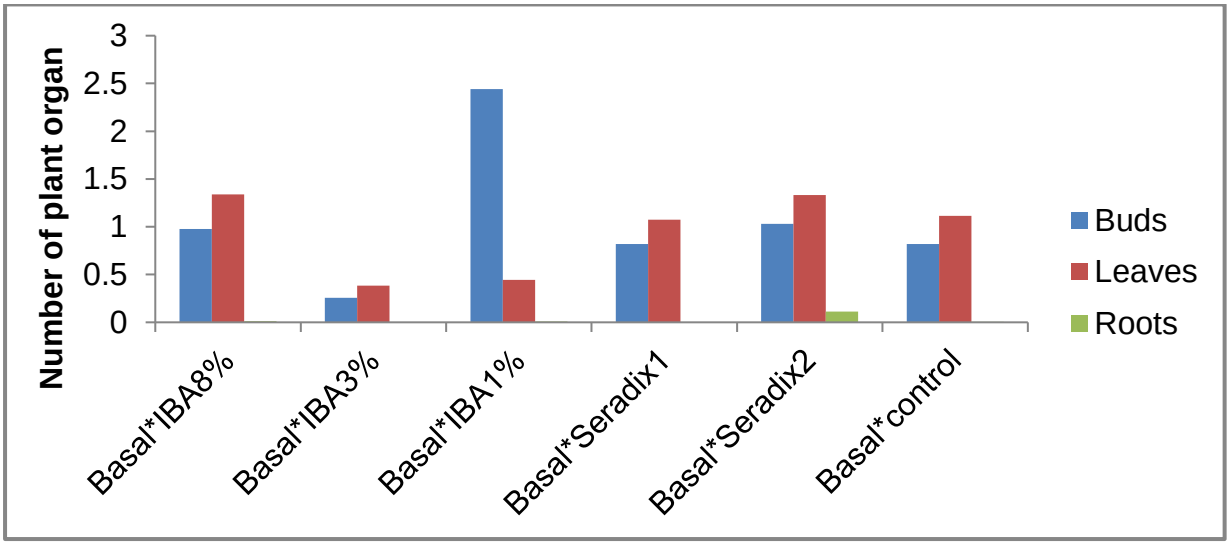

Figure 3.1: The effect of IBA, seradix no.1 and seradix no.2 on basal cuttings of *L. sericea*

At both the Mothong heritage site and UNISA campus, all experiments were undertaken to study the effects of different growth hormone levels and effects of growth media on the rooting success of both tip and basal cuttings of *L. sericea*. Basal cuttings of *L. sericea* did not respond positively to treatment of growth media, growth hormones (Figure 3.1) under greenhouse and shade net growth environments. In comparison to basal cuttings, there were no significant differences in the rooting of tip cuttings treated with the growth hormones. Percentage rooting was very low for the controls as well as the treatments at 0 to 2% for all the measured parameters (Figures 3.1 and 3.2). Changing the growth media did not affect rooting of the cuttings. In contrast to the reported positive influence of auxins on rooting of cuttings of peach (Tworkoski and Tadeka, 2007) and apple fruit tree (Christensen *et al*., 1980), our study found no significant effect of the growth hormones on the rooting of cuttings of *L. sericea* compared to controls.

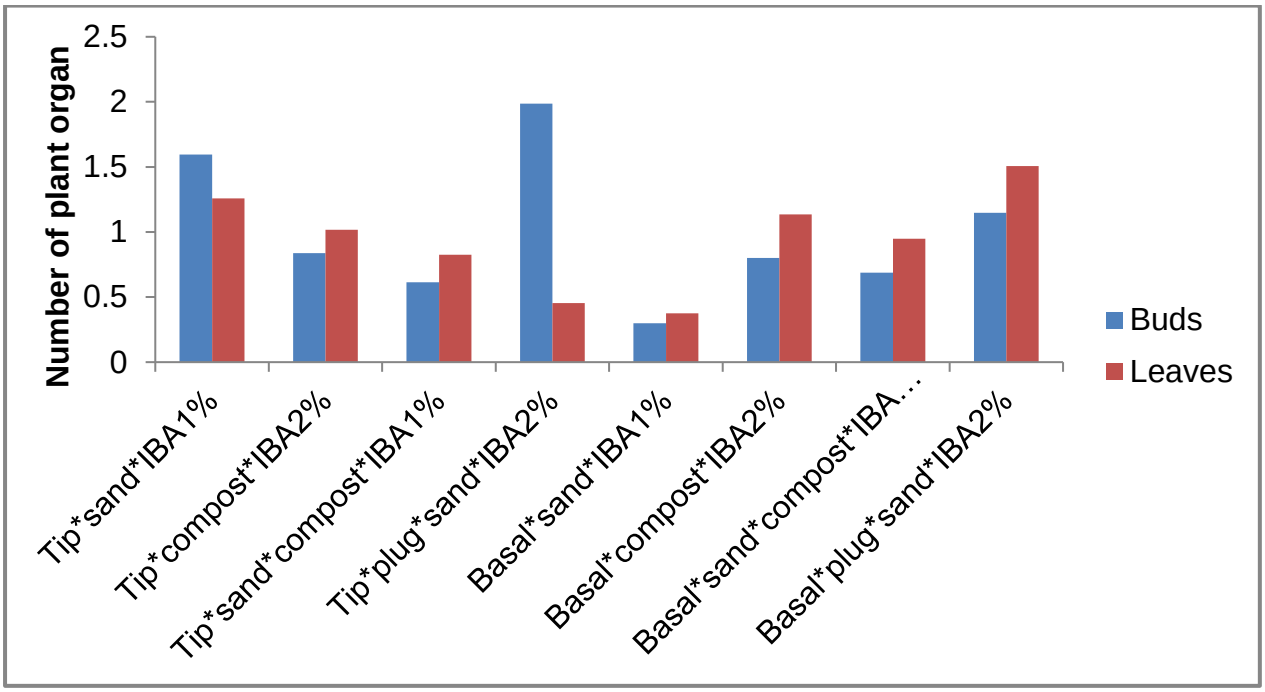

Figure 3.2: The effect of various growth media on tip cuttings of *L. sericea*

3.4. CONCLUSION AND RECOMMENDATIONS

In general, growth hormones such as Indole butyric acid (IBA), seradix no.1 and seradix no.2 applied at different levels had no significant effect on *L. sericea* cuttings. The general success rate was very low at ~1-5% for all the measured parameters for both cutting types and all the different media used. Based on the results from this study, it is not recommended that *L. sericea* be propagated by tip or basal cuttings, although investigation in different seasons might have yielded better results. This plant belongs to the Rosaceae family, with best germination in spring and autumn. This research can be further expanded to include for instance other seasons, other growth hormones, irrigation scheduling, pest control and fertilizer application in combination with hormones and media.

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CHAPTER FOUR

EFFECTS OF PRE-SOWING TREATMENTS AND TEMPERATURE ON SEED GERMINATION OF *LEUCOSIDEA SERICEA*

4.1. INTRODUCTION

Germination, the process that initiates a plant's life cycle, is a highly complex major event that begins with the imbibition of water, followed by an increase in respiratory activities and mobilisation of nutrient reserves, and finally resulting in initiation of growth in the embryo (Fenner and Thompson, 2005; Busso, 2013; Baskin and Baskin, 2014). Externally, a seed is considered germinated when the seed coat bursts, and the cotyledons, hypocotyl and the roots form (Busso, 2013). Internally however, germination is initiated when the metabolism of the seed embryo is activated under certain conditions, which leads to the formation and emergence of the first true leaves (Mcmahon *et al*., 2002; Hodge, 2014). Successful germination requires certain set of conditions such as appropriate environmental conditions (light, relative humidity and gases), the seed dormancy if any must be overcome, and the seed embryo must be viable (Hodge, 2014). Germination begins with the water uptake and then culminates with the emergence of the embryonic radicle. However, germination is often used to indicate its completion. For example, 70% germination means that 70% of a seed population has germinated. To improve seed germination percentage and its rate, various treatment methods have been reported in the literature for the propagation of some plants (Dayamba *et al*., 2010).

Various pre-sowing seed treatments are available in literature aimed at breaking dormancy, unifying and improving seed germination percentage. Soaking plant seeds is a treatment used widely in plant propagation. The aim of pre-sowing seed treatments such as soaking is to improve, unify and increase seed germination (Finch-Savage, 2013). Seeds that are dry at or after maturation must absorb water through the seed coat to initiate the germination process (Kimball *et al*., 2008). Since seeds loose a considerable amount of water during or after maturation, during storage or processing (up to 5-15% of their weight) imbibition is required as this process causes the seed to expand and consequently rupture the seed coat. Water in the seed also has an effect on the metabolism of the embryo which enables it to ensure growth (Kimball *et al*., 2008).

Among other abiotic factors that affect germination of seeds is temperature (Motsa *et al*., 2015). According to Kettenring and Galatowitsch (2007), it is not known whether freshly matured seeds are physiologically dormant and how this dormancy affects seed germination at different temperatures.

From a commercial production perspective, it is important to understand if, for example, pre-sowing treatments are required or not across different growing temperatures for successful germination of *L. sericea* seeds. Different pre-sowing treatments such as smoking and soaking in water have been employed successfully to germinate seeds of some plants under glasshouse conditions (Dayamba *et al*., 2010). Smoke has proven to increase germination percentage in several studies (De Lange and Boucher, 1990; Brown *et al*., 1993; Baxter *et al*., 1994). Smoke as a germination cue has not only been used on species from fire-prone ecosystems but also on species from fire-free ecosystems (Dayamba *et al*., 2010). In light of this information, smoke water treatment was used to treat *L. sericea* seeds. Oldwood is commonly propagated from seeds by homeowners and nurseries. However, no scientific study has been undertaken to determine factors that affect germination of *L. sericea* seeds. The specific objectives of this chapter were to evaluate the interactive effects of smoking, soaking (with cold and hot water), leaching and various temperature regimes on the germination of seeds of *L*. *sericea*

4.2. MATERIALS AND METHODS

4.2.1. Seed collection and preparation

Seeds of *L. sericea* (achenes) were collected from their natural habitat at Florida Clearwater (26°9'26"S 27°54'10°E) in early March 2013 from a dense, thicket formed by *L. sericea* trees along the river. In this study, the term “seeds” mean achenes due to the nature of *L. sericea* fruits. The seeds collected were dried at room temperature and stored prior to experimentation. The nut-like seeds of *L. sericea* were still enclosed at the base of the star shaped, yellow-green seeds. Before drying the seeds, the individual achenes were separated from the flower stalk (Figure 4.1). The seeds were then stored in brown paper bags in the laboratory at ± 20°C and approximately 50% humidity before experimentation.

Figure 4.1: The seeds of *L sericea*

4.2.2. Seed treatments

4.2.2.1 Soaking

4.2.2.1.1 Soaking in hot water

Seeds were immersed in boiled (100°C) distilled water for 2min to prevent seed damage. Cold water was immediately added to the seeds and left to soak for 24hrs.

4.2.2.1.2 Soaking in cold water

Seeds were immersed in cold distilled water (room temperature) for 24hrs.

4.2.3. Leaching

In order to remove germination inhibitors which may prevent seed germination, *L. sericea* seeds were put under a tap with running water for 2hrs for three days.

4.2.4. Smoke water treatment

An instant smoke seed primer disc obtained from the Kirstenbosch Botanical Garden in Cape Town, South Africa, was used. Fifty millilitres of water was added to a glass beaker containing the instant smoke seed primer disc (Figure 4.2) and the seeds were then soaked for 24hrs (as recommended by the manufacturer).

