The morphology and chemical composition of the trichomes of *Withania somnifera* (Solanaceae)

by

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EXAMINERS COPY

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COLLEGE OF AGRICULTURE, ENGINEERING AND SCIENCE

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Abstract

For centuries, plants have been used in the cosmetic, culinary and medicinal industries. Recently however, the use of plants in the medicinal industry has increased due to the widespread awareness of the harmful effects of synthetic drugs on humans. *Withania somnifera* (Dunal.) is an evergreen perennial shrub found in the drier parts of Africa, particularly South Africa and Asia. Since the phytochemical compounds within the extracts of *W. somnifera* act upon both the nervous and reproductive systems, it is used to treat a wide variety of ailments such as arthritis, stress, ulcers, and tremors. This species has therefore been cultivated to extract the phytochemicals produced. The aim of this study was to characterise the micromorphology of the foliar trichomes of *W. somnifera* as well as to elucidate the location and composition of the secretory products. Stereomicroscopy and scanning electron microscopy (SEM) were used to characterise the foliar trichomes. A series of histochemical and phytochemical tests were performed to determine the location and composition of the compounds that are responsible for the healing properties of the extracts of *W. somnifera*. Trichome density and length was also determined in three developmental stages of the leaves. Histochemically stained leaf sections and SEM showed the presence of four morphologically distinct trichome types: glandular capitate, non-glandular dendritic, non-glandular bicellular and non-glandular multicellular. Uniseriate, glandular capitate trichomes consisted of a six-celled secretory head, single-celled stalk and a single basal cell. Secretions from the glandular heads of capitate trichomes were visible on the leaf surface during ESEM and histochemical staining. Non-glandular dendritic trichomes, which appeared to emanate from single basal cells, consisted of 2-4 celled stalks and varying branch numbers. These dendritic trichomes exhibited cuticular warts which are involved in the "Lotus-Effect". Uniseriate, non-glandular bicellular and multicellular (3-6 cells) trichomes also appeared to emanate from single basal cells. Glandular capitate and non-glandular dendritic trichomes were aggregated on the mid-vein of young and mature leaves, possibly to protect underlying vasculature. Histochemical staining and phytochemical testing revealed the presence of two major phytochemical compounds of medicinal importance, i.e. alkaloids and phenolic compounds. These compounds are used to treat a wide variety of ailments, such as dysentery, TB, paralysis, asthma and inflammation, and also act as chemical deterrents in plants. The results of this study explain possible roles of glandular capitate, non-glandular dendritic, non-glandular bicellular and non-glandular multicellular trichomes based on their morphology and foliar distribution. Future studies should aim at determining the biosynthetic pathways, as well as the modes of secretion of alkaloids and phenolic compounds.

Conferences contributions and attendance from this dissertation:

Munien, P., Naidoo, Y., Naidoo, G., 2012. The micromorphology and histochemistry of the leaf trichomes of *Withania somnifera* (Solanaceae). International Conference on Advances in Plant Sciences (ICAPS 2012), Chiang Mai, Thailand (Funded by Dr. Y. Naidoo).

Munien, P., Naidoo, Y., Naidoo, G., 2012. Morphology and chemical composition of the foliar trichomes of *Withania somnifera* (Solanaceae). 50^{th} Meeting of the Microscopy Society of Southern Africa, (MSSA 2012) University of Cape Town (Personal funds).

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List of Abbreviations:

1-c St	1-celled stalk
2-c St	2-celled stalk
3-c St	3-celled stalk
B	Bicellular trichome
Bc	Basal cell
Br	Branches of dendritic trichome
Bm	Broad mite
c	Chloroform
C	Glandular capitate trichome
Cm	Cellular microfibrils
Cs	Cuticular striations
Cu	Cuticle
Cw	Cuticular warts
Cyt	Cytoplasm
D	Dendritic trichome
Dc	Druse crystal
ESEM	Environmental Scanning Electron Microscope
h	Hexane
m	Methanol
M	Multicellular trichome
N	Nuclues/Nuclei
P	Plastid
Pc	Prismatic crystal
Sec	Secretory material
SEM	Scanning Electron Microscope
Sh	Secretory head of glandular capitate trichome
Sm	Stomata
St	Stalk
TEM	Transmission Electron Microscope
V	Vacuole
Vs	Vesicle
W	Cell wall

CHAPTER 1

INTRODUCTION

1.1. Traditional medicine and medicinal plants

Plants have been used for centuries, not only for medicinal purposes, but also in the cosmetic and culinary industries (Joy *et al*., 1998; Lange *et al*., 2000; Debnath *et al*., 2006 Gairola *et al*., 2008; Mahesh and Satish, 2008). In South Africa, there are over 30 000 plant species, of which approximately 3 000 are used in African traditional medicine (van Vuuren, 2008; van Wyk, 2008). Many of these medicinal species are grown and harvested with the main aim of improving informal economic markets through the production of tea, tablets, ointments and tonics. The commercialization of medicinal species in South Africa has increased rapidly over the last 15 years, accompanied by an increase in scientific research on these plants (van Wyk, 2008). Factors that have driven the increased interest in herbal medicines are the increasing world population, increased cost of living, higher rates of unemployment, increased awareness of the harmful effects of synthetic medication on humans, and the widespread acceptance and accessibility of traditional medical systems (Erasto *et al*., 2005; Khan *et al*., 2006). The increased acceptance of traditional medicine has also led to developed countries utilizing medicinal plants in the treatment of both old and emerging sicknesses. However, the commercialization of these species leads to overharvesting which results in many becoming endangered (Joy *et al*., 1998; Hoareau and DaSilva, 1999; Jäger and van Staden, 2000; Williams *et al*., 2000).

The past few decades have seen traditional medicine being supported by phytochemical and pharmacological studies (Jäger and van Staden, 2000, Debnath *et al*., 2006). Micropropagation of the species has also increased due to the widespread interest of maintaining current populations and protecting genetic diversity (Jäger and van Staden, 2000, Amin, *et al*., 2013). Phytochemical, micromorphological, pharmacological and micropropagation studies have also been undertaken simultaneously to ensure that the micropropagated plants have the same, or enhanced medicinal traits compared to the wild varieties (Jäger and van Staden, 2000, Debnath *et al*., 2006).

1.2. Description of *Withania somnifera* (L.) Dunal

Withania somnifera is an evergreen, perennial shrub (Fig. 1) found in the drier parts of South Africa, India, Pakistan, Mumbai, Sri Lanka, Congo, Egypt, Morocco, Jordan and Afghanistan (Atal and Schwarting, 1961; Khan *et al*., 2006; Singh *et al*., 2010; Kumar and Kumar, 2011). It is commonly known as Indian ginseng or Ashwagandha and belongs to the

family Solanaceae (Rahman *et* al., 2003; Khan *et al*., 2006; Senthil *et al*., 2009; Chatterjee *et al*., 2010; Kumar *et al*., 2010a; Kumar *et al*., 2010b; Singh *et al*., 2010). The plant produces greenish or yellowish flowers and red "berry-like" fruit (Scartezzini and Speron, 2000; Kumar *et al*., 2010a; Kumar and Kumar, 2011; Uddin *et al*., 2012). Contained within the fruit are yellow seeds that are 2.5 mm in diameter (Uddin *et al*., 2012). The leaves are simple and ovate and up to 10 cm in length (Scartezzini and Speron, 2000; Uddin *et al*., 2012). The leaves and roots are used in traditional medicine as both external applications and tonics (Atal and Schwarting, 1961; Senthil *et al*., 2009; Chatterjee *et al*., 2010; Singh *et al*., 2010). Concoctions of *W. somnifera* act upon the nervous and reproductive systems, having rejuvenative effects on the whole body (Chatterjee *et al*., 2010; Ram *et al*., 2012). It is therefore widely used to improve vitality, as well as to aid in the recovery of various illnesses (Khan *et al*., 2006; Chatterjee *et al*., 2010; Kumar and Kumar, 2011; Ram *et al*., 2012).

Medicinal studies were conducted on *W. somnifera* as early as 1911, as this species was used as a sedative in Ayurvedic medicine (Atal and Schwarting, 1961; Scartezzini and Speron, 2000; Chaurasiya *et al*., 2007). Previous studies showed the presence of numerous alkaloids which are responsible for the plants' sedative properties. The major alkaloid is withanine (Atal and Schwarting, 1961). There exists morphological variation within this species, especially between wild and cultivated plants (Atal and Schwarting, 1961). Drugs prepared from the alkaloids of different *W. somnifera* plant parts had both positive and negative sedative action on different animal species (Atal and Schwarting, 1961). This indicates that there may also be chemical variation in this species, related to both morphological and geographical variations (Atal and Schwarting, 1961; Mehboob, 2010).

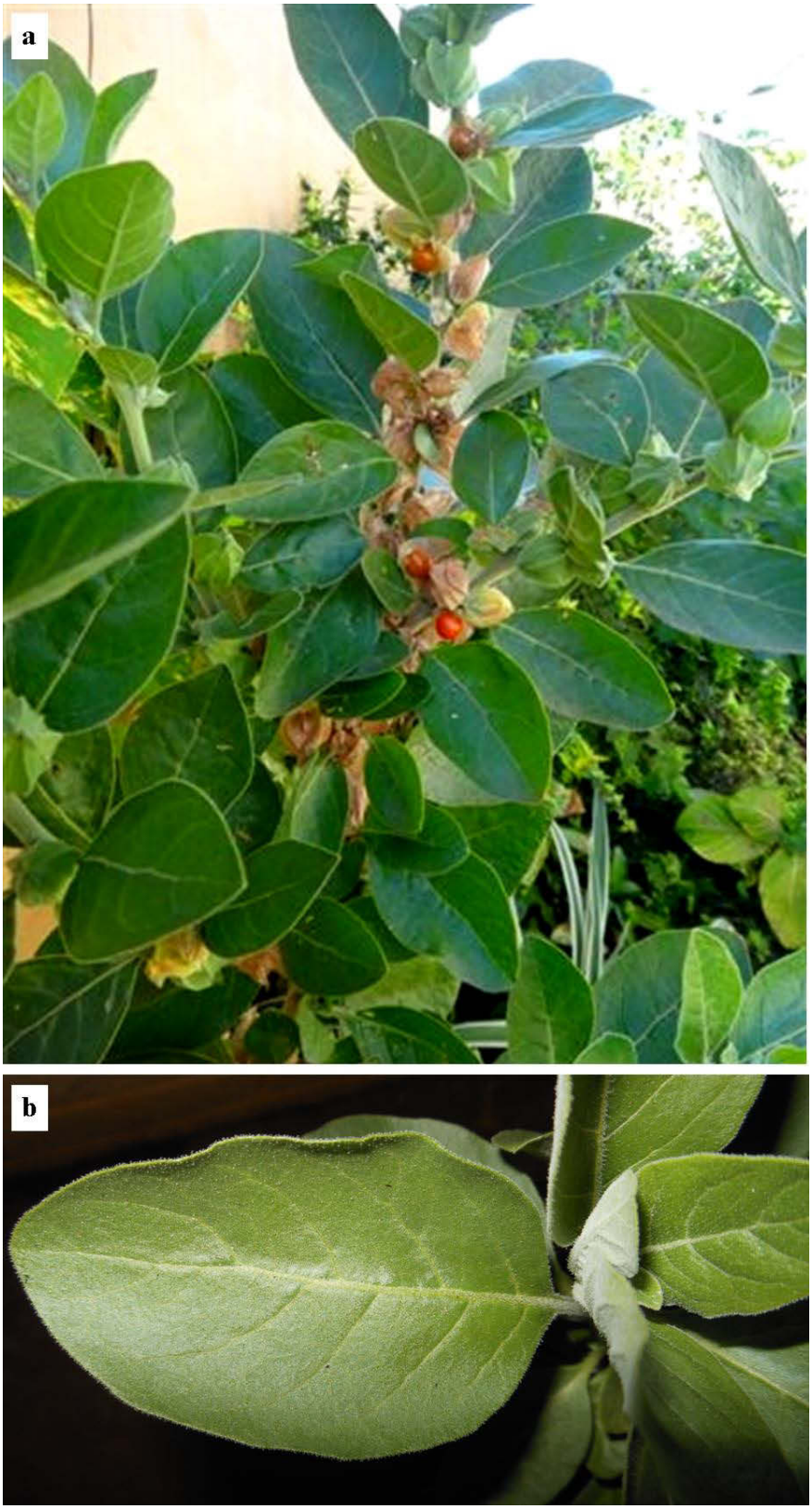

Figure 1. a) *Withania somnifera* containing red fruit, b) fully expanded mature leaf of *W. somnifera* showing a pubescent surface (Courtesy of Prof. G. Naidoo).

1.3. Rationale for this study

Much research has been done on the composition of the phytochemicals produced by *W. somnifera*, some in great detail; however, little or no work has been done on the foliar micromorphology and ultrastructure, as well as the mode of secretion of these phytochemicals. This study therefore will aim to describe the micromorphology of the trichomes present on the adaxial and abaxial surfaces of the leaves, determine the possible site of synthesis and the composition of phytochemicals in the secretions. Trichome morphology might assist as a useful diagnostic characteristic within the family.

1.4. Aims and Objectives

The aims and objectives of this study were to:

i. describe the micromorphology and distribution of the foliar trichomes using light, stereo and scanning electron microscopy.
ii. determine trichome density and dimensions in emergent, young and mature leaves**.**
iii. describe the ultrastructure of the foliar trichomes by examining ultra-thin sections using transmission electron microscopy.
iv. elucidate the location and composition of the phytochemical compounds produced by the trichomes using a variety of histochemical and phytochemical tests and thin-layer chromatography.

CHAPTER 2

LITERATURE REVIEW

2.1. Family Solanaceae

The family Solanaceae, also known as the nightshade or potato family, consists of approximately 90 genera and 3 000 species (Jacobs and Eshbaugh, 1983). Members of this family are usually herbaceous or woody, inhabiting a wide range of habitats. Some of the economically important genera are *Solanum* (potato and eggplant), *Lycopersicon* (tomato), *Physalis* (edible fruits such as Cape gooseberry and Chinese lantern) and *Capsicum* (chilli peppers) (D' Arcy, 1992). Species of this family flaunt an array of morphological variation which is brought about by gene flow during the simultaneous flowering seasons of species. This genetic variation has posed problems for taxonomists and has thus resulted in "poorly defined taxa" (Jacobs and Eshbaugh, 1983). Solanaceae consists of two major subfamilies: Solanoideae, consisting of widely distributed species and Cestroideae, consisting of species restricted to America and Australia. The two largest genera of Solanaceae are *Lycianthes* and *Solanum*, with the latter comprising of approximately half of the total species of the family. *Solanum* is divided into five subgenera and 55 sections, consisting of approximately 1 500 species (D' Arcy, 1992).

Most species of Solanaceae produce alkaloids which, although poisonous to humans, offer commercial as well as medicinal value (Adedeji *et al*., 2007; Kumar *et al*, 2011). *Solanum* has been reported to produce steroidal saponins and glycoalkaloids. These secondary metabolites are important, both medicinally and ecologically. Species of this genus possess antimycotic, antiviral and cytotoxic properties (Khan *et al*., 2006; Kumar *et al*., 2011).

2.2. Genus *Withania* (L.) Dunal

The genus *Withania* comprises approximately 23 species, most of which are distributed between the east Mediterranean region and South Asia, especially India (Vashistha *et al*., 2010). *Withania somnifera* is the most important and commonly used medicinal species. It produces the highest amount of withanolides, a group of steroidal lactones that are unique to a few species of *Withania* (Chaurasiya *et al*., 2009; Senthil *et al*., 2009). *Withania coagulans* and *W. aristata* are also used to treat a wide variety of medical conditions owing to the very numerous healing properties of these withanolides. Due to the importance of withanolides in the medicinal industry, many studies are being undertaken to investigate the biological effects of the extracts of species

belonging to the genus (Khan *et al*., 2006; Martín-Herrera *et al*., 2007; Mathur and Argrawal, 2011).

2.3. Withanolides

The molecular skeleton of withanolides is defined as 22-hydroxy ergostane-26-oic acid-26, 22-olide. Modifications of the carboxylic skeleton or the side chains result in many new structural variations of withanolides. These variants are often referred to as modified withanolides or ergostane-type steroids related to withanolides. Withanolides are classified according to their chemical nature. These groups include withanolide glycosides, withaphysalins, physalins, nicandrenones, jaborols, acnistins, perculactones and withajardines (Mathur and Argrawal, 2011).

