

Micromorphology and phytochemistry of the foliar secretory structures of *Stachys natalensis* Hochst. and development of an *in vitro* propagation protocol

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ABSTRACT

Many members of the genus *Stachys* have been used as ornamental plants, or as edible foods and, in a number of cultures, as traditional medicine. One such species, *Stachys natalensis* Hochst., is a perennial, straggling shrub with aromatic leaves that are covered with hairs. Despite its widespread use for a number of reasons, little information has been available on this plant thus far, its foliar secretory apparatus, or the nature and potential therapeutic value of these secretions. Hence, the aims and objectives of the present study were to elucidate key micromorphological features of the leaf secretory structures of *S. natalensis*, using both light and electron microscopy and to determine the location and chemical composition of the exudates using various histochemical and phytochemical techniques. An additional aim was to establish an *in vitro* micropropagation protocol for the sustained and high-yielding production of this elusive and often difficult to cultivate species. Furthermore, the foliar micromorphological fidelity between field and *in vitro* propagated material was compared.

Electron micrographs indicated the presence of glandular and non-glandular trichomes on both abaxial and adaxial foliar surfaces of field grown plants. Greater trichome density was observed on the abaxial surface with trichome distribution decreasing as the leaf developed ($p < 0.05$). Uniseriate, unbranched and striated non-glandular trichomes, present on the foliar surfaces of *S. natalensis* are known to play a role in physical plant defence mechanisms against herbivory. The presence of an elevated cellular pedestal and striated micro-ornamentation on the stalk served as characteristic features of the non-glandular trichomes. Peltate and capitate glandular trichomes were also identified on the foliar surfaces across all developmental stages. Peltate glands consisted of a short stalk and a multicellular head containing two to eight secretory cells. Two types of capitate trichomes were observed. Type I capitate trichomes consisted of a striated stalk, cutinized neck cell and bulbous head which may be uni- or bi-cellular; Type II capitate trichomes were comprised of a wide base, long and tapering, septate stalk, neck cell and a multicellular secretory head cell. The process of secretion differed between the peltate and capitate glandular trichomes. The peltate and Type II capitate trichomes included a porous cuticle which facilitated the release of secretion to the exterior. Cuticular rupture at weak points of the equatorial plane of the secretory head was observed in Type I capitate trichomes. Qualitative histochemical staining of leaf sections and preliminary phytochemical tests revealed the presence of alkaloids, lipid components, terpenoids and complex polysaccharides concentrated in the glandular trichome head cells and leaf crude extracts, respectively. The perceived therapeutic benefits of this plant are likely to lie within this suite of secondary metabolites. *Stachys natalensis* plant extracts also contained considerable levels of total phenolic compounds (3.43 ± 0.01 mg GAE/g dry material) and flavonoids (3.04 ± 0.01 mg QE/g dry material). The methanolic extracts demonstrated significant free radical scavenging ability (49.49 ± 3.87 ug/ml) which indicates the potential for its use as a natural antioxidant.

In vitro propagation protocol using axillary bud explants was developed for this species. A multi-step decontamination treatment involving explant immersion in 1% and 3% NaClO, followed by 0.1% HgCl₂ was the most efficient method for explant decontamination, resulting in overall explant survival of 48%. All media preparations resulted in > 70% bud break within three weeks with cultures initiated on Medium C (MS supplemented with 0.5 mg/l BAP and 0.5 mg/l IBA) showing the highest percentage of bud break. Growth medium B (0.5 mg/l kinetin and 0.5 mg/l IAA) showed the greatest total shoot multiplication, number of shoots/explant (9.1 ± 3.6) and height/explant (50.2 ± 5.0 mm) compared to other PGR combinations after 12 weeks. The addition of exogenous auxin (2 mg/l IAA) to MS medium allowed for 64% of plantlets to produce adventitious roots in five weeks, after which rooted plants were acclimatized. Acclimatized plantlets (92 ± 4.2 %) did not show any gross morphological abnormalities compared to field-grown plants, apart from the presence of visibly longer non-glandular trichomes. The peltate and both subtypes of capitate glandular trichomes of acclimatized plants were morphologically similar to their field-grown counterparts. Trichome density on acclimatized plants was greater on the abaxial surface of emergent leaves and this density decreased with leaf maturity, as was observed with field-grown plants. This study appears to be the first investigation of the micromorphology of the foliar structures of *S. natalensis*. Future studies on morphological aspects of secretory structures should include cytochemical investigations to determine the exact mechanism and origin of glandular secretions. Further analyses regarding the composition of the glandular essential oils and its potential pharmacological efficacy are required. With an effective *in vitro* propagation protocol being presently established, further optimisation with respect to the type and concentration of exogenous PGRs, explant type or even various routes of organogenesis can be investigated. This may provide a means of enhancing plantlet production, maintaining superior-selected genotypes, and thus potentially maximising the yield of putative pharmacologically-important secondary metabolites.

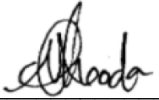
DECLARATION 1

The experimental work described in this dissertation was carried out in the School of Life Sciences, University of Kwa-Zulu Natal, Durban, from January 2012 to December 2013, under the supervision of Dr Y. Naidoo and Dr M. Nakhooda.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

As the candidate's supervisor I have approved this dissertation for submission.

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Co-supervisor

DECLARATION 2

I, declare that

1. The research reported in this dissertation, except where otherwise indicated, is my original research.
2. This dissertation has not been submitted for any degree or examination at any other university.
3. This dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
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Signed:

CONFERENCE/WORKSHOP CONTRIBUTIONS

1. Kalicharan, B., Naidoo, Y. and Nakhooda, M. (2012) The micromorphology and histochemical characteristics of the secretory structures of *Stachys natalensis* Hochst. 50th Annual Conference of the Microscopy Society of Southern Africa (MSSA), University of Cape Town, South Africa.

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LIST OF ABBREVIATIONS

2-iP	6- γ - γ -[dimethylallylamino]-purine
BA (synonym. BAP)	6-benzyladenine or 6-benzylaminopurine
BSH	Bi-cellular secretory head
CT	Capitate trichome
CP	Cellular pedestal
CR	Cellular rupture
DPPH	2,2-diphenyl-1-picrylhydrazyl
ES	Epidermal stalk
FAA	Formaldehyde-acetic acid-ethanol
GAE	Gallic acid equivalent
HgCl₂	Mercuric chloride
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
MTH	Multicellular trichome head
MS	Murashige and Skoog
NC	Neck cell
NAA	1-naphthaleneacetic acid
NaClO	Sodium hypochlorite
NG	Non-glandular trichome
PT	Peltate trichome
PC	Porose cuticle
PGRs	Plant growth regulators
PSP	Pre-secretory phase
QE	Quercetin equivalent
S	Stalk
SC	Stalk cell
SEM	Scanning electron microscopy
SO	Secretory opening
spp.	Species
SS	Stalk septation
WHO	World Health Organisation

CHAPTER 1: INTRODUCTION

1.1. TRADITIONAL MEDICINE IN SOUTH AFRICA

The socio-economic status of many African countries limits provision of basic healthcare to the general population. According to the World Health Organisation (WHO), approximately 80% of developing countries rely on traditional medicine as their primary source of medical care (WHO, 2002; Chan, 2003). Amongst rural populations, the use of traditional remedies sourced from medicinal plants is high, mainly due to the inaccessibility, unavailability and cost of western medicines (Patwardhan, 2005). In addition, the traditional methods for treating illness and disease have existed for far longer than conventional methods, with information on plant use and preparation being verbally passed down from generation to generation (Mander *et al.*, 2007; Da Silva *et al.*, 2011). Studies conducted by Mander *et al.* (2007) indicate that in many instances, ethnomedicine is a preferred choice over conventional drugs by both rural and urban consumers due to its combined use with ritual and divination.

The traditional medicine trade in South Africa is a flourishing industry with over 27 million consumers (Dold and Cocks, 2002). It is supported by the country's rich plant biodiversity of approximately 30 000 indigenous species, which represents 10% of the world's floral diversity (Street and Prinsloo, 2013). It is almost impossible to characterise all medicinally important plants in South Africa, with current reports defining at least 4000 species as ethnobotanically significant and over 700 species as commercially utilised in ethnomedicines (Mander *et al.*, 2007; Van Wyk *et al.*, 2009). Due to the growing interest in medicinal plants by researchers and those in industry, many indigenous botanical resources have been screened for their pharmacologically important phytochemicals (Street and Prinsloo, 2013). This preliminary data can be used for the scientific validation and standardization of traditional medicines and to determine the efficacy and safety of herbal remedies (Patwardhan, 2005). Additionally, it may facilitate the conservation of exploited plant species, which remains a serious threat to biodiversity (Dold and Cocks, 2002; Firenzuoli and Gori, 2007).

1.2. OVERVIEW OF THE GENUS *STACHYS*

Stachys is one of the largest genera in the flowering family Lamiaceae, comprising of more than 300 different species (El Beyrouthy *et al.*, 2009; Salimi *et al.*, 2011). The distribution of the genus spans parts of Europe, the Mediterranean and South-West Asia, North America and southern Africa (El-Beyrouthy *et al.*, 2009; Khanavi *et al.*, 2009). *Stachys*, derived from the Greek word for "ear of grain",

was named by Linnaeus in *Species Plantarum* in 1753 in recognition of its distinctive spike-like inflorescence. Many members of this genus have been used as ornamental plants, edible food sources and as therapeutic agents in traditional medicine (Govil *et al.*, 2006; Piozzi & Bruno, 2011).

1.3. DESCRIPTION OF *STACHYS NATALENSIS*

Stachys natalensis Hochst. is a perennial, aromatic shrub that may be erect or straggling and up to two metres in height (Figure 1.1). It is found in grassy and woody areas along the east coast of southern Africa as well as in certain areas of Swaziland and Zimbabwe. *Stachys natalensis* retains familial traits such as bilabiate flowers and thin, opposite leaves. The flowers are white with lilac markings and are arranged in verticillasters (false whorls) (Hawke, 2005). The leaves are narrowly cordate with crenate margins and are covered with hairs on both foliar surfaces as well as along the multiple, branched stems (Pooley, 1998).



Figure 1.1 *Stachys natalensis* Hochst. growing in Reservoir Hills, Durban, South Africa.

[A] Grassland area in which *S. natalensis* parent material was obtained.

[B] Whole plant material.

[C] White flowers with lilac markings and distinct inflorescence of *S. natalensis*. Bar: 5 mm

1.4. RESEARCH RATIONALE AND MOTIVATION

Foliar hairs, known as trichomes, originate from epidermal cells and may vary in appearance, size and distribution among different plant species (Werker, 2000). Most glandular trichomes secrete biologically active compounds which may be responsible for the curative properties of the plant. It is imperative to categorize these therapeutic compounds and understand the link and mode of action of the plant structures in which they are contained and secreted (Naidoo *et al.*, 2009). Several studies validate the use of *Stachys* spp. for the treatment of an assortment of ailments (Govil *et al.*, 2006; Goren *et al.*, 2011). However, there appears to be no published reports on *S. natalensis* or its chemical composition to date.

Many *Stachys* spp. are used in ethnomedicine in the form of extracts or decoctions made from the aerial parts of the plant and roots (Piozzi and Bruno, 2011). Removal of these parts requires whole plant destruction (Mander *et al.*, 2007). The high demand for plant organs for traditional medicine and novel drug development merits the use of plant tissue culture techniques for the production of preferred plant genotypes or specific plant organs. Furthermore, *in vitro* propagation may alleviate the pressure of over-harvesting medicinally important species (Legkobit and Khadeeva, 2004). Micropropagation has many advantages over conventional propagation techniques including: rapid mass multiplication of plants irrespective of climate or season, generation of pathogen free plants and selective propagation of specific plant organs and metabolites (Tripathi and Tripathi, 2003; Rai, 2010). An efficient *in vitro* micropropagation protocol for *S. natalensis* is required as the plant is rather inaccessible (mainly hidden or entwined with other similar straggling plants) and often difficult to source due to poor seasonal growth. Additionally, there are many discrepancies in the taxonomic evaluation of the genus *Stachys* due to their variable morphology and wide habitat (Dinc & Ozturk, 2008). Trichome morphology may be used as a useful, preliminary classification tool to resolve complex phylogenetic relationships within the genus (Salmaki *et al.*, 2009). Plant tissue culture is an effective means to maintain the plant genotype and reduce the risk of taxonomic discrepancies.

Although the foliar characteristics and therapeutic properties of many *Stachys* spp. are well documented, there have been no descriptive or experimental studies conducted on the morphology or phytochemical constituents of *S. natalensis*. There are few reports on the micropropagation of *Stachys* spp. (Legkobit and Khadeeva, 2004; Ghiorghita *et al.*, 2011) but no reference to the propagation of *S. natalensis* plantlets *en masse*.

1.5. AIMS OF THE PRESENT STUDY

This study was undertaken to describe key micromorphological features of the foliar structures of *S. natalensis*, elucidate composition and localisation of leaf exudates and generate a reliable *in vitro* micropropagation protocol for *S. natalensis*.

1.6. OBJECTIVES

The objectives of this study were as follows:

- a) Identify and describe the micromorphology of the secretory structures associated with *S. natalensis* using stereomicroscopy, scanning electron microscopy (SEM) and light microscopy.
- b) Quantitatively determine trichome density between the adaxial and abaxial surfaces of emergent, young and mature leaf specimens of field-grown *S. natalensis* samples.
- c) Identify the location of compounds of interest within the leaf tissue using various histochemical stains.
- d) Investigate the composition and nature of exudates utilising preliminary qualitative phytochemical tests.
- e) Assess the total phenolic content, total flavonoids content and antioxidant activity of the crude extract of *S. natalensis* using the Folin–Ciocalteu method, aluminium chloride colorimetric assay and DPPH (diphenyl-1-picrylhydrazyl) assay respectively.
- f) Design and implement a reliable and efficient *in vitro* clonal propagation protocol using axillary buds via direct organogenesis.
- g) Determine trichome density between the adaxial and abaxial surfaces of emergent, young and mature *in vitro* propagated plants and compare to that of field-grown plants.

CHAPTER 2: LITERATURE REVIEW

The plant foliar surface contains a plethora of secretory tissues and microstructures that vary in size, shape, function and arrangement amongst different plant species (Werker, 2000). Secretory tissues are important defining characteristics for many plant families and may be classified into two broad categories based on their location and mechanism of secretion (Svoboda and Svoboda, 2001). The first type, intercellular cavities or secretory ducts, exude substances within the plant body (endogenous secretion) (Fahn, 1988). Examples of such tissues are the laticifers (latex secreting cell system) present in members of the family, Apocynaceae (*Vinca sardoa*), Caricaceae (*Carica papaya*) and Asclepiadaceae (*Gomphocarpus physocarpus*) to name a few (Agrawal and Konno, 2009; Konno, 2011). The second type is involved in exogenous secretion which is the release of substances to the plant surface (Fahn, 1988). Examples of plant structures involved in exogenous secretion include: hydathodes (salt-secreting glands), nectaries, gum and mucilage secreting glands and different types of trichomes (Svoboda and Svoboda, 2001). The term “trichome”, from the Greek word ‘*trikoma*’ (growth of hair), refers to a bristle-like appendage that originates and extends outward from the epidermal cells on plant vegetative and reproductive organs (Levin, 1973). They invariably differ in size, morphology, location and composition amongst plant species, hence, classification is most often based on a combination of characteristic features (Werker, 2000).

2.1. TRICHOME MORPHOLOGY

Plant trichomes may be separated into two distinct types: non-glandular and glandular (Wagner, 1991; Werker, 2000). These trichome types have been described using various terminologies including straight, hooked, dendritic and spiral (Levin 1973; Fahn, 1988; Dalin *et al.*, 2008). Payne (1978) first published a glossary of trichome terminology which described both non-glandular and glandular types by virtue of their shape and appearance (Figure 2.1). Later reports elaborated upon this general terminology and suggested that glandular trichomes may also be differentiated based on the nature and localisation of substances that they secrete or store (Wagner, 1991).

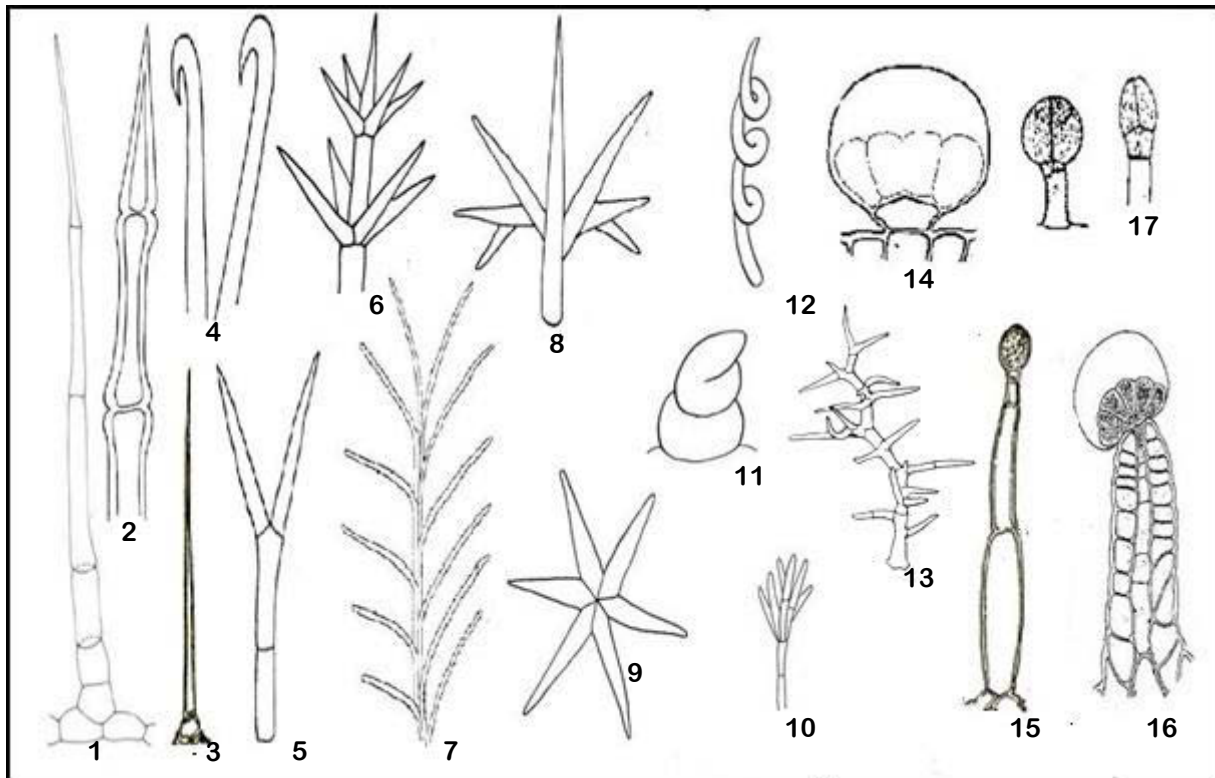


Figure 2.1 Early descriptions of the diverse types of non-glandular and glandular trichomes found on plant surfaces. These include: [1] attenuate, [2] uniseriate hair with osteolate cells, [3] acicular, [4] ucinuate, [5] furcate, [6] abietiform, [7] plumose, [8] pedate, [9] stellate, [10] penicillate, [11] heliciform, [12] spiral, [13] dendritic, [14] peltate, [15] uniseriate capitate, [16] multiseriate capitate and [17] bicellular capitate. Adapted from Payne (1978) and Roe (1971).

2.1.1. Non-glandular trichomes

Non-glandular trichomes display great diversity with regard to their anatomy and structure (Wagner, 1991; Kolb and Muller, 2004). Most non-glandular trichomes are simple, branched or stellate (Choi and Kim, 2013). They may be unicellular or multicellular. Multicellular trichomes may be further classified as uniseriate (single row of cells), as seen in some *Salvia* and *Teucrium* spp., or multiseriate, (several rows of cells) as noted in some *Helianthus* spp. (Werker, 2000; Grubescic *et al.*, 2007; Aschenbrenner *et al.*, 2013). Non-glandular trichomes may differ in length and symmetry with the apex tapering, rounded or hooked (Payne, 1978). The general soft or spiky appearance of non-glandular trichomes can be attributed to the deposition of cell wall constituents such as lignin and cutin (Werker, 2000). Trichomes may contain striations or warty micro-ornamentation that may traverse the entire length or parts of their surface (Ascensao *et al.*, 1995; Bhatt *et al.*, 2010a).

Diverse morphologies of non-glandular trichomes may exist on different organs of the same plant such as those on *Dracocephalum moldavicum*, reported by Dmitruk and Weryszko-Chmielewska

(2010). Those authors found that the calyx and stem contained unicellular, bent trichomes covered in micropapillae whilst multicellular, long spiky trichomes were found only on the corolla surface. Conversely, different types of non-glandular trichomes can be found on the same plant organ such as the unicellular, unbranched hairs and stellate trichomes found on the leaves and stems of *Stachys lavandulifolia* (Rezakhanlo and Talebi, 2010).

2.1.2. Glandular trichomes

Glandular trichomes are very common throughout the eudicotyledonous angiosperms (Levin, 1973; Bhatt *et al.*, 2010a). They are morphologically diverse and are involved in the biosynthesis and secretion of a range of substances such as nectar, mucilage, secondary metabolites, polysaccharides, resin and essential oils (Levin, 1973; Lange and Turner, 2013). Glandular varieties can be differentiated on the basis of their anatomy, function, mode and mechanism of secretion, type of exudate and location on the plant vegetative or reproductive organs (Serrato-Valenti *et al.*, 1997; Serna and Martin, 2006). Glandular trichomes can be unicellular or multicellular as well as uniseriate or multiseriate (Werker, 2000). Two morphologically distinct secretory trichome types, peltate and capitate trichomes, are often described (Maleci-Bini and Giuliani, 2006; Marin *et al.*, 2012; Naidoo *et al.*, 2013).

Peltate trichomes generally consist of a basal epidermal cell, a diminutive stalk cell and a multicellular secretory head (4 - 16 cells) with a large subcuticular space for the storage of secretory substances (Fahn, 1988). Peltate glands are regarded as long-term glandular structures as the secretion is only released upon the rupture of the trichome cell wall (Tissier, 2012). In aromatic families such as the Lamiaceae, Geraniaceae, Cannabaceae and Asteraceae, these trichomes are well known for the production and secretion of essential oils (Werker, 2000; Favi *et al.*, 2008; Tissier, 2012; Boukhris *et al.*, 2013).

Capitate trichomes are much more variable in structure amongst different species. Werker (1993, 2000) identified two general types of capitate trichomes. The first consisted of a basal epidermal cell, unicellular stalk, a neck cell and a uni-, bi- or multicellular head cell with a small subcuticular space for storage of exudates. The second type is similar to the first with the exception of one or two basal epidermal cells and a bulbous, unicellular head cell with a subcuticular space (Maleci-Bini and Giuliani, 2006; Jia *et al.*, 2012). Capitate trichomes are regarded as short term glandular trichomes, since the period of secretion (containing polysaccharides, polyphenols or essential oils) is brief, mainly active during early organ development (Giuliani and Maleci-Bini, 2008). Numerous studies have highlighted the structural diversity of glandular trichomes among different plant taxa and even

between species (Ascensao *et al.*, 1999; Corsi and Bottega, 1999; Giuliani and Maleci-Bini, 2008; Marin *et al.*, 2012; Rusydi *et al.*, 2013).

2.2. TRICHOME FUNCTIONS

The secretory functions of trichomes are often the defining characteristics in many plant genera such as the salt glands of *Avicennia*, nectaries of *Passiflora* and oil secretory glands in *Mentha* (Wagner, 1991; Werker *et al.*, 1993). Trichomes serve a variety of functions which are dependent on the trichome type and location (Wagner, 2004).

2.2.1. Plant defence against herbivory

Trichomes often function as sensors to detect leaf activity and prepare the plant for insect attack (Werker, 2000). In many instances, trichomes serve as physical or chemical deterrents against insect herbivory (Tian *et al.*, 2012). Trichomes on the surface of *Glycine max* (soybeans) for example, inhibit insect movement across the plant. Thus, larvae are unable to access the epidermis for nourishment. Insects may eat through the trichome but suffer from little weight gain and subsequent death due to the poor nutritional value conferred by the cellulose and lignin enriched trichomes (Dalin *et al.*, 2008). Trichome density also plays a factor in resistance to herbivory (Tian *et al.*, 2012). In a study by Riddick and Wu (2011), the survival rate of the lady beetle *Stethorus punctillum* was related to the trichome density of *Phaseolus lunatus* (lima bean) leaves. The study indicated that younger leaves had a dense distribution of hooked trichomes which impaled *S. punctillum* larvae resulting in death due to starvation, desiccation and numerous puncture wounds. Trichome production may also be induced in many plant species as a direct response to insect damage (Levin, 1973; Howe and Jander, 2008).

Secondary metabolites are produced as a by-product of normal plant growth and development. Glandular trichomes may store or induce production of these secondary metabolites such as flavonoids, terpenoids, and alkaloids in response to insect or pathogen attack (Wittstock and Gershenzon, 2002). The defensive compounds may poison, repel or immobilise insects. Tian *et al.* (2012) determined that growth of *Helicoverpa zea*, an agricultural pest, was severely affected by the presence of glandular trichomes on wildtype *Solanum lycopersicum* plants compared to its hairless mutant. It was hypothesized that the glandular secretions contained toxic/sticky substances which acted as chemical deterrents by entrapping *H. zea*. Other examples include the *Sitophilus zeamais* weevil which showed significant toxicity to eucalyptol (1,8-cineole), isolated from the essential oil of

Vernonia amygdalina (Adeyemi, 2011) and *Aphis nerii*, an arthropod fatally immobilized in the sticky exudate of the glandular trichomes from *Sicana odorifera* (Cucurbitaceae) (Kellog *et al.*, 2002). Plants may also employ indirect defence mechanisms by releasing volatile organic compounds (stored in secretory structures) which attract predators of the insects that attack the plant surface (Pare and Tumlinson, 1999; Howe and Jander, 2008). Examples include glandular structures of *Zea mays* and *Gossypium* plants, which produce volatiles that attract predatory wasps when damaged by moth larvae (Pare and Tumlinson, 1999).

2.2.2. Pollination

Trichomes are involved in other plant-insect interactions in addition to plant defense. Many trichome-derived compounds are utilized as attractants for species-specific pollination (Caissard *et al.*, 2004). Reis *et al.* (2004) described the pollination mechanism of *Stanhopea lietzeii* and *S. insignis*. These orchid species exude fragrant oils which attract neotropical bees. Trichomes are also involved in specialised mechanisms of insect capture for pollination. The downward-facing trapping flower trichomes of *Aristolochia* spp. facilitate the easy entrance of insects into flowers but impede their escape until the flowers modify their inner surface post-pollination (Oelschlägel *et al.*, 2009).

2.2.3. Protection against water loss, UV-B and light damage

Trichomes play an important role in maintaining plant physiology upon exposure to environmental stress (Yan *et al.*, 2012). In cases of drought or elevated temperatures, they reflect light, thus lowering the temperature over the leaf surface, which reduces water loss through transpiration (Abdulrahman and Oladele, 2011). Perez-Estrada *et al.* (2000) investigated trichome density of *Wigandia urens* under different seasonal and environmental parameters. These authors found that trichome density was higher during the dry season and on plants that grew in sun exposed areas. Furthermore, decreased trichome density was noted in response to irrigation and shade treatments. These observations implied that trichomes were involved in limiting water loss through transpiration and reflecting solar radiation. Trichomes play a role in protection against UV-B radiation by accumulating flavonoids and other UV absorbing compounds (Yan *et al.*, 2012). Studies indicated that UV-B radiation induces an increase in trichome density in *Nicotiana tabacum* and *Arabidopsis thaliana* which is important for the protection of underlying plant tissues against the detrimental effects of UV-B radiation (Barnes *et al.*, 1996; Yan *et al.*, 2012).

2.3. TRICHOME EVOLUTION AND TAXONOMIC POTENTIAL WITHIN THE LAMIACEAE

In terms of the evolution of secretory structures, many studies propose opposing hypotheses. Fahn (1988, 2002) suggested that secretory structures originally developed within vascular plant organs (as seen in early pteridosperms) and subsequently were scattered in cells of internal vascular tissues (gymnosperms) and then found on the plant surface (present day angiosperms). Bearing this in mind, the glandular trichomes would represent the most advanced and evolved form of the secretory structures. Other studies indicate that glandular trichomes may have evolved independently from stomata or non-glandular trichomes. Carpenter (2006) investigated the morphology of specialised structures on the foliar surfaces of early angiosperms. That author detected the presence of intermediate forms of trichomes and stomata and hypothesised that the specialized trichome structures may be homologous to early stomatal complexes or derived from stomata over multiple evolutionary events. Tissier (2012) provided an alternative hypothesis in that glandular trichomes may have evolved from their non-glandular counterparts via the differentiation of apical cells into secretory cells. The study described the capitate trichomes of *Solanum lycopersicum* and *S. habrochaites* which differed morphologically from the non-glandular type by the presence of a single secretory cell at the trichome tip. To date, there is little molecular support for these hypotheses. Moreover, a single evolutionary occurrence cannot account for the extensive glandular trichome diversity amongst plant families (Tissier, 2012).