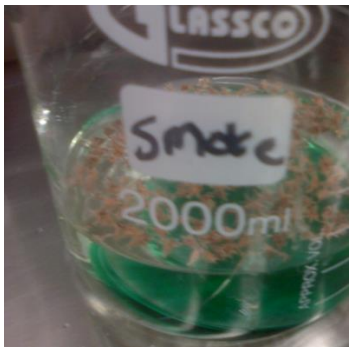

Figure 4.2: Seeds of *L. sericea* in smoke water

4.2.5 Control treatment

A set of the collected seeds were not exposed to any of the above treatments (untreated seeds) and therefore served as control.

4.2.6 Germination trial

A laboratory germination study was conducted in five incubators (NÜVE, Turkey). A randomised complete block design (RCBD) with five constant temperatures ranging from 10ºC to 30ºC with a 5ºC increment, and five pre-sowing treatments over a period of five weeks was investigated The germination trial layout is provided in Table 4.1. The experiments were conducted during September 2013 at the University of South Africa (UNISA), Florida, Science campus. A preliminary study was done during which *L. sericea* seeds were germinated in incubators to test seed viability. After seed viability was confirmed, *L. sericea* seeds were exposed to five controlled, constant temperature regimes of 10ºC, 15ºC, 20ºC, 25ºC and 30ºC. In total, 30 seeds were used in five treatment combinations (temperature and pre-sowing treatments) with each treatment replicated three times. A folded white germination filter paper (C140, Lasec) was run through water in a container and thereafter the excess water was left to run off, so that the germination paper was moist, since the germination period was long (Figure 4.3). The germination paper was then placed in a small, airtight plastic container and the treated seeds were placed in the germination paper and then incubated. All the seeds were germinated in the dark. The seeds were supplemented with 5mL of distilled water at the end of each week to keep the germination paper moist so as to prevent the seeds from drying out. The seeds were checked and counted every seven days for nine weeks and returned into the incubator immediately after data collection. Weekly recordings were done for the number of germinated seeds in each treatment. The starting and finishing dates of germination were also recorded to determine the germination percentage as well as the survival score. At the

end of the germination period, the germination percentage and germination percentage were calculated. Seeds with mould were continuously removed from the experiment to encumber spreading the infection.

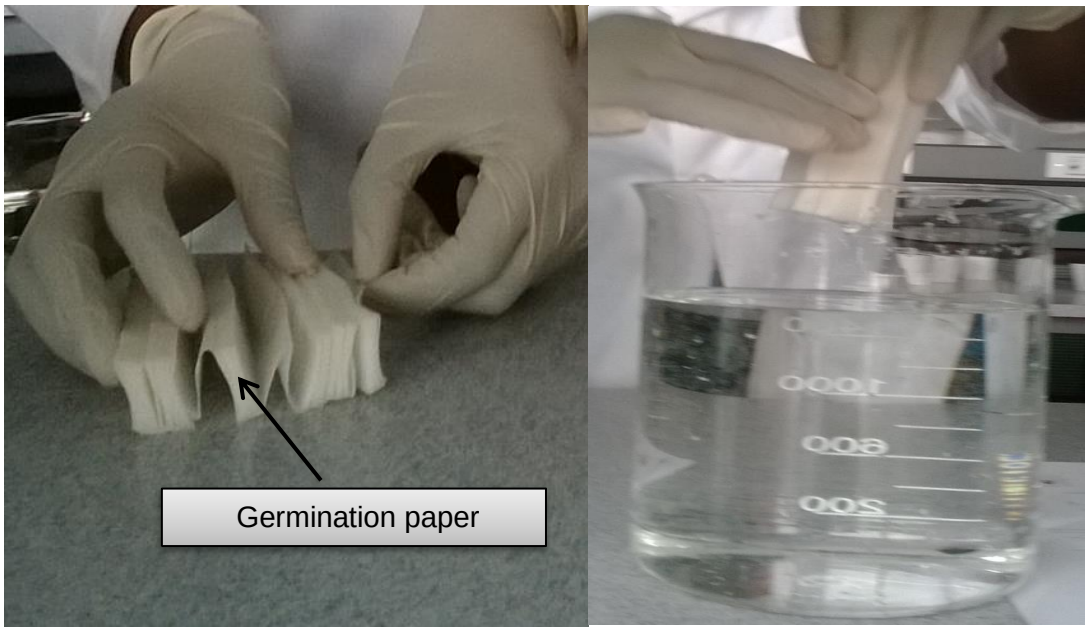

Figure 4.3: Preparation of germination paper prior to sowing

Table 4.1: Seed germination treatment summary; 10°C, 15°C, 20°C, 25°C and 30°C

Treatment	**Temperature (ºC)**				
	10	15	20	25	30
1.Leaching (LC)	LC+10ºC	LC+15ºC	LC+20ºC	LC+25ºC	LC+30ºC
2.Smoking (SM)	SM+10ºC	SM+15ºC	SM+20ºC	SM+25ºC	SM+30ºC
3.Soaking hot (SH)	SH+10ºC	SH+15ºC	SH+20ºC	SH+25ºC	SH+30ºC
4.Soaking cold (SC)	SC+10ºC	SC+15ºC	SC+20ºC	SC+25ºC	SC+30ºC
5. Control (C)	C+10ºC	C+15ºC	C+20ºC	C+25ºC	C+30ºC

4.2.7. Data analysis

All data collected were subjected to factorial analysis of variance (ANOVA) to determine differences between treatments and the treatment means were separated using Duncan's Multiple Range Test (DMRT) at $p<0.05$. All data collected were analyzed using STATISTICA software 10 program 2010 (StatSoft Inc., Tulsa, OK, USA). Germination percentages were determined by counting the number of seeds that germinated in each treatment combination from the total amount of seeds planted and the germination percentage (percentage of seeds germinated per week) were

determined by observing the number of days from the beginning of the experiment to the day(s) when germination was initiated.

4.3. RESULTS AND DISCUSSION

4.3.1. Effect of pre-sowing treatments on seed germination of *L. sericea*

In this study, smoke water treatment applied at pre-sowing significantly ($P<0.05$) affected the percentage of seed germination of *L. sericea* compared to the untreated seeds (control) (Table 4.2). Smoking treatment (SM) had the highest mean percentage of germinated seeds at 50.2% when compared to soaking in cold water (SC), which had the lowest mean germination percentage at 20.6% (Table 4.2). Similarly in a seed germination test by Mofokeng *et al*. (2012) pre-sowing treatments were found to affect seed germination when smoke water treatment was applied to plant seeds compared to control. Positive germination response was also reported when dormant seeds of red grass were exposed to plant-derived smoke and aqueous extracts of smoke (Baxter *et al*., 1994). Drewes *et al*. (1995) enhanced seed germination of *Lactuca sativa* L. when the seeds were treated with plant-derived smoke extract compared to control. In addition, the importance of smoke cue in enhancing seed germination of 26 species of the Ericaceae family in the fynbos has been reported by Brown *et al*. (1993). In this study, the pre-sowing treatments investigated allowed some germination, but none were any better than smoke water treatment. However, untreated seeds germinated better compared to soaking (cold water (SC)), soaking (hot water (SH)) and leaching (LC).

Table 4.2: Effect of pre-sowing treatments on seed germination of *L. sericea.* Values (M±S.E.) with dissimilar letters in a column are significantly different at $P<0.05$. All values are presented as an average of three replicates

Pre-sowing treatment	Germinated seeds (n=30)	Percentage germination (%)
Smoking (SM)	15.12±0.49a	50.2
Leaching (LC)	8.67±0.44c	28.9
Soaking cold (SC)	6.17±0.47d	20.6
Soaking hot (SH)	6.52±0.49d	21.7
Control (C)	12.05±0.58b	40.2

4.3.2. Effect of temperature on seed germination of *L. sericea*

The germination of *L. sericea* seeds was significantly (P<0.05) affected by temperature. The optimum temperature range for seed germination was from 15°C to 20°C with a higher mean percentage of >37.5% when compared to the lowest overall germination percentage of 20% for 30°C (Table 4.3). In a similar study by Zhou *et al*. (2012), the highest germination of *Magnolia officinalis* seeds occurred between 15°C and 25°C. In another study, Mofokeng *et al*. (2012) successfully germinated *Bowiea volubilis* seeds at temperatures between 15°C and 20°C. In another study by Araya (2005), bush tea seeds germinated best at temperatures ranging from 10°C to 25°C. Basra *et al*. (2005) reported that the optimum germination temperature for most plant species is between 15°C and 30°C. In this germination experiment, seed germination was affected by temperature and generally, the seeds germinated best at the temperature range of 15°C to 20°C whereas there was a low germination percentage at 25ºC which declined further at 30°C.

Table 4.3: Effect of temperature on seed germination of *L. sericea*. Values (M±S.E.) with dissimilar letters in a column are significantly different at P<0.05. All values are presented as an average of three replicates.

Temperature (°C)	Germinated seeds (n=30)	Percentage germination (%)
10	9.65±0.61b	32.2
15	11.26 ±0.67a	37.6
20	11.25±0.58a	37.5
25	10.35±0.49b	34.5
30	6.00±0.65c	20.0

4.3.3. Effect of time on seed germination of *L. sericea*

The number of germinated seeds increased linearly and steadily from week one up to the last week of the experiment, week five. The germination percentage varied considerably with different temperature and pre-sowing treatments combination from 16.9% germination in week one to almost double the amount two weeks later (Table 4.4). However, the germination percentage was affected by seed rot caused by mould during the experimental period.

Table 4.4: Effect of time on seed germination *L. sericea* seeds. Values (M±S.E.) with dissimilar letters in a column are significantly different at P<0.05. All values are presented as an average of three replicates.

Time in Weeks	Germinated seeds (n=30)	Percentage germination (%)
Week one	5.09±0.63e	16.9
Week two	9.04±0.62d	30.1
Week three	9.97±0.58c	33.2
Week four	11.76±0.52b	39.2
Week five	12.57±0.42a	41.9

4.3.4. Interactive effect of pre-sowing treatments and temperature on seed germination of *L. sericea*

The interaction of pre-sowing treatments and temperature significantly (P<0.05) affected seed germination percentage of *L. sericea* when compared to their respective controls (Figure 4.4). Temperature affected seed germination percentage between smoking treatment and control in a similar trend. In general, seeds treated with smoke water, germinated best across all the temperatures when compared to the other pre-sowing treatments and the lowest seed germination was obtained at 30°C across all the pre-sowing treatments (Figure 4.4). Leaching (LC) and soaking seeds with either hot (SH) or cold water (SC) affected seed germination negatively with a lower germination percentage than the control especially at 10°C and 30°C. Interestingly, the highest germination percentage was obtained at a temperature range of 15°C and 20°C where the seeds germinated up to an average of 53% and 50% respectively when treated with smoke water compared to control with 43% germination at 15°C (Figure 4.4). Similarly in a study by Mulaudzi *et al*. (2009), germination percentage of *Alepidea amatymbica* and *Alepidea natalensis* was improved when seeds were treated with smoke water and incubated at a germination temperature of 25°C. The maximum percentage seed germination of lettuce was obtained when the seeds were treated with smoke extract and incubated at 20°C (Drewes *et al*., 1995). The effect of temperature on seed germination depends largely on the plant species, the variety, the environment and the duration of the growing season (Baskin and Baskin, 2014). *Leucosidea sericea* seeds germinated better at a temperature range of 15°C and 20°C. In agreement with our findings, Pons and Fenner (2000) and Baskin and Baskin (2014) reported that the optimum temperature for seed germination is between 15°C

and 30°C for most plant species. However, *L. sericea* germination percentage fluctuated across the temperatures and the lowest germination percentage was recorded at 30°C in SH followed by SC, whereas the highest was at 15°C for the smoke treatment followed by 20°C.