Previous studies have revealed that the extracts of *W. somnifera* contained alkaloids, steroidal lactones, saponins and withanolides (Jayaprakasam *et al*., 2003; Khan *et al*., 2006; Chaurasiya *et* al., 2007; Senthil *et al*., 2009; Uddin *et al*., 2012). *Withania somnifera* is also known to contain the largest number of withanolides. This was due to the diverse functional groups and regio-/stereo-forms (Chaurasiya *et* al., 2007). The medicinal properties of *W. somnifera* were due to the presence of withanolides in the leaves and roots (Senthil *et al*., 2009; Uddin *et al*., 2012). Examples of these withanolides are withaferin A, which is known to possess anti-inflammatory and immunosuppressive properties, withanolide D which is believed to have antitumor activity and sitoindosides VII and VIII which were known to antioxidants (Dhar *et al*., 2006; Khan *et al*., 2006; Chaurasiya *et* al., 2007).

2.4. Phytochemical studies

Phytochemical studies aid in identifying the specific classes of compounds that are of medicinal value. To date, random phytochemical screening of plants has proven most successful in discovering biologically active compounds e.g. antibiotics (Mahesh and Satish, 2008). This is due to the small percentage of the total species that have been screened for phytochemicals with potential medicinal value. Species that are selected for phytochemical screening are usually those that are predicted to possess antimicrobial activity (Hadi and Bremner, 2001). *Withania somnifera* also appeared to the candidate for phytochemical studies. Consequently it has been chosen by the author as it had been cited in Ayurvedic medicine and for its numerous alkaloids.

2.5. Previous studies on *W. somnifera*

Cancer is one of the major diseases affecting human life and with current treatments being potentially harmful and expensive, natural alleviants are being sought. The antioxidant properties of *W. somnifera* leaf extracts were used to determine their effect on human tumor cell lines for breast and lung tissue and cells of the central nervous system. Three specific groups of withanolides: withaferin A, physagulin D and viscosalactone B showed antiproliferative activity against all four of these cell lines demonstrating that the species had the potential to reduce tumor growth or prevent cancer altogether (Jayaprakasam *et al*., 2003; Khan *et al*., 2006; Kumar and Kumar, 2011).

Many studies have demonstrated the antioxidant and antimicrobial properties of *W. somnifera* extracts leading to the widespread use of this species in Ayurvedic systems to improve longevity and memory, and counteract stress. These extracts have thus been incorporated into the treatment of diseases such as Alzheimer's, Parkinson's and diabetes. Antioxidant behaviour of the extracts were a result of the production of withanolides such as sitoindosides VII-X and withaferin A, which was reported to contain the "oxidative free radical scavenging enzymes" superoxide dismutase, catalase and glutathione peroxidase (Bhattacharya *et al*., 1997).

Methanol extracts of *W. somnifera* have shown antimicrobial activity against *Candida albicans* (Mahesh and Satish, 2008). A monomeric glycoprotein, WSG, also showed antimicrobial activity against bacterial and phytopathogenic fungi. It is important however, to consider that antimicrobial, antifungal and antibacterial activities differ depending on the plant parts that are used (Mahesh and Satish, 2008).

2.6. Trichome types and functions

Trichomes were generally described as 'hair-like' appendages present on the surfaces of leaves, stems, roots and floral structures. Trichomes appeared to differ in size, shape, location, capability to secrete and function; both between species and plant organs. There was also variability in the type and density of trichomes between the two sides of leaves, bracts, sepals and tepals (Wagner, 1991, Johnson, 1975; Werker, 2000: Wagner, 2004; Adedeji *et al*., 2007; Gairola *et al*., 2008; Ishida *et* al., 2008; Boix *et al*., 2011; Kim *et al*., 2011).

Due to the diversity of trichomes and the different methods of classification, trichome classification has been quite subjective. However, trichomes could be classified into two major

categories, non-glandular and glandular (secretory). These were further sub-divided according to their morphological characteristics (Kim *et al*., 2012). Glandular trichomes might be capitate, peltate, sub-sessile or branched. Non-glandular trichomes might be unicellular, multicellular or branched (Fahn, 1988; Gairola *et al*., 2008; Kryvych *et al*., 2011; Osman; 2012). Typically, glandular trichomes consisted of a terminal secretory head, a stalk and a base, all comprised of one or many cells. In some cases, a neck cell might also exist between the secretory head and the stalk. Glandular trichomes have also been characterised according to the compounds that they excrete and the mode of secretion (Fahn, 1988; Werker, 2000; Gairola *et al*., 2008).

According to Adedeji *et al.* (2007) and Osman (2012), trichomes have been an integral part of comparative studies as they usually showed unique morphological and distributional patterns among taxa. Simple trichome types might serve to prevent water loss, influence pollination or act as a mechanical barrier to herbivory. Glandular, secreting trichomes might be involved in the production and secretion of chemicals to ward off pests or attract pollinators. Plant protection by glandular trichomes could occur in four ways: by obstructing the movement of pests into leaf tissue; by capturing pests so that overall movement across/within leaves are prohibited; by producing and/or secreting volatile or non volatile secondary metabolites; as well as by producing proteins that directly poison or actively deter pests. In some desert species, glandular trichomes have been reported that they secreted high amounts of exudates onto the foliar surface to protect against water loss (Wagner, 1991; Fahn; 1986; Fahn, 1988; Ishida *et* al., 2008; Wagner, 2004; Lev-Yadun, 2006; Gairola *et al*., 2008; Kim *et al*., 2011; Kryvych *et al*., 2011; Weinhold and Baldwin, 2011).

Figure 2. Stalked-glandular (A-O) and non-glandular (1-14) trichomes of Solanaceae (adapted from Adedeji *et al*., 2007).

(A-I) unicellular stalk, unicellular to multicellular head trichomes.

(J-O) bicellular to multicellular stalk, unicellular to multicellular head trichomes.

(1) Spine like trichome.

(2) unicellular short trichome.

(3) unicellular long trichome.

(4) unicellular hooked trichome.

(5) bicellular trichome (large basal cell).

(6) bicellular trichome (normal basal cell).

(7) bicellular trichome (large basal cell, apical cell hooked and pointed).

(8) bicellular trichome (found on petals),

(9) bicellular hooked trichome.

(10) multicellular trichome.

(11) multicellular trichome (large basal cell, 2shrivelled cells).

(12) multicellular trichome (narrow topmost cell).

(13) multicellular trichome (shrivelled middle cell).

(14) multicellular trichome (one cell branching).

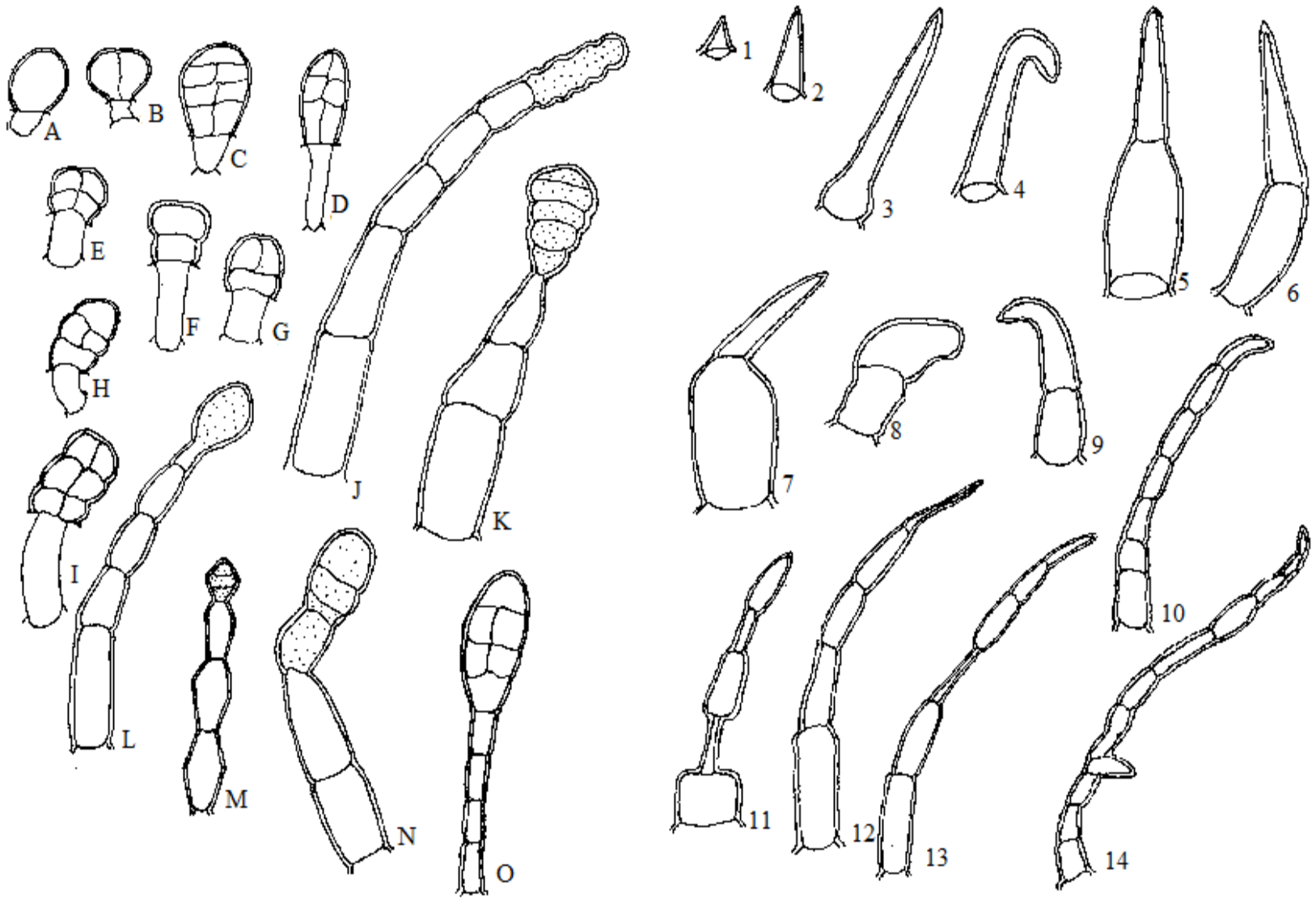
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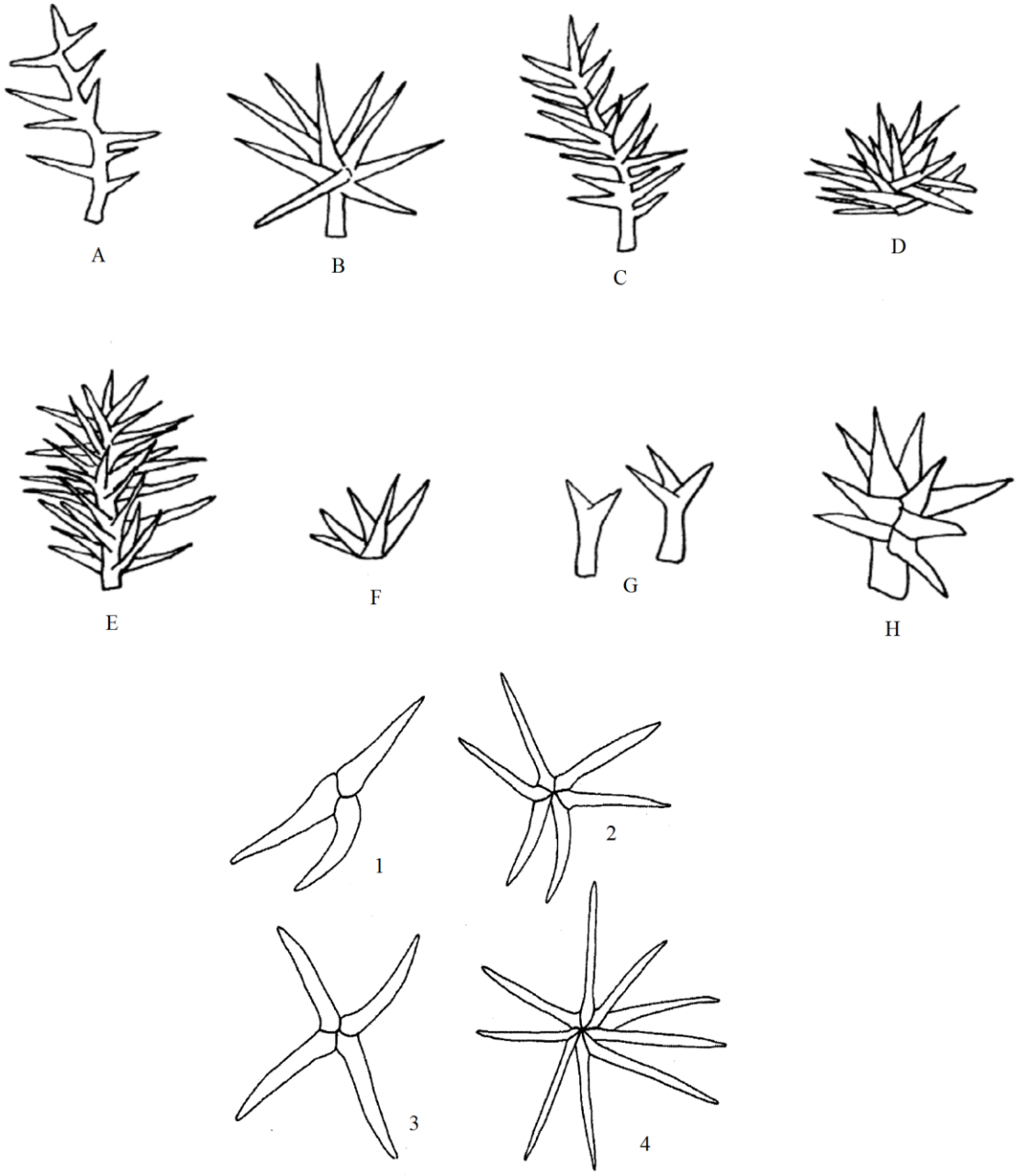
Figure 3. Non-glandular branched (A-H) and non-glandular stellate trichomes of Solanaceae (adapted from Batterman and Lammers, 2004; Adedeji *et al*., 2007).

(A) dendritic branched trichome.
(B) multangulate branched trichome.
(C) multangulate-dendritic branched trichome.
(D) echinoid branched trichome.
(E) echinoid-dendritic branched trichome.
(F) stellate branched trichome.
(G) furrcate branched trichome.
(H) multiserriate- multangulate branched trichome.
(1) triradiate stellate trichome.
(2) tetraradiate or H-shaped stellate trichome.
(3) - (4) multiradiate stellate trichome.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2.7. The importance of foliar trichomes

Apart from the functions that trichomes served to plants and humans, these appendages also serve as a taxanomic tool for the classification of different species (Johnson, 1975; Wagner 1991; Wagner, 1994; Gairola *et al*., 2008). In the Coniferae for example, the variation of trichomes on the twigs of *Picea* allowed for the identification and characterisation of the different species (Johnson, 1975). Categorising plant species or families according to the unique trichome types they contain will assist in the classification of new species. The author has also predicted that *W. somnifera* would produce the results which would be trichome specific in structure and histochemical studies for the purpose of classification.

Secondary metabolites, such as alkaloids, are produced and secreted by glandular trichomes when the leaves of *W. somnifera* are wounded by herbivores. The pungent smell of these alkaloids discouraged herbivores from feeding on the plant parts (Lev-Yaden and Ne'eman, 2003; Chaurasiya *et al*., 2007). These secretions have been reported to protect against phytophagus arthropods by producing toxic acylsugars (Kim *et al*., 2011; Weinhold and Baldwin, 2011).

The essential oils that confer therapeutic properties are also synthesised and stored in glandular trichomes (Lange *et al*., 2000; Boix *et al*., 2011; Kim *et al*., 2011). Leaves of all species of Solanaceae contained trichomes, with trichome type and distribution varying among species. Many of these species are found to contain both glandular and non-glandular trichomes on both foliar surfaces, with *Solanum torvum* being the only species within the family to possess stellate (star-shaped) non-glandular trichomes (Adedeji *et al*., 2007; Weinhold and Baldwin, 2011).

Kumar *et al*. (2010b) found that non-glandular branched trichomes were abundant on the leaves of *W. somnifera*. These trichomes are randomly distributed, and more dense on the abaxial surface. Although Kumar *et al*. (2010b) also mentioned the presence of glandular trichomes on the leaves; there is no indication of the distribution and density of these trichomes. The foliar trichomes varied in length and are between 100 and 450 μm wide (Kumar *et al*., 2010b).