The Lamiaceae is one of the largest families among the angiosperms, containing more than 240 genera (Giuliani and Maleci-Bini, 2008). Micromorphological studies with regard to epidermal structures, in particular the trichomes, have been widely utilised to describe similarities and differences amongst the various taxa (Ascensao *et al.*, 1995; Gairola *et al.*, 2009; Baran *et al.*, 2010; Dunkic *et al.*, 2012). Both glandular (peltate and capitate) and non-glandular trichomes are characteristic features of species of the family (El-Beyrouthy *et al.*, 2009; Bhatt *et al.*, 2010b; Dunkic *et al.*, 2012). The presence, type and morphology of trichomes and phytochemical characteristics of their secretions may be used as discriminative markers at species or genera level (Ascensão *et al.*, 1995; Celep *et al.*, 2011). Many members such as *Ocimum*, *Stachys*, *Mentha* and *Salvia* spp. are also well known for the production of essential oils and various secondary metabolites within the glandular trichomes which may be utilised as chemotaxonomic indicators for species classification (Maleci-Bini and Giuliani, 2006; Naidoo *et al.*, 2013). *Stachys* is considered as one of the largest genera within the Lamiaceae, with many taxonomic discrepancies and problems in nomenclature (Giuliani and Maleci-Bini, 2012). Trichome presence on vegetative and/or reproductive structures, coupled with its

typology has presented an efficient classification tool to delimit closely related species or subspecies (Giuliani and Maleci-Bini, 2008) as well as differentiate between sections (Dinc and Ozturk, 2008).

2.4. STACHYS: HISTORY AND BIOLOGICAL ACTIVITY

The genus *Stachys* comprises of more than 270 species and is known by a variety of common names including woundwort, betony and lamb's ears (Piozzi and Bruno, 2011; Thomas, 2011). Many species of the genus have been historically utilised by numerous cultures in folklore and for the treatment of a wide variety of illnesses. The use of *Stachys officinalis* (betony) for the treatment of headaches, wounds and abscesses is perhaps one of the earliest accounts of the genus (Thomas, 2011). The Spanish adage, "He has as many virtues as betony" bears testament to its value. In fact, physicians documented at least 47 medicinal uses of the plant including: anti-inflammatory, anti-bacterial, anti-nephritic and anxiolytic effects. In addition, the early Anglo-Saxons believed that the plant warded off evil spirits and therefore propagated it in churches (Thomas, 2011).

In recent times, the therapeutic use of *Stachys* spp. has increased worldwide. In Iranian traditional medicine, herbal infusions of *Stachys lavandulifolia* and aerial parts of *S. inflata* and *S. recta* are used to treat skin infections, rheumatism, indigestion and gastro-intestinal disorders (Khanavi *et al.*, 2009; Pirbalouti and Mohammadi, 2013). *Stachys thunbergii* and *S. aethiopica* are used in South African traditional medicine for the treatment of hysteria and insomnia and to reduce fever caused by delirium (Stafford *et al.*, 2008). *Stachys officinalis*, known as 'Ranilist' and 'Betonica' in Bulgaria and Italy respectively, is used as an antiseptic and diuretic (Leporatti and Ivancheva, 2003). Table 2.1 indicates the scientific support for the many medicinal uses of *Stachys* spp. In most cases, studies show a diverse range of active compounds in the secretions or crude extracts including flavonoids, glycosides, phenolic compounds, di-terpenoids and volatile and essential oils which support the use of this genus in traditional medicine (Duman *et al.*, 2005; Goren *et al.*, 2011; Govil *et al.*, 2006).

Table 2.1 Review of phytochemical studies on the essential oils and crude extracts of some *Stachys* spp. (2000-2013).

Species	Medicinal use	Reference
<i>Stachys byzantina</i> , <i>S. inflata</i>	Anti-inflammatory activity	Maleki <i>et al.</i> (2001); Khanavi <i>et al.</i> (2005)
<i>S. cretica</i> L. subsp. <i>Symrnaea</i> , <i>S. germanica</i> , <i>S. spinulosa</i> , <i>S. euboica</i> , <i>S. menthifolia</i>	Anti-microbial activity	Skaltsa <i>et al.</i> (2003); Ozturk <i>et al.</i> (2009)
<i>S. officinalis</i> , <i>S. alpina</i> , <i>S. palustris</i> , <i>S. recta</i> , <i>S. sylvatica</i> , <i>S. lavandulifolia</i>	Antioxidant activity	Bilusic-Vundac <i>et al.</i> (2007); Rahzani <i>et al.</i> (2012); Šliumpaitė <i>et al.</i> (2013)
<i>S. recta</i> , <i>S. alpina</i> subsp. <i>dinarica</i> .	Hepato protective activity	Baran <i>et al.</i> (2010); Kukic-Markovic <i>et al.</i> (2011)
<i>S. tibetica</i> , <i>S. lavandulifolia</i>	Anti anxiety (anxiolytic) and sedative	Monji <i>et al.</i> (2011); Kumar <i>et al.</i> (2013)

2.5. BIODIVERSITY IN SOUTH AFRICA

Recently, there has been a considerable increase in the global use of traditional remedies for a wide variety of ailments. Furthermore, many industrial and pharmaceutical companies, research organizations and tertiary institutions are interested in the development of traditional medicine and bioprospecting compounds of interest from established and potential medicinal plants.

South Africa has one of the richest plant biodiversities in the world, with many species regarded as economically or medicinally important (Afolayan and Adebola, 2004). The current demand for specific whole plants by traditional healers and the increased monetary reward offered to traders that are able to source these plants result in the exploitation of certain species, to the extent that they are only found in protected habitats (Wiersum *et al.*, 2006). Plant resources are rapidly declining as a

result of environmental changes to natural ecosystems and the illegal overharvesting of plants with potential medicinal value (Afolayan and Adebola, 2004; Canter *et al.*, 2005). This leads to reduced endemic populations, loss of biodiversity, habitat loss and environmental degradation (Canter *et al.*, 2005). There are many initiatives for the assessment of threatened species as well as *in situ* and *ex situ* conservation strategies under implementation. Although conventional cultivation for many plants is desirable as it reduces harvesting pressure on threatened or rare species, there are several limitations e.g. many medicinal plants do not produce seeds or seeds are too small and/or difficult to germinate (Sharma *et al.*, 2010). For instances where seeds are used for conventional propagation, plant yield is highly heterozygous and shows variable growth (Sharma *et al.*, 2010; Amujoyegbe *et al.*, 2012). Medicinal plants are not often acquiescent to vegetative propagation via cuttings, thus mass propagation of cultivars is often limited due to low germination rates (Canter *et al.*, 2005). Furthermore, vegetative propagation may result in the presence of systemic bacteria and fungi which may negatively affect the quality of the plant and any herbal end product derived from it (Amujoyegbe *et al.*, 2012). A viable option is the utilization of plant biotechnology to offset the loss of significant species and for the efficient regeneration of medicinal and economically important plant species (Canter *et al.*, 2005). Plant tissue culture, or *in vitro* micropropagation, has been successfully used for continuous, mass multiplication of elite and disease-free plants, obtained quickly and economically (Sharma *et al.*, 2010).

In vitro micropropagation has many advantages over conventional propagation methods. These include enhanced rates of multiplication using relatively small amounts of starting plant material, a shorter shoot cycle that yields an exponential increase in shoot production, rapid *in vitro* production of secondary metabolites, plant regeneration irrespective of season or climate, maintenance of germplasm stocks indefinitely and the rapid propagation of genotypes that are disease- and pathogen-free and genetically identical to parent plants (Bhowmik and Matsuz, 2001; Tripathi and Tripathi, 2003; Arikat *et al.*, 2004). The method is also useful for the propagation of sexually sterile species (triploids) and seedless plants such as bananas and to control experimental parameters such as light intensity and temperature (Rai, 2010). Clonal propagation affords pharmaceutical and agricultural industries an alternate and fast solution to the issue of procuring large amounts of plant matter (Tasheva and Kosturkova, 2013). This simultaneously reduces the pressure of overharvesting plants and maintaining natural resources.

2.6. PLANT TISSUE CULTURE

Tissue culture is a collective term referring to procedures used to maintain and grow plant cells, tissues or organs on suitable media under aseptic conditions. It is based on the concept of totipotency, whereby each single plant cell has the capability to express the full genetic potential of the plant (Rao and Ravishanker, 2002). An overview of early developments in the field of plant cell culture is found in Table 2.2. In 1902, Haberlandt developed the concept of *in vitro* cell culture by attempting to cultivate various differentiated plant cells in a glucose-enriched salt solution (Rai, 2007). The experiment garnered little success, possibly due to the relatively simple nutrients utilised and the lack of sterile conditions (Sussex, 2008). In subsequent years, other researchers were successful in maintaining plant growth via callus induction (White, 1939), introducing the use of plant growth regulators (Went, 1935; Miller *et al.*, 1955) and successfully inducing growth using different explant material (Ball, 1946; Muir, 1954).

Micropropagation is the vegetative propagation of whole plants from explant material (small pieces of plant tissue) using controlled *in vitro* tissue culture methods. Micropropagation techniques were initially developed for the ornamental plant industry and later for the production of elite fruit and vegetable crops (Tasheva and Kosturkova, 2013). Recently, much research has focused on propagation of medicinal plant species *en masse* (Valizadeh and Valizadeh, 2011). Positive results were obtained using shoot tip and meristems of medicinal plants such as *Cinchona ledgeriana*, *Digitalis* spp., *Rauvolfia serpentina*, *Glycyrrhiza glabra*, *Bacopa monnieri* and *Artemisia annua* (Tripathi and Tripath, 2003; Debnath *et al.*, 2006; Sharma *et al.*, 2008).

Table 2.2 Early history and development of plant tissue culture.

Year	Progression	Reference
1838	Proposal of cell theory	Schleiden and Schwann
1902	First attempt at <i>in vitro</i> cell culture	Haberlandt
1922	Successful culture of root and stem tips	Kolte and Robbins
1935	Discovery of first plant growth hormone, indole acetic acid (IAA)	Went
1939	Continuous growing of callus culture by addition of vitamin B	White, Gautheret
1946	Whole plant of <i>Lupinus</i> regenerated by shoot culture	Ball
1954	Single cells from callus culture	Muir
1960	Isolation of protoplast cells	Cocking
1962	Media development	Murashige and Skoog
1970	Single cells to somatic embryos	Backs-Husemann and Reinert
1974	Concept of developmental stages of <i>in vitro</i> tissue culture	Murashige
1984	First industrial production of shikonin (dye and medicinal compound) suspension cultures of <i>Lithospermum erythrorhizon</i>	Mitsui petrochemical
1985	Development of gene transfer system using <i>Agrobacterium tumefaciens</i>	Gheysen
1988	Development of photoautotrophic micropropagation (sugar-free medium)	Kozai and Iwanami

* Adapted from Sussex, 2008; Rai, 2007

2.7. PROCESS OF *IN VITRO* MICROPROPAGATION

Micropropagation may proceed via two pathways: direct or indirect organogenesis. The former uses axillary explants from donor plants for shoot induction, followed by rooting, whilst the latter requires an intermediate callus induction stage (Rai, 2010). Murashige (1974) defined four stages for successful *in vitro* plant tissue culture with an additional “stage 0” added by Debergh and Maene (1981). The entire process illustrated in Figure 2.2 is discussed below.

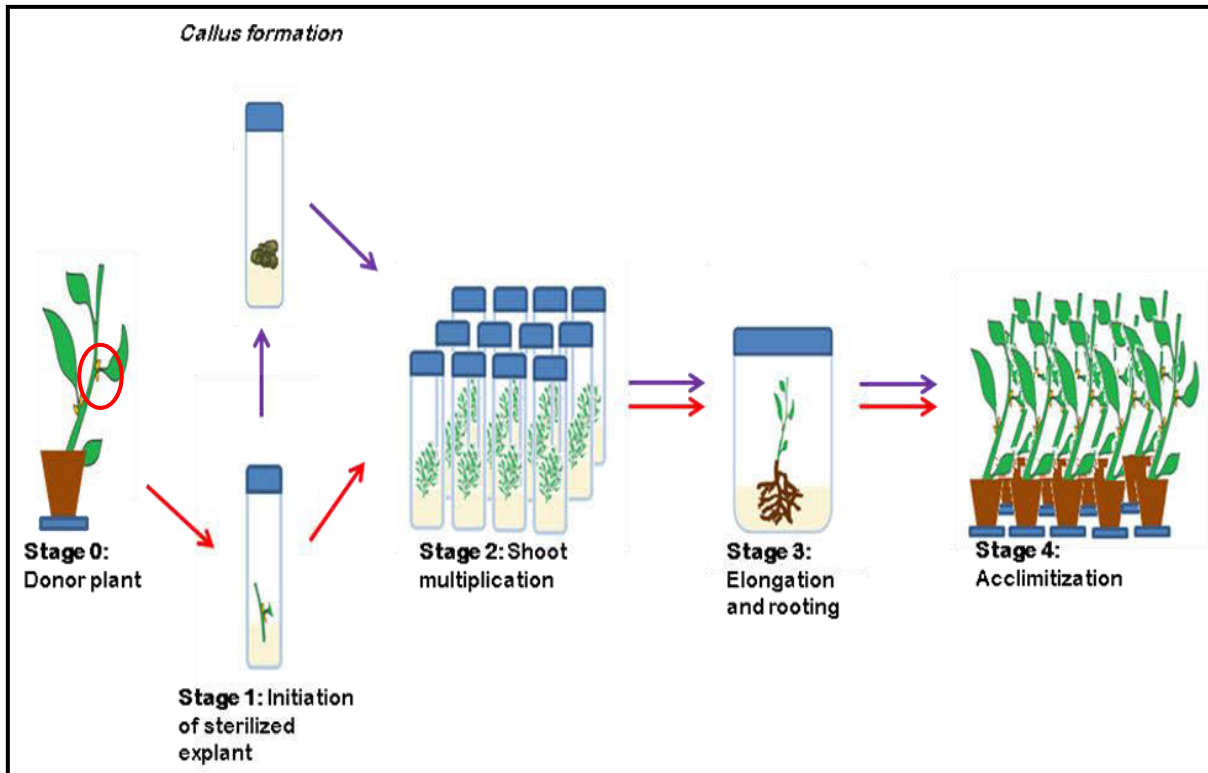


Figure 2.2 General overview of the stages involved in plant micropropagation. Parent plants are selected (stage 1) and explants are isolated and decontaminated before initiation onto suitable growth media (stage 2). Indirect organogenesis results in callus formation with each new cell having the potential for shoot and root formation (purple arrows). Direct organogenesis maximises shoot multiplication over successive subcultures (red arrows), followed by elongation and rooting of plantlets. Micropropagation results in the formation of multiple, acclimatized, true-to-type plantlets (stage 4). Adapted from Medina *et al.* (2004).

2.7.1. Stage 0: Selection and maintenance of stock plant

This primary step in the process requires the selection of stock material which should be cultivated under optimum *ex vitro* conditions. Plant material may undergo pre-conditioning by exposure to anti-

microbial agents to reduce the risk of contamination. Studies showed that pre-treatment of *Dryopteris cristata* rhizomes with Nystatin significantly reduced exogenous and endogenous fungal contamination (Zenkteler and Kwasna, 2007) whilst Mng'omba *et al.* (2007) determined that the application of a systemic fungicide to the *Uapaca kirkiana* parent plant before stock plant collection effectively lessened the fungal infestation during *in vitro* culture. In the case of field-grown *S. natalensis* plants, the hairy leaves are prone to grime accumulation, which may require the application of a systemic fungicide to reduce microbial load on stock material.

2.7.2. Stage I: Initiation and establishment of an aseptic (sterile) culture

This stage involves isolation of explants, surface sterilization and establishment of explants on suitable growth medium (Murashige and Skoog, 1962).

a. Explant isolation

Explants may be obtained from almost any part of the plant, including vegetative or reproductive parts (Yildiz, 2012). Micropropagation of a number of plant species has been successfully achieved using a range of explants, such as axillary buds (*Coleus forskohlii*, *Metha piperita*), rhizome (*Curcuma domestica*, *Costus speciosus*) and cotyledon (*Panax ginseng*) (Chaturvedi *et al.*, 2007). Successful shoot regeneration of *Withania somnifera*, a well known medicinal plant was obtained using different explant types such as the node, internode, hypocotyl and embryo (Kulkarni *et al.*, 2000). However, consideration must be given to the route of organogenesis required in the study as explant source may often result in different morphogenic responses. Komalavalli and Rao (2000) observed that shoot multiplication was achieved using axillary node, cotyledonary node and shoot-tip leaf explants whilst other explant types (leaf, petiole, and root) induced callus formation in *Gymnema sylvestre*. The authors attributed these observations to the variation in levels of plant hormones present in the excised explant before *in vitro* culture. Shoot tips and auxiliary buds are extensively used as explant material for direct shoot induction in a variety of species (Chauhan *et al.*, 2012; Kosar and Mahmoud, 2012; Shahzad *et al.*, 2012) and are viable explant options in the present study as callus production is not required. Additional factors such as the age of the donor plant and size of explant remain important considerations for successful culture and may also affect the initiation potential of the culture. The general consensus indicates that while larger explants may have better survival capabilities and are able to withstand harsher modes of sterilization, smaller material limits viral and microbial contamination (Bhojwani and Razdan, 1996; Yildiz, 2012). Studies also indicate that the

propagation potential of juvenile tissues is often higher compared to mature tissues (Le Roux and Van Staden, 1991; Mohebodini *et al.*, 2011).

b. Surface sterilization

It is a pre-requisite for tissue culture techniques to be carried out under sterile conditions. In many cases, treatment of stock plants at stage 0 is insufficient to eradicate surface contaminants. Microbial growth rates far exceed plant cell division. Thus, surface sterilization endeavours to remove all microorganisms that will thrive under *in vitro* conditions whilst still maintaining explant viability. There are a variety of disinfectants such as ethanol, hydrogen peroxide, bromine water, mercuric chloride, sodium hypochlorite and silver nitrate to name a few. Sodium hypochlorite (NaClO) is a widely used disinfectant due to its potency against microorganisms and some viruses (Oyebanji *et al.*, 2009; Yildiz, 2012). In a study by Mihaljevik *et al.* (2013) on the effectiveness of various decontaminating agents on axillary buds of a cherry cultivar, sodium hypochlorite was shown to facilitate 80% aseptic explant survival. In fact, many research articles have evaluated its use in place of sterilization equipment (Peiris *et al.*, 2012). Whilst mercuric chloride (HgCl₂) is an effective decontaminating agent, it is highly toxic to human health and to fragile plant material at high concentrations (Moghaddam *et al.*, 2011). However, it has been used for the explant decontamination of Lamiaceae species such as *Orthosiphon stamineus* (Rashid *et al.*, 2012) and *Ocimum gratissimum* (Saha *et al.*, 2012) and may potentially be of use for the decontamination of *S. natalensis*.

2.7.3. Stage II: Shoot multiplication

The multiplication stage follows that of initiation once explants have been successfully established on suitable media and are flourishing. This stage involves repeated sub-culturing of *in vitro* material for shoot multiplication until desired numbers of regenerated plantlets are obtained to continue to stage III. Most often medium constituents for shoot multiplication remain the same as for initiation with the only modification being that of plant growth regulators for maximum shoot induction (Diab *et al.*, 2011; Hussain *et al.*, 2012). Vinterhalter and Vinterhalter (1999) observed that higher sucrose concentrations in growth media also triggered morphogenesis by the formation of axillary buds and adventitious root branching in *Dracaena fragrans* and *Ceratonia siliqua*. However, the effect of carbon sources on shoot multiplication is beyond the scope of the current study.

a. *Plant growth regulators (PGRs)*

Plant growth regulators are vital constituents in media preparation. There are four main groups of plant regulators that are used in *in vitro* culture: auxins, cytokinins, gibberellins and abscisic acid. They are involved in cell division and induce shoot or root production depending on the proportion of cytokinins and auxins as shown in Figure 2.3.

Auxins, derived from the Greek word “*auxien*” meaning to grow or increase, often act antagonistically with other growth regulators. There are many different auxins used in tissue culture for induction of cell division and elongation, rooting and somatic embryogenesis (Simon and Petrasek, 2011). Indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) are natural auxins found at the growing shoot apex of plant tissues and are routinely used in the micropropagation of many species (Arikat *et al.*, 2004; Rai, 2010). The "direct inhibition hypothesis" indicates that auxin from apical buds travels down shoots to inhibit axillary bud growth. This encourages shoot growth and restricts lateral branching. Cytokinins move from the roots into the shoots resulting in lateral bud growth. Removal of the apical bud results in release of axillary bud inhibition which increases lateral growth (plants grow more densely) (Booker *et al.*, 2003).

The effect of cytokinins on plant tissue is variable, especially in collaboration with auxins. Cytokinins are involved in the stimulation of cell division and direct morphogenic responses (George *et al.*, 2008). Although natural cytokinins such as zeatin and 2-iP are utilised in plant tissue cultures, synthetic cytokinins such as 6-benzyloaminopurine (BAP) and kinetin are more commonly used for their effectiveness in multiple shoot induction (Makunga and Van Staden, 2008; Valizadeh and Valizadeh, 2011; Jana *et al.*, 2013). The choice of specific growth hormones in a study may require consideration of PGRs that are applicable to other related species. For example, Zuzarte *et al.* (2010) and Kara and Baydar (2012) utilised benzyladenine (BA) in combination with (IBA) for shoot multiplication of *Lavandula pedunculata* and *L. angustifolia* respectively. Other examples include the use of kinetin for shoot multiplication of *Ocimum americanum* (Kumar and Jhanwar, 2013) and *Ocimum basilicum* (Gopal *et al.*, 2014). Available literature on the micropropagation of *Stachys* spp. is limited. In a study by Ghiorghita *et al.* (2011) the efficiency of BA in combination with IAA, IBA and 1-naphthaleneacetic acid (NAA) for the propagation of *Stachys sieboldii* was acknowledged. Thus the inclusion of synthetic cytokinin and exogenous auxin to plant culture may prove beneficial for the micropropagation of *S. natalensis*.

Gibberellins are involved in growth regulation, breaking seed dormancy, enzyme induction, callus induction and internode elongation. Of the almost twenty gibberellins known, GA₃ is most often used. Abscisic acid is used only for somatic embryogenesis and for culturing woody species. Gibberellins share an antagonistic relationship with that of abscisic acid in regulating plant growth (Razem *et al.*, 2006; Rai, 2010). The current study focuses on the generation of an efficient, simple propagation protocol via direct organogenesis. Therefore, the effect of gibberellins and abscisic acid will not be evaluated in the current study.

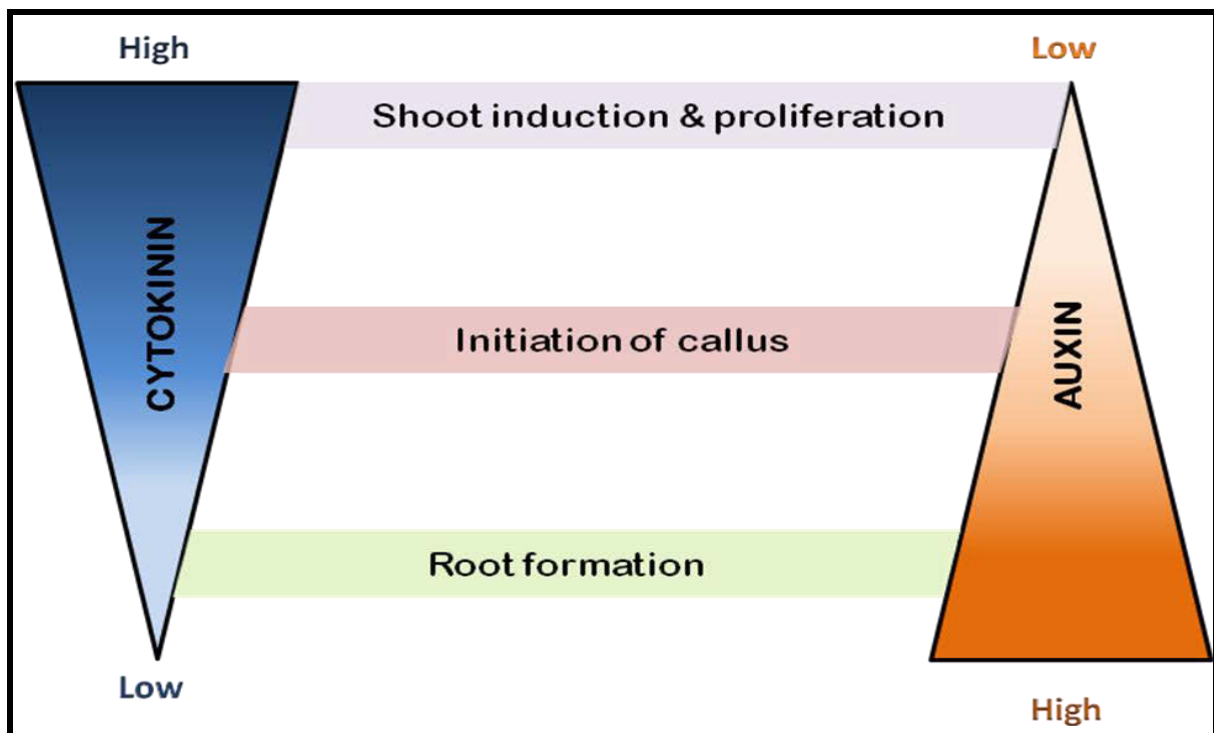


Figure 2.3 The effect of auxin to cytokinin interaction on *in vitro* plant growth. Auxin alone induces cell expansion but no cell division. Upon addition of cytokinin, cells expand and differentiate. At equal levels of both hormones, parenchyma cells form undifferentiated callus. Higher cytokinin to auxin levels induce shoot proliferation, whilst higher auxin levels induce root formation. Adapted from Weber (2013).

2.7.4. Stage III: Rooting

This stage involves the transferral of shoots obtained in stage II onto suitable rooting medium. Rooting media may be slightly modified with regard to composition of basal medium and plant growth regulators (increased levels of auxins) for root induction (Hussain *et al.*, 2012). This is however, dependant on the plant species. For example, the best response for root induction in *Achyranthes bidentata* (Hossain *et al.*, 2013) and *Mentha spicata* (Fadel *et al.*, 2010) was achieved on

full strength MS medium. On the contrary, Baque *et al.* (2010) observed a significant inverse relationship between MS strength and adventitious root growth in *Morinda citrifolia*. In fact, many other studies have reported greater root induction on half-strength MS medium (Abou Dahab *et al.*, 2004; Goel *et al.*, 2009; Asghari *et al.*, 2012; Saha *et al.*, 2012). It is generally accepted that the addition of auxins play a role in root initiation and development (Pop *et al.*, 2011). Studies report that the addition of exogenous IAA to the culture medium of propagated *Ocimum gratissimum* (Gopi *et al.*, 2006) and *Calendula officinalis* (Victorio *et al.*, 2012) significantly increased the rate of rooting. Upon successful rooting, plantlets are evaluated for their survival potential and prepared for *ex vivo* conditions.

2.7.5. Stage IV: Establishment of plantlets in soil

In vitro tissue culture conditions are ideal for rapid and maximum growth of the plantlets. However, these controlled conditions (ample light and high humidity) cause them to be vulnerable to disease, soil pathogens, low moisture and nutrient levels and stress prevalent in natural environments (Kaur *et al.*, 2011; Panigrahi *et al.*, 2013). Once plantlets have rooted sufficiently, they are removed from the growth medium, rinsed and transferred to organic compost, potting soil, vermiculite or perlite (Kaur *et al.*, 2011; Hussain *et al.*, 2012). The plantlets may also be placed in a mist chamber or exposed to antitranspirants to control relative humidity and water loss thus ensuring that plants develop normally and become autotrophic (Marin-Velazquez and Antonio, 2003).

CHAPTER 3: MATERIALS AND METHODS

3.1. SAMPLE COLLECTION AND PREPARATION

Whole *S. natalensis* plants were collected from Durban, KwaZulu-Natal (29° 47'12.462; 30° 55' 45.0156). A voucher specimen was prepared and deposited at the Ward Herbarium, University of KwaZulu-Natal, Westville Campus. Randomly selected leaf specimens at different developmental stages i.e. emergent (<10 mm leaf length), young (10 mm – 25 mm leaf length) and mature (>30 mm leaf length), were used for each sample preparation batch (n=10)

3.2. STEREOMICROSCOPY

Entire leaf samples were used to investigate surface structures located on the adaxial and abaxial leaf surfaces. A Nikon AZ100 Stereomicroscope and NIS-D Elements image software (Nikon Instruments Inc., USA) were utilised to observe and capture images of different types of foliar trichomes and their distribution.