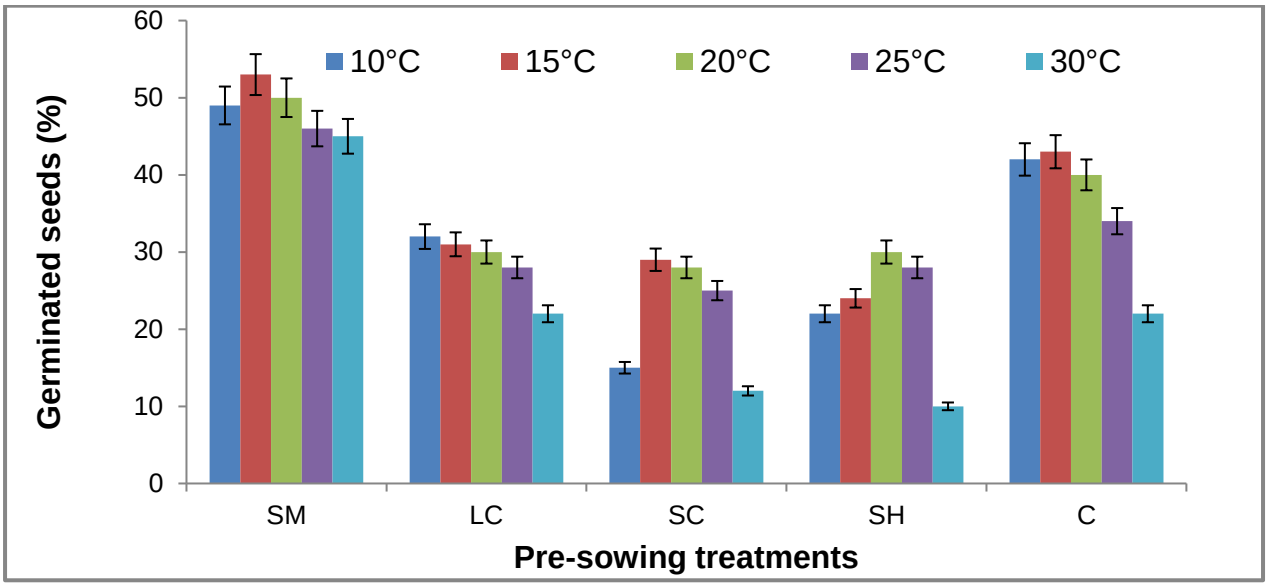

Figure 4.4: Interactive effect of temperature and pre-sowing treatment on the germination percentage of *L. sericea* seeds.

4.3.5. Interactive effect of time and temperature on seed germination of *L. sericea*

In general, the percentage of seed germination increased linearly with time regardless of the temperature and the highest percentages of germinated seeds were observed at week five at 15°C and 20°C (Figure 4.5). The turning point was reached during week three at 15°C and only a slight increase was observed from week four to week five at 20°C. It would therefore seem that the optimum time for germination was reached at week five for the temperatures tested. According to Hegarty (1973) and Bierhuizen and Wagenvoort (1974), seed germination percentage linearly increases with temperature. *Leucosidea sericea* seeds germination percentage increased linearly with time regardless of the incubation temperature (Figure 4.5), however, it was proportional to the increases in temperature from 10°C through to 20°C (Table 4.3).

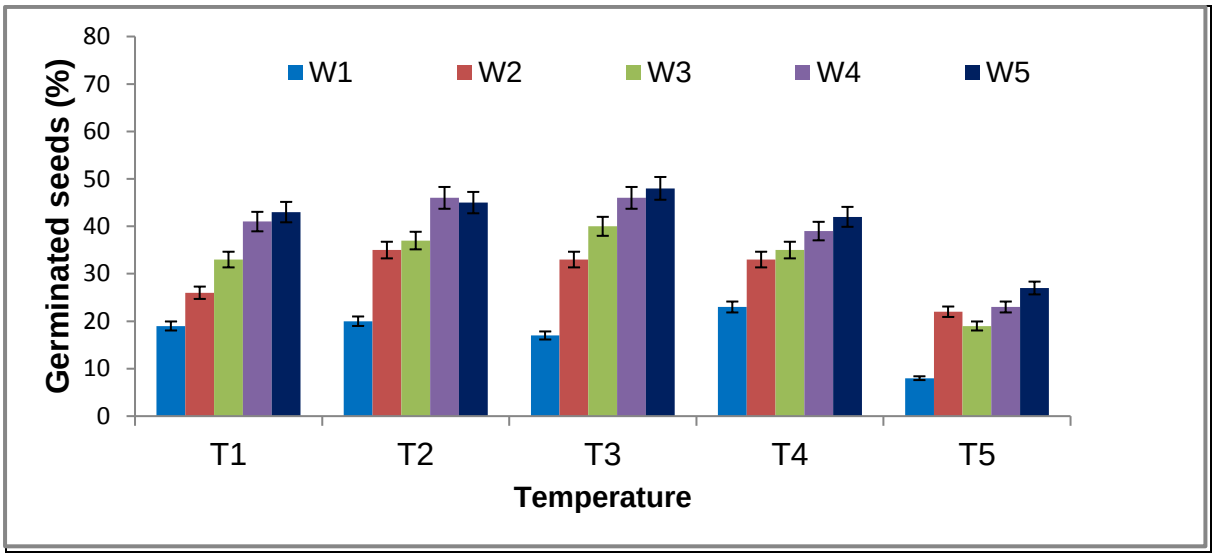

Figure 4.5: Effect of time and temperature on the germination percentage of *L. sericea* seeds. W1= Week one, W2=Week two, W3= Week three, W4= Week four, W5= Week five.

4.3.6. Interactive effect of time and pre-sowing treatments on seed germination

The interaction of time and pre-sowing treatment also significantly affected seed germination of *L. sericea* compared to control (Figure 4.6). The highest germination percentage (65%) was obtained with seeds treated with smoke water at week five and was followed by control with 48% at the same time period (week five) (Figure 4.6). In all the pre-sowing treatments, the highest germination percentage was achieved after week five. This suggests that, the longer the germination time the higher the germination percentage. In other words, regardless of the pre-sowing method, the period of germination was an important factor determining the seed germination percentage of *L. sericea* seeds. The highest seed germination percentage (65%) was observed for smoke water treated seeds after a period of five weeks (Figure 4.6). The lowest seed germination percentage in all weeks was noted for seeds that were treated with hot water (SH), which might suggest that the seeds were affected by the temperature of the hot water.

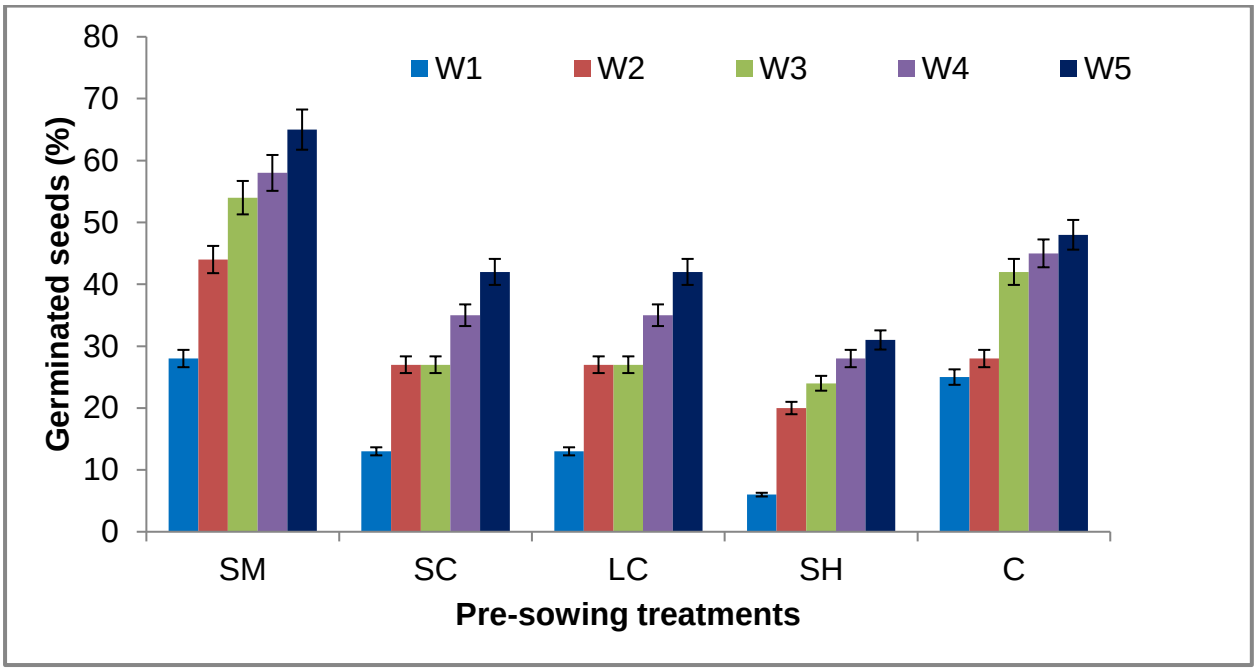

Figure 4.6. Interactive effect of time and pre sowing treatments on germination of *L. sericea* seeds. W1= Week one, W2=Week two, W3= Week three, W4= Week four, W5= Week five.

4.4. CONCLUSIONS AND RECOMMENDATIONS

For successful seedling establishment, temperature and the time of seed sowing were determined to play a major role. Pre-sowing treatments can also play a major role in commercial farming as these methods optimize germination and ensure uniform crop maturity, which is a desirable factor for commercial farmers. *Leucosidea sericea* flowers from August to December (Palgrave *et al*., 2002; Grant and Thomas, 2011) with the very small fruit (about three mm) which is available from December to January.

In this study the germination results revealed that *L. sericea* seeds were affected by pre-sowing treatments. Of the five constant temperatures 15°C followed by 20°C were the best (Table 4.3). Thirty degrees Celsius had the lowest number of germinated seeds at 20% when compared to the other germination temperatures.

The interaction of temperature and pre-sowing treatments significantly affected seed germination of *L. sericea* compared to controls (untreated seeds) (Figure 4.4). The seeds treated with water either through leaching (LC), soaking in both hot (SH) and cold water (SC) had low germination and high mortality at all the temperature levels when compared to seeds treated with smoke water and the control. Germination percentage was higher for untreated seeds when compared to water treated seeds (leaching, soaking (hot) and soaking (cold)). Smoking had the highest number of

germinated seeds across all temperature levels when compared to the other pre-sowing treatments. This result is in agreement with Roche *et al*. (1997) where significant differences in germination were achieved when smoke water seeds were compared to untreated seeds. Smoke water treatment had the highest germination percentage across all temperatures, but germination was the highest at 15°C with 53% seed germination. However there were no significant differences at 15°C between untreated seeds and smoke water treated seeds. There was a general increase in seed germination with time regardless of the temperature. It can be concluded that the longer the germination period the higher the germination percentage would be (Figure 4.5).

As recorded in Figure 4.6 the germination percentage of *L. sericea* increased with time from week one up to week five, however the germination percentage varied and was slightly reduced when seeds were leached and soaked in cold water and hot water compared to smoke water treatment and the control across the five weeks period. Of the four pre-sowing treatments, smoke water treatment had the highest number of germinated seeds at 65% at week five, followed by control with 58% at week four when pre-sowing treatments interacted with temperature. Germination increased linearly from week one up to week five for all the treatments employed in this study. The germination percentage of untreated seeds deteriorated at week five due to rotting of seeds on the other hand smoking had the lowest amount of rotting hence the highest germination percentage.