Besides the physical attacks from herbivores and other pests, climate might also influence the type and distribution of foliar trichomes. Garg and Varshney (1980) showed that the foliar trichomes of *W. somnifera* increased in length and density on adaxial surfaces of plants grown in a polluted area. Stomatal pores were also reduced by one third in the leaves of plants in an unpolluted area compared with those from a polluted area. This led to the assumption that the

plants grown in polluted areas would have slower growth rates due to the limited Co^2/O^2 exchange between stomata and the atmosphere, which could be an adaptive characteristic of the plants to the environment. Thus seed production and the completion of the life cycle would also be delayed. Studies such as these are becoming prominent due to the increasing worldwide population, increased levels of pollution and climate change (Seinfeld and Pandis, 2012). Another topic of interest is the impact that these foliar micromorphological changes will have on the medicinal properties of species (Gairola *et* al., 2010).

Advances in research on glandular secreting trichomes were also important for the enhanced accumulation of medicinally important exudates. Recombinant DNA approaches, for example, allowed for the genetic manipulation of enzymes that would modify the chemical nature of secretory products to enhance disease resistance or to allow for the improved metabolism of phytochemical compounds for medicinal use (Wagner, 1991; Wagner, 2004). Modification of secretory products might also prove useful to plants by increasing their attractiveness to pollinators.

2.8. Trichome development

Trichomes developed from specialised epidermal cells called protodermal cells. These cells have been reported to undergo differentiation or cell division to form the hair-like projections (Johnson, 1975; Werker, 2000; Wagner, 2004). With the process of trichome differentiation, specialised structures were formed. The cell walls of trichomes might be comprised purely of cellulose or might also become saturated and covered with lignin, carbonates and silicates. These substances within the cell walls determined the stiffness (or softness) of trichomes. Cell walls of trichomes might either be thin or thickened to a degree such that the lumen of the cell decreased in size. In trichomes that are shed, the thickening of cell walls is usually reduced at the site of the breakage (Johnson, 1975). The exterior surfaces of the cell walls might also display various types of ornamentation such as ripples, bumps striations or warts that resulted from changes in microfibril orientation or the accumulation of carbonates and silicates (Werker, 2000). Trichomes are also covered by a cuticular layer extending from the epidermis. With regard to the glandular structure of trichomes, a series of anticlinal and periclinal divisions resulted in the formation of auxiliary cells and glands (Wagner, 1991, Johnson, 1975; Werker, 2000).

Developmental patterns of glandular and non-glandular trichomes varied between species. In some species trichomes might remain metabolically active throughout the life of the organ on

which they are found, whereas in others, the trichomes might senesce before maturity (Johnson, 1975; Wagner, 2004). Trichomes which became senescent might either persist or are shed. This is true for both glandular and non-glandular trichomes, where the non-glandular branched trichomes of *Artemisia dracunculus* (Asteraceae) and the glandular resin-secreting trichomes of *Betula pendula* (Betulaceae) are shed. In some species, trichomes reached maturity far sooner than the organ on which they existed. On the stems of *Melampyrum nemorosum* (Orobanchaceae), for example, trichomes reached maturity over a few days, whilst the trichomes of other species might take up to 30 days to reach maturity (Johnson, 1975). Senescent trichomes that remained on plant organs might play important roles in water absorption, seed dispersal and the mechanical protection of the specific tissue.

One of the key questions related to trichome development was whether the density of trichomes on leaf surfaces remain fixed or changes with age (Werker, 2000). This was also species dependent. The glandular trichomes on leaf surfaces of *Leonotis leonurus* (Lamiaceae) decreased with leaf growth. This was consistent with the 'leaf expansion', which suggested that although trichomes might appear densely packed together on emergent or young leaves, expansion during leaf development resulted in the trichomes which became widely spaced in mature leaves. The leaves of *Ocimum basilicum* (Lamiaceae) however, had their own pattern of differentiation. Although glandular trichomes of *O. basilicum* are produced in the early stages of leaf development, mature leaves still contained meristematic regions. New glandular trichomes are therefore produced via the process of cell division (Werker *et* al., 1993; Ascensão and Pais, 1998; Werker, 2000).

Despite the vast amount of scientific information available on medicinal plants, there is still a need for ongoing studies to make sound decisions related to the commercial opportunities offered by the assortment of plant species (Williams *et al*., 2000). This research should be conducted in a multidisciplinary, collaborative manner and should involve taxonomy, biosystematics, ethnobotany, organic chemistry, agrotechnology, pharmacology, horticulture and studies on the reproductive biology of specific plant species and their relatives. Such research will allow for the determination of suitable species for large scale production, accurate identification and location of important chemicals, development of suitable protocols for micropropagation, and genetic preservation of medicinal plant species (Joy *et* al., 1998; van Vuuren, 2008; van Wyk, 2008). As an initial attempt, the author would address the micromorphology and chemical composition of

the trichomes of *W. somnifera* in order to elucidate some of the trichome characters which might be used in plant taxonomy classification.

CHAPTER 3

MATERIALS AND METHODS

3.1. Collection of leaf material

Leaves of *Withania somnifera* were collected from a private residence in Umhlanga, KwaZulu-Natal (29°43′31″ S, 31°5′9″ E). *Withania somnifera* plants were also grown from seeds in a private residence in Malvern, KwaZulu-Natal (29°53'0" S, 30°55'17" E). Leaves were classified as emergent (5–9 mm), young (10–40 mm) and mature (> 40 mm) based on their length.

3.2. Microscopy

3.2.1. Stereomicroscopy

Stereomicroscopy was used to obtain information on trichome type and density on fresh leaves. Images of adaxial and abaxial leaf surfaces were obtained using a Nikon AZ100 stereomicroscope equipped with a Nikon Fiber Illuminator (Nikon, Japan) as well as the NIS-Elements Software (NIS-Elements D 3.00, SP 4 (Build 502)).

3.2.2. Environmental scanning electron microscopy (ESEM)

ESEM was used to view and image biologically functional leaf material as well as secretions on the foliar surfaces. Sections of approximately 4 mm^2 in area of fresh emergent, young and mature leaves were mounted onto brass stubs using double-sided carbon tape. Adaxial and abaxial surfaces were viewed with a low vacuum Zeiss EVO LS 15 ESEM (at 20 kV and working distance of 7.5 –9 mm) and images were obtained.

3.2.3 Scanning electron microscopy (SEM)

SEM was used to examine the micromorphology of the foliar trichomes of chemically-fixed samples. SEM preparations involved fixing segments of fresh material in 2.5 % glutaraldehyde for 24 hours. The material was subsequently subjected to three 5 minute washes with phosphate buffer, followed by a 1 hour post fixation with 0.5 % osmium tetroxide. Thereafter, the material was subjected to three 5 minute washes with distilled water and dehydrated in 30 %, 50 % and 70 % alcohol (two changes, each of 5 minutes); followed by two changes for 10 minutes in 100% alcohol. The segments were then dried to their critical point in a Hitachi Critical Point Dryer (Hitachi, LTD. Tokyo, Japan), mounted onto brass stubs using double-sided carbon tape and sputter coated with a layer of gold (at a vacuum of 0.1 Torr for 2.5 minutes) using a Polaron SC 500 Sputter Coater

(Quorum Technologies Ltd., United Kingdom) (Kim *et al*., 2011). Samples were viewed using a LEO 1450 SEM (at 5 kV and working a distance of 5–31 mm) and a Zeiss Ultra-Plus FEG-SEM (at 5 kV and a working distance of 3.7–9 mm). Scanning electron micrographs were taken of the adaxial and abaxial surfaces of emergent, young and mature leaves.

3.2.4. Transmission Electron Microscopy (TEM)

TEM was used to study the fine structure of the leaf tissue. TEM preparations involved fixing segments (approximately 2 mm^2) of fresh material in 2.5 % glutaraldehyde for 24 hours. The material was subsequently subjected to three 5 minute washes with phosphate buffer, followed by a 1 hour post fixation with 0.5 % osmium tetroxide (made up in 0.1 M phosphate buffer). Thereafter, the leaf sections were subjected to three additional 5 minute phosphate buffer washes and dehydrated in 20 %, 30 %, 50 % and 75 % acetone (two changes, each of 5 minutes), followed by two changes for 10 minutes in 75 % acetone and four changes for 10 minutes in 100 % acetone. The dehydrated leaf sections were infiltrated with equal parts of Spurr's resin (Spurr, 1969) and acetone for 4 hours, thereafter in whole resin for 24 hours. The leaf sections were thereafter embedded in whole resin using silicone moulds, and polymerized at 70 °C in an oven for 8 hours.

Sections were cut using a Reichert Jung Ultracut-E ultramicrotome (Reichert, Inc., USA) (Boix *et* al., 2011; Kim *et al*., 2011). Survey sections (1–2 μm) were placed on a slide, stained with Toluidine Blue and viewed with a Nikon Eclipse 80i light microscope (Nikon, Japan) equipped with a Nikon DS-Fi1 camera and NIS-Elements imaging software package (NIS-Elements D 3.00, SP 4 (Build 502) (Kumar *et al*., 2010b). Once trichomes were visible in survey sections, ultrathin sections (80 nm) were picked up onto copper grids and post-stained by being placed onto large drops of 2.5 % uranyl acetate. The sections were allowed to stain for 8–10 minutes at room temperature and rinsed with cooled, freshly boiled distilled water. The copper grids were then placed onto drops of Reynold's (1963) lead citrate solution in a closed petri dish with dry NaOH pellets (to prevent moisture or excessive oxygen causing the stain to precipitate) and allowed to stain for 5–8 minutes at room temperature. The grids were then rinsed with distilled water and placed on filter paper. The sections were viewed (at 100 kV) and imaged using a Jeol 1010 TEM (Japan) equipped with an Olympus MegaView III CCD camera (Soft Imaging System GmBH, Münster, Germany).

3.3. Trichome density and length

Selected images obtained from ESEM and SEM were analysed using the iTEM software programme (Soft Imaging System GmBH, Münster, Germany) to count the number of glandular and non-glandular trichomes present on both leaf surfaces, of emergent, young and mature leaves. The area of the leaf surface included in the image was also determined. Thereafter, the lengths and widths of all trichomes were measured. For the purpose of this study, non-glandular bicellular and multicellular trichomes were considered to be no different as both types look morphologically alike on scanning electron micrographs. Trichomes present on the emergent abaxial leaf surfaces were also excluded from the analysis as their dense arrangement poses problems in identification and counting. All data were analysed using PASW 18 statistics version 18.0.3 (SPSS Inc., Chicago, Illinois, USA).

The differences in trichome density in different developmental stages, as well as between adaxial and abaxial surfaces were analysed, such that there were 5 different locations: emergent adaxial, young adaxial, young abaxial, mature adaxial and mature abaxial. Trichome density was compared using a Multivariate Analysis of Variance (MANOVA) using the statistical software package IBM SPSS Statistics for Windows (Version 21.0). Armonk, NY: IBM Corp.). The MANOVA was performed on ranked data as the data was not normally distributed ($p<0.0005$).

A One-way Analysis of Variances (ANOVAs) was also undertaken to compare the lengths of glandular and non-glandular trichomes on both leaf surfaces, in the three stages of development. The assumptions of normality and equality of variances were met for all ANOVA tests. Tukey's Post-hoc tests were used to make pair-wise comparisons of the trichome frequencies and dimensions. A p-value of <0.05 was recognised as being significant.

3.4. Histochemistry

Fresh emergent (80 μm) and young (100 μm) leaf sections were cut using an Oxford vibratome sectioning system. Histochemically stained sections were viewed and images captured with the Nikon Eclipse 80i light microscope. The following histochemical stains were used to identify and locate the various compound groups:

3.4.1. Alkaloids

Sections were stained for 10 minutes each with Wagner's reagent (1.27 g iodine and 2 g potassium iodide dissolved in 100mL dH_2O) and Dittmar reagent (1 g potassium iodide and 1 g sodium nitrate dissolved in 30 mL HCl and 30 mL dH_2O) (Furr and Mahlberg, 1981; Ascensão and Pais, 1987). Alkaloids stained orange/brown.

3.4.2. Lipids, cutin and suberin

Sections were placed in a saturated solution of Sudan Black B for 30 minutes. Excess solution was blotted using paper towel and replaced with 70 % ethanol. Sections were then rinsed with 70 % ethanol and mounted in glycerine. Cutinised/suberised cell walls stained blue. Lipids in cells stained dark blue-black. For control treatments, sections were pre-treated with methanol and chloroform and stained with Sudan Black B for 30 minutes (Ascensão and Pais, 1987). Excess solution was blotted off using paper towel and sections were rinsed with 70 % ethanol before mounting in glycerine.

Sections were placed in Sudan III and IV for 20 minutes. Sections were then rinsed with 70 % ethanol and mounted in 70 % glycerol. Cutinised/suberised walls and lipid inclusions stained orange/red. For control treatments, sections were pre-treated with methanol and chloroform and stained with Sudan III and IV for 20 minutes (Ascensão and Pais, 1987). The sections were then rinsed with 70 % ethanol before mounting in 70 % glycerol.

3.4.3. Esterified pectins

Sections were placed in hydroxylamine hydrochloride solution, as well as in 1 part concentrated HCl and 2 parts 95 % ethanol. Excess solution was blotted using paper towel and sections flooded with ferric chloride in 60 % ethanol and 0.1 N HCl. Esterified pectins stained red.

3.4.4. Lignin aldehydes

Sections were mounted in a drop of saturated aqueous solution of phloroglucinol in 20 % HCl. Lignin aldehydes and cuticle components stained red (colour changes to brown in approximately 30 minutes).

3.4.5. Lipids

Sections were placed in 1% Nile blue at 37 °C for 1 minute and then 1% acetic acid at 37 °C for 1 minute before rinsing with distilled water. Neutral lipids (such as fats, oils and waxes) stained red, while acidic lipids (such as phospholipids) stained blue. For control treatments, sections were pre-treated with methanol and chloroform and stained with 1 % Nile blue for 1 minute followed by 1 % acetic acid blue for 1 minute before rinsing with distilled water (Ascensão and Pais, 1987).

3.4.6. Phenolic compounds

Sections were placed in 10 % aqueous ferric trichloride and a dash of sodium carbonate for 15 minutes at room temperature. In this test, orto-dihydroxyphenols react with ferric ions to produce green or black deposits.

3.4.7. Polysaccharides and unesterified pectins

Sections were placed in an aqueous Ruthenium Red solution (1:5000) for 10 minutes. Acidic polysaccharides stained pink. Unesterified pectins stained pink/red.

3.4.8. Total proteins

Sections were immersed in bromophenol blue (95 % ethyl alcohol with 10 g $HgCl_2$ and 100 mg bromophenol blue per 100 ml) for 15 minutes at room temperature. Sections were then rinsed in 0.5 % acetic acid for 20 minutes and treated with a sodium phosphate buffer (0.1 M, pH 7.0) for 3 minutes. Total proteins stained blue.

3.4.9. Carboxylated polysaccharides, phosphate groups and polyphenols

Sections were placed in Toluidine Blue for 40 seconds. Excess stain was removed using water and sections mounted in 70 % glycerol. Carboxylated polysaccharides stained pink, phosphate groups on macromolecules stained purplish-greenish blue and polyphenols stained blue.

3.4.10 Starch and cellulose

Sections were mounted in KI/I_2 for 10 minutes. Excess stain was then removed using water. Cellulose structures stained blue, Lignified walls and cutin stained yellow.

3.5. Fluorescence Microscopy

Fresh, hand-cut sections of young leaves were viewed and imaged at different wavelengths using a Zeiss LSM 710 confocal microscope. Cells that contain phenolic compounds emit a blue fluorescence at excitation wavelengths between 330 and 380 nm (Ascensão and Pais, 1987). A red fluorescence emitted by sections indicated the presence of plastids (e.g. chloroplasts) within the cells (Ascensão and Pais, 1987).

Fresh hand-cut sections were also stained with 2 % acridine orange for 2 minutes before rinsing with distilled water. The sections were mounted with water, viewed, and imaged at 488 nm using a Zeiss LSM 710 confocal microscope. Acridine orange is a fluorochrome stain that binds to the DNA of cells, thus indicating viability of cells (Mirrett, 1982; Winter *et al*., 2007).