3.3. SCANNING ELECTRON MICROSCOPY (SEM)

Scanning electron microscopy was used to investigate trichome micromorphology and distribution. Leaves were subjected to three different modes of preparation for SEM, i.e. freeze-drying, chemical fixation and cryo-SEM.

3.3.1. Freeze- drying

Fresh leaf sections of approximately 2 mm² were quenched in liquid nitrogen and freeze-dried in an Edwards' Modulyo freeze-dryer for 72 hours (- 40 °C to -60 °C; 10⁻¹ Torr). Samples were mounted onto brass stubs using double-sided carbon adhesive tape and sputter- coated with gold in a Polaron SC500 sputtercoater. Samples were viewed with a Zeiss UltraPlus Field Emission microscope. Areas of interest were identified and images captured using SmartSEM image software (v.0.5 Zeiss, Germany).

3.3.2. Chemical fixation

Young and emergent leaves of *S. natalensis* were hand sectioned (± 2 mm²) and then chemically fixed for two hours in 2.5% glutaraldehyde. The specimens were washed with phosphate buffer and post-fixed with 0.5% osmium tetroxide in a dark cupboard for one hour. Samples were dehydrated by exposure to increasing concentrations of acetone (30%, 50%, 75% and 100%). The samples were critically point-dried using a Hitachi HPC1 critical point dryer, mounted onto brass stubs and sputter-coated with gold. Images were captured as previously described.

3.3.3. Cryo-SEM

Stachys natalensis leaf segments ($\pm 1 \text{ mm}^2$) were mounted on brass stubs with 50:50 colloidal graphite & Tissue-Tek® O.C.T compound (Sakura® Finetek) mounting medium. Samples were quenched in liquid N₂, sublimed at -90 °C for 10 minutes and coated with Platinum (60 sec; 10 mA) under vacuum. Samples were viewed and images captured with Zeiss UltraPlus Field Emission microscope with an additional Quorum PP3000T Cryo-SEM system.

3.4. LIGHT MICROSCOPY

Fresh hand as well as wax-embedded young leaf material was utilised for light microscopy. Sections were prepared as outlined below, mounted onto glass slides and viewed using the Nikon Eclipse 80i light microscope. Images were captured using the NIS-D elements software programme.

3.4.1. Wax embedding

Young leaf tissue was sectioned and immersed immediately in 70% FAA (formaldehyde-acetic acid-ethanol) fixative for 48 hours (Kladnik, 2006). Samples were then dehydrated using a graded ethanol series involving immersion of samples in 70% ethanol for 30 minutes (repeated thrice), immersion in 80% ethanol for two hours, in 90% ethanol for four hours and finally in 100% ethanol for 90 minutes. Four samples were then stained with fast green and safranin in 100% ethanol for 30 minutes. The remaining slides were then placed overnight in 100% ethanol. Samples were then exposed to a gradual xylene: ethanol infiltration (25:50, 50:50, 75:25) for an hour each before immersion in xylene, twice for 15 minutes each time. Pellets of Paraplast Plus® tissue embedding media (McCormick Scientific, USA) were added to the samples and left for three hours. These were replaced with new wax pellets four times after three hour intervals. Hardened wax sections were sectioned using a Reichert Ultracut E rotary microtome to obtain semi-thin sections (1-2 microns).

3.4.2. De-waxing protocol

Slides containing wax sections of leaf tissue were immersed in 100% xylene (x3 washes), 100% ethanol (x3 washes), 70% ethanol (x2 washes) and distilled water for 2 minutes per wash.

3.5. HISTOCHEMISTRY

De-waxed sections of leaf tissue were stained by the following reagents to detect the localization of specific cellular compounds within the leaves:

3.5.1. Ferric trichloride – phenolic compounds

Fresh leaf sections were placed in 10% ferric trichloride (10 g ferric trichloride pellets in 100 ml distilled water, followed by filtration through Whatman™ No. 1 filter paper). A drop of sodium carbonate was added and the sections were left at room temperature for 15 minutes. In this test, ortho-dihydroxyphenols react with ferric ions to produce deep green or black deposits (Johansen, 1940).

3.5.2. Mercuric bromophenol blue – proteins

Leaf sections were immersed in bromophenol blue (100 mg bromophenol blue with 10 g of HgCl₂ and 95% ethyl alcohol) for 15 minutes. Stained samples were rinsed in 0.5% acetic acid for 20 minutes and immersed in a 0.1 M sodium phosphate buffer (pH 7.0) for three minutes. A blue coloration was indicative of total proteins (Mazia *et al.*, 1953).

3.5.3. Nadi reagent – terpenoids and resins

Fresh-hand leaf sections were immersed in two drops of nadi reagent (0.5 ml of a 1% alpha-naphthol solution in 40% ethanol with 0.5 ml of 1% dimethyl-*p*-phenylenediamine HCl in water). Sections were left at room temperature in the dark for 60 minutes. Sections were rinsed in sodium phosphate buffer (0.1 M, pH 7.2), mounted and viewed. Essential oils appear blue whilst resin acids stain red (David and Carde, 1964).

3.5.4. Nile blue – neutral and acidic lipids

Leaf sections were placed in 1% Nile blue for one minute at 37 °C and then in 1% acetic acid for one minute at 37 °C before being rinsed in distilled water. Neutral lipids stained red whilst acidic lipids stain blue (Cain, 1947).

3.5.5. Ruthenium red – mucilage and pectin

De-waxed sections were placed in aqueous ruthenium red solution (1: 5000) for 10 minutes. Mucilage and pectin stains red/ rose pink (Johansen, 1940).

3.5.6. Sudan III – lipids

Fresh-hand sections were immersed in the stain (saturated solution) for 15 minutes and then rinsed briefly with 70% ethanol to remove excess stain. Lipid inclusions stained orange/red (Pearse, 1985).

3.5.7. Toudine blue – general stain

Leaf sections were immersed in a 0.05% aqueous solution of toluidine blue for one minute. Excess stain was washed with distilled water and stained sections were mounted in 70% glycerol and viewed.

This metachromatic stain (two sets of colour reactions) stains phosphate groups on molecules green-blue whilst polyphenols (lignin) stain blue (O'Brien *et al.*, 1964).

3.5.8. Wagner reagent & Dittmar reagent – alkaloids

Fresh-hand leaf sections were immersed in Wagner reagent and Dittmar reagent for a period of ten minutes each. The stained sections were rinsed with 70% ethanol and then mounted. Alkaloids were detected by a brown colour change (Furr and Mahlberg, 1981).

Prepared slides stained with fast green and safranin was also viewed. Standard control slides were carried out simultaneously. Each test was carried out in triplicate. Treated samples were viewed using a Nikon Eclipse 80i light microscope. All images were captured using NIS-D Elements software.

3.6. PHYTOCHEMICAL PROFILE

3.6.1. Solvent extraction

Leaves were harvested and allowed to air-dry for 72 hours. The dried material was then ground to a fine powder. Approximately 8 g of ground material was dissolved in 100 ml of methanol and placed in a round bottomed flask attached to a Soxhlet apparatus. The flask was heated and solvent extraction proceeded for 60 minutes. The solution was filtered, the crude extract was retained and the process was repeated thrice. Successive extractions using hexane followed by chloroform were carried out twice after 30 minute intervals. The resultant liquid was used for qualitative phytochemical tests.

3.6.2. Qualitative phytochemical tests

Phytochemical analyses on the crude extracts (methanol, chloroform and hexane) and negative control (water) were conducted in accordance with standard protocols (Harbourne, 1973; Trease and Evans, 1978; Philip *et al.*, 2011; Damodaran and Manohar, 2012). All tests were replicated twice. Detailed methodology was as follows:

a. Alkaloids

Dragendorff's test: Two drops of Dragendorff's reagent were added to one ml of the extract. A reddish- orange precipitate indicated the presence of alkaloids.

Hager's test: One ml of extract was mixed with one ml of Hager's reagent (1 g of picric acid in 100 ml distilled water). The formation of a yellow precipitate indicated the presence of alkaloids.

b. *Amino acids*

Ninhydrin test: Two drops of Ninhydrin reagent were added to two ml of dilute extract. A deep purple colour change indicated the presence of amino acids.

c. *Anthraquinones*

A few drops of 2% hydrochloric acid were added to one ml of extract. The formation of a red precipitate indicates the presence of anthraquinones.

d. *Carbohydrates*

Benedict's test: Benedict's reagent (solution one made up of 173 g sodium citrate and 100 g sodium carbonate in 800 ml water, filtered and made up to 850 ml added to a second solution composed of 17.3 g copper sulphate and 100 ml distilled water) was added to one ml of extract and heated for two minutes. A red-brown precipitate indicated the presence of monosaccharides.

Molisch's test: Molisch's reagent (15 g α -naphthol dissolved in 100 ml ethanol) was added to five ml crude plant extract. A brown-red colour reaction indicated the presence of polysaccharides.

e. *Cardiac glycosides*

Keller-Killiani test: Five ml of plant extract was mixed with two ml glacial acetic acid. Two drops of ferric chloride were added, followed by the addition of concentrated sulphuric acid such that the acid remains underneath. The presence of glycosides was indicated by the formation of a brown ring at the junction of the two layers and a blue-green ring at the upper surface.

f. *Coumarins*

One ml of 10% NaOH was added to one ml extract. The development of a yellow colour indicates a positive reaction for coumarins.

g. *Flavonoids*

Lead acetate test: A 10% lead acetate solution (three ml) was added to two ml dilute extract. The formation of a white precipitate indicated the presence of flavonoids.

h. *Phenolic compounds/ tannins*

Ferric trichloride test: Two ml distilled water was added to one ml of the extract and a few drops of 10% ferric trichloride. A dark green colour change indicated a positive result for the presence of phenols and/or tannins.

i. *Quinones*

One ml concentrated sulphuric acid was added to equal amount of extract. The development of a red precipitate indicated the presence of quinones.

j. *Saponins*

Foam test: The extract (five ml) was diluted with distilled water to 20 ml. The solution was shaken in a graduated cylinder for 15 minutes. The presence of saponins in the extract was identified by the formation of a persistent two cm foam layer.

k. *Steroids*

Lieberman-Burchard test: Acetic anhydride (two ml) was added to the extract (five ml). One ml of concentrated sulphuric acid was then carefully added to the solution. The formation of a blue-green colour indicated the presence of steroids.

l. *Terpenoids*

Salkowski's test: Five ml of crude extract was mixed with two ml chloroform. Concentrated sulphuric acid (three ml) was then carefully added to the mixture forming distinct layers. The presence of terpenoids was indicated by the formation of a reddish-brown colour at the interface between the two solutions.

3.6.3. Quantification of total phenolics and flavonoids

The total phenolic content was determined using adapted protocols of the Folin-Ciocalteu method (Singleton and Rossi, 1965; Moyo *et al.*, 2013). Total flavonoid content was determined using an adapted aluminium chloride colorimetric assay (Zhishen *et al.*, 1999).

a. *Preparation of plant extract*

Powdered leaf material (2 g) was extracted with 10 ml of 50% aqueous methanol in a sonication bath containing ice-cold water for 60 minutes. The extract was filtered under vacuum through Whatman™ No. 1 filter paper. The filtrate was immediately used for the determination of total phenolics and flavonoids.

b. *Total phenolic content: Folin–Ciocalteu method*

A stock solution of gallic acid was prepared by dissolving 0.5 g of dry gallic acid in 10 ml of ethanol and diluting with distilled water in a 100 ml volumetric flask. Working solutions with concentrations of 0, 50, 100, 150, 250, and 500 mg/l gallic acid were prepared. Gallic acid (0–500 mg/l) was used as the effective range of the assay and for generation of the standard curve.

The sample extract (0.5 ml) was added to 2.5 ml of 1:10 diluted Folin–Ciocalteu reagent. After three minutes, 2 ml of saturated sodium carbonate solution (75 g/l) was added. After two hours of incubation at room temperature in the dark, the absorbance of three randomly selected samples were measured at 765 nm. The results were expressed as milligram gallic acid equivalent (mg GAE)/g dry weight of plant matter.

c. *Total flavonoid content: The aluminium chloride colorimetric assay*

To quantify the total flavonoid content, quercetin was used as the reference. Quercetin stock solution (0.25 mg/ml) was prepared by dissolving 10 mg quercetin in 40 ml of 80% ethanol. Standard working solutions of 0, 25, 50, 75, and 100 µg/ml quercetin were prepared in 10 ml volumetric flasks by adjusting the volume with 80% ethanol (Chang *et al.*, 2002; Ozkok *et al.*, 2010)

Extract (1 ml) or quercetin standards were added to a 10 ml volumetric flask containing 4 ml of distilled water. To the flask, 0.30 ml of 5% NaNO₂ was added and after five minutes 0.3 ml of 10% AlCl₃ was added. After five minutes, 2 ml of 1M NaOH was added and the volume was made up to 10 ml with distilled water. The solution was mixed and the absorbances of three random samples were measured against an ethanol blank at 510 nm. The total flavonoid content was expressed as mg quercetin equivalents (QE)/ g dry weight.

3.6.4. Antioxidant activity

The antioxidant activity of the *S. natalensis* methanolic extract was determined using a modified method of the DPPH radical scavenging assay (Blois, 1958; Karioti *et al.*, 2004; Bilusic-Vundac *et al.*, 2007)

a. Principle of the DPPH (1,1-diphenyl-2-picrylhydrazyl) assay

The assay is based on the reduction of 1,1-diphenyl-2-picrylhydrazyl, a stable free radical. The presence of an odd electron provides a maximum absorption at 517 nm (purple colour). In the presence of an antioxidant (or extract with radical scavenging potential), the electron is paired off. As a consequence, absorption decreases and the degree of discolouration (yellow) provides an indication of the extracts' scavenging potential in accordance with its hydrogen donating ability (Patel and Patel, 2011).

b. DPPH assay protocol

A 0.1 mM methanolic DPPH solution and sample extracts consisting of final concentrations of 0, 1, 2.5, 5, 10, 50, 100, 150 and 250 $\mu\text{g ml}^{-1}$ were freshly prepared before the assay. One ml of each plant extract concentration was added to one ml of methanolic DPPH solution and incubated in the dark for 30 minutes. Thereafter, absorbance was measured at 517 nm using methanol as a blank with a UV-visible spectrometer (Shimadzu, UV-1601; Japan). Ascorbic acid at final concentrations of 1.0, 2.5, 5, 10 and 50 $\mu\text{g ml}^{-1}$ were used as a positive control. A solution of 50% methanol, instead of extract or antioxidant, was used as a negative control. Extract colour which may adversely affect absorbance values was accounted for by subtraction of sample absorbance from sample + DPPH absorbance. The assay was done in triplicate using random extract samples.

The free radical scavenging activity of the extract, determined by the decolouration of the DPPH solution, was calculated according to the formula:

$$\% \text{ scavenging} = \frac{[\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{test extract}}]}{\text{Absorbance}_{\text{control}}} \times 100$$

The effective concentration of sample required to scavenge DPPH radical by 50% (IC₅₀ value) was calculated for the extract as well as ascorbic acid by linear regression analysis between percentage inhibition and sample concentrations.

3.7. MICROPROPAGATION OF *STACHYS NATALENSIS*

3.7.1. Plant collection and decontamination

Field-grown *S. natalensis* plants were obtained from Durban, South Africa (29° 47' 12.462; 30° 55' 45.0156). The plants were pre-treated thrice over a two week period with an aerosol fungicide (Wonder FUNGI GUN™, Active ingredient: 3.0 g/kg Triforine). Axillary bud segments were used as nodal explants (1.0 - 1.5 cm) and were initially surface decontaminated by intermittent washing with tap water for 20 minutes. Due to high levels of plant contamination, samples were washed with distilled water to remove residual media and microbial growth, followed by exposure to a number of decontamination treatments, as outlined in Table 3.1. Samples were rinsed in distilled water after each step of the procedure. Thereafter decontaminated samples were placed onto a suitable growth medium. The experiment was conducted in a randomized design using 25 explants in each of the five treatments.

Table 3.1 Decontamination treatments for *S. natalensis* axillary bud explants.

Treatment	Immersion in 1% NaClO	Immersion in 3% NaClO	Immersion in 0.1% HgCl₂
1	2 × 5 min	-	-
2	-	2 × 5 min	1 × 10 min
3	-	-	1 × 20 min
4	1 × 7 min	1 × 5 min	1 × 10 min
5	1 × 5 min	1 × 2 min	1 × 7 min

3.7.2. Culture conditions and media

a. Initiation and shoot multiplication

Nodal explants (1-2 cm) were cultured on MS medium (Murashige and Skoog, 1962) supplemented with 20.0 g/l sucrose and 12 g/l nutrient agar. Media composition differed with regard to the combination and concentration of plant growth regulators (PGRs) for initiation, shoot multiplication and rooting. Media for bud break (initiation) was as follows: medium A contained a combination of 0.5 mg/l BAP and 0.5 mg/l IAA, medium B contained

0.5 mg/l kinetin and 0.5 mg/l IAA and medium C contained 0.5 mg/l BAP and 0.5 mg/l IBA. There were 50 explants per medium preparation. The same combinations of PGRs were used for multiplication media in a cytokinin: auxin ratio of 2:1. Microshoots (8 - 10 cm) with a minimum of six leaves were chosen for transfer into rooting medium which contained ½ strength MS medium supplemented with 2mg/l IAA as the only PGR. The percent rooting, number of roots per shoot and root length were recorded over five weeks. The pH of all media was adjusted to 5.7 ± 0.1 by the addition of 1 M HCl or 1 M NaOH before decontamination by autoclaving at 121° C (1 kPa) for 20 minutes. Cultures were incubated in a growth room with a 16 hour light/8 hour dark photoperiod, photon flux density of $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ and temperature of $26^\circ \text{C} \pm 0.1^\circ \text{C}$ (day) and $23^\circ \text{C} \pm 0.1^\circ \text{C}$ (night).

b. Acclimatization

Once rooted, plantlets were removed from the medium, rinsed off to remove residual medium and transferred to pots containing organic potting soil and kept in a mist tent (three second spray per six minute interval) for acclimatization. The soil was supplemented with ¼ strength MS salts for a week. After one month, acclimatized plants were transferred to a greenhouse and irrigated daily. The percentage survival was recorded after six weeks.

3.8. STATISTICAL ANALYSES

Glandular and non-glandular trichome counts (per cm^2 leaf surface) for field grown and *in vitro* plants were obtained from SEM micrographs using the iTEM imaging software (Soft Imaging System GmbH, Münster, Germany). A two-way ANOVA statistical test was conducted in IBM SPSS Statistics (version 21.0, IBM®, USA) to determine trichome density across three developmental stages viz. emergent, young and mature as well as between the abaxial and adaxial foliar surfaces. Tukey's post-hoc test was implemented to obtain multiple comparisons of factors investigated. All quantitative data was reported as mean \pm standard error of the mean (SE). Values were regarded as significant at $p < 0.05$. Regression analyses and calculation of EC_{50} values for antioxidant activity were done using IBM SPSS Statistics.

CHAPTER 4: RESULTS

4.1. TRICHOME TYPES AND DISTRIBUTION

The leaf surfaces of *Stachys natalensis* are visibly hispid and bear numerous non-glandular and glandular trichomes of different morphologies (Figure 4.1 A). Non-glandular trichomes primarily occupy the midrib and lateral veins and are especially dense on the abaxial surface (Figure 4.1 B and 4.1 D). The adaxial surface contains a random distribution of colourless glands (Figure 4.1 C). In addition, these glands are pronounced on the abaxial surface and display great structural diversity (Figure 4.2). Transverse section of the leaves show the structural features of the leaf lamina including multicellular peltate and bicellular capitate trichomes (Figure 4.3). The adaxial surface was furnished with many bent non-glandular trichomes and peltate trichomes occurring in rows in epidermal grooves (Figure 4.4).

Emergent leaves appeared to have a dense covering of trichomes which visibly decreased as the leaf matured (Figure 4.5). Trichome density among three growth stages (emergent, young and mature) and two foliar surfaces (abaxial and adaxial) were investigated using a two-way analysis of variance (ANOVA). There were significant differences in both glandular ($F_{(2, 0.678)} = 7.408$; $p < 0.05$) and non-glandular trichome density ($F_{(2, 0.324)} = 10.544$; $p < 0.05$) among the three developmental stages and between the upper and lower foliar surfaces (Figure 4.6 A and B). Multiple comparisons of the three developmental stages using Tukey's post-hoc test indicated significant differences in trichome density between emergent and young leaves and between emergent and mature leaves (glandular and non-glandular: $p < 0.05$). The abaxial surface was found to contain a higher number of both glandular and non-glandular trichomes (Table 4.1).

Plate One

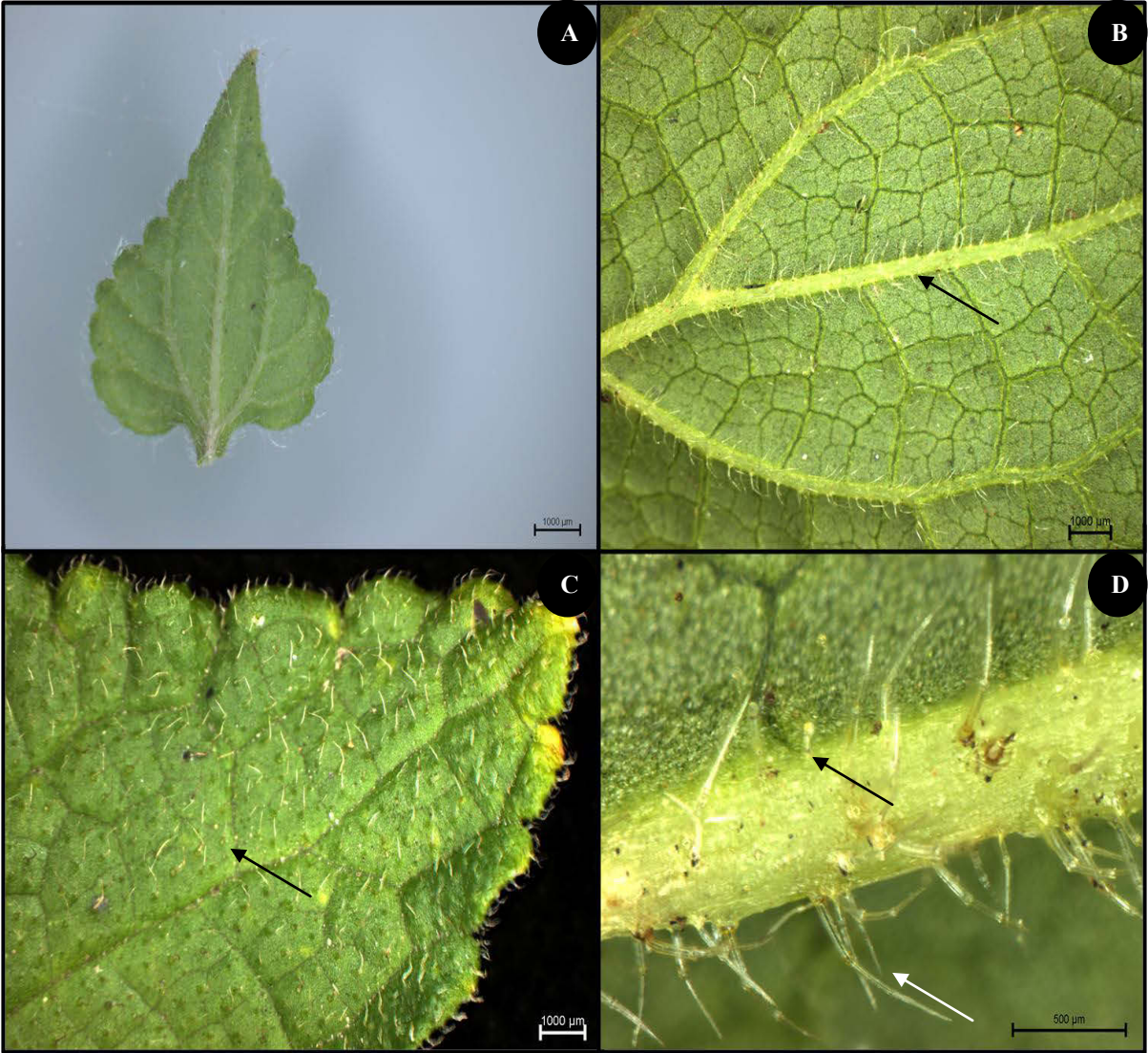
Figure 4.1 Stereo micrographs showing overall surface morphology and trichome distribution on the adaxial and abaxial foliar surfaces of *S. natalensis*.

[A] Hispid abaxial surface of emergent leaf.

[B] Abaxial surface of mature leaf showing non-glandular trichomes which appear frequently on the central midrib and branching veins.

[C] Secretory glands randomly dispersed over the abaxial surface of a young leaf specimen.

[D] Mature leaf showing glandular (black arrow) and non-glandular (white arrow) trichomes on the abaxial surface.



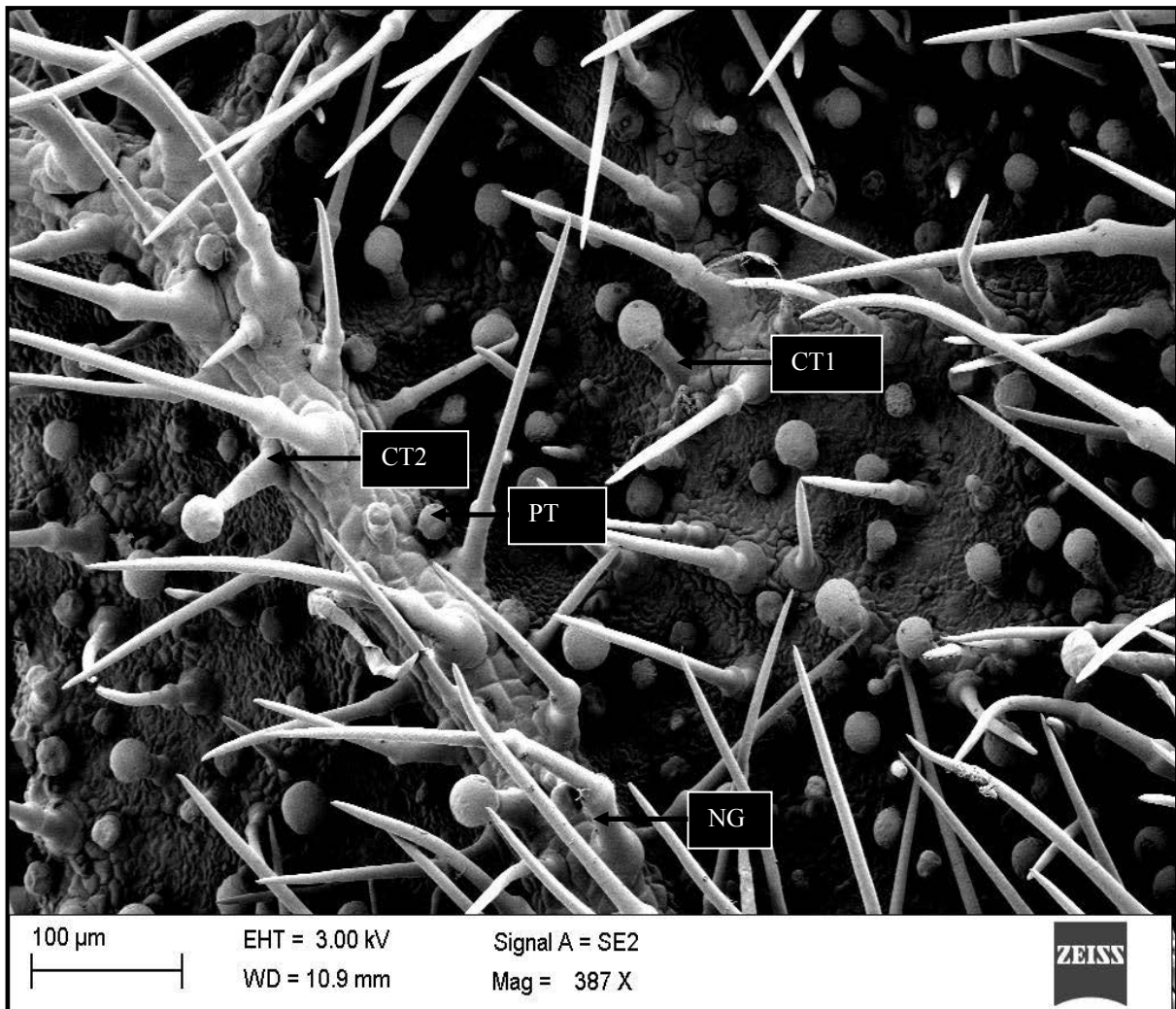


Figure 4.2 SEM micrograph showing an overview of the different types of non-glandular and glandular trichomes on the abaxial surface of an emergent leaf including: elongated non-glandular trichomes (NG) and three visible glandular trichome types viz. a short stalked capitate trichome (CT1), a long stalked capitate trichome (CT2) and numerous peltate trichomes (PT).