Leucosidea sericea is a prolific seed producer and can establish easily in the wild. This is evidenced by the average germination percentage of the control treatments in the study. Smoke water treatment enhanced the germination percentage slightly, which might indicate that the veld fires in the region might be important in increasing germination of this species in the wild. The marked decrease in germination at 30°C shows that the species prefer milder temperatures of spring to germinate in natural conditions. Due to the acceptable germination response of the control treatments, *L. sericea* seeds can be germinated without any pre-treatment at a temperature of around 20°C. As the species is a prolific seed producer, many seeds can be harvested for germination and germination at 48% is therefore acceptable to simplify the germination process in a commercial setting.

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CHAPTER FIVE

DETERMINING MINIMUM INHIBITORY CONCENTRATION OF STORED *LEUCOSIDEA SERICEA* LEAVES AGAINST *PROPIONIBACTERIUM ACNES*

5.1. INTRODUCTION

Leucosidea sericea showed very good antibacterial activity against *P. acnes* in a previous study by Sharma *et al*. (2014). The activity of the extract was ≤15.62µg/mL and the activity of the active compound alpha kosin was ≤1.95µg/mL. For quality control purposes, an anti-bacterial assay was performed on the stored leaves to determine the effect of storage on the anti-bacterial activity against *P. acnes*.

The manner in which acne vulgaris develops (pathogenesis) is multi-factorial and one of the most important factors is inflammation caused by a response to the gram positive bacterium *P. acnes* (Burkhart and Burkhart, 2003) which is capable of biofilm formation. The other three pathophysiologic factors include androgen-mediated stimulation of sebaceous gland activity, keratinization and comedogenesis. Although there is a wide range of acne treatments (Haider and Shaw, 2004) the response of acne to treatment varies considerably. Topical retinoids, topical antimicrobials, isotretinoin and oral antibiotics are the most commonly used methods of treating acne and these methods are typically employed to kill bacteria or inhibit inflammation (Haider and Shaw, 2004; Kim *et al*., 2008). Current therapies available to patients from pharmacies (over the counter) are on the rise and can be classified into five major groups namely: cleansers, leave-on products, mechanical treatments, essential oils and vitamins (Decker and Graber, 2012). These treatments are either used individually or concomitantly depending on the diagnosis and severity of the disease (Leyden 2003; Coenye *et al*., 2012). Since resistance to bacteria is a major setback in the treatment of acne, cures which can inhibit the growth of *P. acnes* and at the same time suppress inflammation might increase the cure of acne.

Literature suggests that plant extracts can possibly be used as therapeutic agents for acne (Chomnawang *et al*., 2007; Kim *et al*., 2008; Tsai *et al*., 2010). Antimicrobial and anti-inflammatory effects of herbs such as rose (*Rosa damascene*), dzhong (*Eucommia ulmoides* oliv.) and yerba mate (*Ilex paraguariensis*) were found to inhibit *P. acnes* at MIC values of 2.5 and 1mg/mL respectively. Although *L. sericea* is used against various ailments, the phytochemical research on this plant is very limited. In a previous study by Sharma *et al*. (2014) ethanolic extracts of *L. sericea* were found to inhibit *P. acnes* at significant MIC values of ≤15.62µg/mL. This study therefore investigated the effect of stored leaves harvested in four seasons on the anti-bacterial activity against *P. acnes*.

5.2. MATERIALS AND METHODS

5.2.1. Plant collection and preparation of the leaf extract

Leaves of *L. sericea* were collected consecutively in four different seasons namely; spring, summer, autumn and winter. The leaves were then air dried and protected from direct sunlight at room temperature. The dried leaves were ground into fine powder and stored at room temperature in airtight containers protected from direct sunlight. The duration of storage was dependent on the time of harvest. A set of leaves were stored for one year eight months (harvested during spring), another set for one year five months (harvested during summer), another for one year four months (harvested during autumn) and for one year (harvested during winter).

The powdered plant materials (three grams) were soaked in 30mL of ethanol for 72hrs and placed on a shaker at room temperature for extraction of the compounds for the anti-bacterial assay. The extracts were filtered and the filtrates were placed under laminar flow to evaporate the remaining solvent to produce 8-10mg of crude ethanol extract.

5.2.2. The anti-bacterial assay protocol

Minimum inhibitory concentration (MIC) values of ethanolic extracts of *L. sericea* samples were determined for anti-bacterial activity against *P. acnes* using a serial dilution method as described by Eloff (1998). The extract stock solution was prepared by weighing 2mg of the ethanolic plant extracts in 2mL Eppendorf tubes. One mL of 10% DMSO (dimethyl sulfoxide- C_2H_6OS) was added to the 2 mg dried leaves and sonicated at 40°C for 5min then 100µL of double distilled water (ddH_2O) was added.

The bacterial concentration used in the assay corresponds to the 0.5 McFarland standards. The bacterial cultures of *P. acnes* were grown on Brain Heart Infusion agar (Merck SA (Pty) Ltd.) and transferred to Nutrient Broth (Merck SA (Pty) Ltd.) using a sterile inoculation loop. The OD (optical density) was then adjusted to 0.132 at 600nm using a Beckmann spectrophotometer. This OD corresponds to the 0.5 McFarland standard which equates to 10^8CFU/mL.

A positive control was prepared by adding tetracycline (Sigma-Aldrich- Kempton Park, South Africa) in a 15mL tube (0.2mg/mL) dissolved in autoclaved dH_2O which resulted

in 2mg of tetracycline in 10mL dH_2O. A volume of 100µL was then added in a serial dilution method over a range to give concentrations of 500-3.9625µg/mL to the plant extracts and positive control wells (Figure 5.1). The bacterial control was used to make sure that the bacterial cells used in the assay were viable. The DMSO control was used as a solvent control to make sure that bacterial inhibition was due to the sample activity and not the solvent vehicle. The medium was used as control to ensure that the media used in the assay was free of contaminating microorganisms and that any change in the growth reagent was due to the *Propionibacterium acnes* cells and not another species (Figure 5.1).

Before the serial dilution assay was undertaken, the 96-well plates with the prepared samples were incubated for 72hrs at 37°C under anaerobic conditions in Anaerocult A (Merck SA (Pty) Ltd.) in the dark. Twenty microlitres of PrestoBlue (resazurin-based solution that functions as a cell viability reagent) was added to determine the MIC values by visually observing the colour change in the wells.

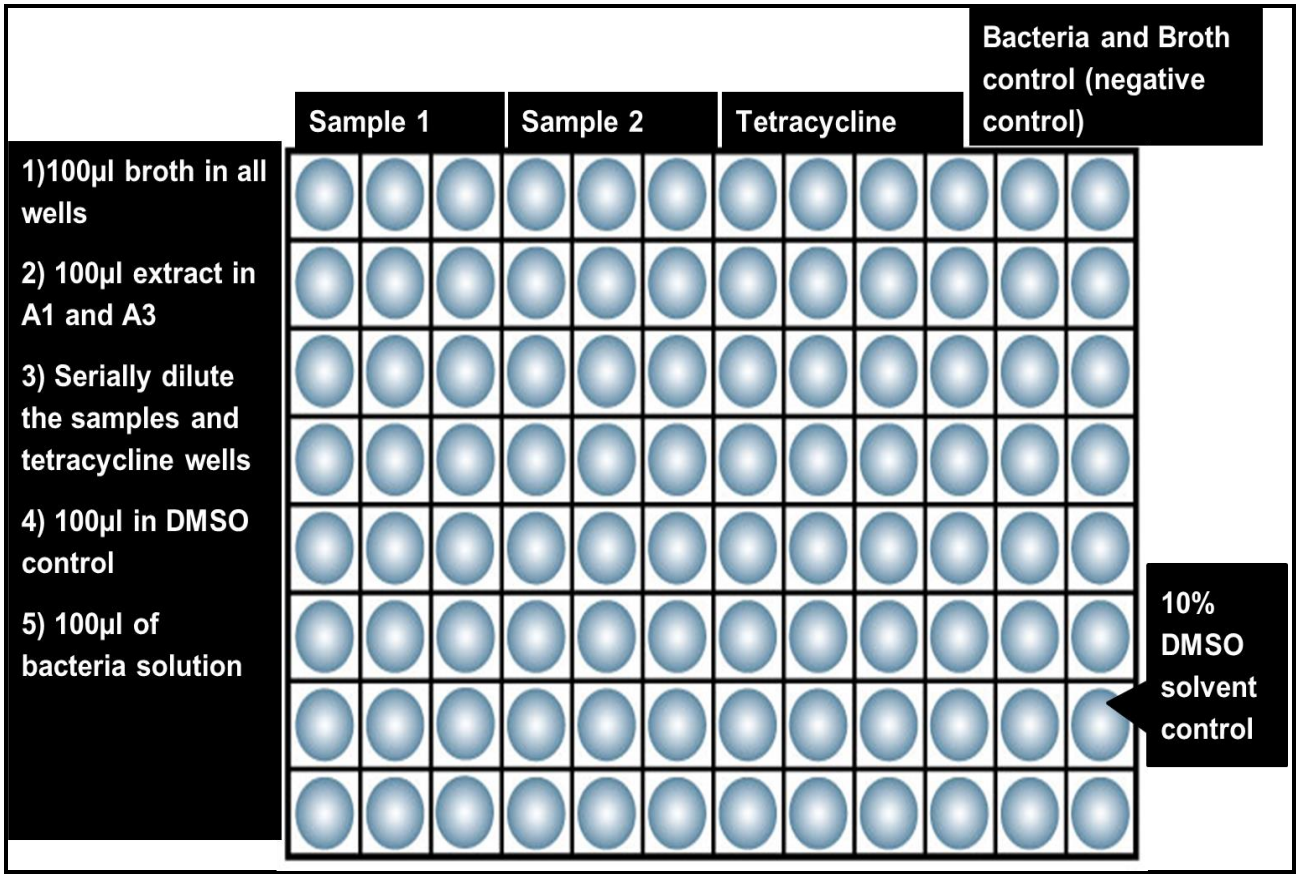

Figure 5.1: Ninety six-well plate layout with sample preparation and controls

Since PrestoBlue is a resazurin-based dye blue in colour, it converts to resorufin (Pink) in the presence of viable bacterial cells. The minimum inhibitory concentration of the samples was defined as the lowest concentration of the plant extract that is required to inhibit the conversion of the PrestoBlue from blue to pink.

5.3. RESULTS AND DISCUSSION

The antibacterial activity of *L. sericea* leaf ethanol extracts against *P. acnes* are summarised in Table 5.1. After the addition of PrestoBlue, the MIC values of tetracycline (positive drug control) was determined to be ≤3.90µg/mL and the extract from leaves harvested in winter inhibited the bacteria at the same concentration. Sharma *et al*. (2014) found corresponding MIC values of ≤3.12µg/mL for tetracycline in a study were *P. acne* was investigated using *L. sericea* leave extracts. The ethanol leave extracts of *L. sericea* exhibited antibacterial activity with MIC values ranging from ≤3.90µg/mL in winter, ≤31.25µg/mL in spring, ≤7.81µg/mL in autumn and ≤15.62µg/mL in summer (Table 5.1). These results are similar to the findings by Sharma *et al*, (2014) where ethanol extracts of *L. sericea* leaves inhibited *P. acnes* at ≤15.62µg/mL which corresponds to the summer extracts of the current study.

Table 5. 1: The MIC values of *L. sericea* extract against *P. acnes* with MIC values in µg/mL

Sample Id	**Minimum Inhibitory concentration (MIC) µg/mL**	**Season**
T5 S2 S1	31.25	Spring
T4 S1 S1	15.62	Spring
T3 S1 S2	15.62	Summer
T4 S2 S2	15.62	Summer
T5 S1 S3	7.81	Autumn
T5 S1 S3	7.81	Autumn
T5 S2 S4	7.81	Winter
T6 S1 S4	3.90	Winter

In the present study, the best activity was exhibited by autumn and winter seasons harvested samples with MIC values of ≤7.81µg/mL and ≤3.90µg/mL respectively, followed by spring and summer with MIC values of ≤31.25 and ≤15.62µg/mL,

respectively (Table 5.1). This is the first study where the effect of storage and season of *L. sericea* leaves was determined on inhibition of *P. acnes.* In a previous study Sharma *et al*. (2014) it was reported that ethanolic leaf extracts of *L. sericea* inhibited *P. acnes* at ≤15.62µg/mL and that alpha kosin inhibited the acne inducing bacteria at ≤1.9µg/mL. From the results a clear distinction can be made in activity in different seasons, with the highest activity obtained in winter.