3.6. Phytochemistry

Leaves were air-dried for 3 months, and then ground to a fine powder. Approximately 10 g of the powdered material was placed in a round-bottomed flask, together with 100 ml methanol (Fig. 4) (Rahman *et al*., 2003). A series of four three-hour extractions were performed by heating and distilling by reflux, using a soxhlet apparatus. The extracts were filtered after each extraction (using Whatman No. 1 filter paper) after each extraction to separate the filtrate from the leaf material, and fresh methanol was added to the leaf material for further extractions. This process was repeated with hexane and chloroform. A variety of phytochemical tests were performed on the methanol, hexane and chloroform extracts to determine the presence of phytochemical compounds.

3.6.1. Alkaloids

Two drops of Dragendroff's reagent were added to 1 ml of extract. A reddish-orange precipitate indicated the presence of alkaloids.

Two drops of Hager's reagent were added to 1 ml of extract. A yellow precipitate indicated the presence of alkaloids.

Two drops of Wagner's reagent were added to 1 ml of extract. A brown precipitate indicated the presence of alkaloids.

3.6.2. Amino acids

A drop of Ninhydrin solution was added to 1 ml of extract. A purple colour indicted the presence of amino acids.

3.6.3. Carbohydrates

A drop of alcoholic solution of α-naphthol was added to 1 ml of extract. The mixture was shaken well, and 0.5 ml of concentrated sulphuric acid was added along the sides of the test tube. A violet-purple ring indicated the presence of carbohydrates.

One ml of extract was boiled in a water bath with 1 ml of Fehling's solution A and B. A red precipitate indicated the presence of carbohydrates.

One ml of Benedict's reagent was added to 1 ml of extract, and allowed to boil in a water bath for 2 minutes. A yellow-red precipitate indicated the presence of carbohydrates.

3.6.4. Cardiac glycosides (Keller Killiani test)

Five ml of extract mixed with 1 ml glacial acetic acid containing a drop of ferric chloride was slowly added to 1 ml of concentrated sulphuric acid, such that the acid remained underneath the mixture. The appearance of a brown ring indicated of the presence of cardiac glycosides.

3.6.5. Flavones and flavonones

Concentrated sulphuric acid (0.5 ml) was added to 1 ml of extract. A yellow to orange colour indicated the presence of flavones. An orange to crimson colour indicated the presence of flavonones.

3.6.6. Phenolic compounds

Two drops of ferric trichloride were added to 1 ml of extract. A dark green colour indicated the presence of phenolic compounds.

Two drops of lead acetate were added to 1 ml of extract. A bulky white precipitate indicated the presence of phenolic compounds.

3.6.7. Sterols

1.5 ml of chloroform was added to 1 ml of extract. A drop of concentrated sulphuric acid was added along the sides of the test tube. A red colour ring at the junction and a green fluorescent layer at the bottom of the solution indicated the presence of cholesterol.

3.7. Thin Layer Chromatography (TLC)

The leaf extracts were analysed using Thin Layer Chromatography (TLC) to separate the compounds present in the leaves. In separate beakers, 0.5 mg of the methanol, chloroform and hexane extracts were dissolved in 5 ml of methanol, chloroform and hexane respectively. Each of the dissolved extracts was then filtered using a Whatman No. 1 filter. A drop of each extract was spotted separately onto a pre-coated silica gel 60 F_{254} TLC plate (Merck). The plate was placed upright in a beaker containing a mobile solution made up of 5 ml toluene, 5 ml glacial acetic acid and 1 ml formic acid. The mobile solution was allowed to run up the plate. Images of the plate were taken at 254 and 366 ultra-violet light. The plate was then sprayed with a solution made up of 0.05 g vanillin, 1 g boric acid, 2 ml sulphuric acid and 100 ml methanol.

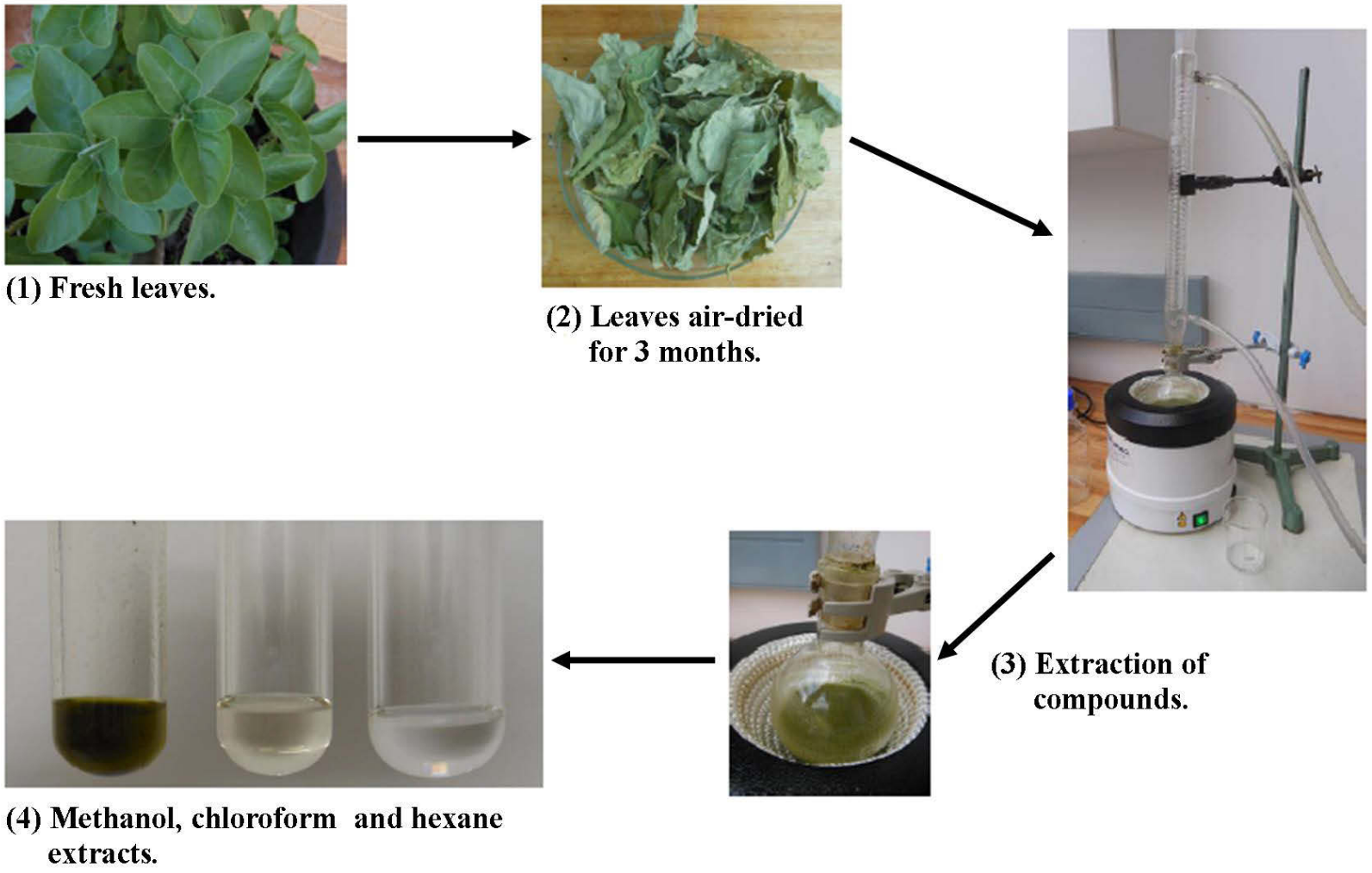


Figure 4. Process used to extract phytochemical compounds as methanol, chloroform and hexane extracts.

CHAPTER 4

RESULTS

4.1. Leaf micromorphology

The leaves of *W. somnifera* contained four morphologically distinct trichome types: glandular capitate, non-glandular dendritic (branched), non-glandular bicellular and non-glandular multicellular trichomes. Stereomicrographs (Fig. 5 & 6) showed non-glandular dendritic trichomes on both adaxial and abaxial foliar surfaces, at all stages of development. Glandular capitate trichomes were not clearly visible on the stereomicrographs however, these were assumed to be the glossy, globular structures as observed on emergent leaves (Fig. 5a–b). Non-glandular bicellular and non-glandular multicellular trichomes were initially observed only on the adaxial surfaces of mature leaves with stereomicroscopy (Fig. 6a) however, both trichome types were later identified on young leaf sections with SEM and histochemical procedures (Fig. 14a–b & 20–25).

Cuticular striations were visible on both foliar surfaces (Fig. 10a & 12b). These striations radiated from the bases of capitate and dendritic trichomes as well as from stomata. Cuticular striations were more frequent on abaxial surfaces and decreased with increasing leaf development. The guard cells of stomata were surrounded by three subsidiary cells of differing sizes, thus the arrangement of stomata on adaxial and abaxial surfaces of *W. somnifera* leaves was classified as anisocytic (Fig. 7a–b & 9b). Stomata on the adaxial surface were closed while those of the abaxial surface were open (Fig. 7a–b).

Broad mites (Fig. 8b) and scale insects (Fig. 8c) were commonly observed on leaves sampled during February 2013. Eggs of broad mites were also present on leaf surfaces (Fig. 8a–b). Movement of these mites was restricted across emergent leaf surfaces due to the intertwining of dendritic trichomes.

Figure 5. Stereomicrographs showing dendritic and glandular capitate trichomes on fresh leaves of *W. somnifera*.

(a) Non-glandular dendritic (D) and glandular capitate (C) trichomes on emergent adaxial surface.

(b) Non-glandular dendritic (D) and glandular capitate (C) trichomes on young adaxial surface.

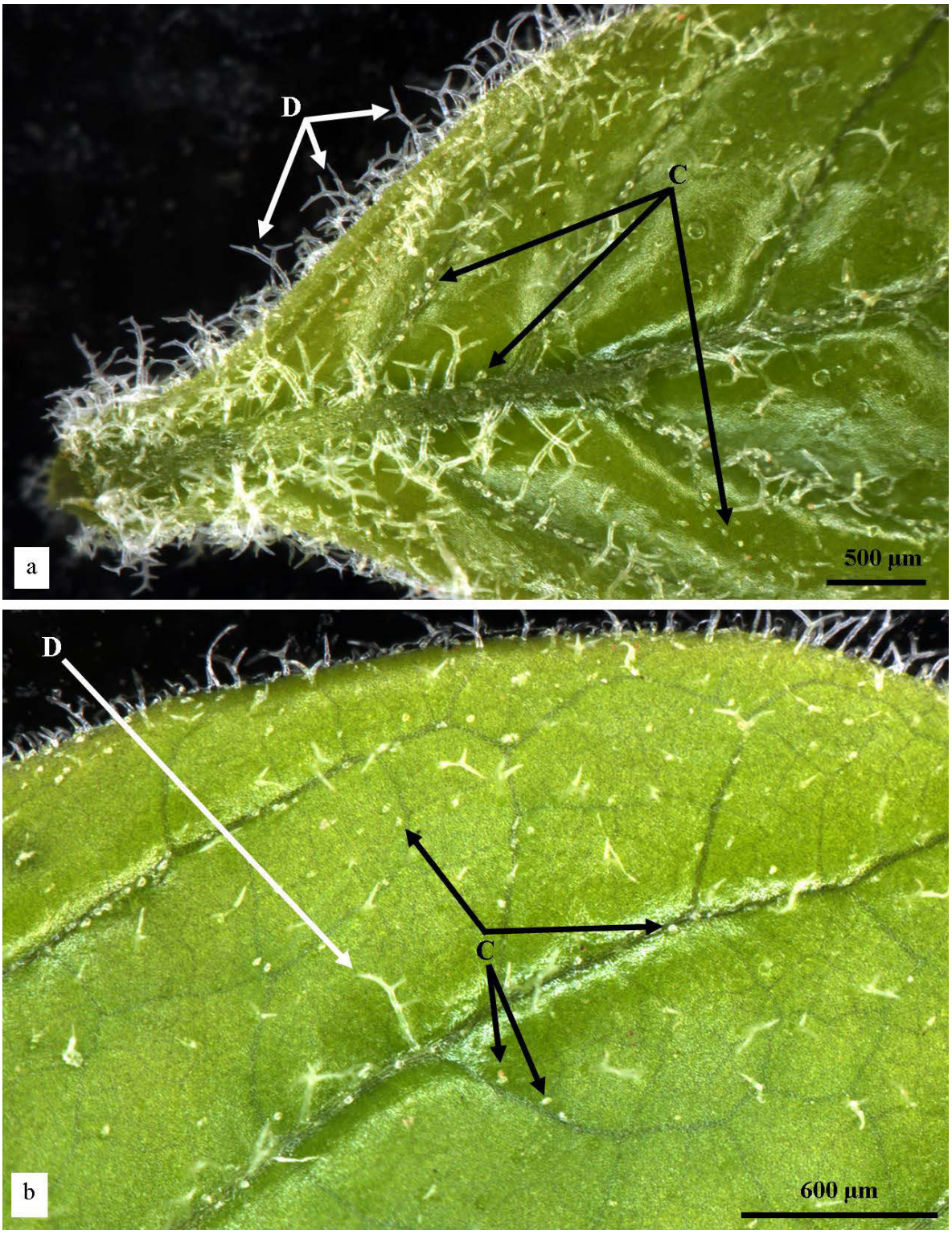

D
C
a
500 µm
D
C
b
600 µm

Figure 6. Stereomicrographs showing non-glandular bicellular and non-glandular dendritic trichomes on mature leaves of *W. somnifera*.

(a) Non-glandular bicellular (B) trichomes on adaxial surface.
(b) Non-glandular dendritic (D) trichomes on abaxial surface.