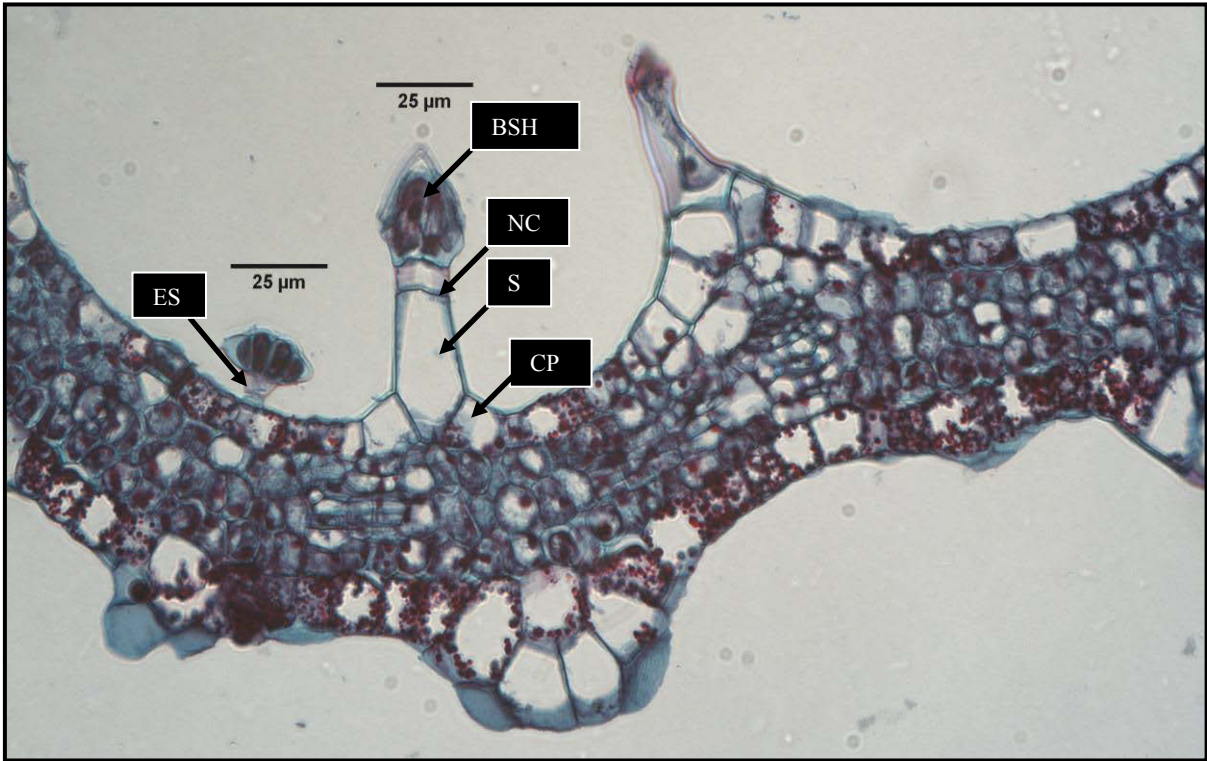


Figure 4.3 Transverse section of young *S. natalensis* leaf stained with fast green and safranin showing overview of the foliar structures. Light micrograph showing glandular trichome features. Note the short epidermal stalk (ES) of multicellular peltate trichomes. Structural features of Type II capitate trichomes include elevated epidermal cells that make up the cellular pedestal (CP), long stalk (S), distinct neck cell (NC) and bi-cellular secretory head cell (BSH).

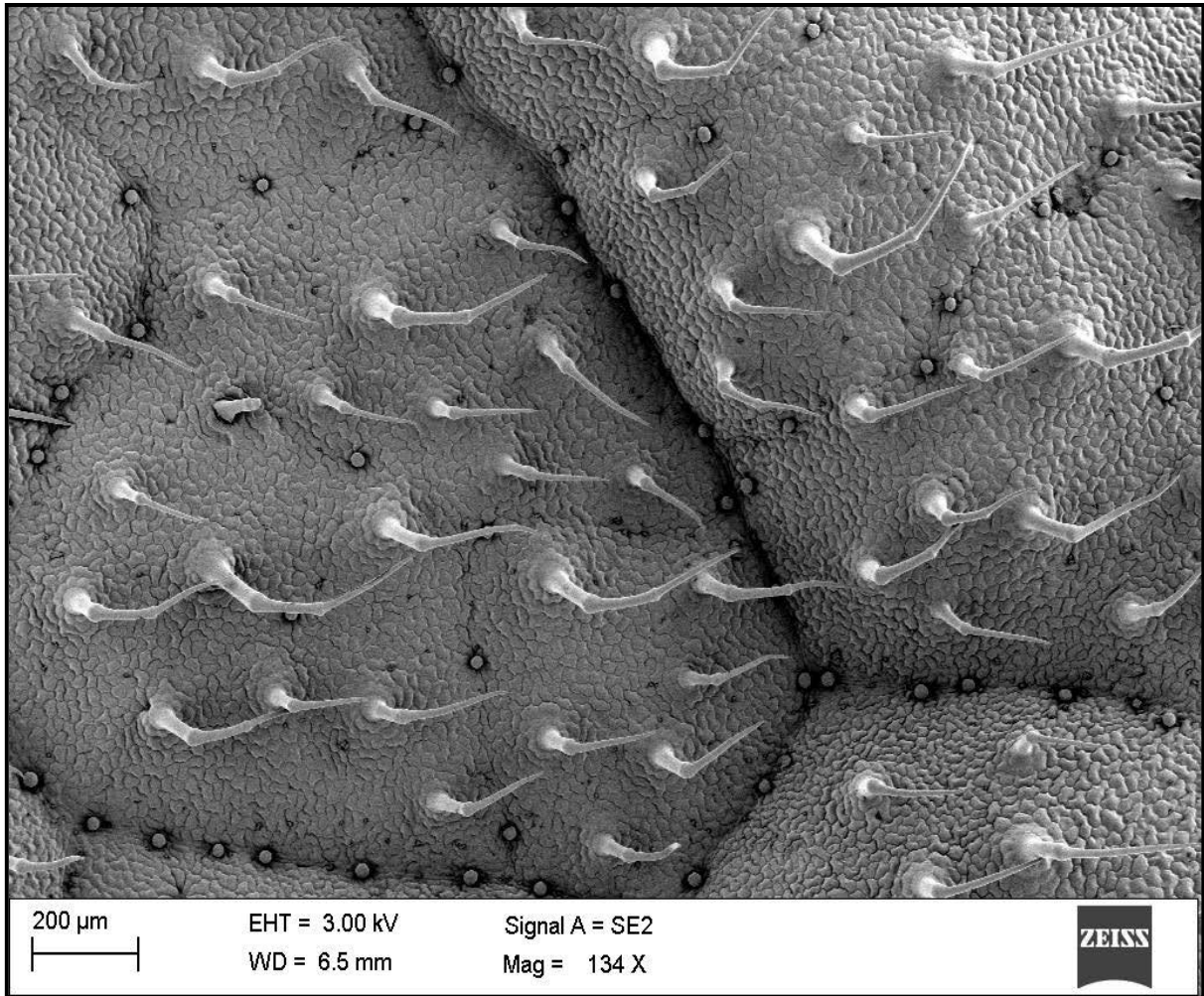


Figure 4.4 SEM micrograph of the adaxial surface of an emergent *S. natalensis* leaf showing miniscule peltate trichomes in epidermal depressions and numerous, bent non-glandular trichomes.

Plate Two

Figure 4.5 SEM micrographs showing trichome distribution on the two foliar surfaces and across three developmental stages of *S. natalensis*.

[A-B] Emergent leaf showing dense distribution of non-glandular and glandular trichomes on the adaxial (A) and abaxial (B) foliar surfaces.

[C-D] Young *S. natalensis* leaf showing trichome distribution on the adaxial (C) and abaxial (D) foliar surface.

[E- F] Mature leaf with visibly sparse non-glandular and glandular trichomes on the adaxial (E) and abaxial (F) foliar surface

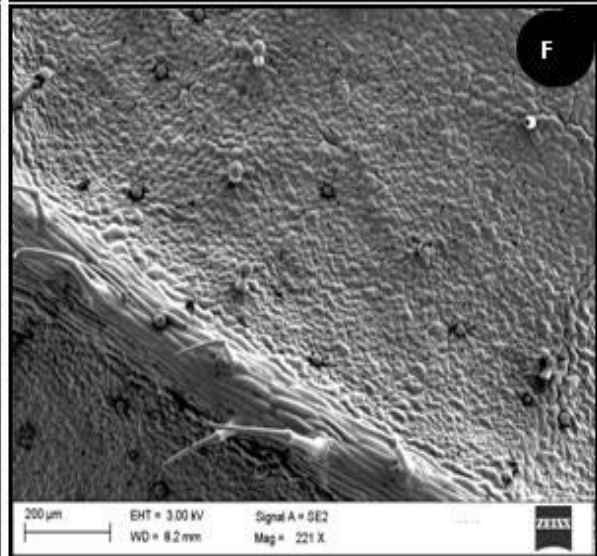
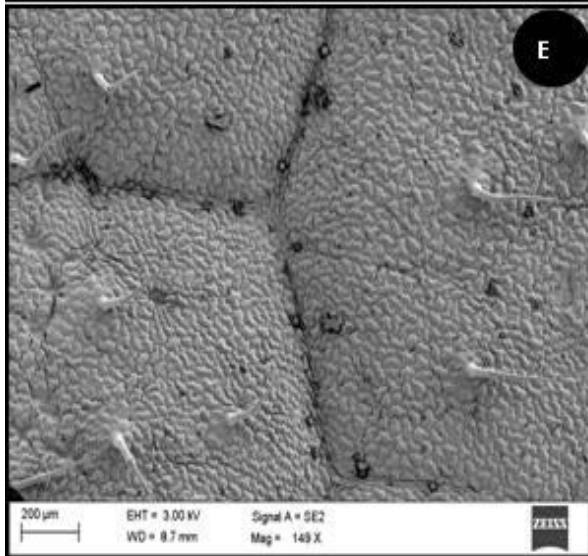
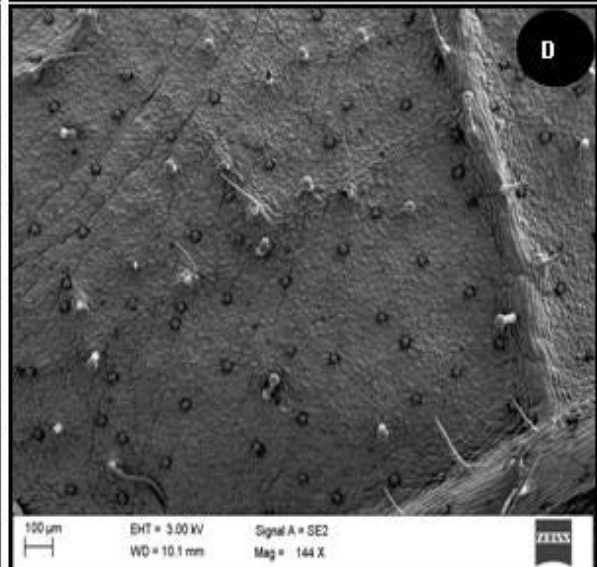
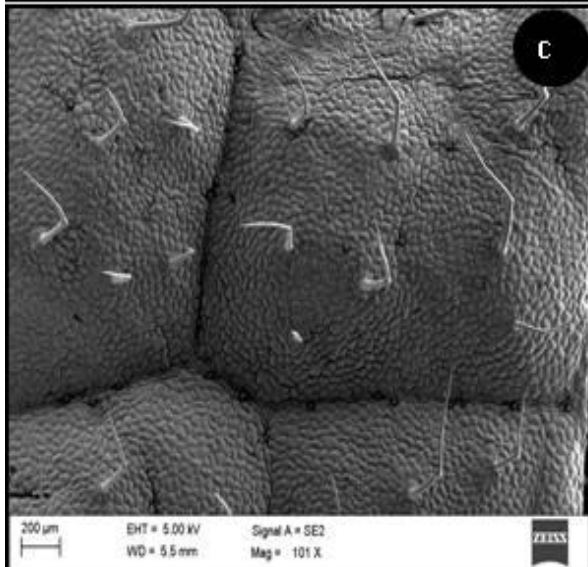
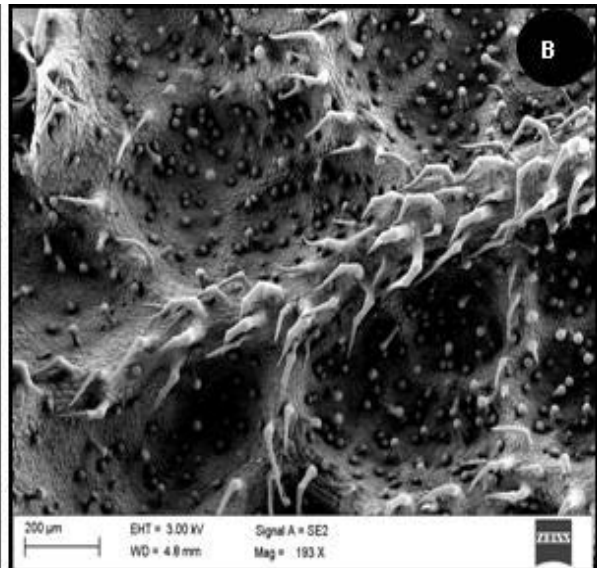
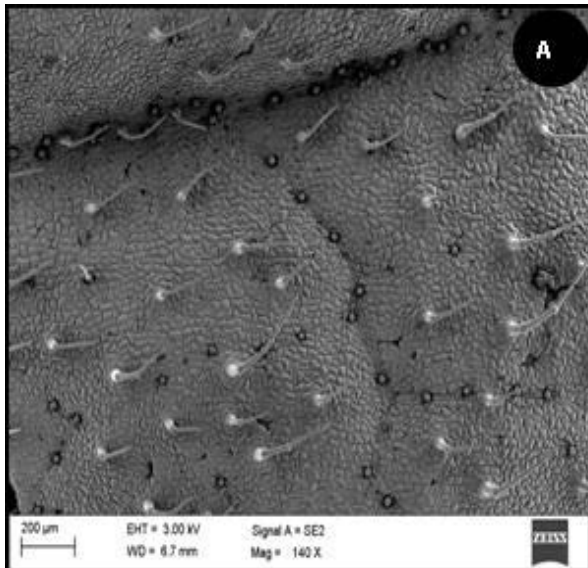


Plate Three

Figure 4.6 Mean non-glandular (A) and glandular (B) trichome density at three stages of leaf development (emergent, young and mature) and on the abaxial and adaxial foliar surfaces of *S. natalensis*.

Values are mean \pm SE (n=20; $p < 0.05$) was recognised as significantly different using Tukey's multiple comparison post-hoc test and denoted by different letters.

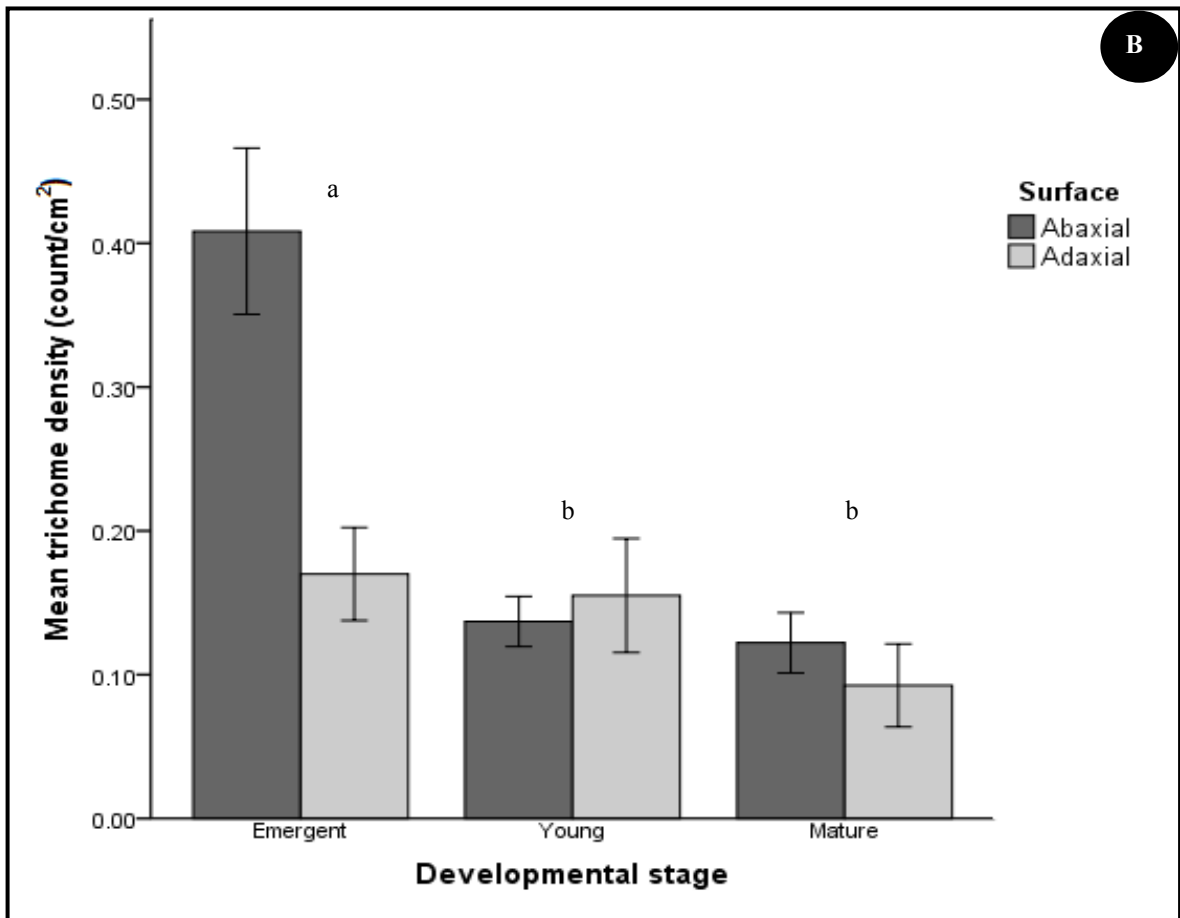
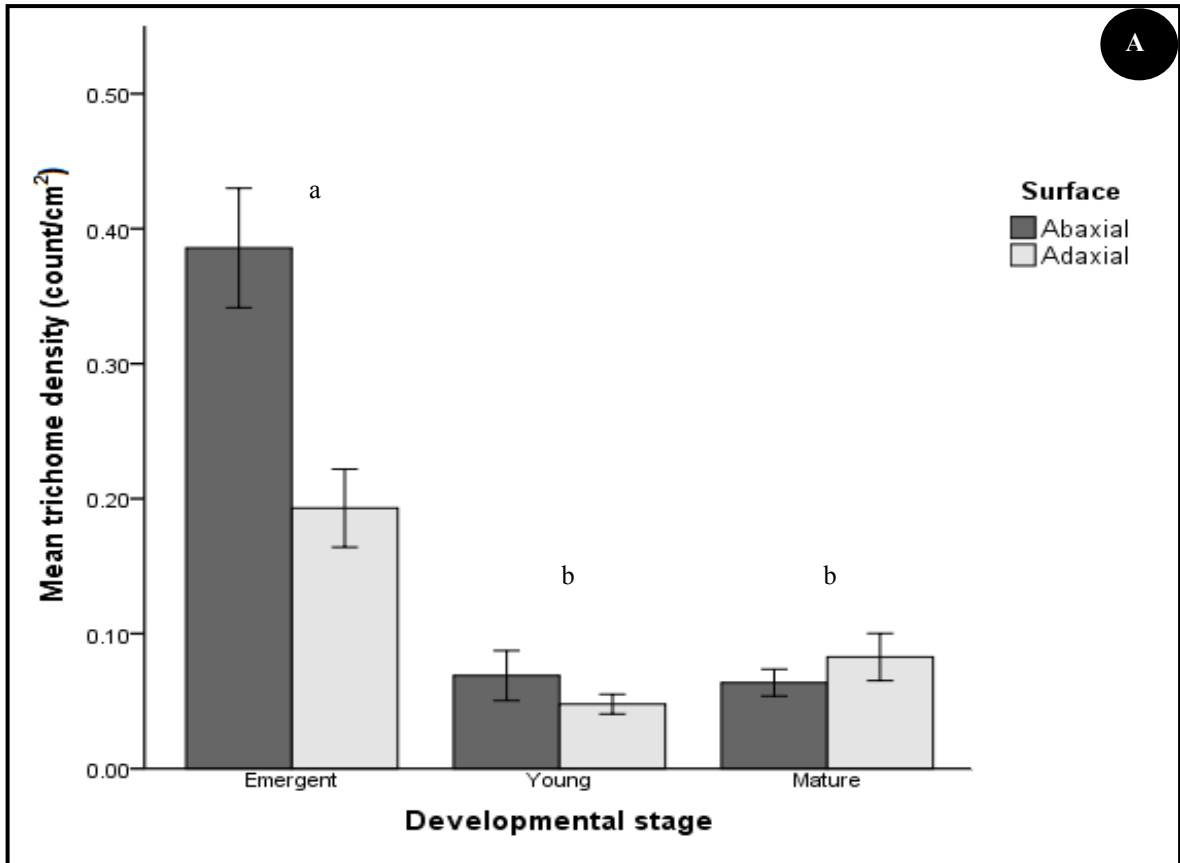


Table 4.1 Mean trichome density on the abaxial and adaxial leaf surfaces of *S. natalensis* plants.

Trichome type	Mean trichome density (count/cm ²)	
	Abaxial	Adaxial
Non-glandular	0.631 ± 0.03	0.108 ± 0.02
Glandular	0.310 ± 0.03	0.139 ± 0.02

Data represents mean ± SE

4.2. TRICHOME MORPHOLOGY

Two morphological types of non-glandular trichome were observed on the foliar surfaces of *S. natalensis* across all developmental stages. Structurally, the first type appeared acicular or acuminate, with the stalk tapering gradually to a sharp point. The other morphological type appeared to be arthrodachylous, jointed or highly septate (Payne, 1978). Both types of non-glandular trichomes were uniseriate and unbranched (Figure 4.7). These trichomes have a wide base attached to the epidermal layer via a multicellular (two-ten cells) epidermal cellular pedestal (Figure 4.7 A - D). The trichome stalk which may be elongated tapering toward the apex or bent from the first or second stalk septation facing the leaf apex has visible striations traversing the entire stalk length of the cellular pedestal (Figure 4.7 E - F). These striations were also observed on glandular structures (Figure 4.9 A).

Glandular trichomes were divided into two types: peltate and capitate trichomes. Peltate trichomes with a large, multicellular head appeared to contain between two to eight head cells (Figure 4.8). Two types of capitate glandular trichomes were observed: Type I and Type II (Figure 4.9). Type I was characterised by a distinctly striated stalk and a spherical shaped head that may be bi-cellular or unicellular, according to Werker (2000) (Figure 4.9 A and Figure 4.11 A) and Type II was observed primarily on the abaxial surfaces of emergent and young leaves. Type II capitate trichome was composed of a bulbous base, long, tapering septate stalk, a distinctive neck cell and a multicellular head cell (Figure 4.9 B). Glandular trichomes were observed in both pre- and post-secretory phases. The former was characterised by accumulation of secretion in the subcuticular space in peltate head cells (Figure 4.10 A). Preliminary observations indicate that septation of the secretory cells facilitates the flow of secretion. Glandular trichomes in post-secretory phase showed a distinct contraction of secretory head cells upon loss of secretion possibly via a porose cuticle (Figure 4.10 B). Two possible mechanisms of secretion were observed in Type I and Type II capitate trichomes. Loss of secretion via cuticular rupture was noted in Type I capitate trichomes (Figure 4.10 D) whilst Type II capitate trichome was shown to secrete substances via pore-like openings on each of the multicellular head cells (Figure 4.10 C).

Plate Four

Figure 4.7 SEM micrographs of the non-glandular trichomes on the foliar surface of *S. natalensis*.

[A-D] appear to show maturation of non-glandular trichomes on the adaxial leaf surface. Note the epidermal cellular pedestal (CP) developing into a multicellular structure and the septation (SS) of the trichome stalk [B, C, D].

[E-F] Distinctive micro-ornamentation (striations) observed on the cellular pedestal (CP) and traversing the length of the trichome stalk (S)

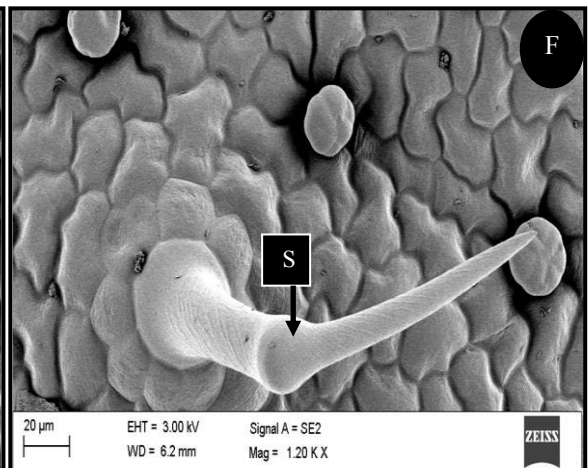
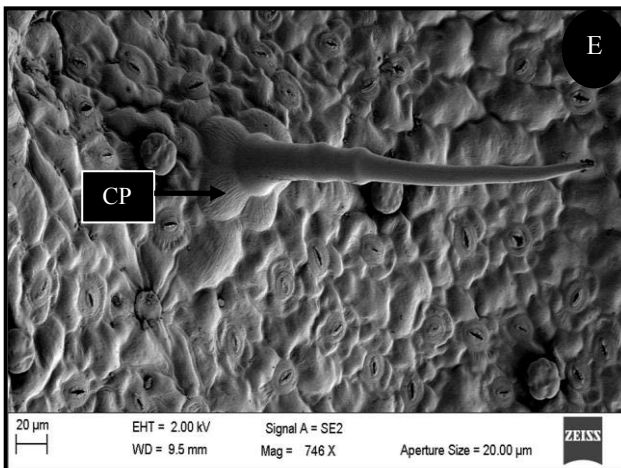
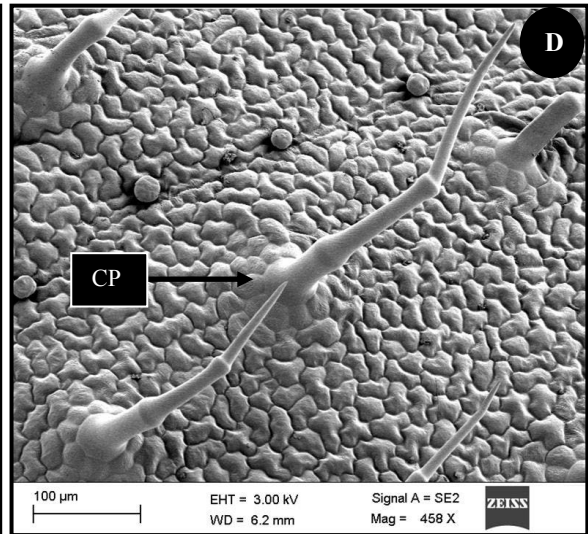
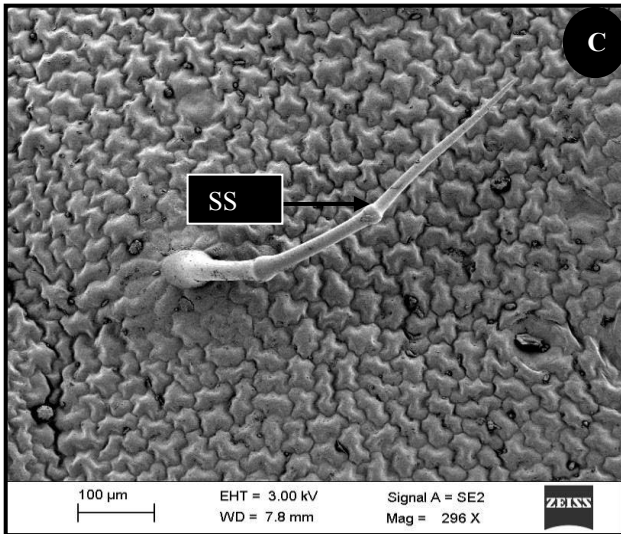
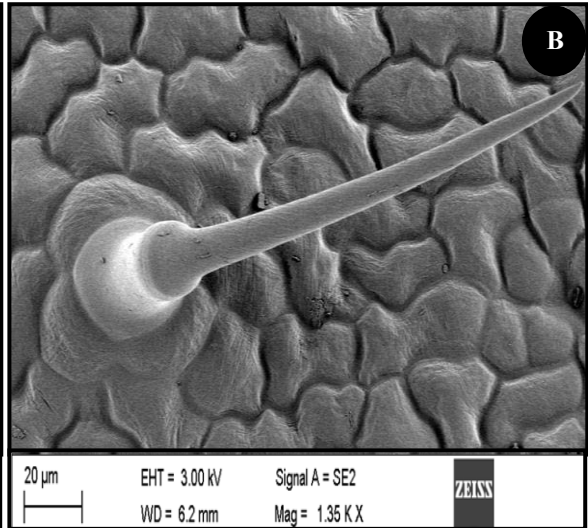
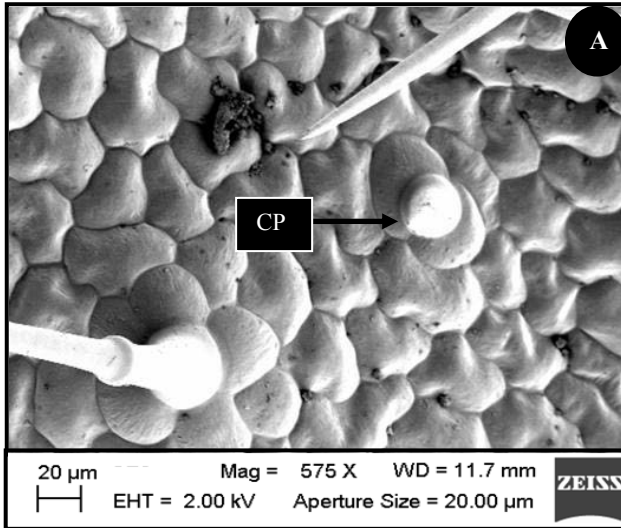