Leucosidea sericea ground leaves were harvested in four different seasons and then stored for one year eight months (harvested during spring), one year five months (harvested during summer), one year four months (harvested during autumn) and then for one year (harvested during winter). Perusal of Figure 5.2 indicates the effect of storage time of leaves harvested in summer and winter seasons on the activity of *L. sericea* against *P. acnes*. The antibacterial activity of the extract showed excellent activity at ≤3.90µg/mL when leaves were harvested in winter and stored for a year. The autumn extract, stored for 17 months inhibited the growth of *P. acnes* at ≤7.8125µg/mL. The other two seasons (summer and spring) inhibited *P. acnes* at ≤15.625 to ≤31.25µg/mL when stored for one year five months and one year eight months, respectively. All the extracts were re-tested after six months to ensure that the length of storage did not affect the activity. The same activity levels were obtained indicating that storage does not have a great influence on the antibacterial activity.

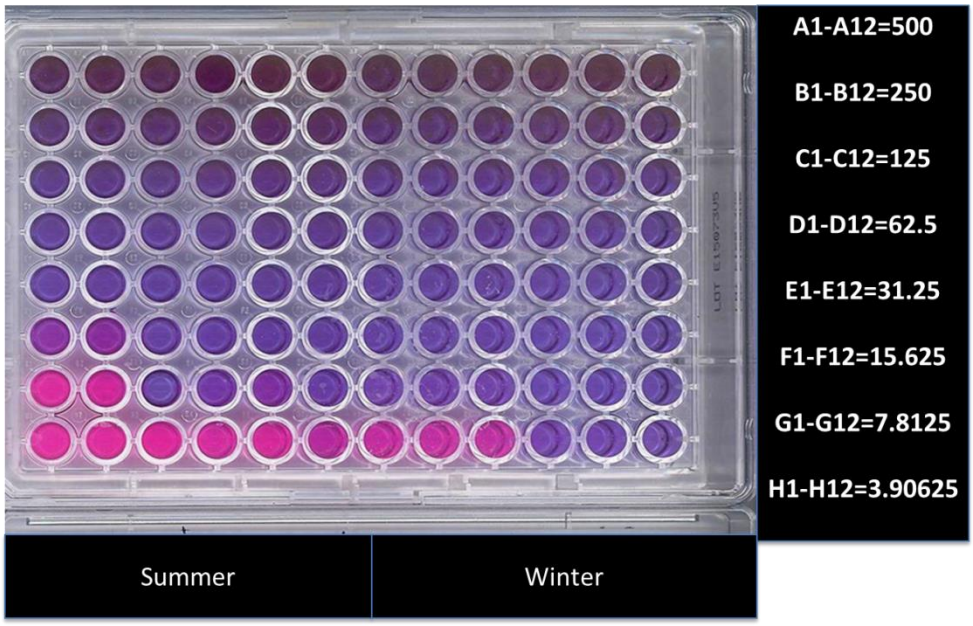

Figure 5.2: Ninety six well plate representing summer and winter seasons with their replications

5.4. CONCLUSIONS AND RECOMMENDATIONS

The antibacterial assay conducted confirms the antibacterial activity of *L. sericea* crude extracts against *P. acnes* as previously reported by Sharma *et al*. (2014). Based on the results obtained, it can be concluded that ethanol plant extracts of *L. sericea* inhibited the growth of *P. acnes* with significant MIC values of ≤3.90µg/mL for winter and lower during autumn, spring and summer. When the leaves that were harvested in different seasons were stored for a year or longer at room temperature similar activity was obtained even with the repetition on the assay. This suggests that storage does not have a negative effect on the antibacterial activity of *L. sericea* leaves for a period of at least one year. Therefore, season has a major effect on activity, with no effect from storage. Since the powdered leaves were stored in a laboratory at room temperature, it implies that this method of storage will be affordable to farmers. It is also evident from the analysis that storage did not affect the activity after storage of up to a year at room temperature, indicative of stable compounds within the plant.

Based on these results, it is recommended that the leaves must be harvested in winter when the antibacterial activity is optimum. The leaves can be stored at room temperature, protected from direct sunlight for up to one year without compromising the antibacterial activity. The antibacterial activity in winter was similar to the positive control, but better when compared to previously reported antibacterial activity by Sharma *et al*. (2014).

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CHAPTER SIX

EFFECT OF SEASON ON THE QUALITY OF CULTIVATED *LEUCOSIDEA SERICEA* LEAVES

6.1. INTRODUCTION

Metabolite refers to any chemical compound formed as an end result of metabolism or a metabolic reaction (Chawla, 2002) and metabolite levels can be regarded as the ultimate response of biological systems to genetic or environmental factors (Fiehn, 2002). Metabolomics denotes an all-inclusive, non-discriminatory high-throughput analysis of intricate metabolites such as those found in plant extracts (Fiehn, 2002). Plants metabolizes more than 200 000 different molecules which are involved in the structure, assembly, and maintenance of tissues and organs, as well as in the physiological processes related to growth, development and reproduction (Borém and Fritsche-Neto, 2014). Metabolomics is used in a wide range of applications such as plant breeding, assessment of crop quality, food assessment, toxicity assessment, nutrition assessment, medical diagnosis, assessment of disease status, pharmaceutical drug developments, yield improvement in crops and fermentation, environmental adaptation, biomarker, gene-function elucidation, integrated systems biology and technological advances in analytical chemistry (Moco *et al*., 2007). Abiotic factors such as ultra violet light, temperature and biotic factors such as parasitism and pathogenic attack influence the development of complex metabolic compounds in plants (Hardy and Hall, 2012).

Metabolomics was used in this study to investigate metabolite variation amongst seasons (winter, summer, spring and autumn) in *L. sericea* leaves. Ethanolic extracts of *L. sericea* and alpha kosin (Sharma *et al*., 2014) were reported to have anti-bacterial activity against *P. acnes*, hence a metabolomic study was then ensued for quality control purposes to determine whether the antibacterial activity of the leaves changes with seasons and storage. The study sought to determine whether variation was due to the active compounds which were previously identified from the plant (Sharma *et al*., 2014).

6.2. MATERIALS AND METHODS

6.2.1. Seasonal Variation

Material was harvested in October 2013 (spring), January 2014 (summer), April 2014 (autumn) and June 2014 (winter). The leaves were then air dried away from direct sunlight at room temperature. The storability of the dried plant material was evaluated at three to four months storage intervals. Metabolomics profiling (sample extraction,

data mining, processing and analysis), as proposed by Maree and Viljoen (2012) was used to determine the effect of season on the quality of cultivated material by using ^{1}H-NMR where the metabolome of *L. sericea* samples were investigated.

6.2.2. Sample preparation and extraction

The plant material was extracted using a direct extraction method. Fifty milligrams of the dried leaves were weighed in 2mL eppendorf tubes and then extracted with 0.75mL of deuterated methanol (CH_3OH-*d4*) and 0.75mL of potassium dihydrogen phosphate (KH_2PO_4) buffer in deuterated water (D_2O) containing 0.1% (w/w) TSP (trimethylsilylpropionic acid sodium salt) and NaOD (deuterated sodium hydroxide) was used to achieve the desired pH (pH 6.0). The samples were then vortexed for 1-2min at room temperature, ultra-sonicated at 30ºC for 20min and then centrifuged at 13 000rpm for 20min. The supernatant from each tube was then transferred into 5mm NMR tubes where 32 scans were performed in a 600MHz NMR spectrometer.

6.2.3. Data analysis

A 600MHz NMR spectrometer (Varian Inc, California, USA) was used to obtain spectral data (^{1}H-NMR) with 32 scans. ^{1}H-NMR metabolomics was used to define the differences and similarities between different samples taking a snapshot of the metabolome of the plant. The spectral data from NMR was then processed using MestReNova software (9.0.1, Mestrelab Research Spain) where the spectral data from NMR was subjected to: phase corrections, baseline correction, referencing and normalising after which the NMR spectral data was binned by peak from 0.00 to 10.0ppm and the regions were divided into 0.04ppm bins. The impurities on the chemical shift range of δ 4.70-4.90 (water peaks) and 3.23-3.36 (methanol peaks) were excluded from the final data for further analysis. The data from MestReNova was converted into an excel sheet which was utilised for further analysis in SIMCA-P software (13.0, Umetrics, Sweden). Multivariate data analysis was performed using SIMCA-P where the data was first analysed using an unsupervised method known as principal component analysis (PCA-X), followed by orthogonal partial least square discriminatory analysis (OPLS-DA), a supervised model. Scores plots, contribution plots and loading plots were used to determine sources of variation and the NMR

values from the plots were then used in conjunction with databases and published literature for annotation.

6.3. RESULTS AND DISCUSSION

A principal component analysis (PCA) of the four seasons provided a clear overview of the similarities and variations between and amongst seasons (Figure 6.1). The principal component analysis provided an unsupervised overview of the metabolome of the plant leaves harvested in spring, autumn, winter and summer seasons, where variation was observed and samples similar in profiles grouped together. Informed by the anti-bacterial assay which resulted with winter having the highest anti-bacterial activity at the MIC value of ≤3.90µg/mL and spring with the lowest activity at ≤31.25µg/mL (Table 5.1 in chapter 5), a decision was taken to further investigate these two seasons only.

The multivariate analyses (both PCA-X and OPLS-DA) on spring and winter were done to determine possible causes of variation (Figure 6.2). After the removal of water and methanol peaks (impurities), spring and winter separated on the x-axis as shown in Figure 6.2. Analysis of the loadings plot in conjunction with the contribution plots reveals that the separation into the two groups can be ascribed to various regions including the lower intensity of the sugar region at ~3-4ppm in samples from winter compared to spring. Figure 6.2 depicts winter grouping on the right of the ellipse and spring on the left of the ellipse. For a more detailed analysis of spring and winter, contribution plots and ^{1}H-NMR peaks were used for winter in order to determine possible sources of variation and the possible reasons for the high anti-bacterial activity and low anti-bacterial activity in these seasons.