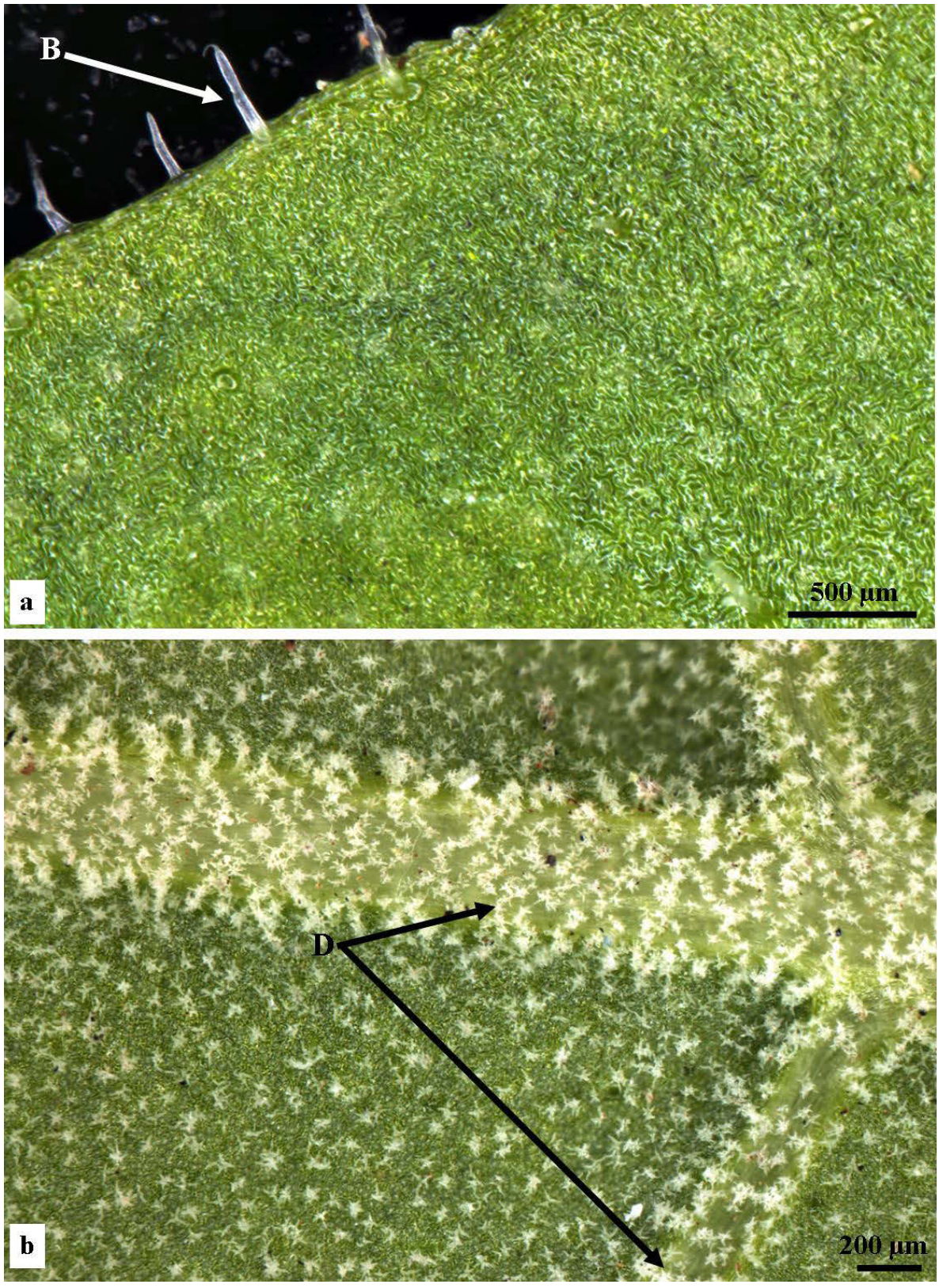
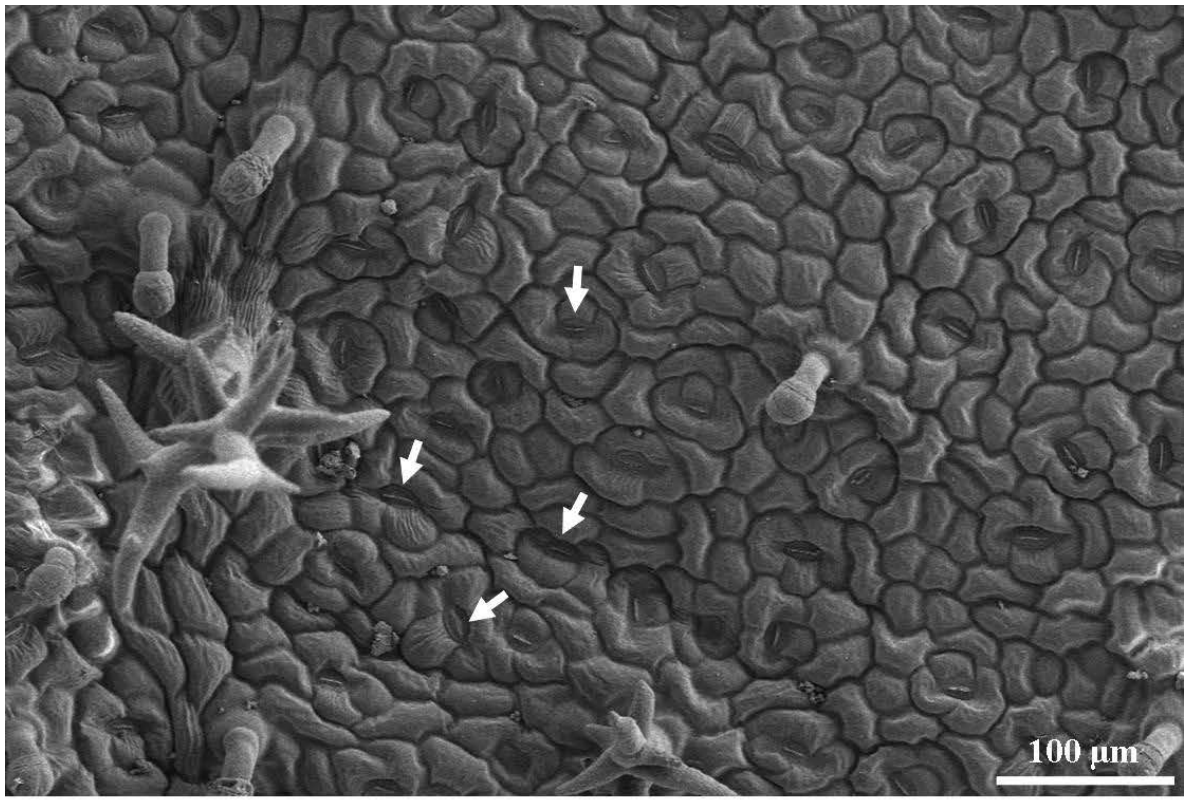

B
a
500 µm
D
b
200 µm

Figure 7. SEM micrographs showing stomata on *W. somnifera* leaves.

a) Closed stomata (indicated by arrows) on the adaxial surface of a young leaf.
b) Open stomata (indicated by arrows) on the abaxial surface of a mature leaf.


100 µm

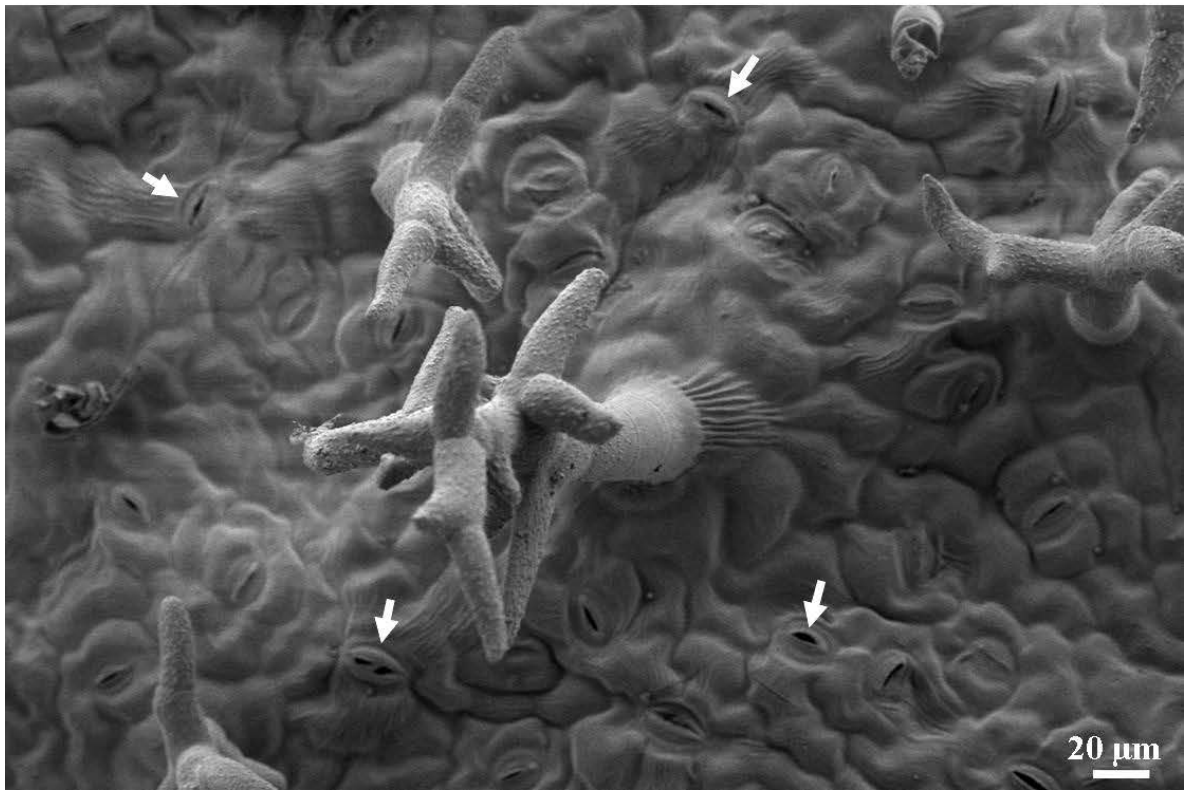
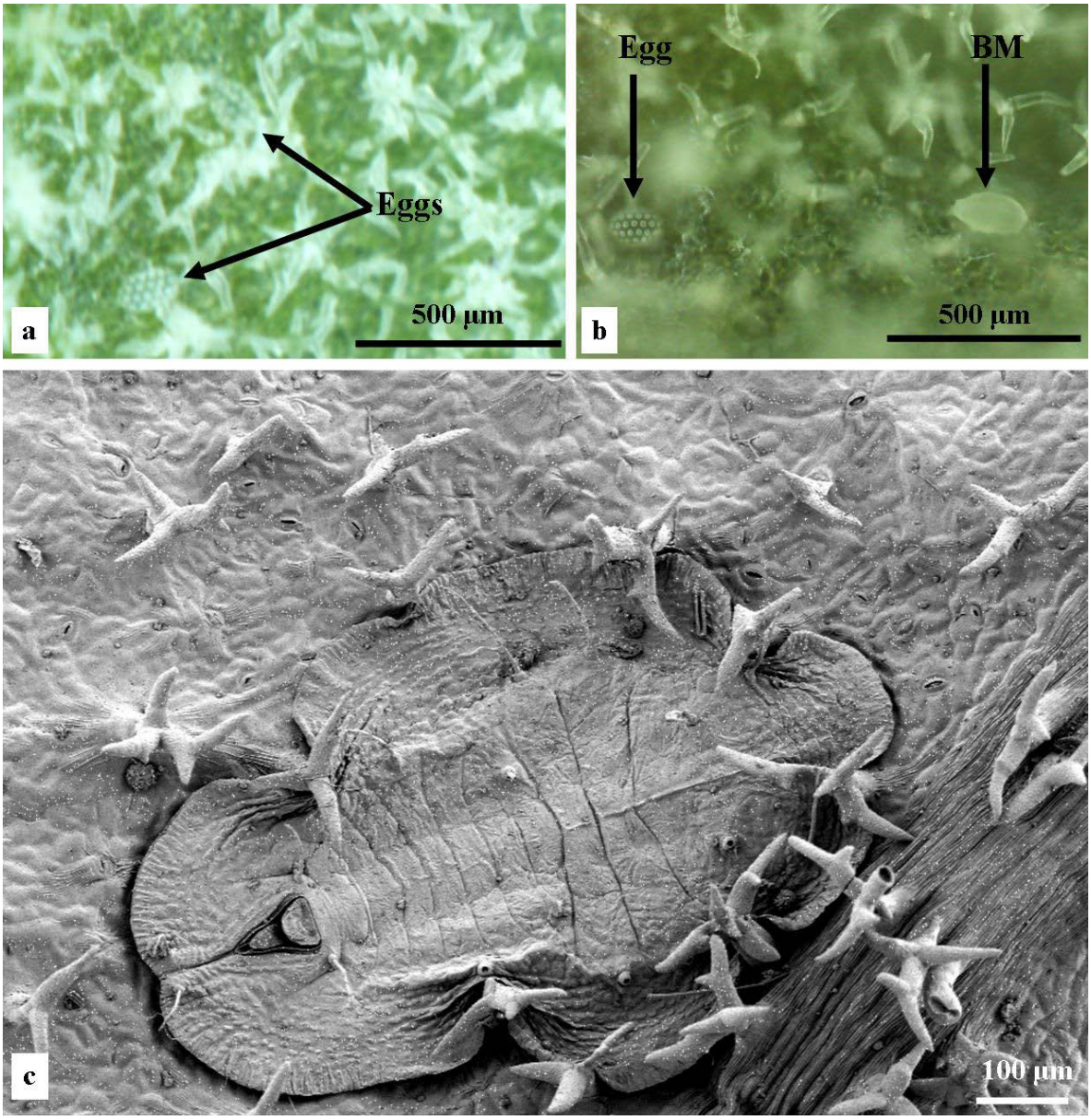

20 µm

Figure 8. Broad mites and scale insects associated with *W. somnifera* leaves.

a) Stereomicrograph showing broad mite eggs on the abaxial surface of a young leaf.
b) Stereomicrograph showing a broad mite egg and a broad mite (BM) present on the abaxial surface of a young leaf.
c) SEM of a scale insect on the abaxial surface of a mature leaf.


Eggs
500 µm
a
Egg
BM
500 µm
b
c
100 µm

4.1.1. Glandular secretory trichomes

ESEM showed that the small structures observed using stereomicroscopy to be glandular capitate trichomes with single-celled stalks and a six-celled secretory head (Fig. 9a–b). The secretions of glandular trichomes were also observed (Fig. 9a). Post-secretory trichomes did not appear to have a definitive rupture in the secretory head. Glandular capitate trichomes (Fig. 9, 10 & 11) on mature leaves of *W. somnifera* were approximately 60.52 ± 7.64 μm in length, with secretory heads having an average diameter of 33.66 ± 5.85 μm. These secretory trichomes were uniseriate and arose directly from single protodermal cells (Fig. 20–25) and were occasionally orientated parallel to the leaf surface (Fig. 9a–b, 10b & 11a). Glandular capitate trichomes did not exhibit micro-ornamentations on outer surfaces. Histochemical staining of leaf sections showed that all cells of capitate trichomes contained one or more phytochemical groups (Fig. 11b–c & 20–25).

Figure 9. ESEM micrographs showing glandular capitate and non-glandular dendritic trichomes on fresh leaves of *W. somnifera.*

a) Glandular capitate trichome with a single-celled stalk (St) and six-celled secretory head (Sh) and a non-glandular dendritic (D) trichome with a 2-celled stalk (St) and 4 branches (Br) on adaxial surface of emergent leaf section.
b) Glandular capitate (C) and non-glandular dendritic (D) trichomes on adaxial surface of young leaf section. Stomata (Sm) are also visible.