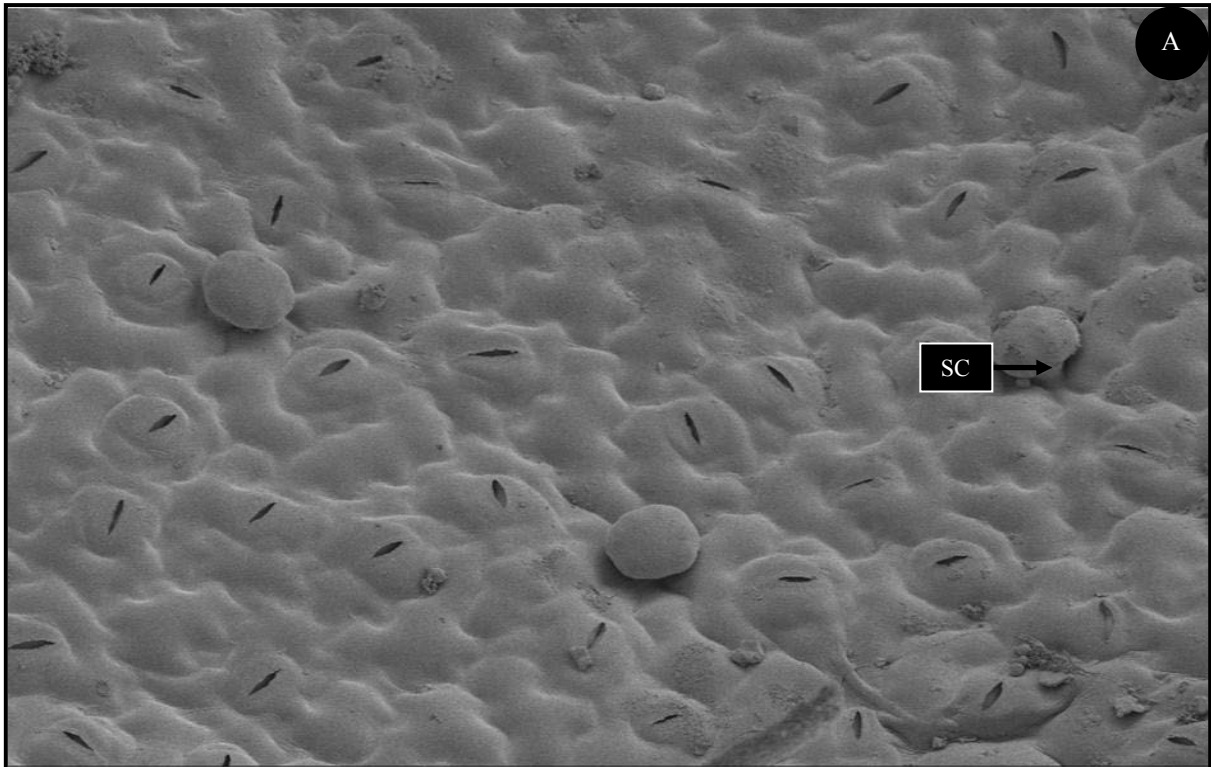
Plate Five

Figure 4.8 SEM micrographs showing the morphology of the peltate trichomes of *S. natalensis*.

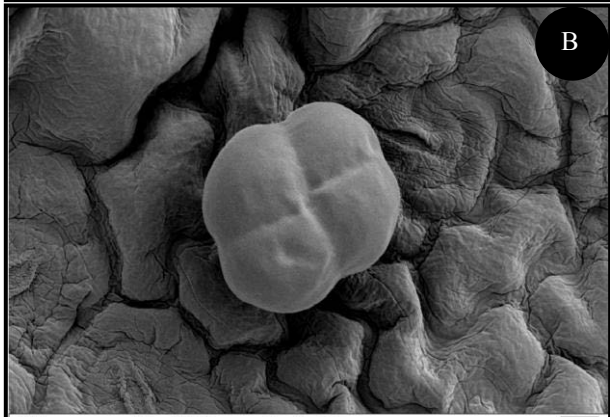
[A] Cryo-SEM image of peltate trichome in epidermal depression with single stalk cell (SC) found on the abaxial surface of a mature leaf.

[B] Peltate trichome with multicellular (four-celled) head found on the abaxial surface of a young leaf.

[C] Multicellular (eight or more cells) head of peltate trichome on the abaxial surface of an emergent leaf specimen.



20 μm EHT = 2.00 kV Signal A = SE2
 WD = 7.8 mm Mag = 835 X Aperture Size = 15.00 μm ZEISS



2 μm EHT = 3.00 kV Signal A = SE2
 WD = 8.1 mm Mag = 5.00 K X ZEISS



2 μm EHT = 2.00 kV Signal A = SE2
 WD = 4.9 mm Mag = 8.13 K X ZEISS

Plate Six

Figure 4.9 SEM micrographs of two types of capitate trichomes found on the foliar surface of *S. natalensis*.

[A] Glandular capitate trichome Type I with bulbous head cell, distinct neck cell (NC) and a shorter, non-septate stalk on the abaxial foliar surface. Micro-ornamentation (striations) is visible on the stalk of mature (M) and developing (inset) capitate Type I trichomes.

[B] Glandular capitate trichome Type II on the adaxial surface characterised by disc-shaped multicellular head (MTH) and elongated, septate stalk (SS) which appears bulbous at the base and narrow toward the apex.

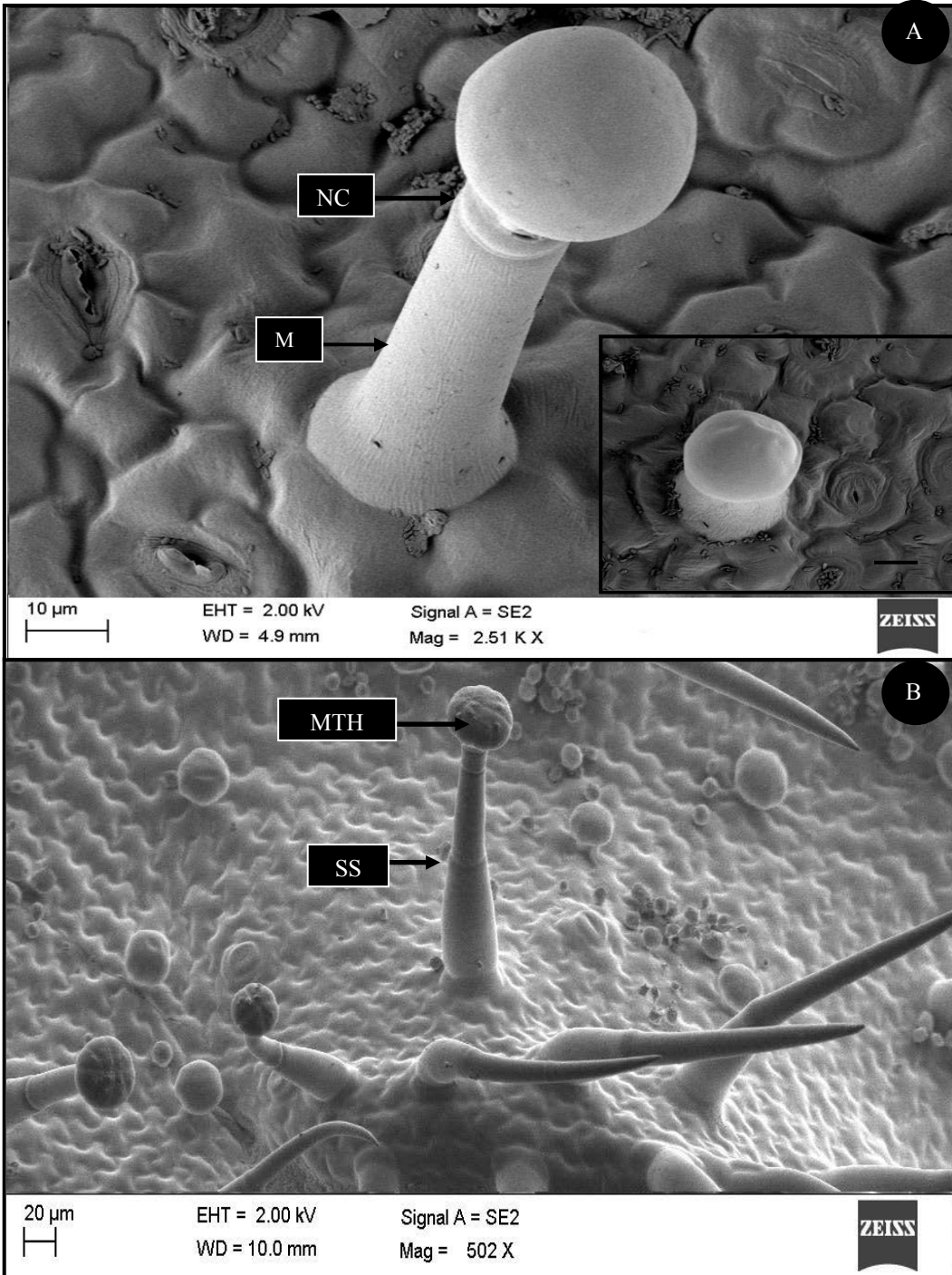


Plate Seven

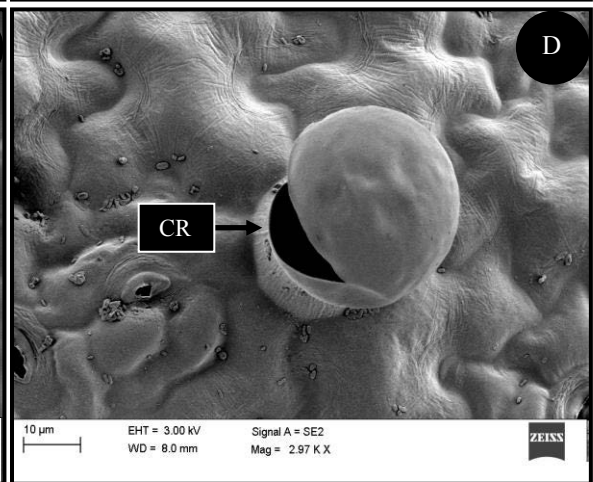
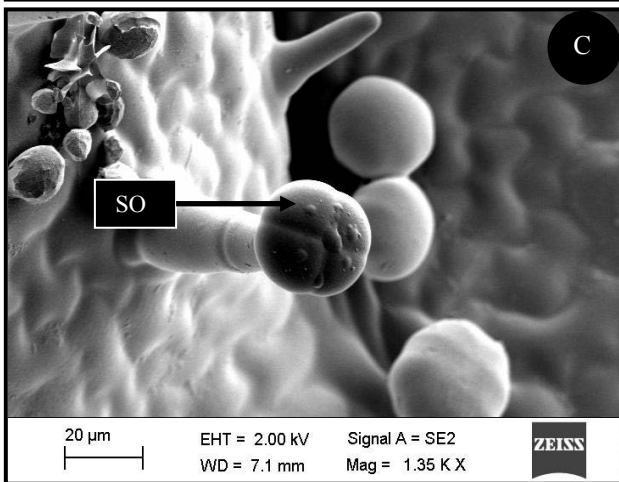
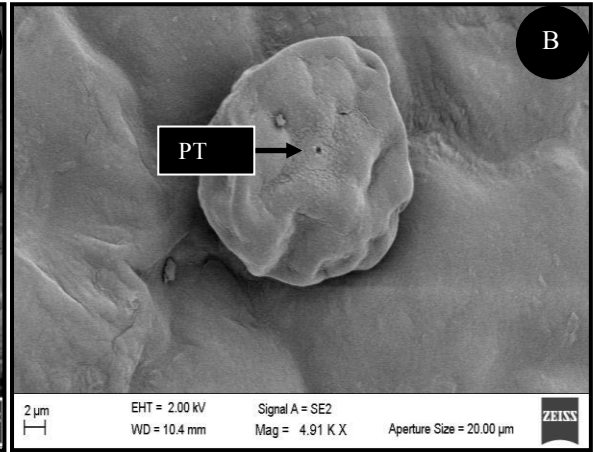
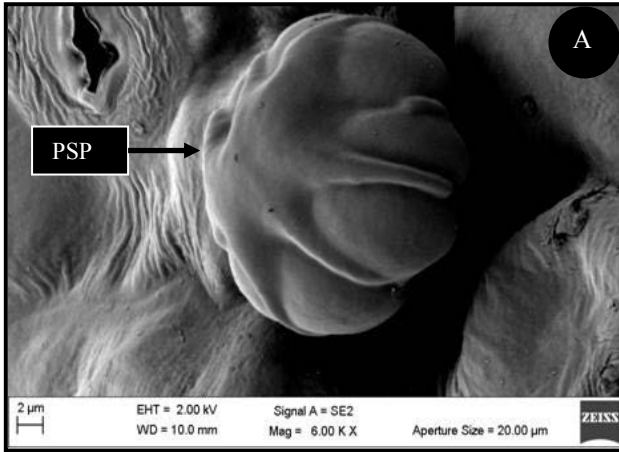
Figure 4.10 SEM micrograph showing secretory processes of the glandular trichomes of *S. natalensis*.

[A] Peltate trichome in the pre-secretory phase (PSP), showing accumulation of secretion within the sub-cuticular space in the glandular head cell.

[B] Peltate trichome in the post-secretory phase. Note the collapse of the peltate trichome (PT) after the release of exudates.

[C] Type II capitate trichome in the process of secretion via openings (SO) on the surface of each of the multicellular head cells. It appears that the glandular cuticle may be highly porose.

[D] Cellular rupture (CR) occurring at fragile regions in the head cell of Type I capitate trichome.



4.3. HISTOCHEMISTRY AND PHYTOCHEMISTRY

Histochemical analyses were conducted on fresh-hand and de-waxed young leaf material showing the localisation of a variety of secondary metabolites in the glandular head cells and cell wall constituents (Figure 4.11).

Alkaloids were detected in the head cells of peltate and Type I capitate trichomes using the Wagner and Dittmar stain (Figure 4.11 A). Positive results in the peltate glands include acidic polysaccharides such as mucilage and pectin identified after staining with ruthenium red (Figure 4.11 B); ferric trichloride positively stained phenolic compounds greenish-black (Figure 4.11 C); mercuric bromophenol blue stained proteins (Figure 4.11 D); nadi reagent stained terpenoids (such as oleoresins) red (Figure 4.11 E). Toluidine Blue stained carboxylated polysaccharides pink & polyphenols blue-purple in glandular head cells (Figure 4.11 F). Acidic lipids were stained blue in peltate glands by the Nile blue stain (Figure 4.11 G). Sudan III was also used to detect lipid molecules in the head cells of the capitate trichomes (Figure 4.11 H) and the presence of suberin or lignin in trichome cell wall components.

Phytochemical tests were performed to determine the main classes of secondary metabolites present in the crude extract of *S. natalensis* which may give a preliminary indication of the therapeutic potential of the plant extract. Hexane was found to be the best solvent for phytochemical extraction as it contained the greatest number of reactive compounds. The leaf extract of *S. natalensis* contained many phytochemicals including: complex carbohydrates, alkaloids, coumarins, phenolics and terpenoids which were detected at high intensities and quinones which showed a slightly weaker reaction (Table 4.2). Total phenolic and flavonoid content in the leaves of *S. natalensis* were 3.43 ± 0.01 mg GAE/g dry material and 3.04 ± 0.01 mg QUE/g dry material, respectively. The radical scavenging activity of *S. natalensis* extract is shown in Figure 4.12. Scavenging activity increases in a dose-dependent manner. The IC_{50} value being the effective concentration to induce a 50% scavenging activity of the DPPH radical of *S. natalensis* extract and ascorbic acid was determined (Table 4.3). The IC_{50} value is negatively correlated with antioxidant potential.

Plate Eight

Figure 4.11 Histochemical staining reactions using semi-thin fresh-hand (A,C,E and H) and dewaxed (B,D, F and G) leaf sections.

[A] Wagner/Dittmar reagent stained alkaloids brown in head cell of long stalked capitate trichome. Bar: 50 μm .

[B] Ruthenium red stained positively for mucilage & pectin in multicellular head of peltate trichome (pink-red). Bar: 25 μm .

[C] Ferric trichloride stained phenolic deposits greenish-black in peltate trichome head cell (arrow). Bar: 25 μm .

[D] Mercuric bromophenol blue stained proteins in peltate head cells positively (blue). Bar: 50 μm .

[E] Positive Nadi reaction in head cells of peltate trichome (red) for the presence of oleoresins. Bar: 50 μm .

[F] Toluidine Blue stained carboxylated polysaccharides pink & polyphenols blue-purple in glandular head cell. Bar: 50 μm .

[G] Positive detection of acidic lipids in peltate glands (blue) by Nile blue stain. Bar: 20 μm .

[H] Sudan III stained lipids orange in head cell of long stalked capitate trichome. Bar: 50 μm .

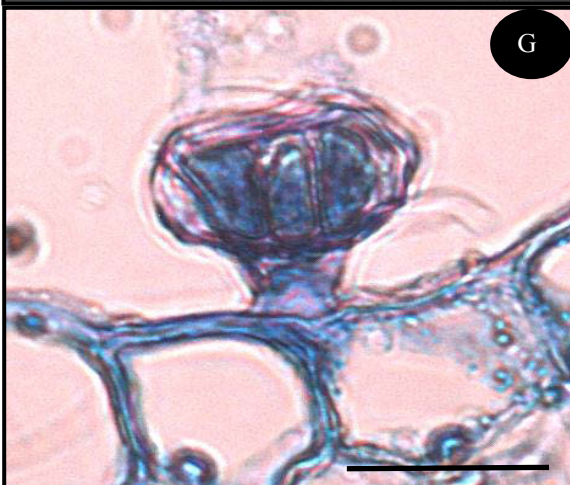
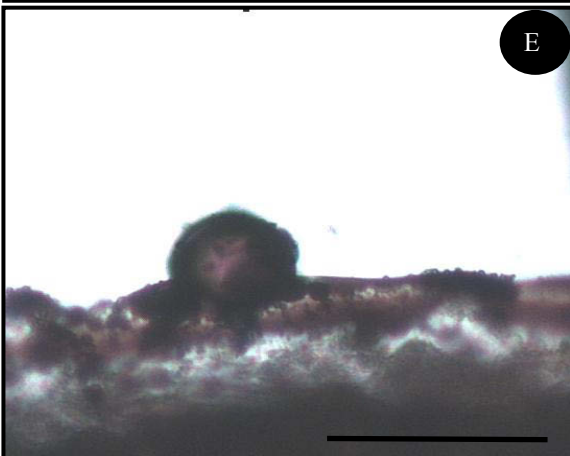
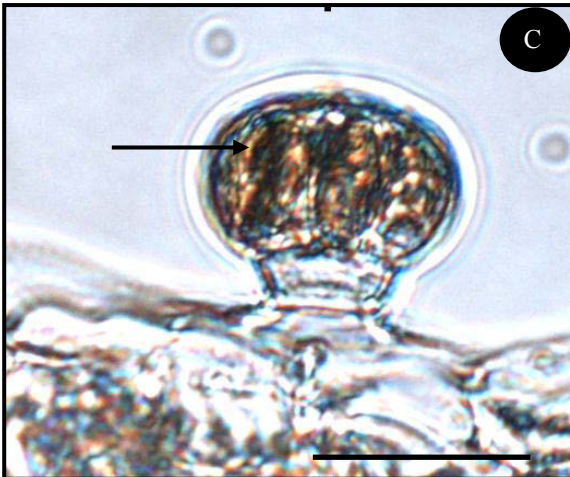
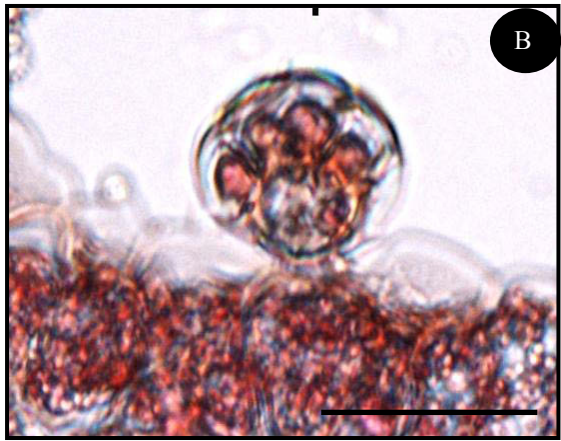
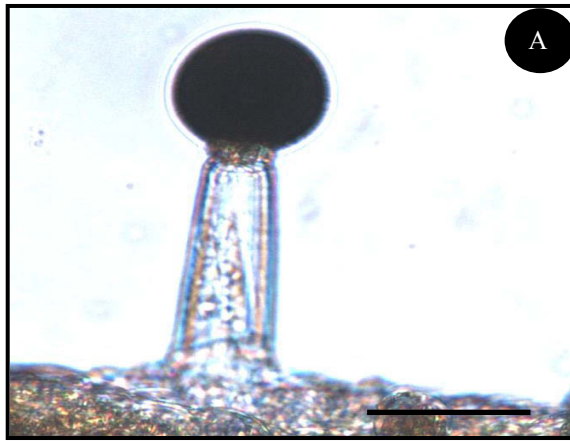


Table 4.2 Preliminary phytochemical profile of the crude extracts (methanol, chloroform and hexane) of powdered leaf material of *S. natalensis*.

Phytocompound	Test	Reaction	Crude extract		
			Methanol*	Chloroform*	Hexane*
Alkaloids	Dragendorffs	Red-orange	++	-	+++
	Hagers	Yellow	+	-	+++
Amino acids	Ninhydrin test	Purple	-	-	-
Anthraquinones	HCl + extract	Red precipitate	-	-	-
Monosaccharides	Benedicts	Red-brown	++	++	-
Polysaccharides	Molisch	Red-brown	-	-	++
Cardiac glycosides	Keller-Killiani	Brown interface and blue-green at upper layer	-	-	-
Coumarins	NaOH + extract	Yellow	-	-	+++
Flavonoids	Lead acetate	White precipitate	-	+++	++
Phenolic compounds	Ferric trichloride	Dark green	++	-	-
Quinones	H ₂ SO ₄	Red	-	-	++
Saponins	Foam	> 2 cm foam layer	+++	-	-
Steroids	Lieberman-Burchard	Red interface and blue ring at upper layer	-	+++	-
Terpenoids	Salkowski's	Red- brown colour	-	++	+++

Intensity of reaction: (+) low intensity, (++) medium intensity, (+++) high intensity

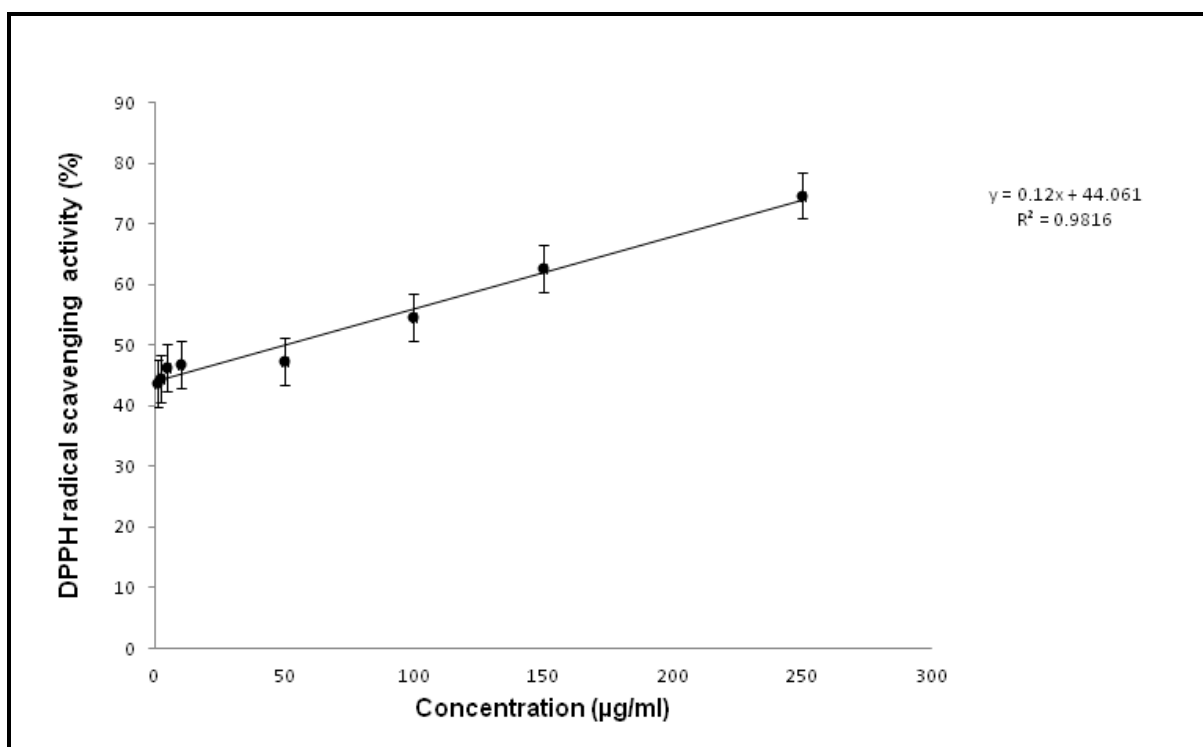


Figure 4.12 Dose-dependent DPPH free radical scavenging activity (%) of the methanolic extract of *S. natalensis*. Bars = SEM, n=3.

Table 4.3 DPPH radical scavenging activity of *S. natalensis* methanolic extracts.

Extract	IC ₅₀ (µg ml ⁻¹)	R ²
<i>S. natalensis</i> leaves	49.49 ± 3.87	0.9816
Ascorbic acid	12.30 ± 2.15	0.8874

Data represents means of three replicates ± standard error.

R² is the coefficient of determination indication the goodness of fit of the model.

4.4. MICROPROPAGATION OF *STACHYS NATALENSIS*

In the present study, axillary bud segments (1 - 2 cm) were used as explant material for the *in vitro* micropropagation of *S. natalensis*. Due to the high levels of explant infection, various decontamination protocols were tested for the introduction of aseptic explants into culture. The effect of different PGRs on initiation and multiplication of *S. natalensis* plantlets were evaluated and microshoots from the most successful treatment were placed onto selected media for rooting and eventually transferred to potting soil for acclimitization. The morphology and trichome density of *in vitro* regenerated plantlets were compared to field-grown plants.

4.4.1. Decontamination and culture establishment

Explant material in culture showed a high level of microbial (both fungal and bacterial) growth which required stringent decontamination treatments (Table 3.1). Low explant survival was noted in treatments that involved low concentrations of NaClO, short immersion intervals and only one type of decontaminating agent. Explant decontamination was accomplished by immersion in 1% NaClO and 3% NaClO for seven minutes and five minutes respectively, followed by immersion in 0.1% HgCl₂ for ten minutes. This protocol resulted in $48 \pm 5\%$ survival of explants and was the only significantly different treatment with regard to explant survival when compared to other decontamination treatments ($F_{(4,15)}=18.353$; $p<0.05$; Figure 4.13). This treatment was employed for the initiation of new cultures as well as to revitalise established cultures showing early signs of contamination (\pm two weeks post-initiation). The percent bud break of initiated cultures in each of the three media preparations is shown in Table 4.4. All media preparations resulted in $> 70 \pm 6\%$ successful bud break within three weeks. Statistical analyses indicated no significant differences among the three media preparations.