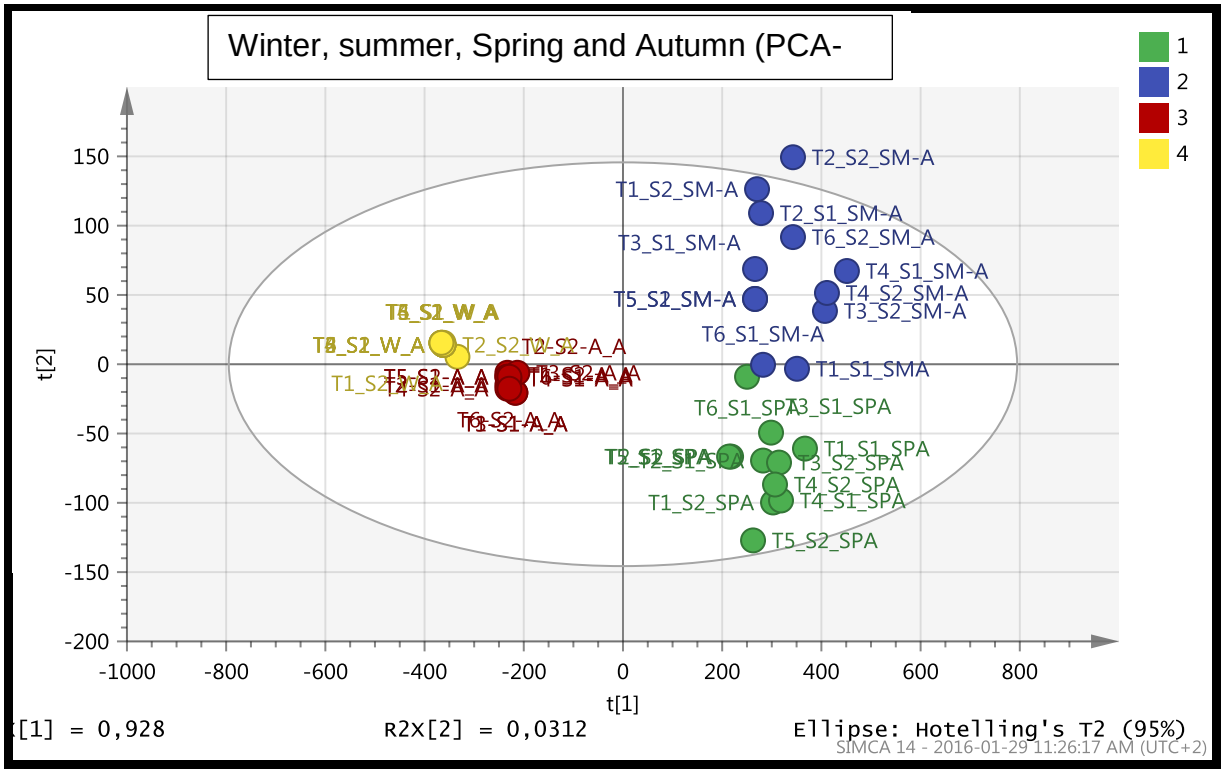

Figure 6.1: Principal component analysis (PCA-X) scores plot of *L. sericea* leaves (R2= 0.977 and Q2= 0.955) classified by seasons: summer (blue), spring (green) autumn (red) and winter (yellow).

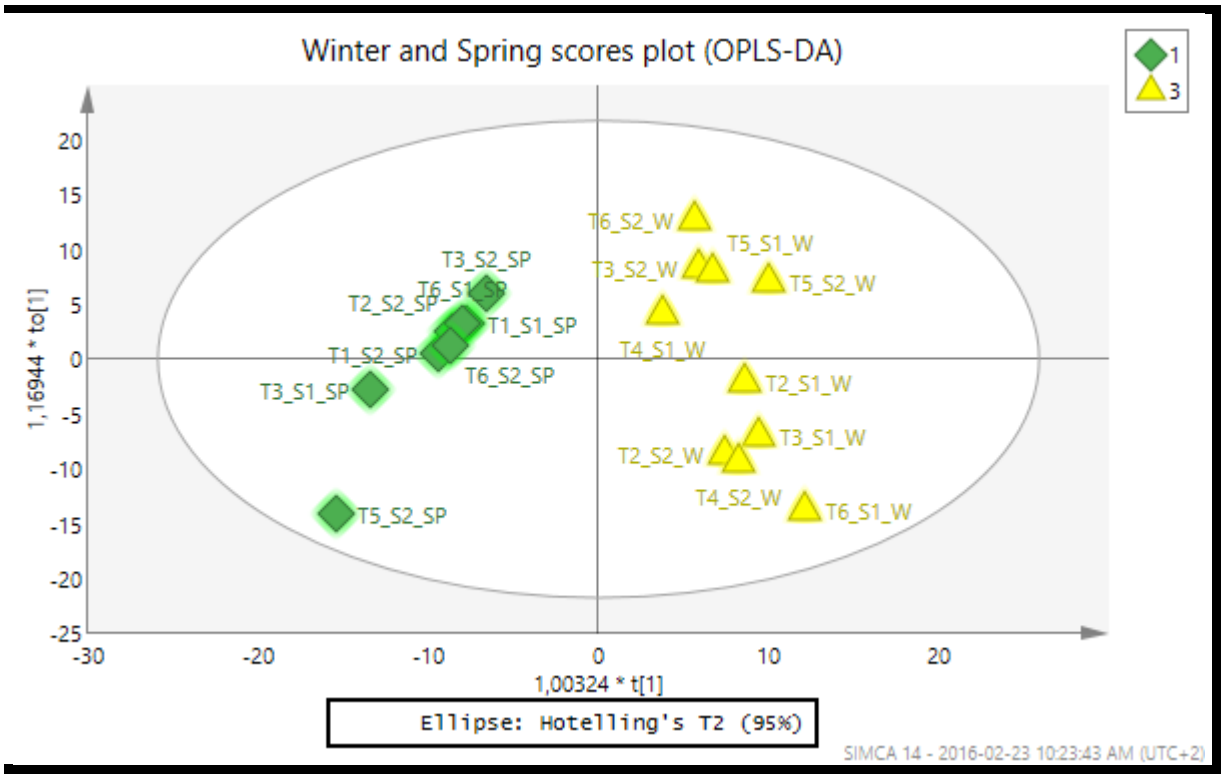

Figure 6.2: Orthogonal partial least square discriminatory analysis (OPLS-DA) scores plot results of *L. sericea* leaves depicting spring (green) and

winter (yellow) The model showed a cross validated accuracy of 95% with R2X=0.914 and R2Y=0.897 for the ethanol extracts.

Compounds that were previously isolated from *L. sericea,* namely: alpha kosin, phytol, phytol acetate, triacontanol, and (E)-3,7,11,15-tetramethylhepadec-2-ene1,17-diol (Sharma *et al.,* 2014) (Table 6.1) were checked against the cause of separation. Through the use of contribution plots, published literature and the ^{1}H-NMR peak data from this study, these compounds were ruled out as the cause of variation due to the absence of most of their peaks in the contribution plots and the NMR spectra. In order to determine the possible cause or causes of variation, contribution plots (Figures 6.3, 6.4 and 6.5) were created for winter from the scores plot (Figure 6.2) above. Figures 6.3 and 6.4 indicates that alpha kosin and phytol were not responsible for variation in the grouping observed in Figure 6.1 and Figure 6.2 above as the peaks associated with these compounds are negatively associated with winter which showed the best activity.

The contribution plot of spring and winter were opposites of each other. For example, the sugar region (~3-4 – 3.9ppm), apha kosin (Figure 6.3) and phytol (Figure 6.4) are positively correlated with spring at the same time negatively correlated to winter, meaning sugar was available in a higher concentration in spring but lower in winter. The same is true for the peaks of the compounds which are positively correlated with winter and not with spring. Falasca *et al*. (2014) observed similar profiles of metabolites from plants harvested in different seasons from the same plant. Using standard analysis methods such as PCA and PLS, Grandizoli *et al*. (2014) was able to pinpoint spectral areas that were responsible for variation in different areas where grapes were grown.

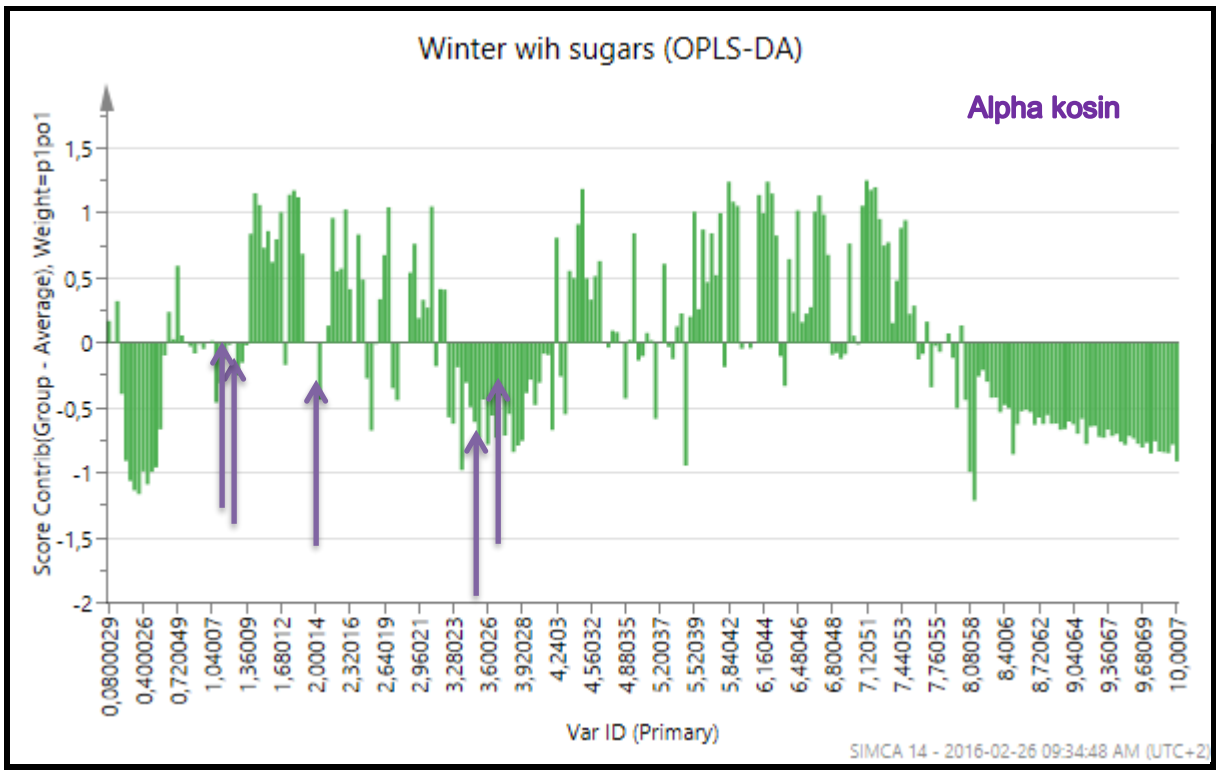

Figure 6.3. Alpha kosin regions indicating that the compound was not responsible for separation.

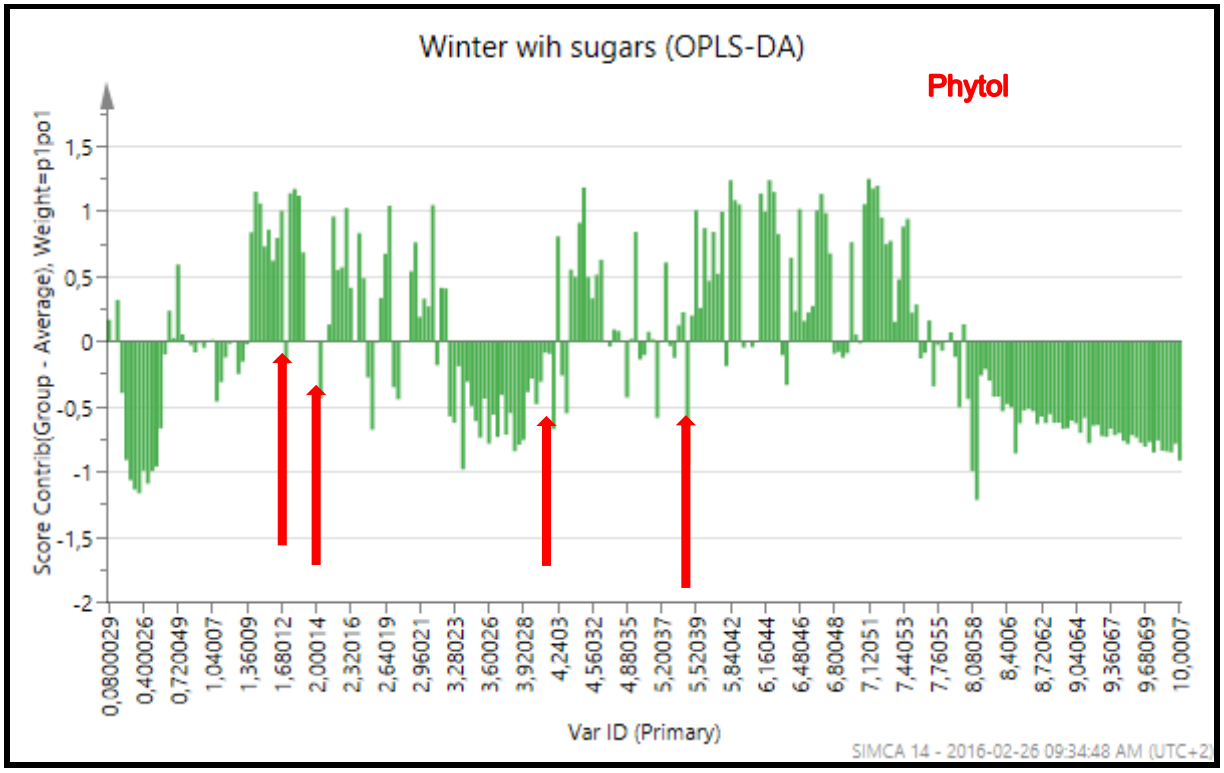

Figure 6.4 Phytol contribution plot regions shown as negative correlation to the separation