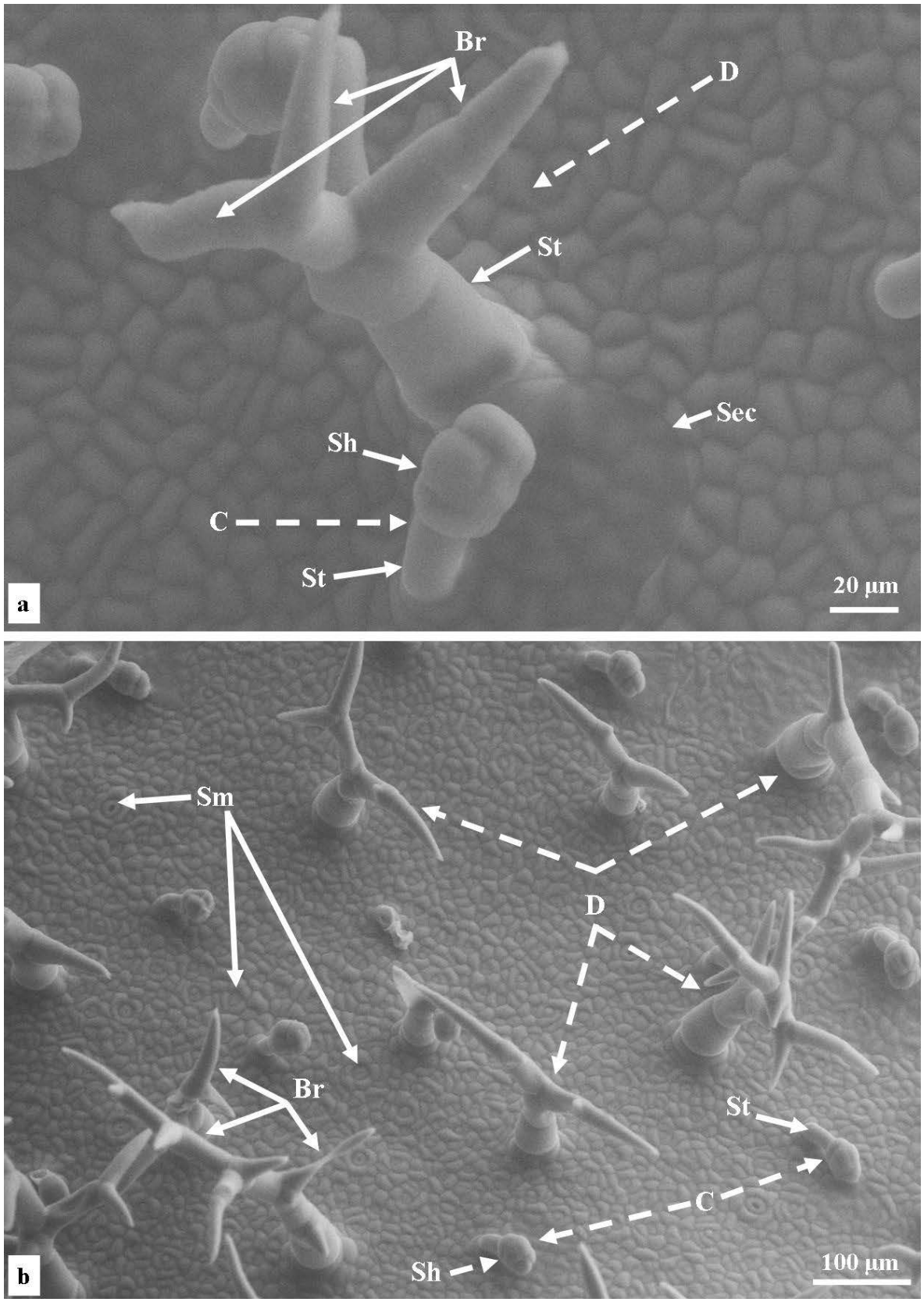
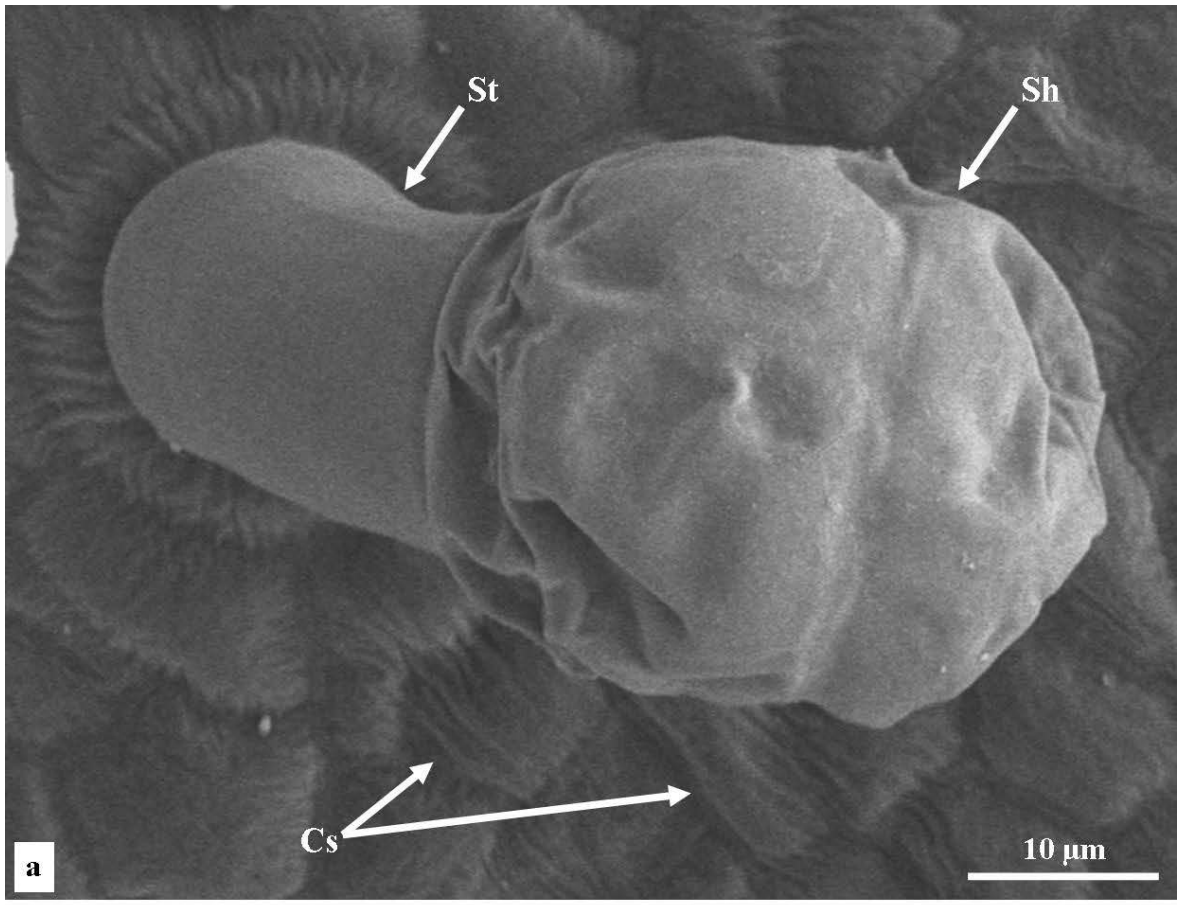

Br
D
St
Sec
Sh
C
St
20 µm
a
Sm
D
Br
St
C
Sh
100 µm
b

Figure 10. SEM micrographs showing glandular capitate trichomes on adaxial leaf surfaces.

a) Glandular capitate trichome with a single-celled stalk (St) and six-celled secretory head (Sh) on emergent leaf surface. Cuticular striations (Cs) are also visible on leaf surface.
b) Glandular capitate trichome adjacent to a non-glandular dendritic trichome. Branches (Br) of dendritic trichome tower over capitate trichome; on young leaf surface.


St
Sh
Cs
a
10 µm

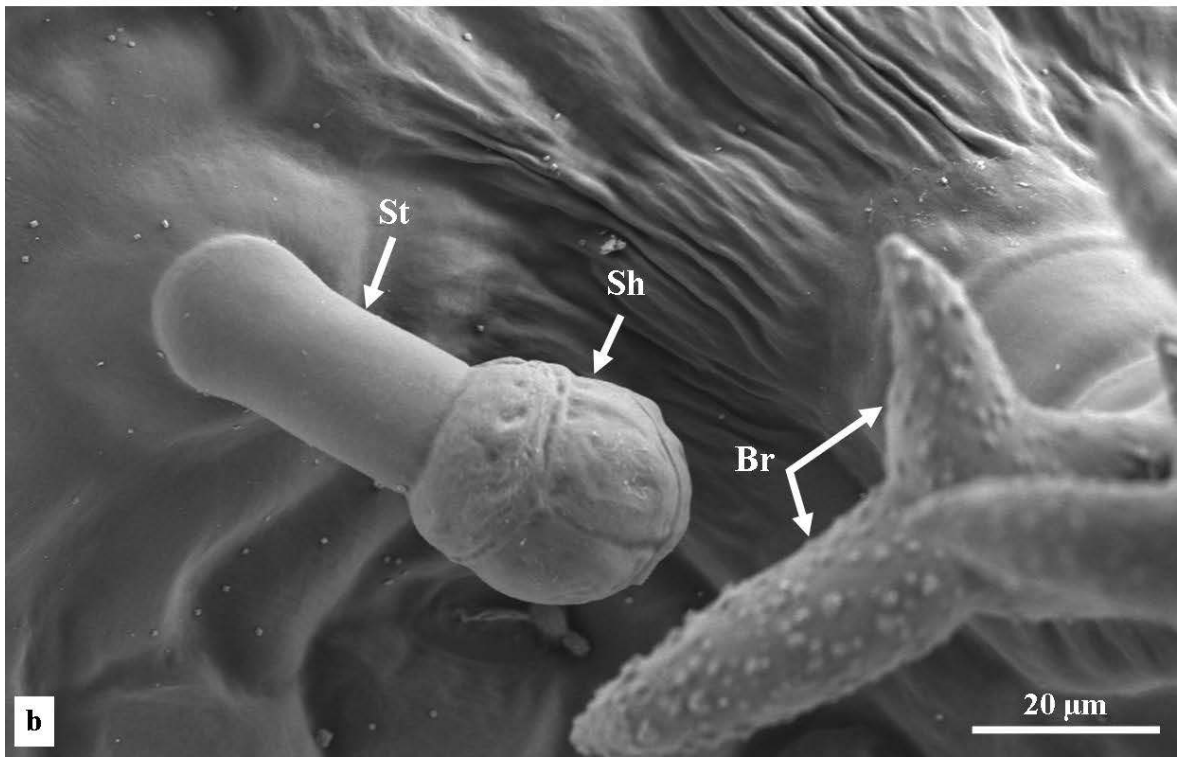
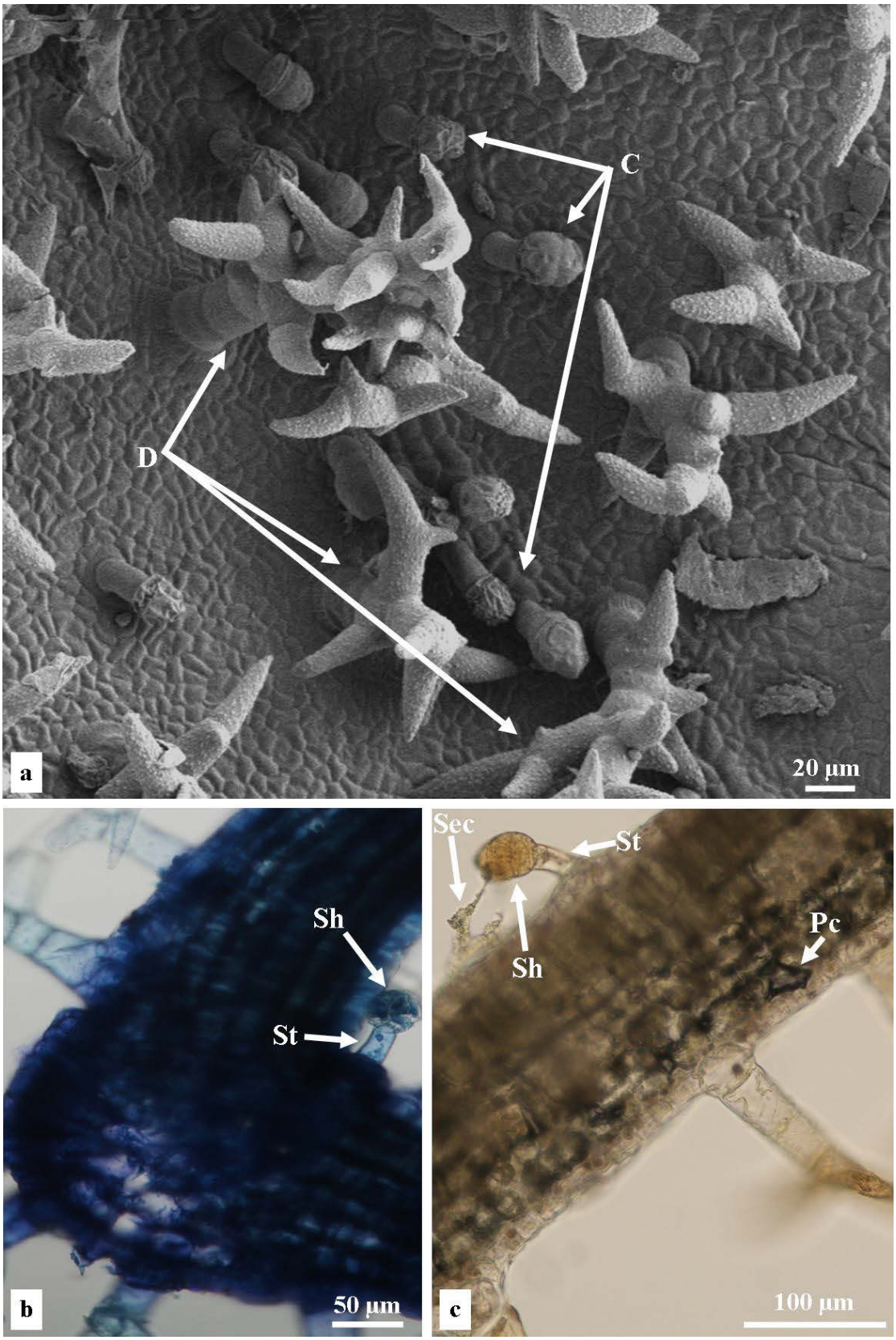

St
Sh
Br
b
20 µm

Figure 11. SEM micrograph and histochemically stained sections showing the structure of glandular capitate trichomes.

a) Six-celled glandular capitate (C) trichomes in close proximity to non-glandular dendritic (D) trichomes on emergent adaxial leaf surface.
b) Glandular capitate trichome with single-celled stalk (St) and six-celled secretory head (Sh) on emergent leaf section stained with Nile blue.
c) Glandular capitate trichome with single-celled stalk (St) and six-celled secretory head (Sh) on emergent leaf section stained with Wagner's and Dittmar reagents. The secretion (sec) of the capitate trichome has been released from the secretory head. A prismatic crystal (Pc) is also present within the leaf section.


C
D
a
20 µm
Sh
St
b
50 µm
Sec
St
Sh
Pc
c
100 µm

4.1.2. Non-glandular trichomes

The non-glandular dendritic trichomes consisted of 2–4 stalk cells and varying branch numbers (Fig. 12a–b & 13a–b). On mature leaves, the average length and width of dendritic trichomes are 125.96 ± 52.11 µm and 155.15 ± 30.06 µm respectively. Non-glandular bicellular and multicellular trichome types were uniseriate with the uppermost cells being tapered (Fig. 14a–b). These trichome types are approximately 138.85 ± 89.55 µm in length and 36.05 ± 10.75 µm in width. Non-glandular multicellular trichomes consisted of approximately 3–6 cells.

All three non-glandular trichome types exhibited microornamentation known as cuticular warts on the outer surface (Fig. 12a–b). Transverse sections cut for histochemical staining showed that the non-glandular dendritic, bicellular and multicellular trichomes originated from single protodermal cells. All three non-glandular trichome types were highly vacuolated (Fig. 13a–b, 14b & 20–25).

Figure 12. SEM micrographs showing non-glandular dendritic trichomes with cuticular warts.

a) Side view of a non-glandular dendritic trichome on mature abaxial leaf surface. Cuticular warts (Cw) are present on the surface.
b) Aerial view of a non-glandular dendritic trichome on emergent adaxial leaf surface. Cuticular warts (Cw) are present on the surface of the trichome and cuticular striations (Cs) are visible on the leaf surface.