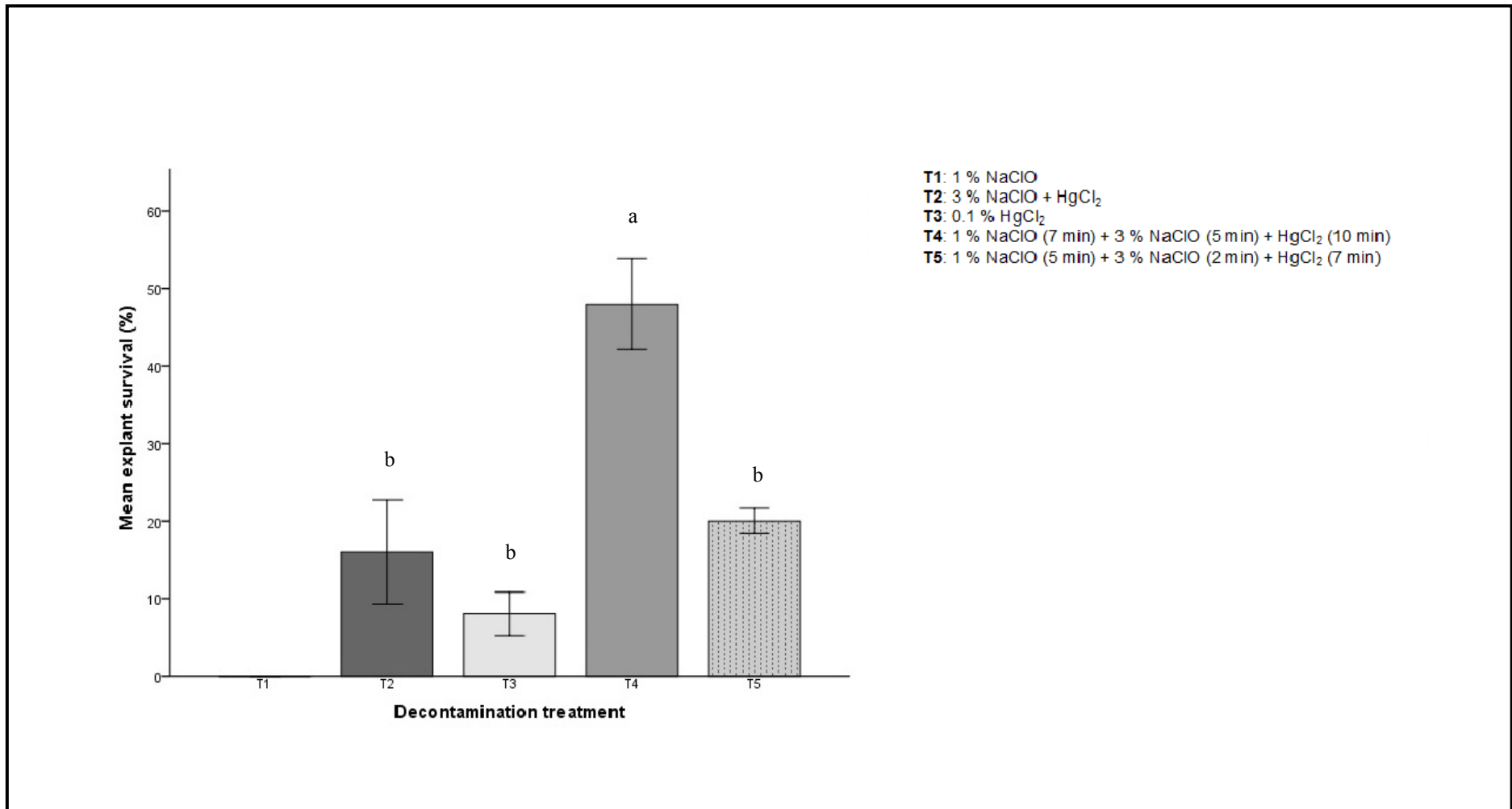


Figure 4.13 The effect of different decontamination treatments on *S. natalensis* explant survival. Bars = mean of four replicates \pm SE (n=25; $p < 0.05$). Mean values sharing the same letter do not differ significantly.

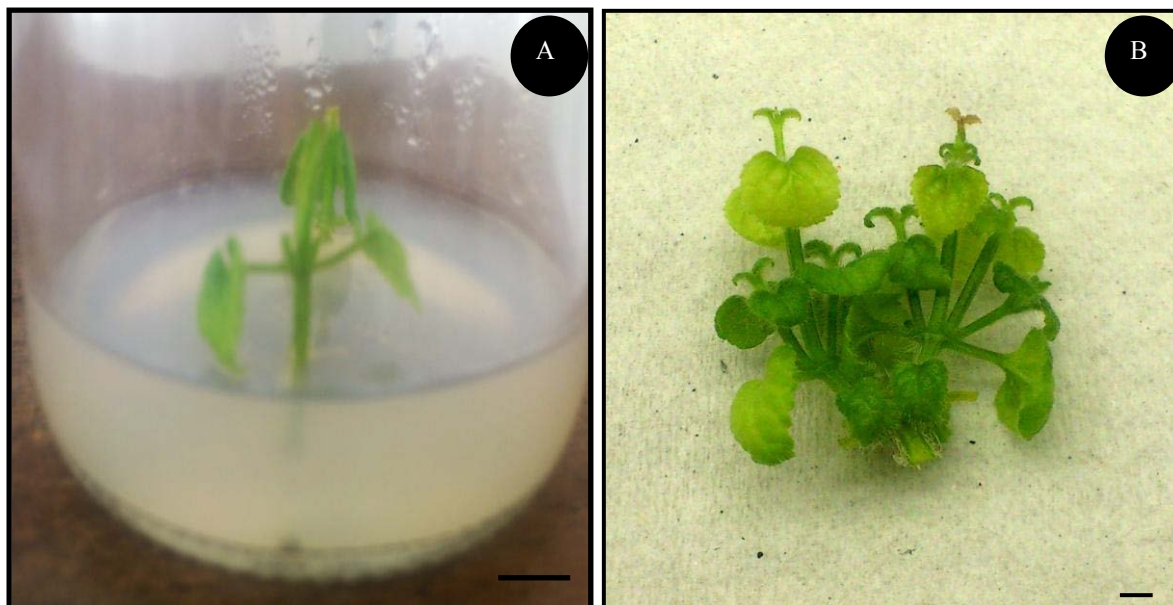


Figure 4.14 Initiation of *S. natalensis* in *in vitro* culture.

[A] Healthy axillary nodal explants cultured on initiation medium to induce bud break. (Bar: 10 mm)

[B] Explants with several shoots formed on initiation medium (BAP + IBA) before subculture onto multiplication media for shoot multiplication. (Bar: 5 mm)

Table 4.4 The effect of different plant growth regulators on successful initiation of axillary buds of *S. natalensis* explants after two weeks *in vitro*, n=25.

Media	PGR combination	% Bud break \pm SE
A	0.5 mg/l BAP + 0.5 mg/l IAA	78 \pm 2 ^a
B	0.5 mg/l Kinetin + 0.5 mg/l IAA	70 \pm 6 ^a
C	0.5 mg/l BAP + 0.5 mg/l IBA	80 \pm 8 ^a

Mean values sharing the same letter within a column do not differ significantly ($p < 0.05$) according to Tukey's post-hoc test.

4.4.2. Shoot multiplication

Growth medium B (kinetin and IAA) showed the greatest total shoot multiplication and elongation (following the bud break stage) compared to other PGR combinations after 12 weeks (Figure 4.15 and 4.16). Mean shoot production per explant and shoot height per explant was assessed using a one-way ANOVA (Table 4.5). There were significant differences in the mean number of shoots produced per explant between cultures in media A and B as well as between media B and C ($F_{(2, 27)} = 11.829$; $p < 0.05$). Plantlets in multiplication medium B showed the greatest shoot production at 9.1 ± 3.6 shoots/explant. Significant differences were noted in the height of shoots per explant between cultures in Media A and B as well as between Media B and C ($F_{(2, 27)} = 34.886$; $p < 0.05$). The greatest mean shoot height of 50.2 ± 5.0 mm was obtained in plantlets grown on medium B.

4.2.3. Rooting and Acclimatization

Plantlets showed significant shoot multiplication and elongation on medium B compared to other growth media and was transferred to medium containing only the PGR IAA (2 mg/l) in order to promote adventitious root formation. Rooting occurred in 64% of plantlets after five weeks. The average number of roots formed per shoot was 5.54 ± 0.43 and the mean root length per shoot was 66.13 ± 3.55 mm. Plantlets with a well-developed root system and root length > 30 mm were selected for acclimatization (Figure 4.17). The 24 *in vitro* propagated *S. natalensis* plantlets placed under greenhouse conditions showed $92 \pm 4.2\%$ survival after six weeks, indicating the efficiency of the micropropagation protocol to form robust, easily acclimatized plantlets with no apparent morphological abnormalities compared to field-grown plants (Figure 4.18).

Plate Nine

Figure 4.15 Shoot multiplication of *S. natalensis* axillary nodal explants in media containing different combinations of cytokinins and auxins after six weeks in culture.

[A] Medium A supplemented with 2 mg/l BAP and 1 mg/l IAA showing relatively slow shoot multiplication (Bar: 10 mm).

[B] Medium B supplemented with 2 mg/l kinetin and 1 mg/l IAA showing significant multiplication and elongation of healthy microshoots (Bar: 10 mm).

[C] Medium C supplemented with 2 mg/l BAP and 1 mg/l IBA showing significant microshoot multiplication in plantlets. Elongation of plantlets appears to be limited compared to medium B at the same stage. (Bar: 10 mm)



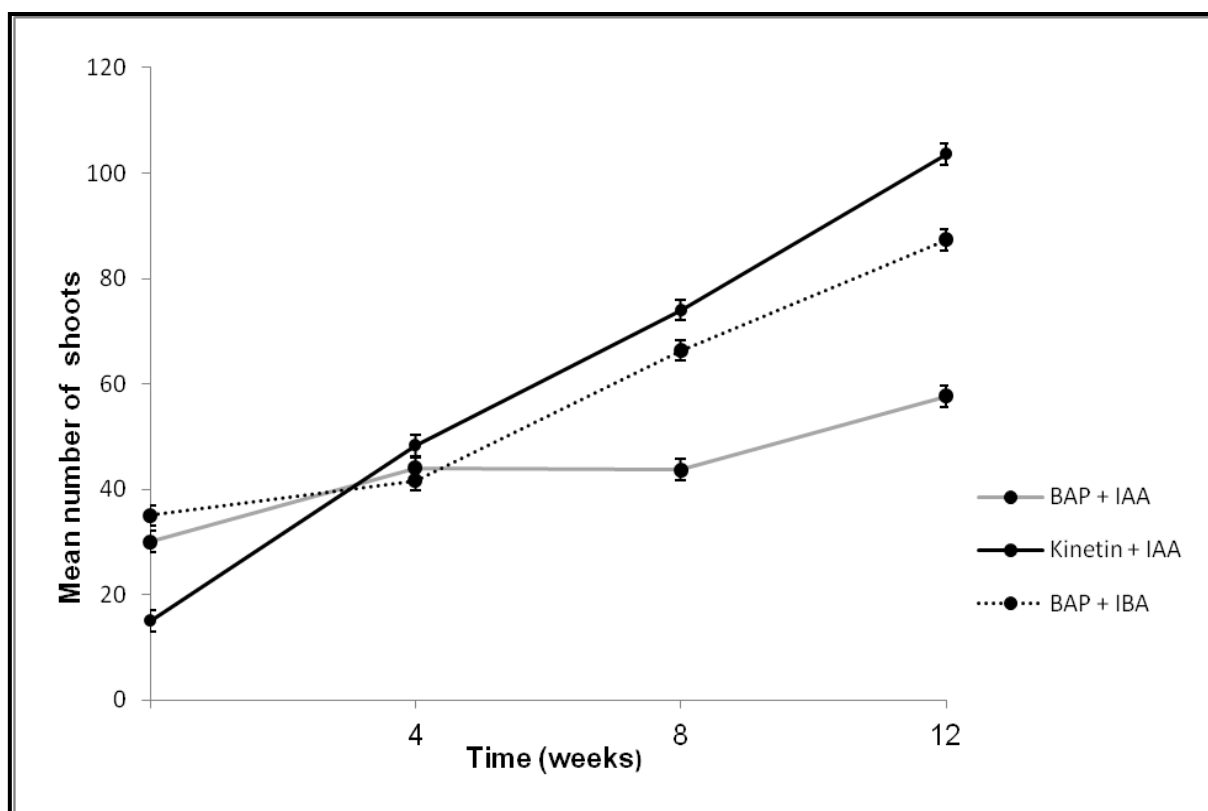


Figure 4.16 The effect of different PGR combinations used in the multiplication stage on the mean number of total shoots produced from all surviving explants over a period of 12 weeks. Medium A (2 mg/l BAP + 1 mg/l IAA), medium B (2 mg/l kinetin + 2 mg/l IAA) and medium C (2 mg/l BAP + 1 mg/l IBA). Values represent means of three replicates and bars = SE of the mean (n>15).

Table 4.5 The effects of different plant growth regulators (PGR) on the mean number (\pm SE) of shoots produced per explant and shoot height after six weeks, n=10.

Media	PGR combination	Mean number of shoots/explant	Mean shoot height/explant (mm)
A	2 mg/l BAP + 1 mg/l IAA	3.6 \pm 1.4 ^a	18.7 \pm 2.2 ^a
B	2 mg/l Kinetin + 1 mg/l IAA	9.1 \pm 3.6 ^b	50.2 \pm 5.0 ^b
C	2 mg/l BAP + 1 mg/l IBA	4.8 \pm 2.3 ^a	15.4 \pm 1.3 ^a

Mean values sharing the same letter within a column do not differ significantly ($p < 0.05$) according to Tukey's post-hoc test.

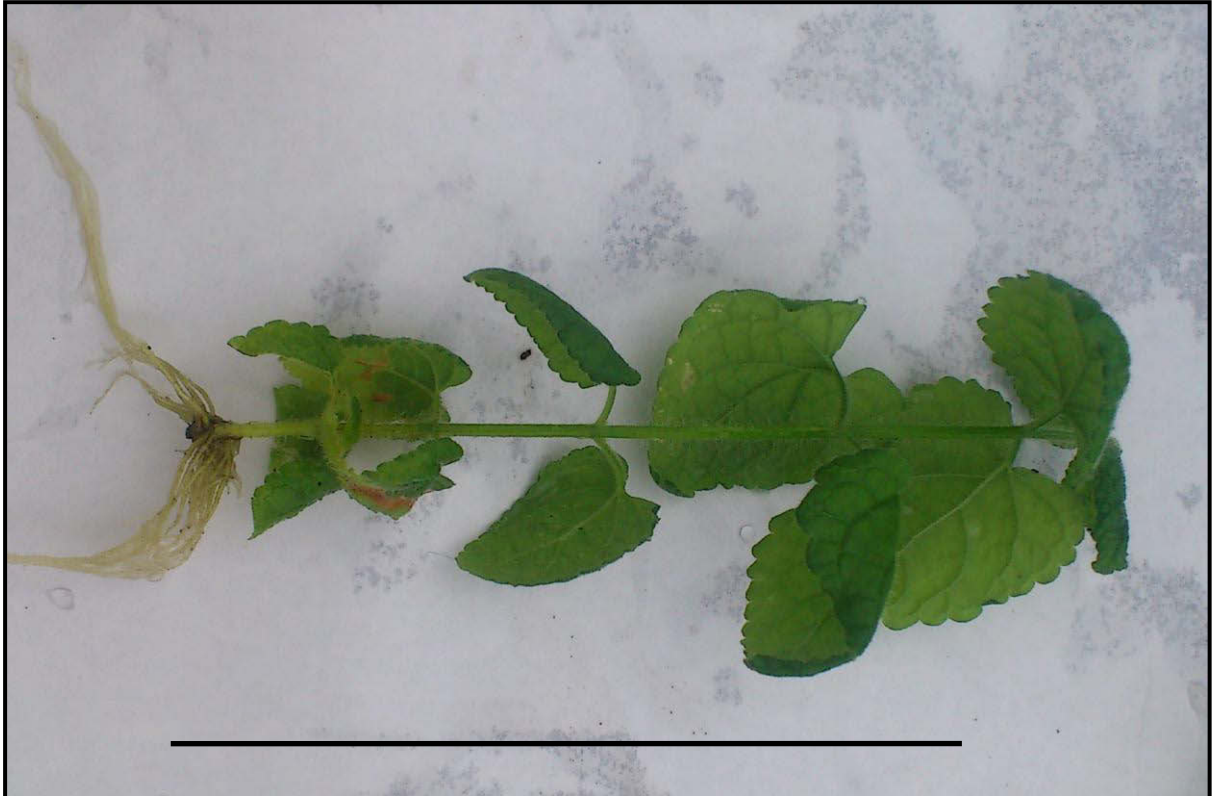


Figure 4.17 *In vitro* propagated plantlet after five weeks in culture before acclimatization. Bar: 80 mm.



Figure 4.18 Acclimatized *S. natalensis* plantlets in the mist tent after four weeks before transfer to the greenhouse.

4.5. MICROMORPHOLOGY OF SECRETORY STRUCTURES OF *IN VITRO* PROPAGATED *S. NATALENSIS* PLANTS

Acclimatized, healthy *in vitro* propagated plantlets showed no visible differences in morphology compared to field-grown plants. Scanning electron microscopy was used to investigate the micromorphology of foliar secretory structures present on the abaxial and adaxial surfaces of emergent, young and mature plantlets. Since the process of *in vitro* propagation through the exogenous supply of PGRs maintains plantlets in the juvenile stage for active growth and regeneration, investigations for mature leaves were carried out after four weeks post-acclimatization. Field-grown and *in vitro* propagated plants may be referred to as wildtype and acclimatized plants respectively.

4.5.1. Non-glandular trichomes

Glandular and non-glandular trichomes were found on both foliar surfaces of emergent, young and mature (i.e. following acclimatization) *in vitro* propagated plants. Non-glandular trichomes of acclimatized plants appeared uniseriate and unbranched. These trichomes consisted of an elongated, septate stalk attached to the leaf epidermis via a multicellular basal pedestal similar to their field grown counterparts. Micro-ornamentation of the trichome stalk was also observed on *in vitro* non-glandular trichomes. Interestingly, the length of the non-glandular trichomes of acclimatized plants appeared to be greater than those found on the surface of wildtype plants (Figure 4.19).

4.5.2. Glandular trichomes

Three types of glandular trichomes, peltate trichome and two types of capitate trichomes were observed on the foliar surfaces of acclimatized plants (Figure 4.20). Peltate trichomes consisted of a multicellular head attached to the epidermal layer via a single epidermal cell. Capitate trichome Type I (striated stalk with a uni-, bi or multicellular head) and Type II (septate stalk, distinct neck cell and multicellular head cell) were observed on both foliar surfaces of acclimatized plants as was the case with wildtype plants.

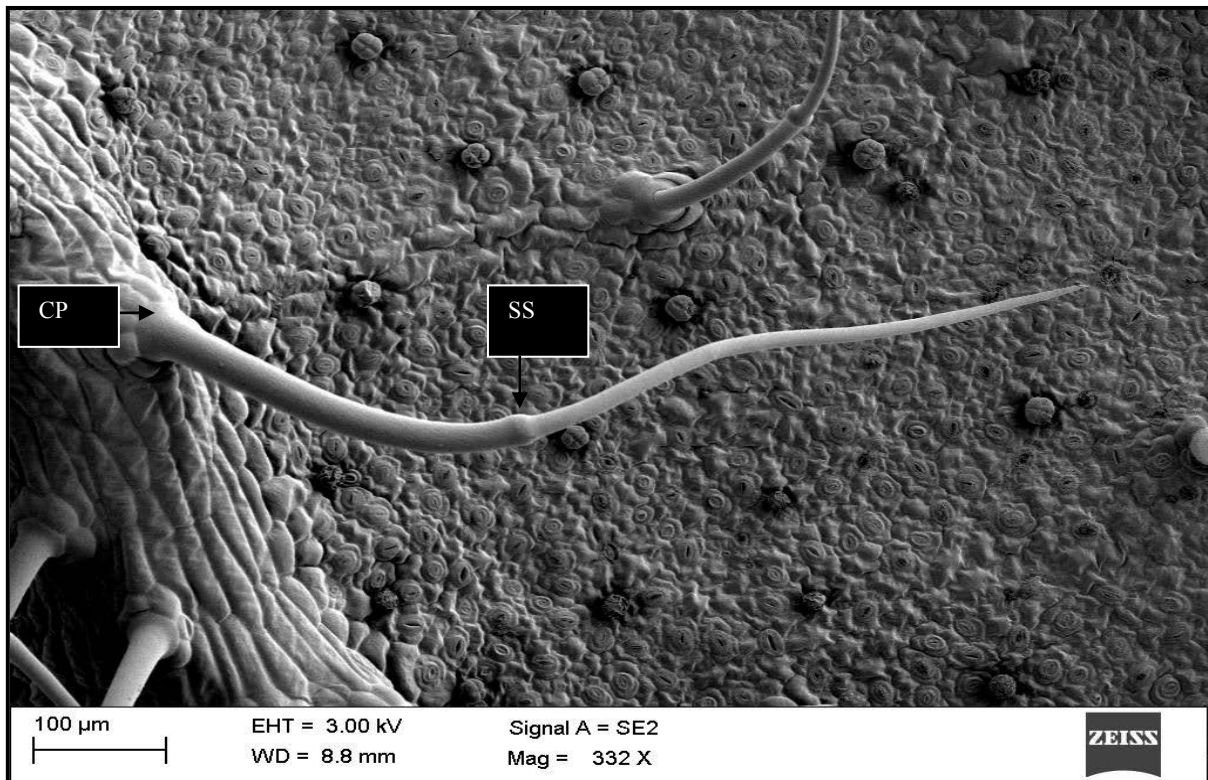


Figure 4.19 SEM micrograph of elongated non-glandular trichome on abaxial surface of freeze-dried emergent leaf of acclimatized *S. natalensis* plants. Note the multicellular epidermal cellular pedestal (CP) and septation of the stalk (SS).

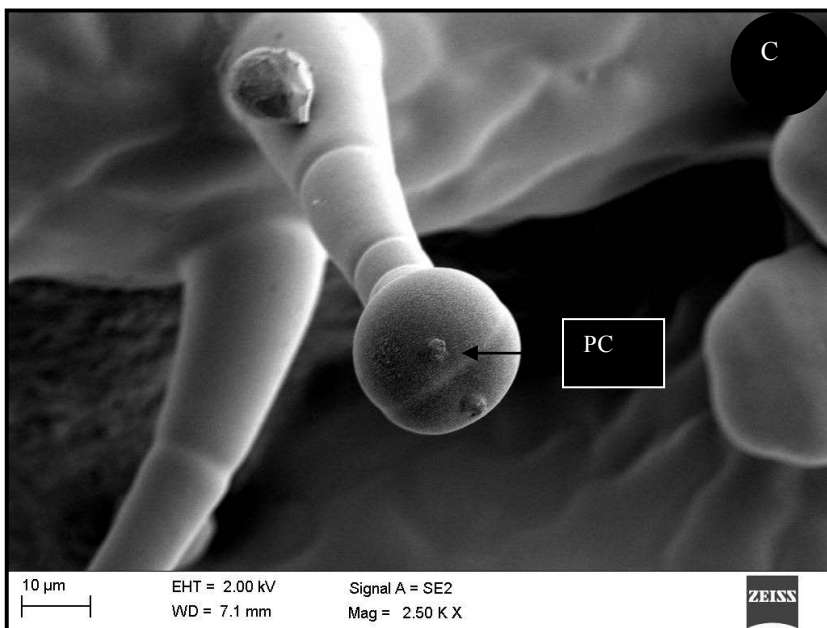
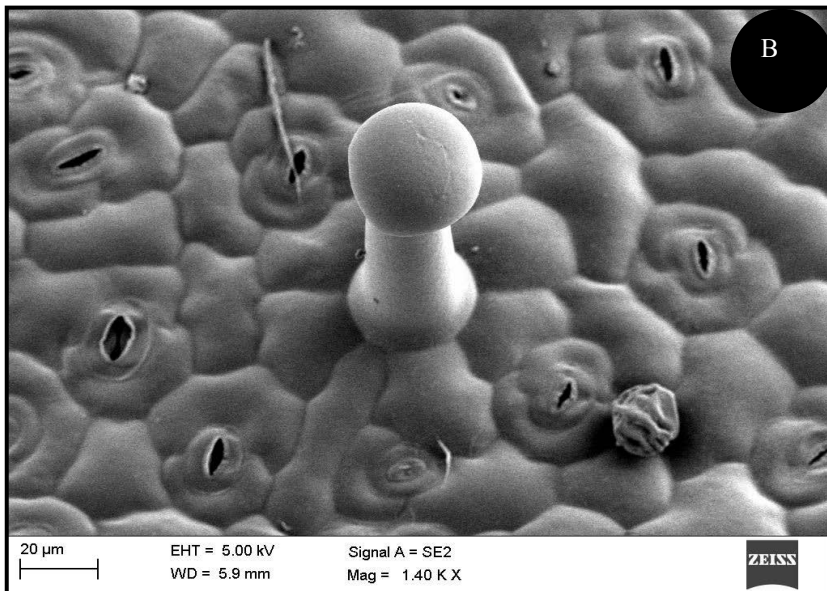
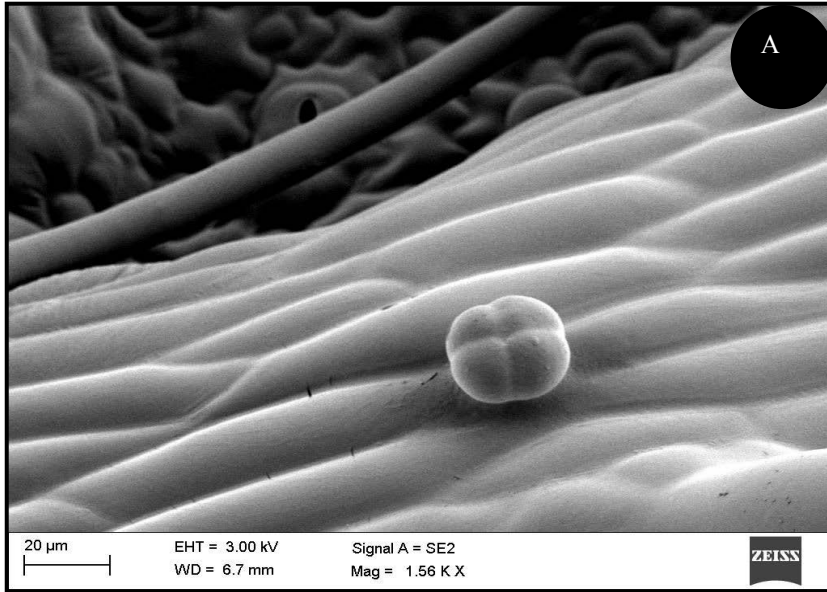
Plate Ten

Figure 4.20 SEM micrographs of glandular trichomes on the foliar surface of acclimatized *S. natalensis* plants.

[A] Peltate trichome on abaxial surface of cryo-prepared emergent leaf with multicellular (four celled) secretory head.

[B] Type I capitate trichome on the abaxial surface of a cryo-prepared young leaf.

[C] Type II capitate trichome on the adaxial surface of cryo-prepared emergent leaf with bi-cellular head. Note secretion of exudates via the porose cuticle (PC) covering the glandular head cells.



4.5.3. Trichome distribution and density

Emergent leaves of acclimatized plants appeared to have a dense covering of trichomes which visibly decreased as the leaf matured (Figure 4.21). Trichome density among three growth stages (emergent, young and mature) and two foliar surfaces (abaxial and adaxial) of acclimatized plantlets were investigated using a two-way ANOVA (Figure 4.22). All assumptions of the analyses were satisfied. There were significant differences in both glandular ($F_{(2,54)} = 91.718$; $p < 0.05$) and non-glandular trichome density ($F_{(2,0.037)} = 155.696$; $p < 0.05$) among the three developmental stages. Multiple comparisons of the three developmental stages using Tukey's post-hoc test indicated that these differences in trichome density existed between emergent and young leaves, emergent and mature leaves and young and mature leaves (Glandular and non-glandular: $p < 0.05$). The abaxial surface was found to contain a higher number of glandular trichomes whilst the adaxial surface had a slightly greater non-glandular trichome density (Table 4.6).