Table 6.1: ^{1}H chemical shift values for compounds isolated from *L. sericea*

Phytol acetate	Triacontanol	Phytol	Alpha Kosin	(E)-3,7,11,15-tetramethylhepadec-2-ene1,17-diol
0.83-0.88	0.88	1.67	1.159	0.86-0.883
2.00	1.26	1.96	1.190	1.42-1.10
2.06	2.16	4.15	1.223	1.65
4.58	3.63	5.4	1.245	1.97
5.34			1.254	2.01
			2.112	3.62
			3.716	4.12
			3.522	5.43

The compounds that were annotated as responsible for variation in winter were compounds with peaks representing those of glucose (negatively associated), kaempferol and quercetin (positively associated) or similar compounds (Figure 6.5). The annotation was done using the contribution plots, literature, databases and the ^{1}H-NMR spectra.

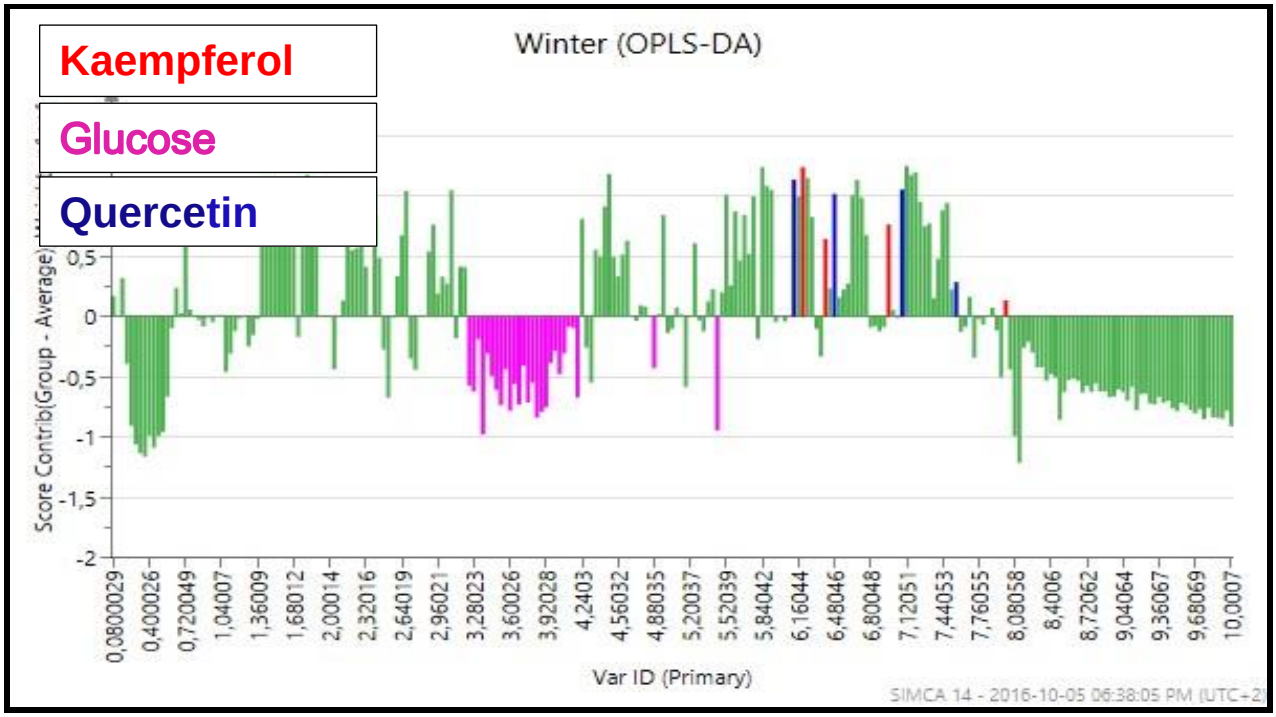

Figure 6.5: Contribution plot showing variables that were responsible for winter grouping. Red arrows represent annotated kaempferol, and blue quercetin whilst purple represents glucose ^{1}H-NMR chemical shifts

Possibly, kaempferol and quercetin ^{1}H-NMR peaks were observed in winter (Figure 6.5) as the NMR regions of the peaks of these compounds match the NMR regions positively associated with the winter contribution plot. Kaempferol and (±)-catechin were found to be the most promising acne treatment compounds by Falcocchio *et al*. (2006) where kaempferol inhibited GehA (glycerol-ester hydrolase A) an enzyme recognised as one of the major factors in the pathogenesis of acne, at IC_{16} of 1.4×10^{-4} M and IC_{50} of 2.3×10^{-4} M. Kaempferol and quercetin demonstrated antibacterial activities against *P. acnes* in a study by Lim *et al*. (2007) where minimum inhibitory concentrations for both compounds were less or equal to ≤64µg/mL against *P. acnes*. Furthermore, the two compounds exhibited a synergic inhibition of *P. acnes* growth when kaempferol and quercetin were combined with erythromycin and clindamycin (Lim *et al*., 2007). Kaempferol is a naturally occurring flavonol found in a variety of plants such as vegetables in the cruciferous family (e.g. broccoli, cabbage, kale) and beans, endive, leek and fruit trees such as grapes and strawberries (Calderon-Montano *et al*., 2011). Kaempferol has a wide variety of uses ranging from dietary, epidemiological to pharmaceutical (Calderon-Montano *et al*., 2011). In a study by Calderon-Montano *et al*. (2011) kaempferol peaks were found to have ^{1}H-NMR chemical shifts at δ 6.2, δ 6.4, δ and δ 6.9 and at δ 8.0.

Similarly, in this study the ^{1}H-NMR chemical shifts were at δ 6.2, δ 6.3, and δ 6.9 and at δ 8.0 (Figure 6.5). Quercetin ^{1}H-NMR peaks absorbed at δ 7.6, δ 6.9, δ 6.5 and δ 6.2 (Table 6.2). Quercetin is said to be the most abundant flavonol in vegetables and fruits with numerous beneficial effects such as antibacterial, antiviral, anti-oxidant, antiproliferative, anti-inflammatory, and anti-carcinogenic (Wollenweber and Jay, 1988 and Pedersen *et al*., 1993). Figure 6.6 shows the NMR profiles for winter and summer and it is clear that some compounds are present in higher concentrations in winter. These peaks once again confirm the possibility of kaempferol and quercetin or very similar compounds as they show peaks in these regions. Figure 6.7 shows the areas of keamperfol and quercetin (obtained from our NMR data) and the peak shapes that correspond to those of kaempferol and quercetin in literature. Figure 6.8 shows kaempferol and quercetin chemical structures.

Table 6.2: NMR regions of quercetin and kaempferol compounds (literature and actual ppm values)

Quercetin	Quercetin study	Kaempferol	Kaempferol study
6.198	6.2	6.18	6.2
6.417	6.5	6.39	6.4
6.887	6.9	6.89	6.9
6.902	7.6	6.90	8.0
7.537		6.91	
7.682		8.07	
7.685		8.08	
7.686		8.09	

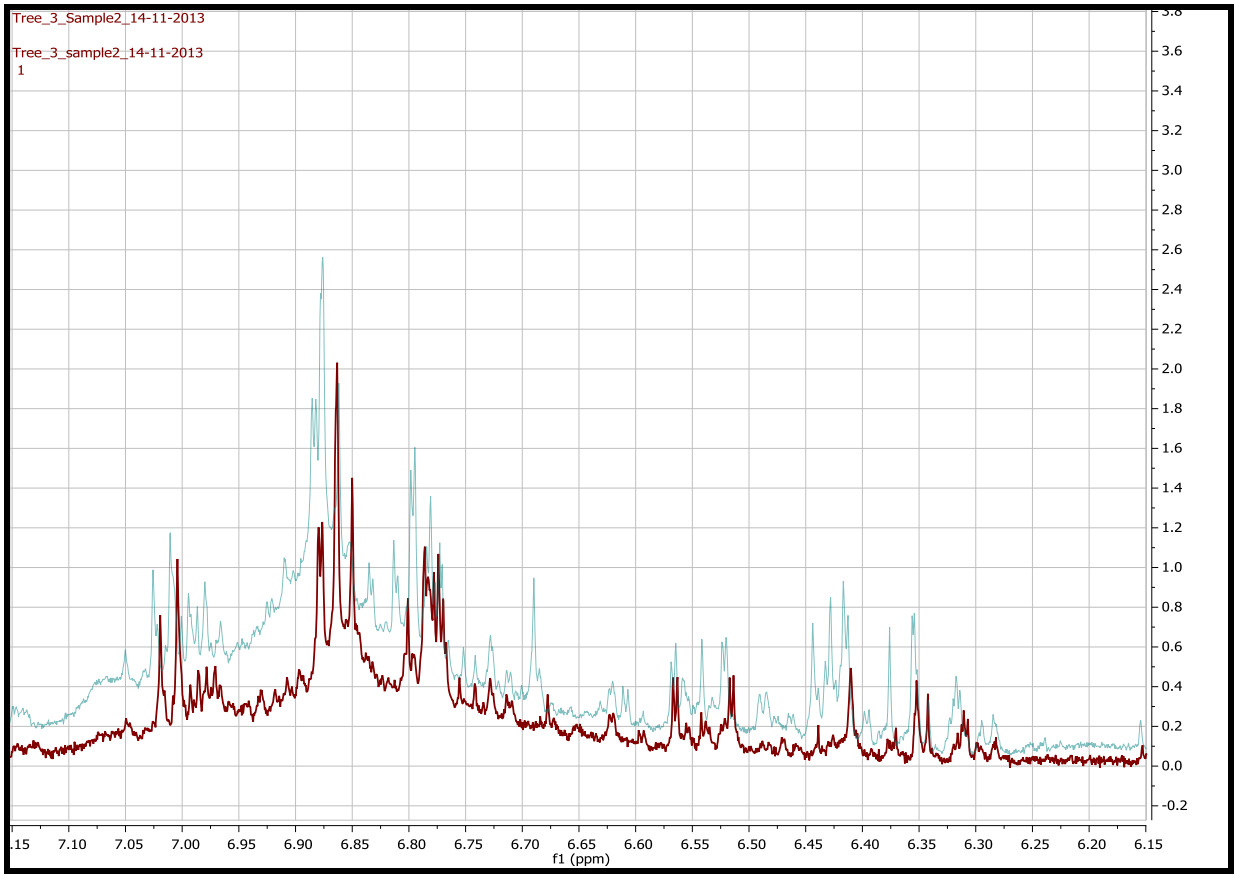

Figure 6.6 NMR regions of winter (blue) and summer (red) indicating regions related to kaempferol and quercetin.