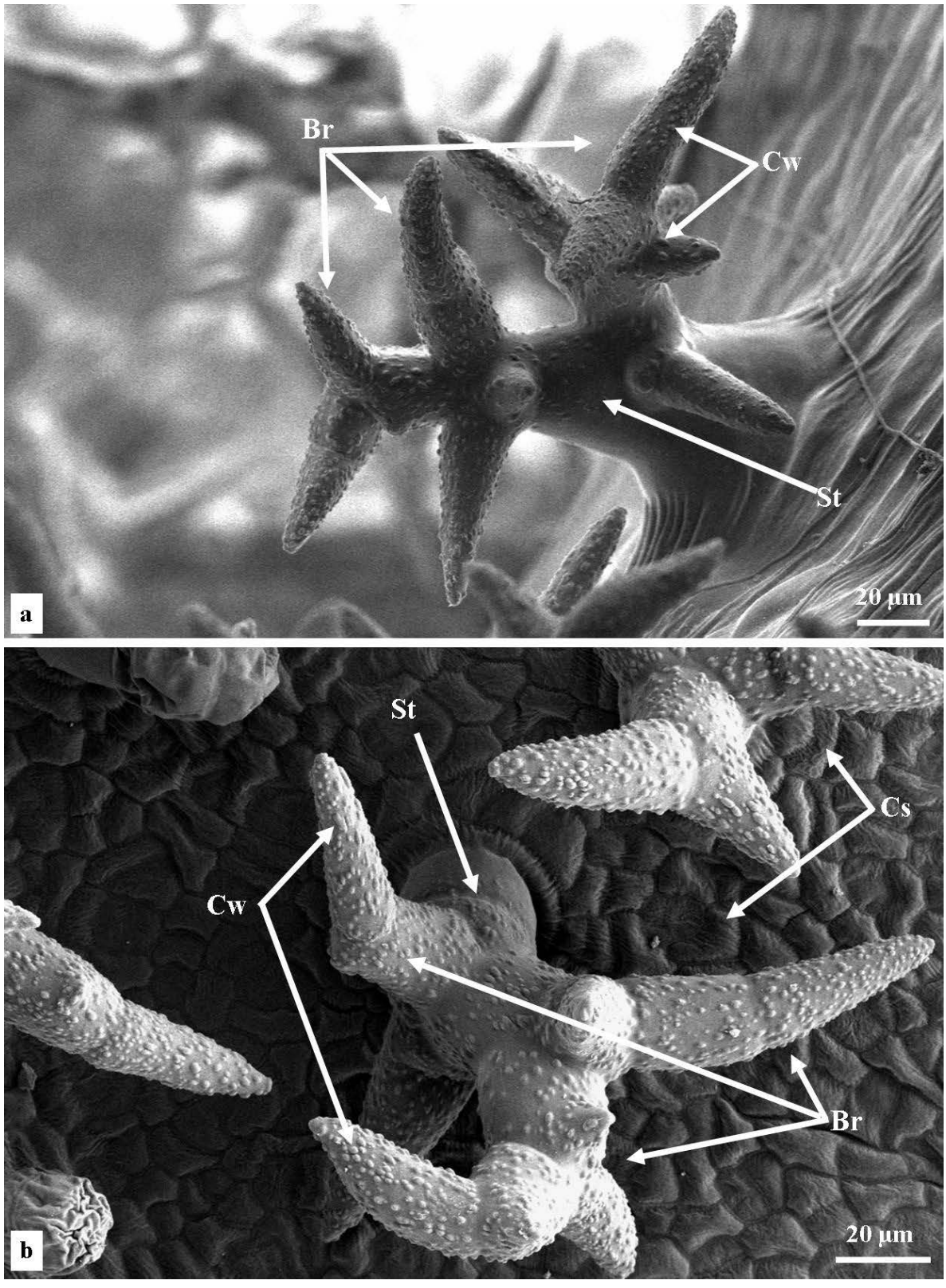
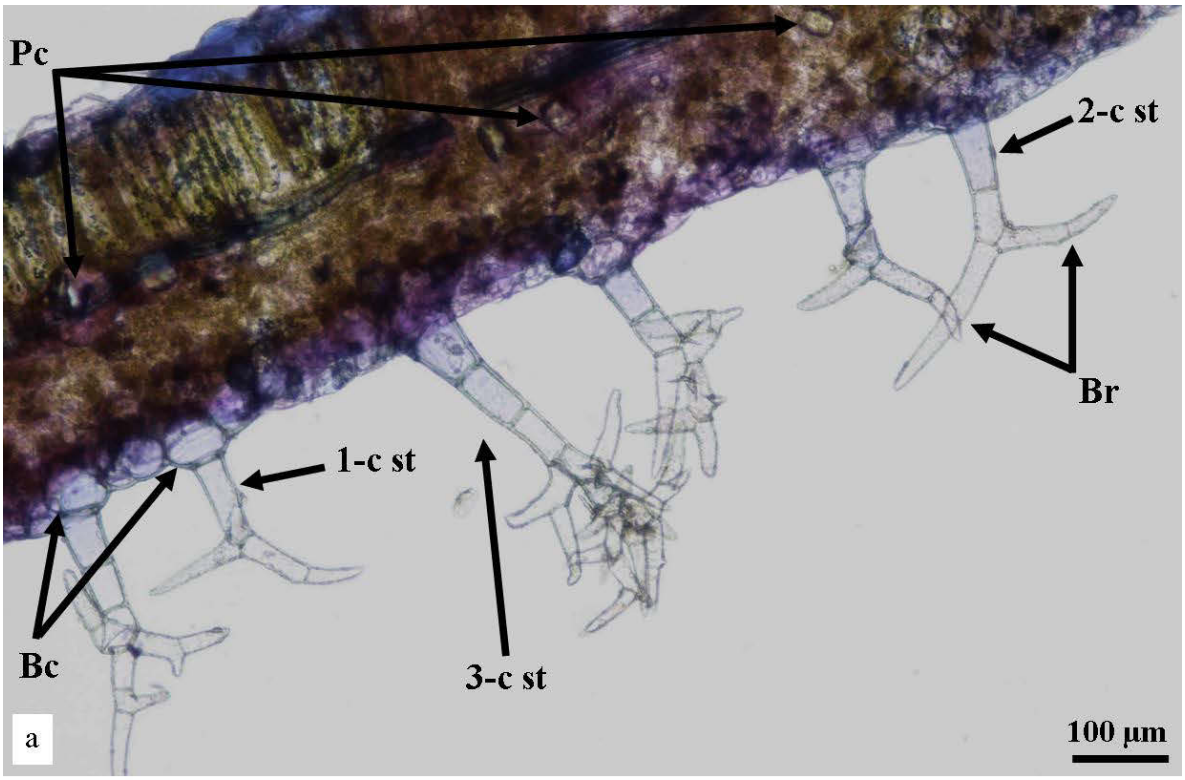

Br
Cw
St
20 µm
a
St
Cs
Cw
Br
20 µm
b

Figure 13. Non-glandular dendritic trichomes on young leaves.

a) Toluidine blue stained section containing non-glandular dendritic trichomes with various stalk cell numbers (1-, 2- & 3-c st) and prismatic crystals (Pc).
b) Section stained with Sudan III and IV containing a non-glandular trichome with a single-celled stalk, emanating from a single basal cell (Bc).


Pc
2-c st
Br
1-c st
Bc
3-c st
a
100 µm

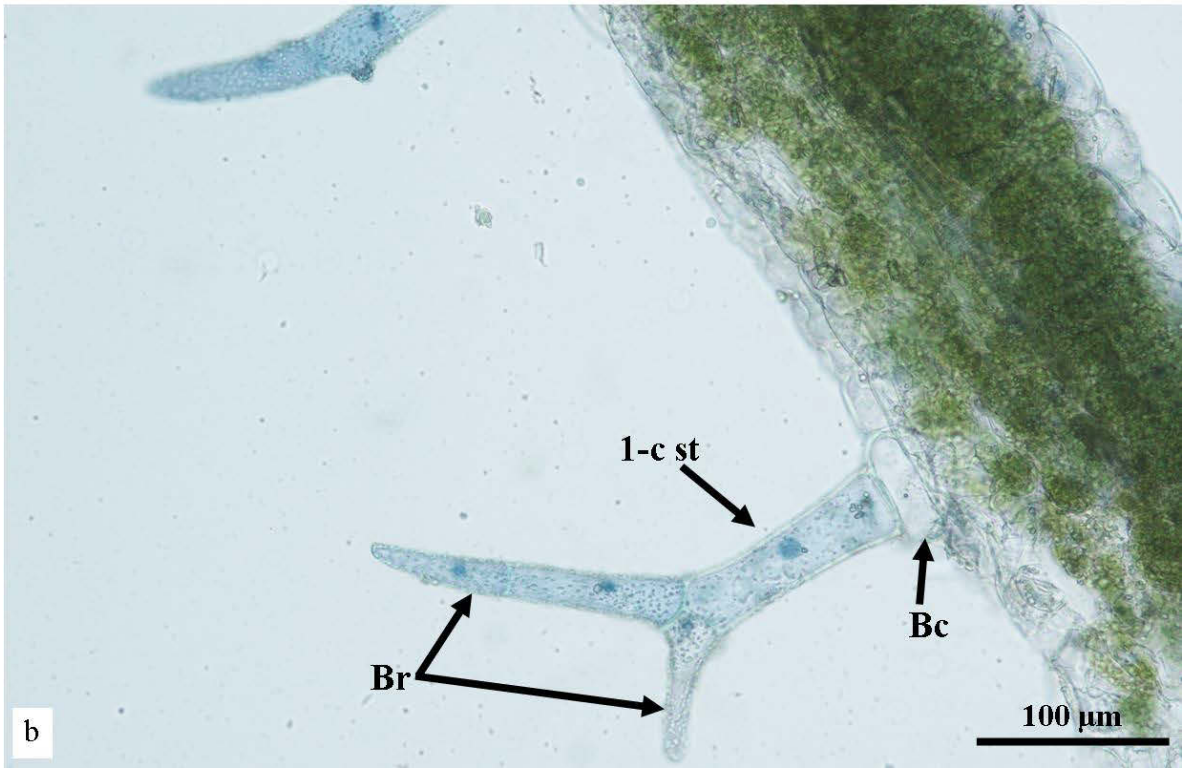
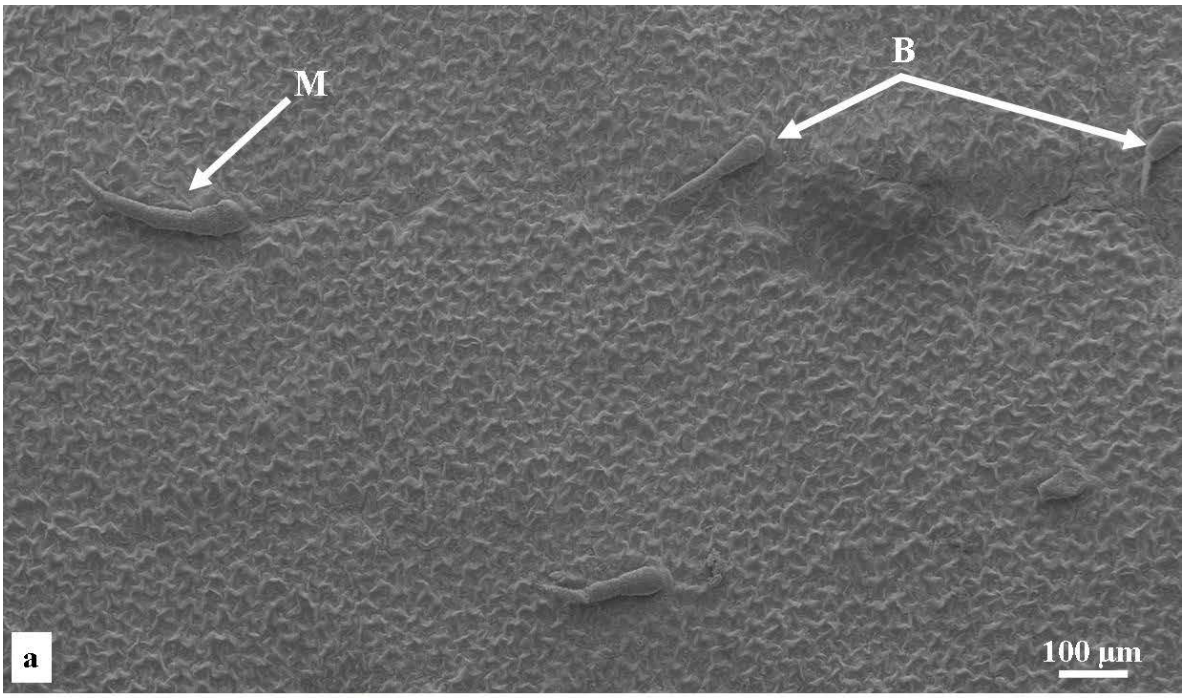
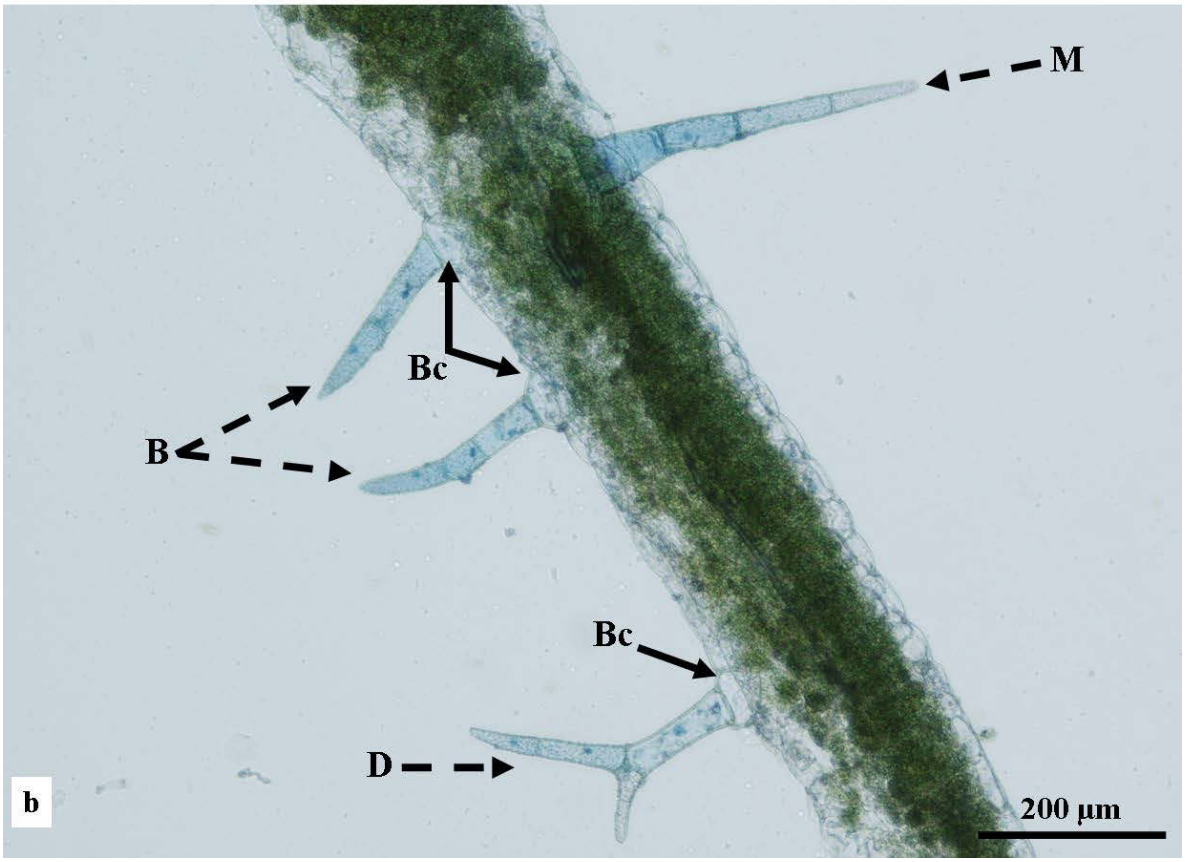

1-c st
Bc
Br
b
100 µm

Figure 14. SEM micrograph and histochemically stained section showing the structure of non-glandular bicellular and multicellular trichomes.

a) Bicellular (B) and multicellular (M) trichomes on adaxial surface of mature leaf.
b) Section stained with Sudan black containing a 4-celled uniseriate multicellular (M) trichome, bicellular trichomes emanating from single basal cells (Bc) and a non-glandular dendritic trichome (D) with a single-celled stalk, emanating from a single basal cell (Bc).


M
B
a
100 µm


M
Bc
B
Bc
D
b
200 µm

4.2. Trichome distribution and density

On close inspection of the images obtained of adaxial and abaxial surfaces, trichome density appeared to decrease with progressive leaf development (Fig. 15a–f). Emergent leaves possessed a greater density of trichomes when compared to mature leaves. Abaxial surfaces also appeared to contain a higher trichome density than adaxial surfaces. Foliar trichomes on leaves at all stages of development appeared predominantly on the mid-vein, with non-glandular dendritic trichomes appearing to 'arch over' glandular capitate trichomes. On emergent leaves that were sampled around February of 2013, trichomes were so dense that it was difficult to view the leaf surface. Young and mature leaves sampled at this time also appeared to contain a higher trichome density as compared to those sampled during May–July of 2012.

The MANOVA statistical test confirmed that the density of glandular capitate trichomes was not the same across the five locations: emergent adaxial, young adaxial, young abaxial, mature adaxial and mature abaxial surfaces ($df=4$, $F=44.519$, $p<0.0005$). According to the Tukey's test, glandular capitate trichome density was significantly different between the adaxial surfaces of emergent and mature leaves ($p<0.0005$) and the abaxial surfaces of young and mature leaves ($p=0.023$). The density of glandular capitate trichomes also differed between the adaxial and abaxial surfaces of young leaves ($p<0.0005$). The difference in glandular trichome density is also demonstrated in Figure 16, where glandular trichomes were absent on abaxial leaf surfaces at all stages of development. With respect to the adaxial surfaces, the marked decrease of glandular trichome density from emergent (34 ± 36 /mm²) to young (25 ± 15 /mm²) and mature (4 ± 5 /mm²) is also shown in Figure 16.

The density of non-glandular dendritic trichomes was also significantly different across the five locations ($p<0.0005$). The Tukey's test showed these differences to be between the adaxial and abaxial surfaces of young leaves ($p=0.009$) and mature leaves ($p<0.0005$). Figure 16 also showed the significant differences in dendritic trichome densities between adaxial and abaxial leaf surfaces, however there was no direct pattern across emergent, young and mature leaves. When comparing adaxial leaf surfaces, emergent leaves (19 ± 15 /mm²) have a greater density of dendritic trichomes compared to that of mature leaves (10 ± 16 /mm²), with the density on young leaves being intermediate (7 ± 3/mm²).

There was no significant difference in the densities of non-glandular bi-/multi-cellular trichomes across the five different locations (p=0.157). This was confirmed by the Tukey's test where p<0.0005 across all comparisons. However, Figure 16a & b showed bi-/multi-cellular non-glandular trichomes to be present only on emergent adaxial (1 ± 2 /mm²), mature adaxial (1 ± 1 /mm²) and young abaxial leaf (1 ± 2 /mm²) surfaces.