Trichome density between field-grown and acclimatized plants at different developmental stages (emergent, young, mature) was determined using a two-way ANOVA. A greater glandular trichome density was detected on acclimatized plants compared to field-grown plants ($F_{(1,54)} = 7.707$; $p < 0.05$; $\text{Mean}_{(\text{field-grown})} = 0.235$; $\text{Mean}_{(\text{in vitro})} = 0.387$). Non-glandular trichome density was higher in field-grown as compared to acclimatized plants ($F_{(1,54)} = 33.243$; $p < 0.05$; $\text{Mean}_{(\text{field-grown})} = 0.131$; $\text{Mean}_{(\text{in vitro})} = 0.030$). In both cases, trichome density was significantly higher on emergent leaves, decreasing as the leaf matured (Figure 4.23).

Table 4.6 Mean trichome density between the abaxial and adaxial leaf surfaces of acclimatized *S. natalensis* plants.

Trichome type	Mean trichome density (count/cm ²)	
	Abaxial	Adaxial
Non-glandular	0.073 ± 0.03	0.074 ± 0.005
Glandular	0.202 ± 0.01	0.122 ± 0.005

Plate Eleven

Figure 4.21 SEM micrographs showing trichome distribution on the adaxial surface of acclimatized *S. natalensis* leaves at three developmental stages.

[A] Emergent, [B] young and [C] mature leaves show the presence of both non-glandular and glandular trichomes. Trichomes appear to be more densely distributed in younger leaves, compared with mature leaves.

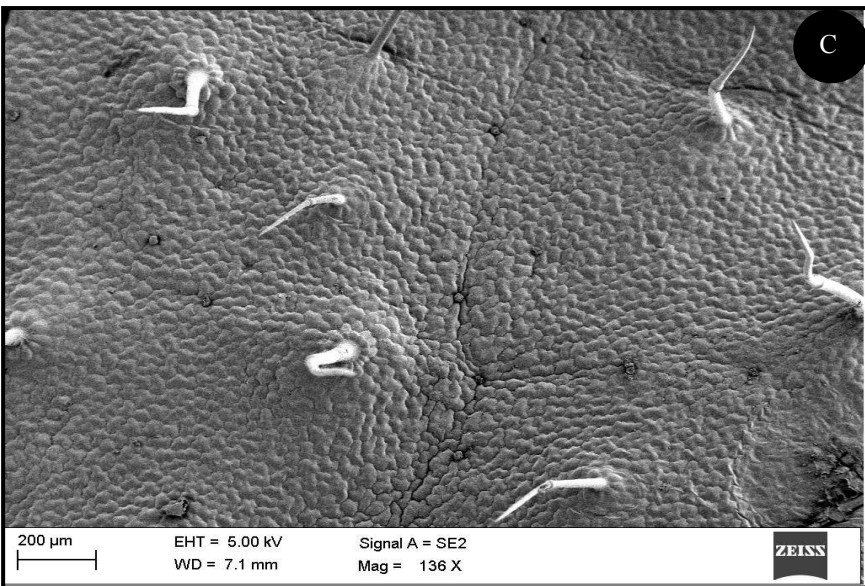
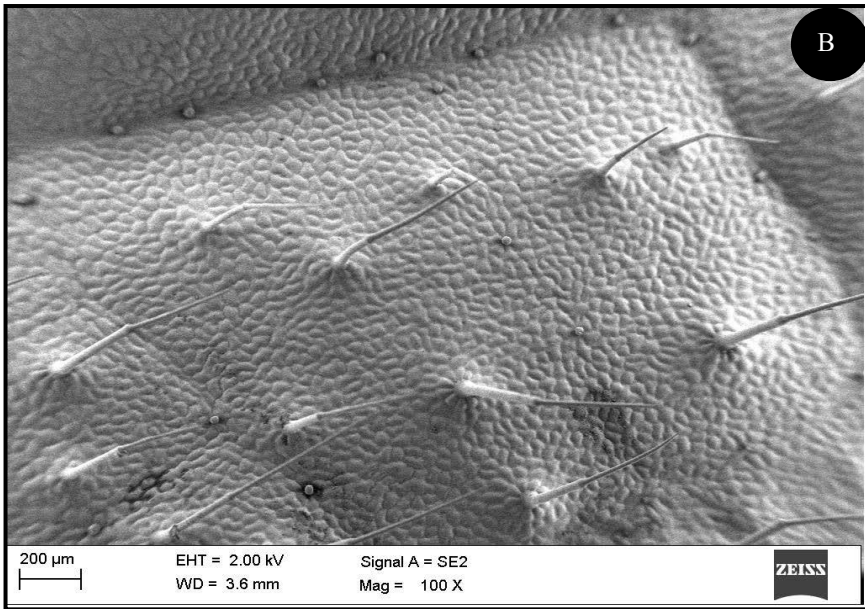
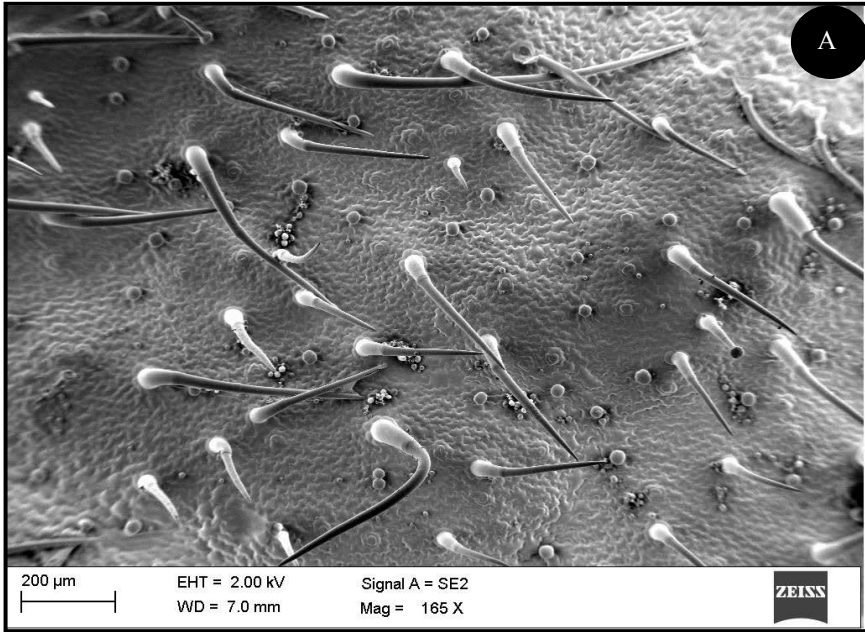


Plate Twelve

Figure 4.22 Mean non-glandular [A] and glandular [B] trichome density at three stages of leaf development (emergent, young and mature) and on the abaxial and adaxial foliar surfaces of acclimatized *S. natalensis* plants.

Values are mean \pm SE (n= 20; $p < 0.05$ was recognised as significantly different using Tukey's multiple comparison post-hoc test and denoted by different letters).

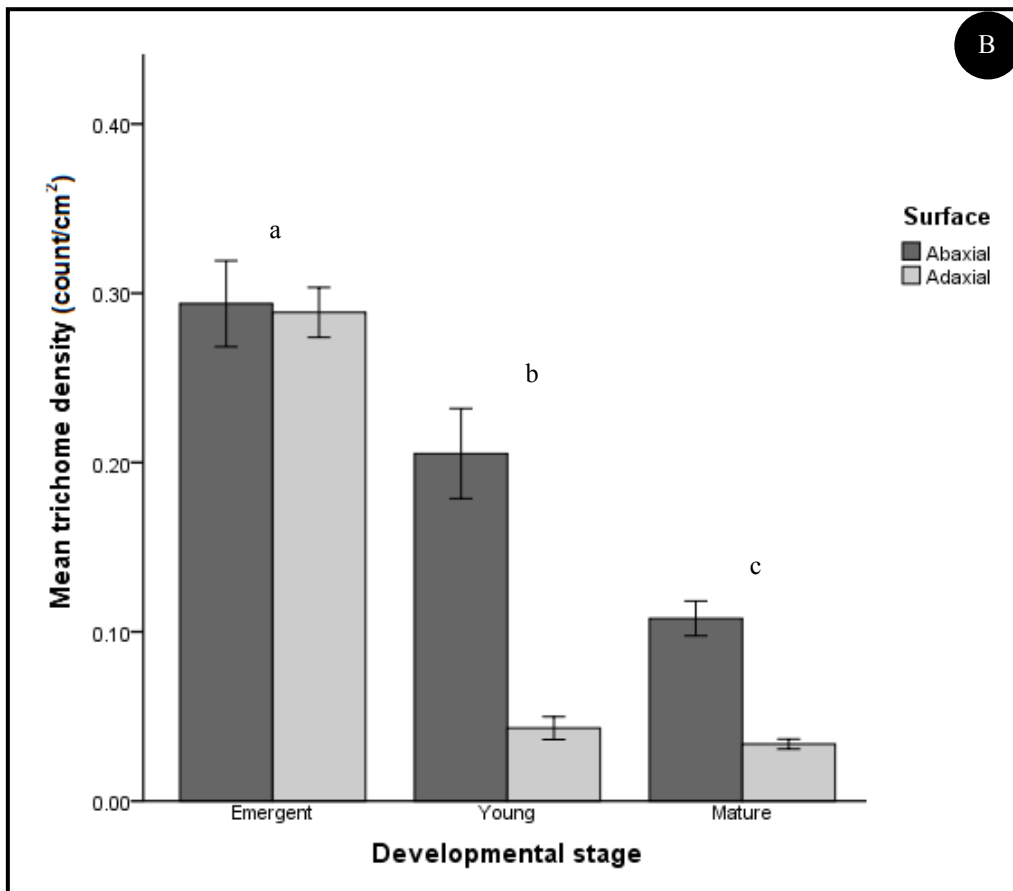
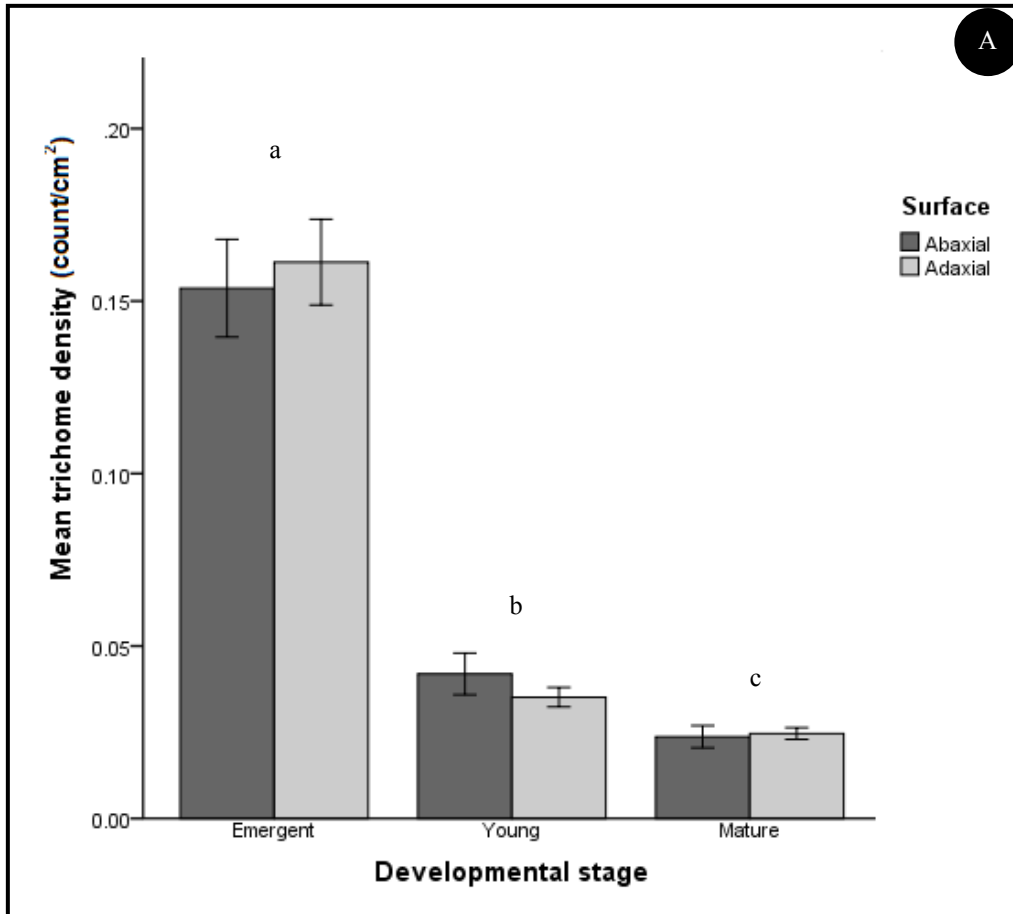


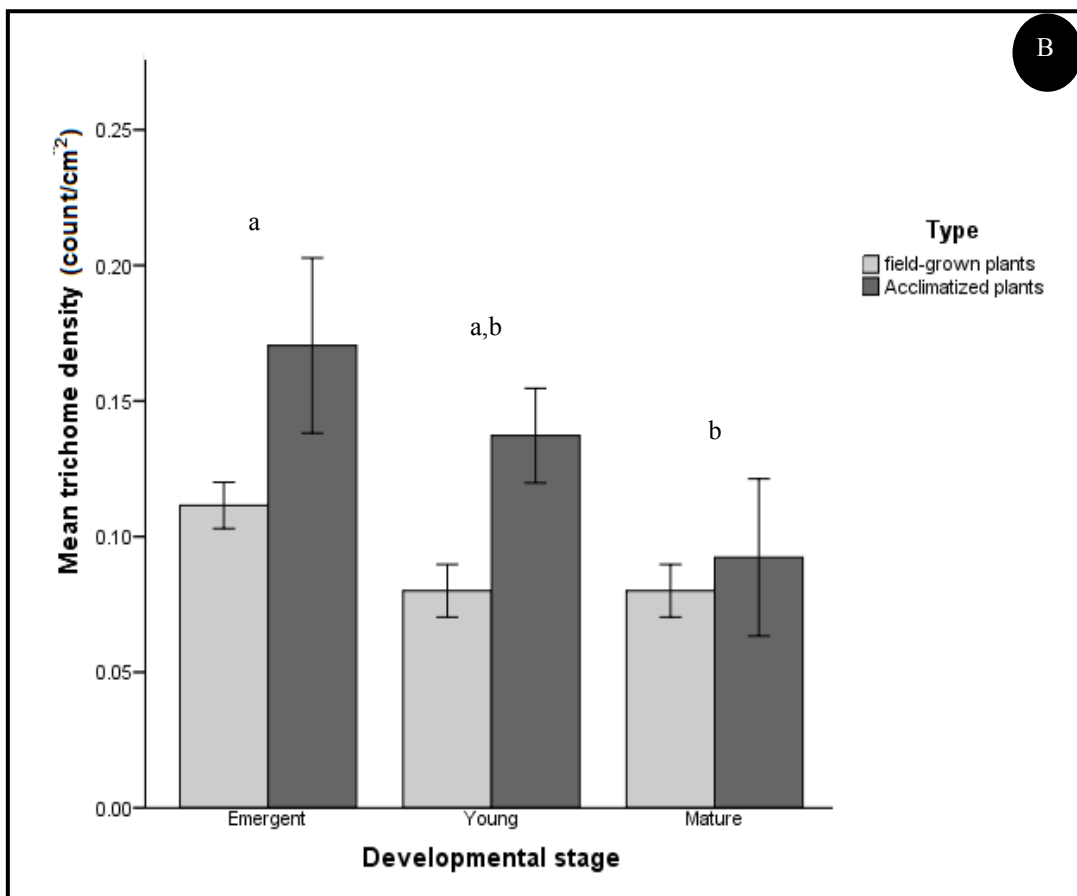
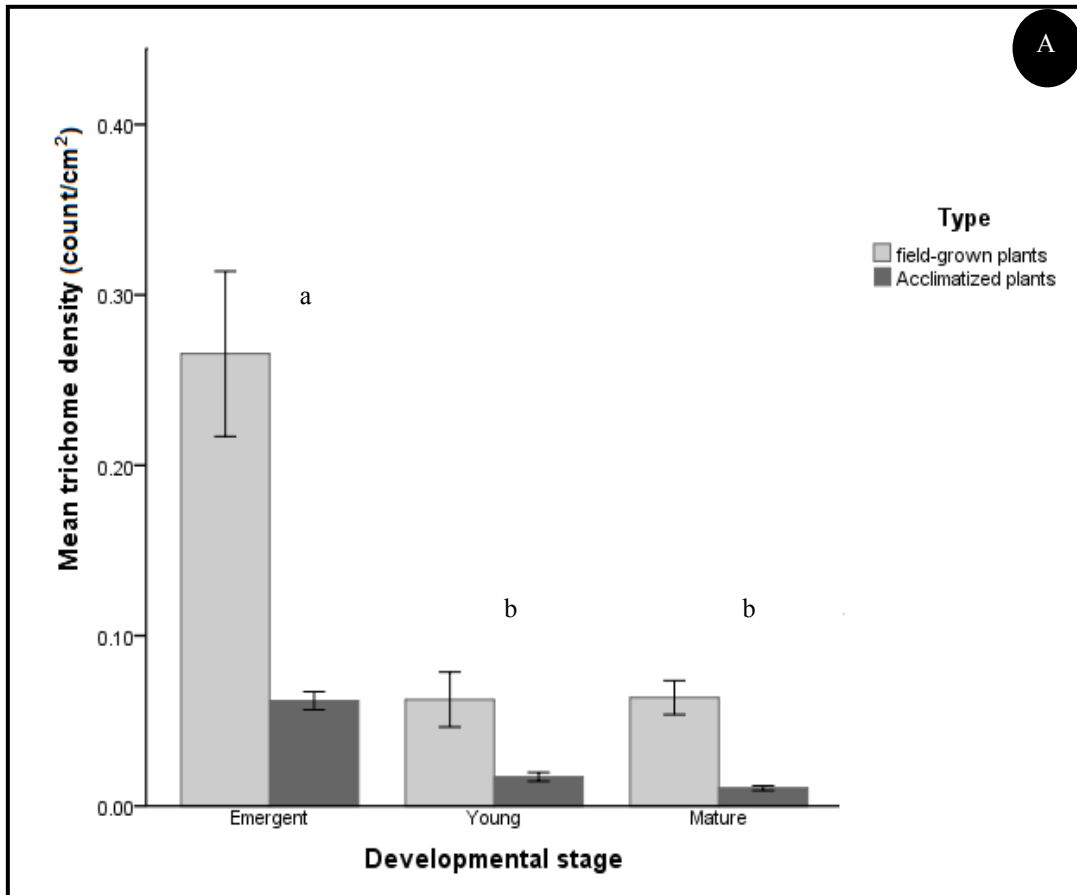
Plate Thirteen

Figure 4.23 Mean trichome density of field-grown and acclimatized *S. natalensis* plants.

[A] Mean non-glandular trichome density at three stages of leaf development (emergent, young and mature)

[B] Mean glandular trichome density at three stages of leaf development (emergent, young and mature)

Values are mean \pm SE (n= 20; $p < 0.05$ was recognised as significantly different using Tukey's multiple comparison post-hoc test and denoted by different letters).



CHAPTER 5: DISCUSSION

This study was undertaken to investigate the micromorphology of secretory structures found on the foliar surfaces of *Stachys natalensis* Hochst. and the histo- and phytochemical characteristics of the exudates. In addition, an efficient micropropagation protocol for the species was developed and the micromorphology of *in vitro* propagated plants was assessed.

5.1. TRICHOME DISTRIBUTION AND MICROMORPHOLOGY

The indumentum of *Stachys natalensis* Hochst contains a spread of both glandular (secretory) and non-glandular trichomes on both foliar surfaces, as seen in most species belonging to the Lamiaceae family (Werker *et al.*, 1993; Bhatt *et al.*, 2010a; Rusydi *et al.*, 2013). In the present study, glandular and non-glandular trichomes were more densely distributed on the abaxial surface compared to the adaxial surface with trichome density progressively decreasing with leaf maturation. Significantly higher glandular trichome densities on the abaxial surface have also been observed in *Trichosanthes cucumerina* (Adebooye *et al.*, 2012), *Artemisia annua* (Kjaer *et al.*, 2012) and *Mentha spicata* (Choi and Kim, 2013) whilst higher non-glandular densities were noticed in Lamiaceae species such as *Dracocephalum moldavicum* (Dmitruk and Weryszko-Chmielewska, 2010), *Plectranthus laxiflorus* (Bhatt *et al.*, 2010a) and *Isodon rubescens* (Liu and Liu, 2012), amongst others.

The phenomenon of leaf expansion has been used in the literature to account for the gradual decrease in trichome density from young leaves to older specimens and is often reported in species of the Lamiaceae (Ascensao *et al.*, 1995; Valkama *et al.*, 2004). It refers to the increase in the leaf surface area as it matures whilst trichome density remains more or less constant. This results in a greater dispersal of trichomes across the leaf surface. Valkama *et al.* (2004) reported that foliar trichomes of some plants are present very early during leaf development, with trichome density remaining constant throughout leaf growth. In other plants, new trichomes are produced throughout leaf development (Maffei *et al.*, 1989). Werker *et al.* (1993) hypothesized that trichome production would continue for however long there are meristematic regions present. The question of trichome functionality at different developmental stages is addressed by many contrasting studies. Werker (2000) showed that trichomes are still functional in mature leaves whilst Gairola *et al.* (2009) reported that trichomes often senesce and are lost in later stages of leaf development as they have little or no functional role. However, the functional role of trichomes may be species-specific or depend on the location of trichomes on different plant organs (Werker, 2000). The non-glandular trichomes of *S. natalensis* were erect or bent toward the leaf apex with a high trichome density occurring on the mid-rib and margin. The greater trichome distribution on younger leaves of *S. natalensis* suggested that trichomes

serve a protective function over their glandular counterparts during early development which may or may not be required at later stages. Many studies show significant increases in trichome density upon exposure to adverse conditions such as mechanical damage or herbivory (Molina-Montenegro *et al.*, 2006; Jaime *et al.*, 2012; Loughner *et al.*, 2012), water loss (Abdulrahman and Oladele, 2011) and environmental stresses (Bhatt *et al.*, 2010b; Adebooye *et al.*, 2012).

The non-glandular trichomes of *S. natalensis* were uniseriate and unbranched, appearing acuminate (long, slender and tapering toward the apices) to arthrochylous (jointed) in form. These were typical of the non-glandular trichomes of other *Stachys* spp. found worldwide including: *Stachys cretica* L. subsp. *vacillans*, *S. ehrenbergii* and *S. distans* from Lebanon (El-Beyrouthy *et al.*, 2009), *S. ballotiformis* and *S. megalodonta* from Iran (Salmaki *et al.*, 2009) and *S. recta* from Italy (Giuliani *et al.*, 2008). Non-glandular trichomes of *S. natalensis* contained two distinctive morphological attributes. The first was a multicellular basal pedestal which is composed of approximately two to ten epidermal cells depending on the stage of differentiation. These raised cells serve to support the trichome (Ascensao *et al.*, 1999) and to provide a point of attachment that “anchors” the trichome to the epidermal surface (Bhatt *et al.*, 2010a). It is a characteristic trichome feature in many plants such as *Ceratotheca triloba* (Naidoo *et al.*, 2012a), *Plectranthus tomentosa* (Kim, 2013) and *Stachys alopecuros* (Venditti *et al.*, 2013a). Trichome development generally proceeds from a single, enlarged and protruding protodermal cell to form uniseriate multicellular trichomes like those of *S. natalensis*, after a series of periclinal divisions (Werker, 2000). The latter are a result of cell divisions that occur parallel to the length of the pre-existing cell (Werker, 2000).

The second characteristic refers to unique striations occurring on the trichome surface of *S. natalensis* from the basal pedestal up to and including the stalk. These are referred to as “micro-ornamentation” and may appear as warts, papillae or striations arising from the cell wall or cuticle on the trichome surface (Werker, 2000; Gairola *et al.*, 2009; Rusydi *et al.*, 2013). Interestingly, studies conducted on trichome morphology of *Stachys* spp. by Salmaki *et al.* (2009) and El-Beyrouthy *et al.* (2009) indicate that in most cases papillate micro-ornamentation is a characteristic feature. The striated trichomes of *S. natalensis* may serve as a defining characteristic but further research is required to evaluate its usefulness as a taxonomic feature. The function of such ornamentation remains unclear. Werker (2000) suggested that micro-ornamentation serves as an indicator of trichome maturation. Striated micro-ornamentation has been identified in trichomes on the young and mature *S. natalensis* leaf surface which suggests that trichome production is maintained throughout leaf development but due to leaf expansion trichome density decreases overall (Werker, 2000; Liu and Liu, 2012). Literature also reports on the involvement of cuticular micro-ornamentation in a self-cleaning mechanism known as

the “Lotus Effect” which is attributed to the hydrophobic nature and structure of trichome surfaces (Barthlott and Neinhuis, 1997). Trichome micro-ornamentation may aid in the removal of grime, water or pathogenic microorganisms and compensate for the accumulation of particles due to the rough leaf surfaces (Barthlott and Neinhuis, 1997). Naidoo *et al.* (2014) hypothesized that cuticular micro-ornamentation may also facilitate the flow of exudates from secretory glands.

The structural and functional aspects of glandular trichomes within the Lamiaceae is well documented (Werker *et al.*, 1993; Serato-Valenti *et al.*, 1997; Ascensao *et al.*, 1998; Ascensao *et al.*, 1999; Giuliani and Maleci-Bini, 2008; Marin *et al.*, 2012; Rusydi *et al.*, 2013). The glandular hairs observed on the foliar surfaces of *S. natalensis* are of two distinct types: peltate and capitate. Many *Stachys* spp., much like other species within the family, do not display any uniformity with regard to the type of glandular trichomes they possess. Foliar surfaces may be covered by both peltate and capitate trichomes, only peltate, only capitate or in rare cases neither type (Huang *et al.*, 2008). In fact, *S. plumosa*, *S. sylvatica*, *S. byzantina* and *S. recta* subsp. *recta* have capitate trichomes only (Maleci-Bini and Giuliani, 2006; El-Beyrouthy *et al.*, 2009) whilst *Stachys* subgenus. *Betonica* possesses only peltate glands (Giuliani and Maleci-Bini, 2012).

The peltate trichomes of *S. natalensis* were uniseriate and comprised of a basal cell, a very short unicellular stalk and a multicellular secretory head (approximately 50 µm in diameter) that contains two to eight secretory cells. Morphologically similar peltate trichomes were noted in other *Stachys* spp. including; *S. cretica*, , *S. neurocalycina*, *S. nivea* and *S. alopecuros* (El-Beyrouthy *et al.*, 2009; Venditti *et al.*, 2013a). Other species differed in terms of the number of secretory head cells (4-16 cells in *S. officinalis*) and the development of a fairly elongated (40 - 60 µm length) stalk cell, as seen in *S. germanica* and related species *Prasium majus*, *Sideritis romana* and *Scutellaria galericulata* (Giuliani and Maleci-Bini, 2008).

The Type I capitate trichomes of *S. natalensis*, widespread within the family Lamiaceae and genus *Stachys*, are composed of a uniseriate stalk, neck cell and a bulbous secretory head (Werker, 2000). They occur on both foliar surfaces across all developmental stages of *S. natalensis* and other species including: *Stachys palaestina* (El-Beyrouthy *et al.*, 2009), *Salvia smyrnea* (Baran *et al.*, 2010) and *Ballota undulata* (Osman, 2012). The Type II capitate trichome with an elongated striated stalk and distinct neck cell bearing a variable number of secretory cells identified in this study shares similarities with the capitate trichomes described in many species of *Stachys*, *Sideritis*, *Prasium* and *Scutellaria* (Giuliani and Maleci-Bini, 2008). This Type II capitate trichome was generally found only on the plant reproductive organs of the Lamiaceae (Giuliani and Maleci-Bini, 2008). However,

Giuliani *et al.* (2011) later identified the exception in the multicellular capitate trichomes of *Sideritis italica* which occurred on the leaves. In the present study, *S. natalensis* was found to also possess this capitate trichome on the foliar surfaces. Further investigations of *Stachys*, *Sideritis* and other related species are required to re-assess the occurrence and distribution of this capitate trichome as a potential taxonomic marker.