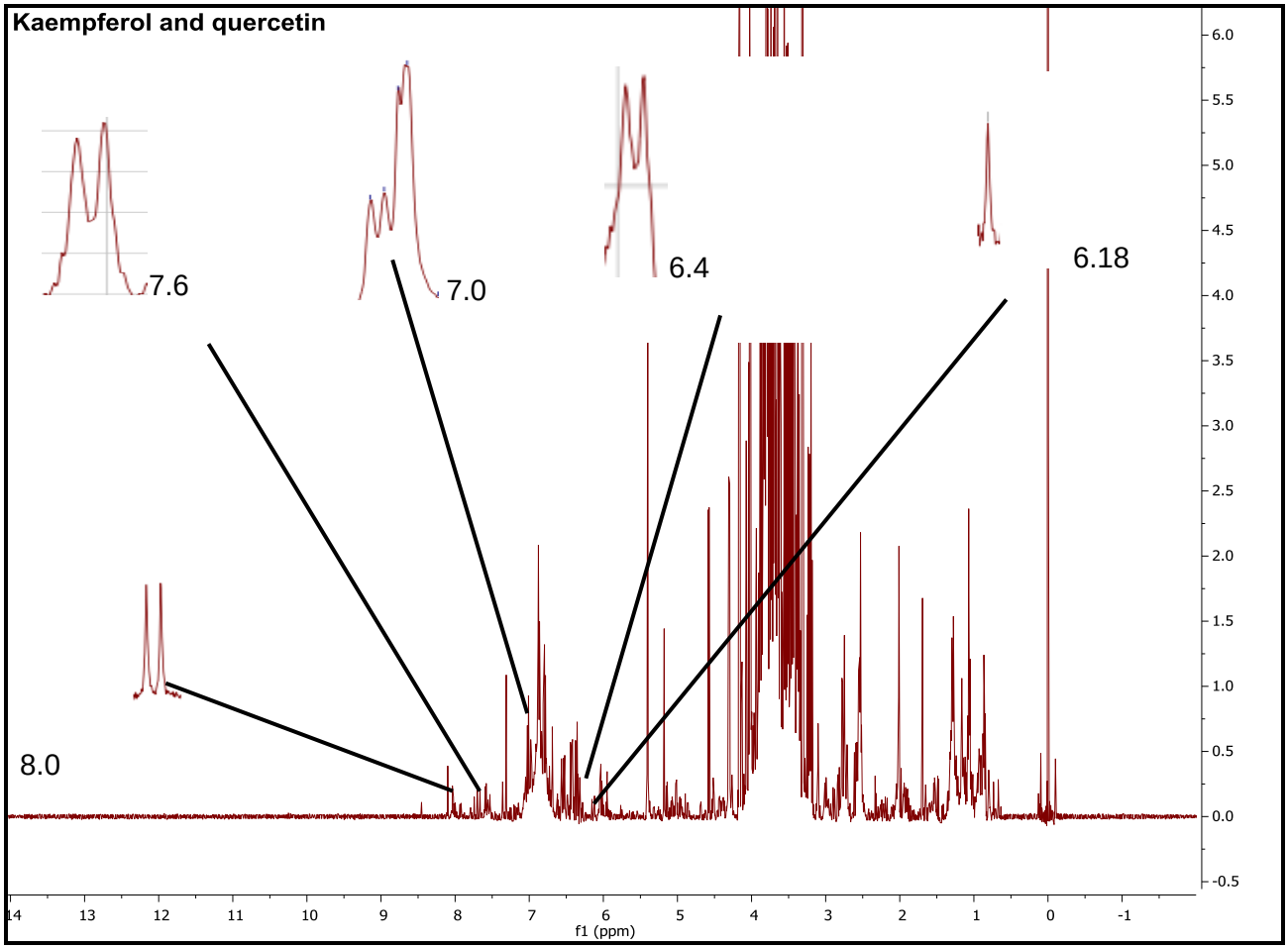

Figure 6.7: Annotated kaempferol and quercetin NMR peaks (6.18, 6.4, 7.0 and 7.6 relate to kaempferol and 6.18, 6.4, 7.0 and 8.0 relate to quercetin peaks.)

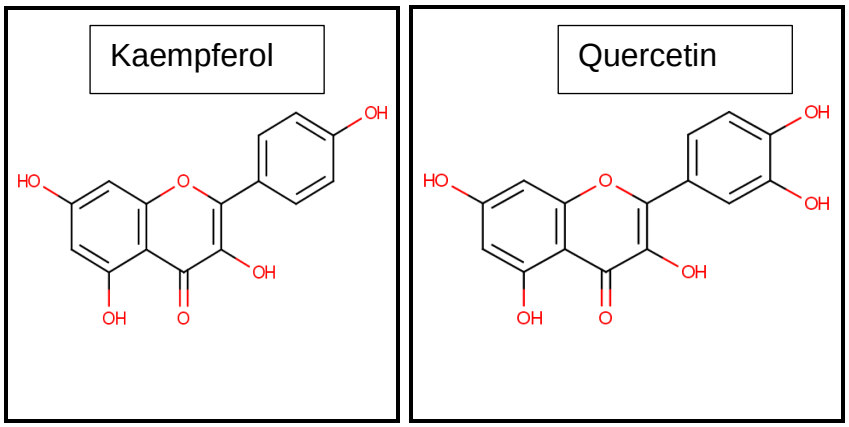

Figure 6.8: Kaempferol ($C_{15}H_{10}O_6$) (left) and quercetin ($C_{15}H_{10}O_7$) (right) chemical structures (http://www.hmdb.ca)

6.4. CONCLUSSIONS AND RECOMMENDATIONS

NMR-based metabolomics was applied to *L. sericea* leaves to successfully detect differences in the metabolome of the plants amongst seasons. The aim of the study was to determine whether there will be differences in the metabolites of the leaves

harvested in different seasons. Multivariate data analysis (PCA and OPLS-DA) performed on ^{1}H-NMR data led to a clear separation of samples according to seasons; summer, spring, autumn and winter. Based on the antibacterial assay results in chapter five, spring and winter were analysed further as they exhibited lowest and highest antibacterial activities respectively. The possible source of variation was determined to be a higher concentration of kaempferol and quercetin or similar compounds in winter. This do however seem very possible as these compounds exhibit anti-acne activity at ≤64µg/mL, (Lim *et al*., 2007), with an additional synergistic effect with other anti-bacterial compounds. It is recommended that further research be conducted on the leaf extracts to isolate the compounds and confirm their chemical identities.

It is therefore recommended that leaf material is harvested in winter, as the highest activity was obtained in this season at ≤3.9µg/mL.

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CHAPTER SEVEN

GENERAL CONCLUSIONS AND RECOMMENDATIONS

7.1. GENERAL CONCLUSIONS

The study investigated different propagation methods as well as quality parameters in production and storage of *L. sericea* for commercial purposes.

Based on the investigations from this study, poor results were obtained with cutting propagation. It is therefore not recommended that *L. sericea* be propagated from cuttings as the general success rate was very low, at less than 2.5% rooting ability of both basal and tip cuttings. This research can be further expanded to include for instance, irrigation scheduling and fertilizer application in combination with hormones and media if clonal material with specific characteristics will be needed in future.

For successful seedling establishment, temperature and pre-sowing treatments played major roles in the germination of *L. sericea* seeds according to results obtained in this study. These two factors (temperature and pre-sowing) optimized germination. The seeds of *L. sericea* germinated better at a temperature range of 15°C to 20°C compared to the other temperatures (10°C and 30°C). Of the five constant temperatures, 15°C followed by 20°C were found to be the best for optimum germination of *L. sericea* seeds (Table 4.3). Thirty degrees Celsius had the lowest number of germinated seeds at 20% when compared to other germination temperatures. The seeds treated with water either through leaching, soaking in both hot and cold water had low germination and high mortality at all the temperature levels when compared to control (untreated seeds) and seeds treated with smoke water, which had significantly the highest germination percentage compared to control, is the best for the propagation of *L. sericea*. The interaction of temperature and pre-sowing treatment had significant effect of seed germination of *L. sericea* (untreated seeds). Seeds treated with smoke water had a significantly higher germination percentage (53%) compared to control (43%) (Figure 4.4) and is the best pre-sowing treatment fo the propagation of *L. sericea*. Similarly, the interaction of period of incubation (time in weeks) and temperature also significantly affected the germination percentage of *L. sericea* seeds (Figure 4.5). The highest percent of germinated seeds was 43% on week five at a temperature of 20°C and the lowest was 8% on week one at 30°C incubation temperature (Figure 4.5). In general, the duration of incubation significantly affected seed germination of *L. sericea* because regardless of the temperature the percent of germinated seeds was highest in week five i.e. germination percentage fluctuated from week one up to week five for all the temperatures and treatments employed in this study (Figures 4.5 and 4.6).

Therefore, it was not surprising that the interaction of pre-sowing and time also affected seed germination. Of the four pre-sowing treatments, smoking had the highest number of germinated seeds at week five (65%), followed by control with 58% at week four (Figure 4.6). The germination percentage of untreated seeds deteriorated at week five due to seed rot, on the other hand smoking had the lowest amount of seed rot hence the highest germination percentage, although it is difficult to explain why. However, it is highly probable that chemicals in the smoke water treatment might have affected microorganisms responsible for seed rot. Smoking treatment had the best effect on seed germination of *L. sericea* with 44% germination in two weeks and a total of 65% germination when the germination period was extended to week five. Time in weeks was positively correlated to germination, and from this experiment it is concluded that the longer the germination period the higher the germination percentage, up to week five as this was the time period investigated in this study.

The ethanol plant extracts of *L. sericea* inhibited the growth of *P. acnes* with significant MIC values of ≤3.90µg/mL in winter with lower activity for summer and spring. When the leaves harvested in different seasons were stored for a year or longer at room temperature, the activity remained the same and therefore without any effect on activity due to storage. Storage for at least one year did not have any negative effect on the anti-bacterial activity of *L. sericea* leaves. Therefore, season has a major effect on activity, with no effect observed for storage.

7.2. GENERAL RECOMMENDATIONS

Based on these results, it is recommended that the leaves must be harvested in winter when the anti-bacterial activity is optimum. The leaves can be stored at room temperature protected from light, for at least a year without compromising the anti-bacterial activity. The anti-bacterial activity in winter was similar to the positive control, and better than what was previously reported by Sharma *et al*. (2014). However, Sharma *et al*. (2014) reported the ethanol leaf extract's anti-bacterial activity to be ≤15.62µg/mL and from this study, the same MIC values were observed from leaves harvested in summer and an even better inhibition of the bacteria was observed in winter with ≤3.90µg/mL. There were differences in the metabolome of the plants amongst the four seasons. The previously isolated compounds did not contribute to the separation observed between summer and winter. Peaks with higher intensity were observed in

regions 6.2-2.9 and 7.2-8.1 during winter when compared to summer profiles. These peaks correlate well to the NMR regions of kaempferol and quercetin or similar compounds and were annotated as possible sources of variation between summer and winter. These compounds are known to have anti-acne activity at MIC ≤64µg/mL (Lim *et al*., 2007). The anti-acne activity of these compounds possibly contributes to the better activity in winter as the concentration of these compounds increased in winter.

7.3. FUTURE RESEARCH

It is recommended that further research be conducted on the leaf extracts to isolate the compounds and to confirm their structures. Confirmation of these compounds would also finally support the seasonal variation and increase in activity. It is however clear, that some compounds are found in higher concentration during winter, which probably contributed to the lower activity observed in winter with a possible additive or even synergistic effect with previously isolated compounds.

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