Both peltate and capitate glandular trichomes have been shown to accumulate secretory material in a subcuticular space of the glandular secretory head in the family Lamiaceae (Ascensão *et al.*, 1995, Turner *et al.*, 2000; Ventrella and Marinho, 2008; Naidoo *et al.*, 2013). The subcuticular space is formed by the detachment of the cuticle from the cell wall (Ascensao *et al.*, 1999; Tissier, 2012). The exact mechanism of secretion accumulation within the subcuticular space is currently unknown and requires ultrastructural investigations of the glandular trichomes of *S. natalensis*, which has not been included in the scope of this study. The release of secretion in glandular trichomes can occur via cuticular rupture at fragile regions of the secretory head (Jia *et al.*, 2012; Naidoo *et al.*, 2013) through a singular apical pore or multiple micropores (Ascensão *et al.*, 1999; Naidoo *et al.*, 2012b) or even by means of diffusion through the cuticle (Caissard *et al.*, 2012). Release of secretion in *S. natalensis* seems to occur via different mechanisms depending on the type of glandular trichome (Figure 4.11). The peltate and Type II capitate trichomes appear to have a porose cuticle which facilitates release of droplets of secretions to the exterior. This has also been observed in *S. galericulata*, *S. germanica* (Giuliani and Maleci-Bini, 2008) and *S. smyrnea* (Baran *et al.*, 2010). In the present study, cuticle elevation resulting from the build-up of secretion in the sub-cuticular space, followed by cuticular rupture at weak points at the equatorial plane of the secretory head cell was observed in Type I capitate trichomes. Cuticle rupture may also be due to external factors such as high temperature, low humidity or mechanical damage (Ascensao *et al.*, 1995; Ventrella and Marinho, 2008; Baran *et al.*, 2010). A similar phenomenon of cuticular rupture was noted in *Plectranthus grandidentatus* (Mota *et al.*, 2013) and *Teucrium salviastrum* (Cavaleiro *et al.*, 2002).

5.2. HISTOCHEMICAL AND PHYTOCHEMICAL PROFILE OF *S. NATALENSIS*

Histochemical tests are useful for the *in situ* localization of the major chemical classes of metabolites present in plant secretions. The histochemical results in this study indicate that the secretion in both the peltate and Type II capitate trichomes of *S. natalensis* include an oleoresin containing terpenoid and lipid inclusions positively stained by the nadi reagent and sudan III. Carboxylated and acidic polysaccharides and small amounts of alkaloids, phenolics and proteins were detected by Wagner and

Dittmar reagent, ferric trichloride and mercuric bromophenol blue, respectively (Figure 4.12). The Type I capitate trichome contained smaller amounts of terpenoids and a mixture of polysaccharides and phenolic compounds. With regard to secretion composition, literature indicates that peltate trichomes typically produce and store the bulk of terpenoids in the form of essential oils (Ascensao *et al.*, 1997; Tissier, 2012) whilst capitate trichomes often contain a complex mixture of polysaccharide, lipid and protein substances (Ascensao and Pais, 1998; Huang *et al.*, 2008). Some exceptions have been identified within the genus *Stachys* and in other plant families which is in agreement with the results obtained in this study, e.g. polyphenols and polysaccharides are present in the peltate trichomes of *S. officinalis* and *S. alopecuroides* (Giuliani and Maleci-Bini, 2008), *Micromeria thymifolia* (Marin *et al.*, 2013) and *Thymus quinquecostatus* (Jia *et al.*, 2012).

The presence of lignin, cutin and pectin was detected in the thickened cell walls of non-glandular trichomes using toluidine blue, sudan III and ruthenium red, respectively. They were also identified in the trichome head, neck cell and stalk of *S. natalensis* capitate trichomes. Neck cells with cutinized walls are also present in members of the Boraginaceae (*Cordia verbenacea*, Ventrella and Marinho, 2008), Pedaliaceae (*C. triloba*, Naidoo *et al.*, 2012a) and other Lamiaceae (*S. italica*, Giuliani *et al.*, 2011). The thickened neck cells of capitate trichomes are hypothesized to prevent apoplastic backflow of secretions, the potentially phytotoxic composition of which may pose a threat to the rest of the plant if released (Serrato-Valenti *et al.*, 1997; Werker, 2000; Baran *et al.*, 2010). Cutin, a versatile cuticular component, is involved in numerous cellular functions required for plant growth and development, whilst pectins are a substantial source of oligosaccharides that are involved in chemical defensive response mechanisms to insect herbivory or pathogenic attack on the plant (Curvers *et al.*, 2010). Non-cellulosic polysaccharides such as lignin provide a framework of strength to trichomes to tolerate high levels of mechanical stress (Bischoff *et al.*, 2010).

The crude extracts and essential oils of *Stachys* species contain many biologically active compounds that exert various pharmacological effects and provide a basis for the use of *Stachys* spp. in ethnomedicine (Kukic *et al.*, 2006a). Preliminary qualitative phytochemical tests on crude extracts of *S. natalensis* indicated the presence of complex carbohydrates, alkaloids, saponins, steroids, terpenoids, phenolics and flavonoids as the main constituents (Table 4.2). In a study by Maa *et al.* (2012) complex carbohydrates were extracted from *Stachys floridana* and tested for its hepatoprotective activity. Results demonstrated that extracted material significantly protected against acute hepatotoxicity induced in mice by carbon tetrachloride (CCl₄).

The presence of alkaloids within the genus *Stachys* is variable. In most species such as *Stachys aethiopica*, *S. inflata*, and *S. byzantina*, alkaloids are either absent, or present in low quantities that are undetected using phytochemical tests (Khanavi *et al.*, 2009). In the case of *S. natalensis*, the converse is true as shown by the strong positive reaction for alkaloids with Wagner and Dittmar reagent. In other *Stachys* spp. e.g. *S. palustris* and *S. officinalis*, the alkaloid-like compounds, stachydrine and betonicine have been detected (Kartsev *et al.*, 1994). Saponins, part of a diverse range of plant glycosides, have been investigated for their significant anti-cancer and anti-cholesterol activity (Guclu-Ustundag and Mazza, 2007). Rezazadeh *et al.* (2009) identified saponins, flavonoids and terpenoids in the aerial parts of *Stachys athorecalyx* which may be responsible for the alleviation of acute and chronic induced inflammation in Wistar rat models. It has also been suggested that the anxiolytic effect of extracts of *S. lavandulifolia* may be attributed to the phenylpropanoid or terpenoid content in the aerial parts of the plant (Monji *et al.*, 2011). Terpenoids are the largest class of secondary metabolites and are implicated in plant chemical defence mechanisms (Wittstock and Gershenzon, 2002). They have received scientific interest due to their involvement in a variety of biological activities including: anti-cancer, anti-microbial, anti-spasmodic, anti-inflammatory and anti-hyperglycemic activities (Thoppil and Bishayee, 2011; Lange and Akhemi, 2013). *Stachys parviflora*, *S. alpina* subsp. *dinarica*, *S. balansae* and *S. germanica* subsp. *salviifolia*, amongst other species recognised for their medicinal value, all contain terpene hydrocarbons as the dominant constituent in the plant crude extract and essential oil (Khanavi *et al.*, 2005; Kukic *et al.*, 2006a; Venditti *et al.*, 2013b). The presence of these secondary metabolites in the crude extract of *S. natalensis* has been established by preliminary phytochemical methods. Further research is justified to determine the quantitative total of these compounds in both crude extract and essential oil in comparison to other related species.

Phenolic compounds and flavonoids were detected within the leaf extract of *S. natalensis* by both preliminary qualitative and quantitative tests. Phenolic compounds include simple phenols, phenolic acids, coumarins, flavonoids and condensed tannins, amongst others, which are involved in pollination mechanisms, plant pigmentation and plant defence strategies (Blainski *et al.*, 2013). Phenolic compounds present in glandular secretory structures may be implicated in plant organ protection against UV-B radiation resulting from sunlight exposure (Combrinck *et al.*, 2007). Flavonoids are involved in inflammatory processes and possess antibacterial and antifungal properties (de Sousa-Araujo *et al.*, 2008). The total phenolic content (3.43 ± 0.01 mg/g dry weight) in *S. natalensis* was substantially lower in comparison to species such as *S. officinalis* (61.2 ± 5.6 mg/g dry weight, Sliumpaite *et al.*, 2013) and *S. inflata* (92.2 ± 0.04 mg/g dry weight, Sohi *et al.*, 2011). Conversely, studies on *S. recta*, *S. salviifolia* and *S. pilifera* detected low phenolic content of < 3 mg/g

dry weight (Bilusic-Vundac *et al.*, 2007; Moein *et al.*, 2012). The Folin-Ciocalteu assay is a routinely employed method to estimate total phenolic compounds in samples. However, it is non-specific to phenolics and detects other readily oxidised samples which may account for elevated or reduced levels of phenolic compounds (Ainsworth and Gillespie, 2007). Greater total flavonoid content (3.04 ± 0.01 mg/g dry weight) was obtained in *S. natalensis* leaf extract compared to *S. lavandulifolia* (4.02 ± 0.02 mg/g dry weight, Bouayed *et al.*, 2007) and other medicinally important herbs *Melissa officinalis* (0.45 mg/g dry weight), *Salvia officinalis* (0.26 mg/g dry weight) and *Mentha piperita* (0.25 mg/g dry weight) (Atanassova *et al.*, 2011).

Many studies have shown that phenolics, especially flavonoids, are potent antioxidants by way of their capacity for free radical scavenging, inhibition of peroxidation and chelation of transition metals (Bilusic-Vundac *et al.*, 2007; Tibiri *et al.*, 2010; Amari *et al.*, 2014). Cai *et al.* (2006) verified that increased phenolic content (phenolic acids, flavonoids, coumarins, quinines and curcuminoids) corresponded to an increased antioxidant activity in 112 selected Chinese medicinal plants known for their anti-cancer potential. In the present study, *S. natalensis* methanolic extract showed a propensity to quench DPPH radicals in a dose-dependent manner as demonstrated by the increase in radical scavenging activity (Figure 4.13). The IC_{50} value obtained for the free radical scavenging activity of *S. natalensis* methanolic extract ($49.49 \pm 3.87 \mu\text{g ml}^{-1}$) was approximately four-fold higher than the standard ascorbic acid antioxidant ($12.30 \pm 2.15 \mu\text{g ml}^{-1}$), indicating the considerable antioxidant activity of the *S. natalensis* extract. In comparison to *S. natalensis*, variable results were obtained for other *Stachys* spp. *Stachys palustris*, *S. recta* and *S. alpina* possessed substantial antioxidant activity (Bilusic-Vundac *et al.*, 2007) whilst *S. byzantina* and *S. plumosa* demonstrated a larger IC_{50} value ($640 \mu\text{g ml}^{-1}$ and $101.61 \mu\text{g ml}^{-1}$ resp.) and by extension reduced antioxidant potential (Erdemoglu *et al.*, 2006; Kukic *et al.* 2006b). In a study on the antioxidant activity of selected *Stachys* taxa, Bilusic-Vundac *et al.* (2007) determined that the ethanolic extract of *S. salviifolia* ($IC_{50} = 9.87 \mu\text{g ml}^{-1}$) was slightly more effective than the methanolic extract ($IC_{50} = 10.7 \mu\text{g ml}^{-1}$) in terms of antioxidant potential. Therefore, it is of future interest to investigate the effect of different solvent extracts on the antioxidant activity of *S. natalensis*. A major drawback exists in the comparison of the antioxidant activity of related plant species. There are many types of antioxidant assays (DPPH, FRAP and ABTS) employed using different solvent extracts (methanol, ethanol, dichloromethane) or essential oils, diverse standard antioxidants (quercetin, ascorbic acid, butylated hydroxyanisole) or varying units of measurement (Háznagy-Radnai *et al.*, 2006; Wojdylo *et al.*, 2007; Conforti *et al.*, 2009; Khanavi *et al.*, 2009; Sliumpaite *et al.*, 2013).

The histo- and phytochemical profile of different taxa within the genus *Stachys* has intensified over recent years, indicating the multitude of pharmacological and biological effects exerted by the plant extracts and the glandular trichome-derived essential oils (Kukic *et al.*, 2006b; Giuliani and Malecibini, 2012; Venditti *et al.*, 2013b). The preliminary phytochemical profile of *S. natalensis* shares similar morphological and phytochemical characteristics with other medicinally important species within the genus which suggests that *S. natalensis* leaves may be good source of phytochemicals. However, *S. natalensis* is difficult to procure due to its poor seasonal growth. Thus, the introduction of this species into aseptic culture and development of an efficient protocol for its propagation *en masse* is undeniably important in order to provide a continuous supply of plant material.

5.3. MICROPROPAGATION OF *STACHYS NATALENSIS*. HOCHST.

Currently, there appears to be limited published literature concerning the *in vitro* propagation of *Stachys* spp. and no protocol for *S. natalensis*, to our knowledge. Thus, a micropropagation protocol was developed for the species using axillary nodal explants. Various decontamination procedures and different combinations of PGRs were tested for their usefulness in initiating shoot and root production in aseptic plant cultures. Acclimatized *S. natalensis* plantlets were evaluated for their micromorphological fidelity compared to field-grown *S. natalensis* plants at three developmental stages (emergent, young and mature).

Tissue culture initiation and production of aseptic *S. natalensis* nodal explants was severely hampered by the onset of persistently high levels of fungal and bacterial infection. Explant source is an important consideration for *in vitro* aseptic culture and growth. *Stachys natalensis* explants were derived from field-grown plant material, which may account for the high levels of microbial contamination and the inefficiency of general decontamination protocols that were successful in other related species (Legkobit and Khadeeva, 2004). Microbial contamination in plant cultures may arise due to inefficient surface sterilisation of the explant as observed in *S. natalensis*, resilience of endophytic microorganisms, or poor aseptic techniques in the laminar flow (Kumar and Shekawat, 2009; Altan *et al.*, 2010; Mihaljevic *et al.*, 2013). There are many reports on the use of sodium hypochlorite or mercuric chloride (HgCl₂) for explant decontamination in plant tissue culture (Avato *et al.*, 2005; Diab *et al.*, 2011; Boix *et al.*, 2012; Saha *et al.*, 2012; Sen *et al.*, 2013). However, prolonged exposure to HgCl₂, in addition to being hazardous and carcinogenic to human health, has potential negative effects on explant material including; explant browning, loss of viability and explant destruction (Sharifkhani *et al.*, 2011; Moghaddam *et al.*, 2011). This has been demonstrated in

the present study by the low explant survival rate upon exposure to HgCl₂ for extended periods of time.

Sodium hypochlorite is a very effective bactericidal agent, significantly reducing bacterial populations even at micromolar concentrations (Oyebanji *et al.*, 2009; Sen *et al.*, 2013). In a study by Thompson *et al.* (2009) decontamination of shoot apex explants by dilute NaClO (1:3) treatment was not significantly different to a 0.2% HgCl₂ treatment. Moreover, shoot development was more pronounced after the NaClO treatment. Hailu *et al.* (2013) determined that the use of 1.5% NaClO treatment for 20 minutes was most favourable for the sterilisation of nodal explants of *Artemisia annua*. In contrast, the use of 1% and 3% NaClO treatments, alone and in combination with each other, were insufficient to significantly reduce contamination in *S. natalensis* explants. Singh *et al.* (2011) suggested the need for balance between the concentration of the decontaminating agent and time in order to overcome the issues of phytotoxicity and low explant survival. Eeswara *et al.* (1999) resorted to more rigorous sterilisation methods by combining HgCl₂ (0.1%, five minutes) and NaClO (10%, ten minutes) for *Azadirachta indica* explants derived from trees in the wild. The use of different concentrations of sodium hypochlorite (NaClO) in combination with mercuric chloride (HgCl₂) over a shorter time interval appeared to be the best method for the decontamination of *S. natalensis* explants whilst maintaining explant viability. Multi-step procedures involving both NaClO and HgCl₂ for surface decontamination has also been noted in *Orthosiphon stamineus* (Rashid *et al.*, 2012), *Alpinia calcarata* (Sudha *et al.*, 2012) and *Saccharum officinarum* (Danso *et al.*, 2011).

In the present study, successful initiation was achieved in all growth media. However, the highest percentage of bud-break was seen in media supplemented with BAP + IBA. Similar results have been obtained in a study by Koul *et al.* (2010), whereby shoot buds were induced directly on mature leaf tissue growing in MS media supplemented with equal concentrations of BAP and IBA. Many studies support the efficiency of different concentrations of BAP in combination with IBA for initiation of shoots (Laribi *et al.*, 2012; Chae *et al.*, 2013; Shukla *et al.*, 2013). In a study by Chae *et al.* (2013) it was determined that the number of initiated shoots per explant was higher in BAP enriched media as opposed to media containing kinetin and that the combined use of BAP and auxins had a significant influence on shoot initiation and development. Medium C (BAP + IBA) and Medium A (BAP + IAA) achieved similar success for initiation of *S. natalensis* shoots. Similarly, Ghiorghita *et al.* (2011) acknowledged that media containing PGRs in the same combination as media A and C in the current study, was the most efficient at shoot initiation in *Stachys sieboldii* MIQ.

Shoot initiation and multiplication is regulated by the concentration ratio of cytokinin to auxin within the growth medium (Das *et al.*, 2013). Successful multiplication of *S. natalensis* microshoots was achieved in all media preparations (Figure 4.16) but the greatest shoot multiplication was obtained in media supplemented with kinetin + IAA (2:1) (Medium B). Similar results for shoot multiplication have been seen in nodal cultures of *Ocimum americanum* and *Solidago virgaurea* by Kumar and Jhanwar (2013) and Yuvaraj (2013) respectively, and by Nalini (2012) in the shoot tip explants of *Chrysanthemum morifolium*. Conversely, prolific shoot growth was detected in many micropropagated *Stachys* spp. cultured in BAP alone, or in combination with IAA, IBA or NAA compared to other PGR combinations (Legkobit and Khadeeva, 2004; Ghiorghita *et al.*, 2011). This suggests that response to different PGRs is variable within the genus *Stachys*. Nathar and Yathoo (2014) advocated the selection of cytokinins for shoot multiplication according to the following descending order of efficiency: BAP > kinetin > zeatin > adenine. The synthetic cytokinin BAP has been involved in effective shoot multiplication in many species (Bhojwani and Razdan, 1996; Muhammad *et al.*, 2007; Abdelmageed *et al.*, 2011; Aicha *et al.*, 2013). However, in the present study, kinetin + IAA showed greatest success for mass shoot production with the highest number of shoots (9.1 ± 3.6 per explant) and greatest shoot height (50.2 ± 5.0 mm) compared to BAP in combination with IAA or IBA (Table 4.5). Medium A (2 mg/l BAP + 1mg/l IAA) and Medium C (2 mg/l BAP+ 1mg/l IBA) showed significantly reduced shoot multiplication of *S. natalensis* plantlets, compared with Medium B (2 mg/l kinetin + 1 mg/l IAA). At high concentrations, BAP is known to induce callus and arrest growth or promote mass shoot formation and limit plant elongation (Bhojwani and Razdan, 1996; El-Finti *et al.*, 2013; Gonbad *et al.*, 2014; Nathar and Yattoo, 2014). This may be the basis for the observed results in the present study but further investigations of the effect of different concentrations of PGRs on shoot multiplication is required. Plant responses under *in vitro* conditions are thus dependent on plant genotype, explant origin and culture conditions (including media constituents and the combination and concentration of PGRs), all of which may be adapted for each species within the same genus (Namli *et al.*, 2010; El-Finti *et al.*, 2013; Gonbad *et al.*, 2014).

Phytohormones have been implicated in many aspects of root development, especially auxins which are known to play a pivotal role in root initiation, patterning, elongation and gravitropism (Pop *et al.*, 2011; Sevik and Guney, 2013). Auxins are regularly applied exogenously to encourage lateral and adventitious root formation in tissue culture (Gutierrez *et al.*, 2012; Sevik and Guney, 2013). The first identified natural auxin, IAA in combination with ½ strength MS media was used in the present study to promote rooting of *S. natalensis* shoots. This combination has previously been responsible for the greatest percent rooting in a variety of species such as *Hypericum perforatum* (Goel *et al.*, 2009),

Calendula officinalis (Victorio *et al.*, 2012), *Ocimum basilicum* (Asghari *et al.*, 2012), *M. piperita* (Manik *et al.*, 2012) and *Dioscorea alata* (Das *et al.*, 2013). Many studies have indicated that *in vitro* rooting occurs faster and more effectively (in terms of root length and number of roots produced per shoot) in the presence of IBA. This has been attributed to its higher stability and persistence in plant tissues compared to IAA (Ludwig-Muller *et al.*, 2005; Nakhoda *et al.*, 2011; Pop *et al.*, 2013). However, Echeverrigaray *et al.* (2010) showed that media supplemented with IAA significantly increased the number of roots and root length in *Salvia guaranitica* plantlets as compared to BAP and NAA which did not affect rooting parameters. Furthermore, Asghari *et al.* (2012) discovered that certain BAP concentrations led to decreased rooting potential and increased shoot hyperhydricity. Hyperhydricity or vitrification is a physiological and morphological condition whereby shoots appear brittle or water-logged, and the *in vitro* plantlets develop poor vascular systems and stomatal abnormalities (Echeverrigaray *et al.*, 2010; Asghari *et al.*, 2012). Root formation may be induced by any type of auxin, however, the mechanism of auxin action at different concentrations and the effect on morphological and physiological development of *in vitro* plantlets should be considered (Nakhoda *et al.*, 2011). Rooting under the influence of other auxins or in PGR-free media was not evaluated within the scope of the present study and may be of interest for further investigations.

In vitro cultured plantlets exist in microenvironments in which all growth parameters occur at optimum levels. Thus, these plantlets are exposed to severe stress during acclimatization which may result in lack of growth and subsequent plantlet destruction. This may be circumvented by the addition of nutrients during early acclimatization and increasing relative humidity (Hazarika, 2003). The high survival rate of *S. natalensis ex vitro* may be attributed to the optimal growth and developmental conditions of plantlets *in vitro* or the supplementation of ½ strength MS medium to the potting soil. Additionally, plantlets were exposed to gradual decreases in relative humidity in the mist tent which may have promoted adaptation to the harsher environment and maintained a high survival rate of *S. natalensis* under greenhouse and field conditions. All acclimatized plantlets (92 ± 4.2 %) in this study did not show any morphological abnormalities compared to field-grown plants. However, it has been reported that *in vitro* propagated plants often exhibit genetic or micromorphological variation, the occurrence of which is dependent on the plant genotype (Legkobit and Khadeeva, 2004).

An examination of the trichomes of *in vitro* propagated *S. natalensis* plants showed no gross anatomical differences compared to field-grown plants with the exception of observably longer non-glandular trichomes on the *in vitro* plant epidermis. Similarly, Abbaspour *et al.* (2012) determined that the average length of leaf trichomes in transgenic tobacco plants were significantly higher than

those in the non transgenic plants. That study suggested that phytohormones in the culture medium were responsible for the observed differences in trichome length. Further optimisation of the *in vitro* protocol of *S. natalensis* is required to determine the effect, if any, of different PGRs on trichome biosynthesis. Both types (peltate and capitate) and subtypes (capitate Type I and II) of glandular trichomes were observed on the foliar surfaces of acclimatized *S. natalensis* plants across all developmental stages. Morphological characteristics of each type such as; stalk septation and micro-ornamentation, number of secretory head cells and mode of secretion were maintained in both field-grown and acclimatized plants showing the success of micropropagation and the mass production of *S. natalensis* plants.

Stachys natalensis leaves grown *in vitro* showed higher gland densities concentrated on the abaxial surface in accordance with their field-grown counterparts. Similar results were observed for the glandular trichomes of *Origanum vulgare* (Morone-Fortunato and Avato, 2008). The density of non-glandular and glandular trichomes of acclimatized plants decreased as the leaf matured which is in keeping with observations of field grown plants (Figure 4.23). Avato *et al.* (2005) also determined that glandular trichome density of *in vitro* regenerated *S. officinalis* plants changed with the physiological age of the leaf. Additionally, the study mentioned a correlation between the development of efficient micropropagation protocols of certain *Salvia* spp. and the production of secondary metabolites (triterpenes and phenolic compounds). If secondary metabolites are available and extractable in high quantities from a plant part, *in vitro* propagation affords the opportunity of mass producing only that specific organ. In the present study, the efficiency of the micropropagation protocol for *S. natalensis* was supported by the morphological fidelity between field-grown and acclimatized plants. However, it is uncertain if the phytochemical fidelity is retained. This may be the basis for future research on the secondary metabolites of acclimatized plants.

CHAPTER 6: CONCLUSION

This study was undertaken to examine the micromorphology of foliar secretory structures found across the three developmental stages of leaves of *Stachys natalensis* Hochst, i.e. emergent, young and mature. The histo- and phytochemical characteristics of the secretion was evaluated for its potential medicinal properties. In addition, an efficient micropropagation protocol for the species was developed and the micromorphology of *in vitro* propagated plants was assessed and compared to that of field-grown plants.

Non-glandular and glandular trichomes were found on both leaf surfaces and across all developmental stages of *S. natalensis* as seen in many members of the Lamiaceae. The non-glandular trichomes appeared to be uniseriate, unbranched and acuminate to arthrodachylous in structure. These trichomes were characterised by the presence of an elevated cellular pedestal, which served to support the trichome and attach it to the leaf epidermis. Two morphologically distinct types of glandular trichomes were identified in the present study: capitate and peltate trichomes. The micromorphology of *S. natalensis* foliar trichomes was found to be similar to other *Stachys* spp. and related genera in terms of structure and location. An interesting observation was the striated micro-ornamentation on the stalk of the non-glandular and Type I capitate trichomes of *S. natalensis*. In contrast, the majority of *Stachys* spp. that have been studied bear papillate micro-ornamentation. Furthermore, Type II capitate trichomes identified on the foliar surface of *S. natalensis* have appeared only on the reproductive organs of other related *Stachys* spp. and on the foliar surface of *Sideritis italica* (Giuliani *et al.*, 2011). The observations in this study may serve as basic distinguishing features in the taxonomic evaluation of *S. natalensis* in comparison to other species and other closely related genera, such as *Sideritis*. Moreover, the use of phylogenetic analyses derived from DNA sequence data of *Stachys* and related species and genera may provide a viable avenue for further research.

Histochemical tests in conjunction with preliminary phytochemical analyses of *S. natalensis* leaves indicated the presence of various phytochemicals such as terpenoids containing oleoresin, saponins, alkaloids and complex polysaccharides within the glandular trichomes. *Stachys natalensis* plant extracts also contained appreciable levels of total phenolic compounds and flavonoids and demonstrated significant free radical scavenging abilities. This indicates the potential for its use as a natural antioxidant. Although the use of *S. natalensis* in traditional medicine has not been documented, it is significant that the plant contains similar classes of secondary metabolites analogous to well established medicinal plants of the genus.

This study appears to be the first investigation of the foliar structures of *S. natalensis*, thus there is a wide scope for future research in this field. Future studies on the morphological aspects of *S. natalensis* should include ultrastructural investigations of the secretory structures to determine the exact mechanism of secretion and an in-depth evaluation of trichome micromorphology to resolve taxonomic discrepancies across related genera. The preliminary phytochemical results obtained in this study validate the need for an intensive evaluation of the crude extracts for other pharmacological activity such as anti-microbial and anti-inflammatory and acetylcholinesterase (AChE) inhibition. In addition, it will be of interest to isolate and characterise the chemical constituents of the essential oils (possibly by gas chromatography-mass spectroscopy) and compare pharmacological efficacy to the plant crude extract.

If potential medicinal phytochemicals are concentrated in a specific plant organ, *in vitro* propagation affords the opportunity for the mass production of that exact plant part (eg. leaves) and the potential to extract concentrated amounts of phytochemicals. Furthermore, *in vitro* micropropagation circumvents the need to harvest mass amounts of plant material thereby facilitating the conservation of indigenous plant resources. The present study sought to establish an *in vitro* propagation protocol for *S. natalensis* by evaluating the effect of different decontamination procedures and combinations of PGRs for efficient initiation and shoot multiplication. High levels of microbial infection were observed in initiated cultures, probably due to the fact that explants were derived from field-grown stock plants. Decontamination using NaClO and HgCl₂ for short intervals, followed by copious washing with distilled water proved to be the most effective decontamination procedure for the axillary nodal explants of *S. natalensis*. This was in contrast to many established protocols for related species in which NaClO or HgCl₂ was utilised as a singular decontaminating agent. The empirical application of cytokinins and auxins induced shoot multiplication and subsequent rooting. Acclimatized *S. natalensis* plantlets (92 ± 4.2 % survival after six weeks) formed robust plants with no apparent morphological variation compared to field-grown plants. The current study has highlighted the need for the control of parameters such as explant origin and culture conditions for effective plant response under *in vitro* conditions. It also provides a basis for future optimization of this protocol involving manipulation of PGR concentrations, different routes of organogenesis and explants derived from other plant organs.

Acclimatized *S. natalensis* plants appeared to be morphologically analogous to their field-grown counterparts in terms of foliar trichome structure. The only observable differences in acclimatized plants were the presence of slightly more elongated non-glandular trichomes on the leaf epidermis. This difference in trichome length was attributed to the action of PGRs in the culture system.

However, the exact PGR responsible for the change and its mechanism of action requires further investigation. Further research is required to determine efficient methods for isolation and extraction of useful secondary metabolites from glandular trichomes of acclimatized plants.

Although an efficient micropropagation protocol via axillary shoot proliferation has been established for *S. natalensis*, it is well known that *in vitro* cultivation of plants often cause genetic alterations or mutations in progeny which may be hereditary. It is important that the genetic integrity of regenerated plants be assessed by molecular techniques (possibly RAPD markers) which may form part of optimizing the current established protocol for future research.

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