There was no significant difference in the length of glandular trichomes across the leaf developmental stages (df=3, F=0.000, P=1.000). According to the Tukey's test, the length of glandular trichomes only differed between the adaxial surfaces of emergent and young leaves (P<0.0005). This difference however was relatively small. Figure 17a showed that the average length of glandular trichomes on abaxial surfaces of emergent leaves was 65 ± 12 µm whilst that of young leaves was 57 ± 9 µm. There was no difference in glandular trichome length between adaxial surfaces of emergent and mature (p=0.276) and young and mature (p=0.571) leaves. Due to a significantly low number of glandular trichomes on the abaxial surface mature leaves; the Tukey's test did not take into account the comparisons between young abaxial and mature abaxial surfaces. Figure 17b however showed that glandular trichomes on abaxial surfaces of mature leaves (64 ± 3 µm) were much longer than those of young leaves (65 ± 12 µm).

There was also no significant difference in the length of bi-/multi-cellular trichomes across the leaf developmental stages (df=3, F=2.640, P=0.093). Due to there being a significantly low number of this trichome type on leaves, the Tukey's test only showed significant results for the comparison between the adaxial surfaces of emergent and mature leaves. There was no difference in trichome length between these developmental stages (p=0.409). According to figure 17a however, bi-/multi-cellular trichomes were absent on the adaxial surfaces of young leaves whilst the average length of this trichome type on the adaxial surfaces of emergent and mature leaves were 114 ± 38 µm and 157 ± 87 µm respectively. On abaxial surfaces, average length of bi-/multi-cellular trichomes were 69 ± 15 µm and 48 ± 0 µm on young and mature leaves (Figure 17b).

With regard to dendritic trichomes, there was a significant difference in length across the leaf developmental stages (df=4, F=8.189, P<0.0005). The only difference however, occurred between the adaxial surfaces of emergent and young leaves (p=0.002). According to figure 17a, dendritic trichomes on adaxial surfaces were greater in length on emergent leaves (181 ±78µm) as compared to young leaves (124 ± 54 µm). There was no significant difference in the length of dendritic

trichomes between adaxial surfaces of emergent and mature ($p=0.204$) and young and mature ($p=0.673$), as well as the abaxial surfaces of young and mature ($p=0.328$) leaves.

Figure 15. SEM micrographs showing the decrease in trichome density with increasing leaf development.

a) Adaxial surface of emergent leaf.
b) Abaxial surface of emergent leaf.
c) Adaxial surface of young leaf.
d) Abaxial surface of young leaf.
e) Adaxial surface of mature leaf.
f) Abaxial surface of mature leaf.

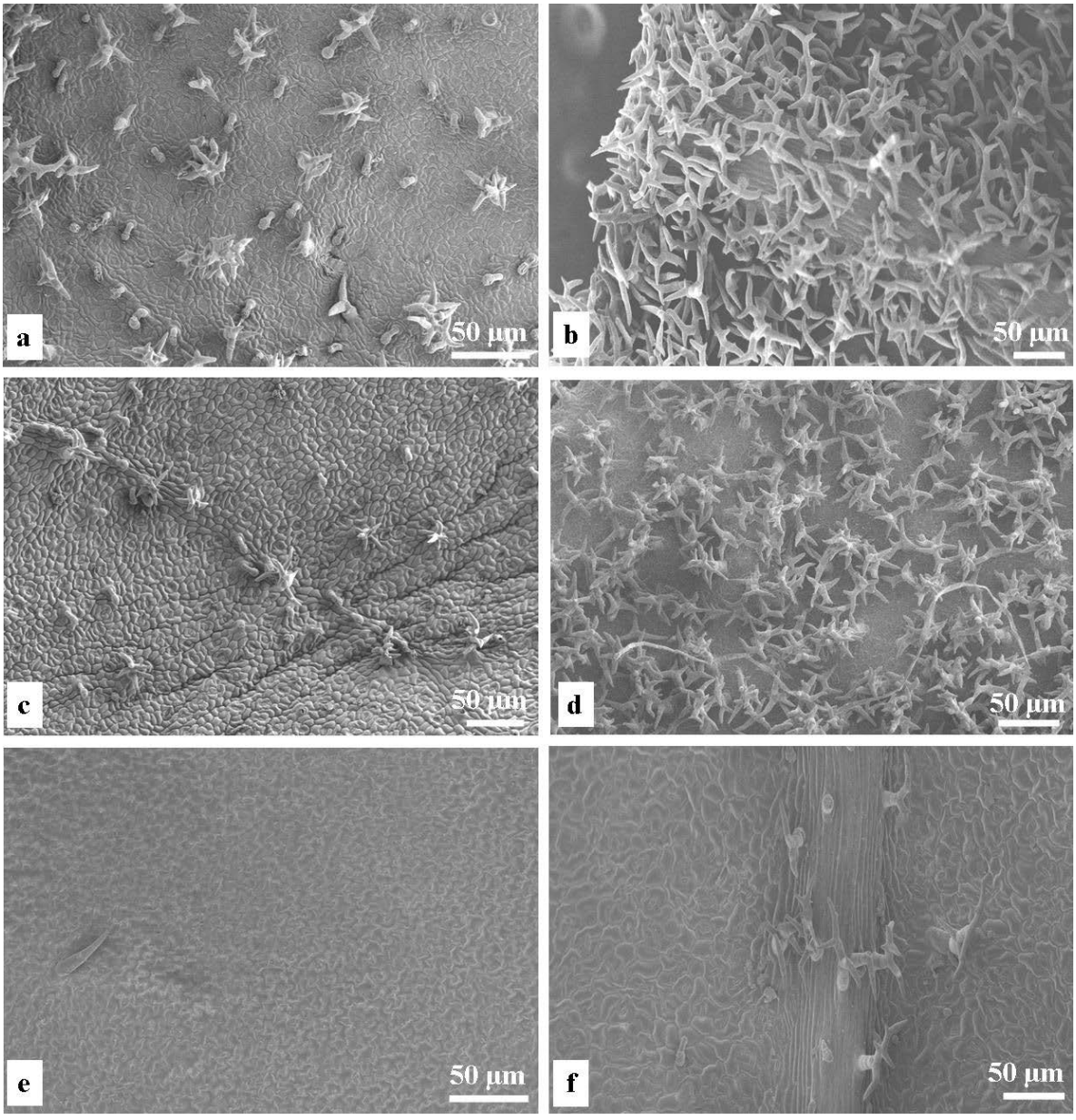

a
50 µm
b
50 µm
c
50 µm
d
50 µm
e
50 µm
f
50 µm

Figure 16. Frequency of glandular capitate (Capitate), non-glandular dendritic (Dendritic) and non-glandular bicellular and multicellular (Bi-/Multi-cellular) trichomes at different leaf developmental stages, emergent, young and mature.

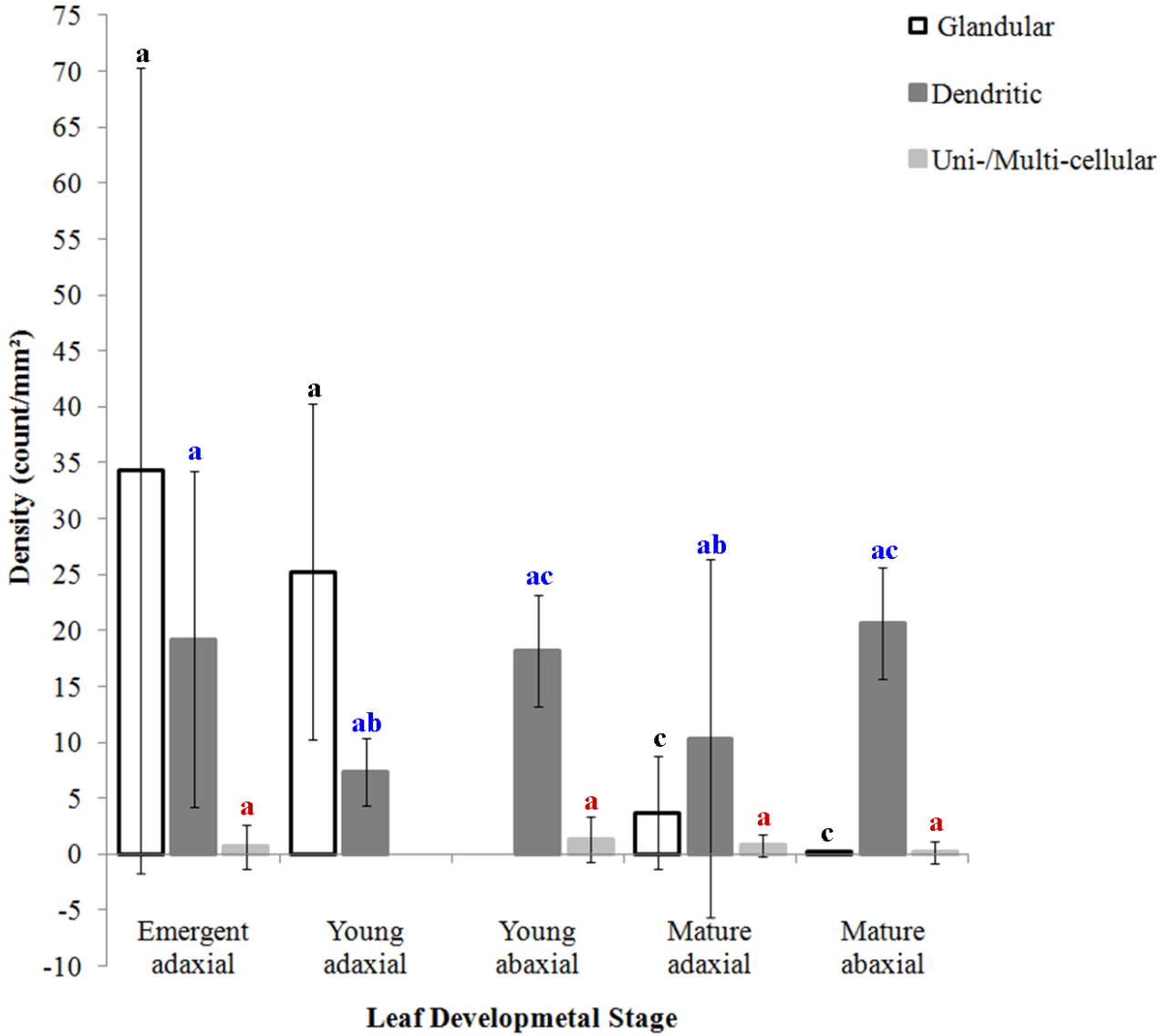
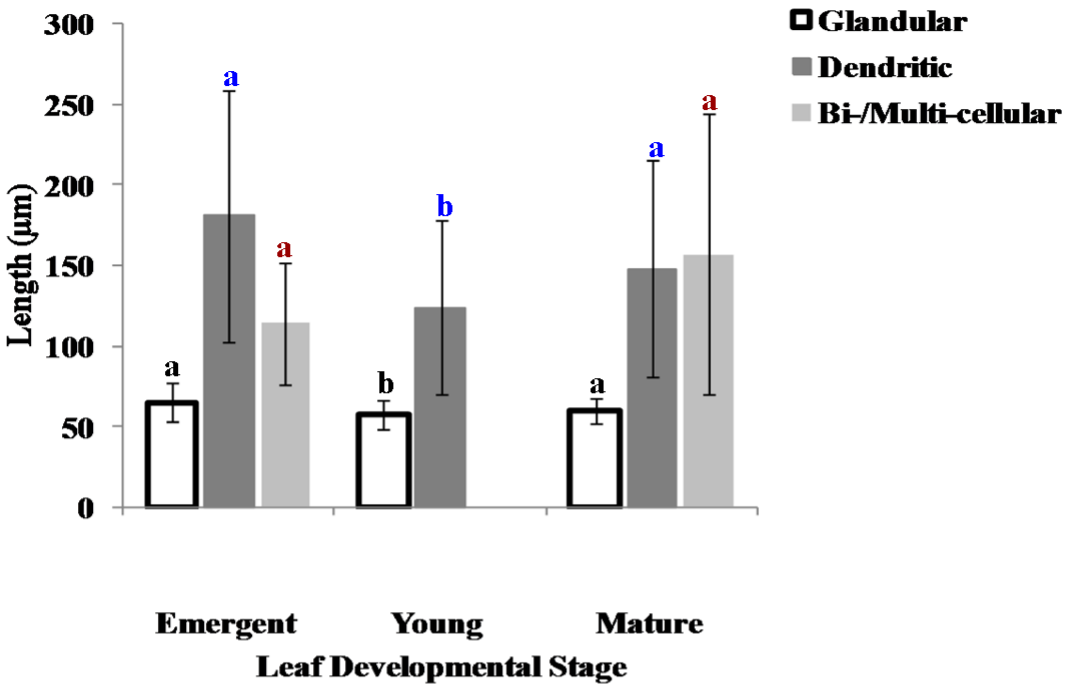

Glandular
Dendritic
Uni-/Multi-cellular
Density (count/mm²)
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
-5
-10
a
a
a
a
ab
a
ac
a
c
ab
a
c
ac
a
Emergent adaxial
Young adaxial
Young abaxial
Mature adaxial
Mature abaxial
Leaf Developmetal Stage

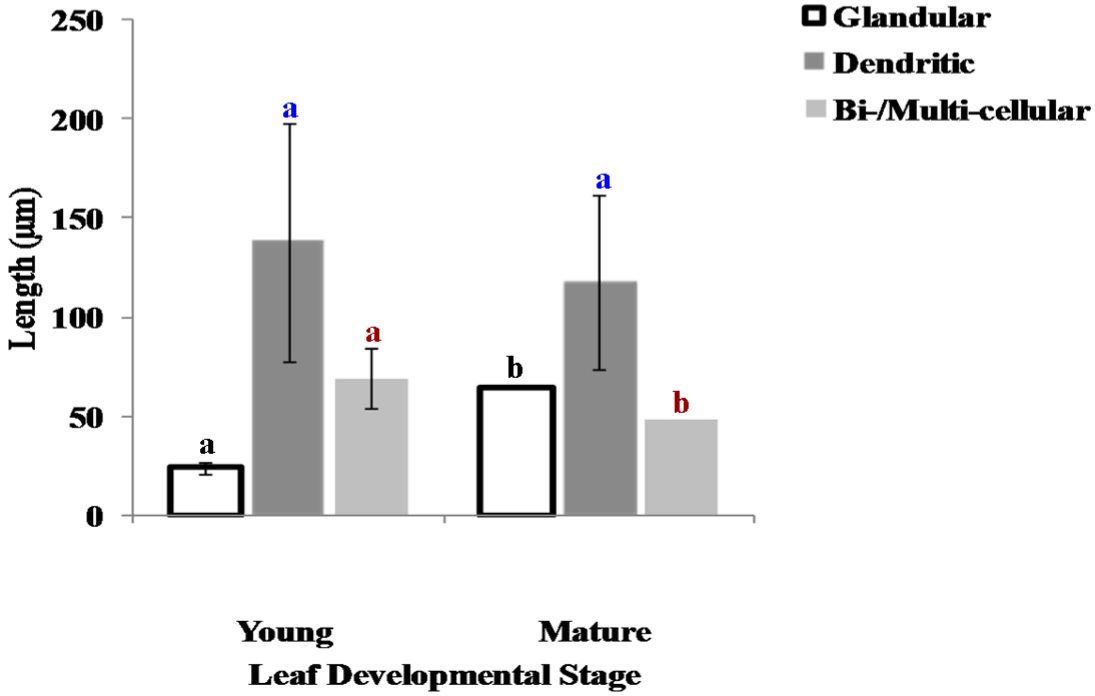
Figure 17. Average lengths of glandular capitate (Capitate), non-glandular dendritic (Dendritic) and non-glandular bicellular and multicellular (Bi-/Multi-cellular) trichomes at different leaf developmental stages, emergent, young and mature.

a) Trichome lengths on adaxial surfaces.
b) Trichome lengths on abaxial surfaces.

a


300
250
200
150
100
50
0
Length (µm)
a
a
a
b
b
a
a
a
Glandular
Dendritic
Bi-/Multi-cellular
Emergent
Young
Mature
Leaf Developmental Stage

b


250
200
150
100
50
0
Length (µm)
a
a
a
b
a
b
Glandular
Dendritic
Bi-/Multi-cellular
Young
Mature
Leaf Developmental Stage

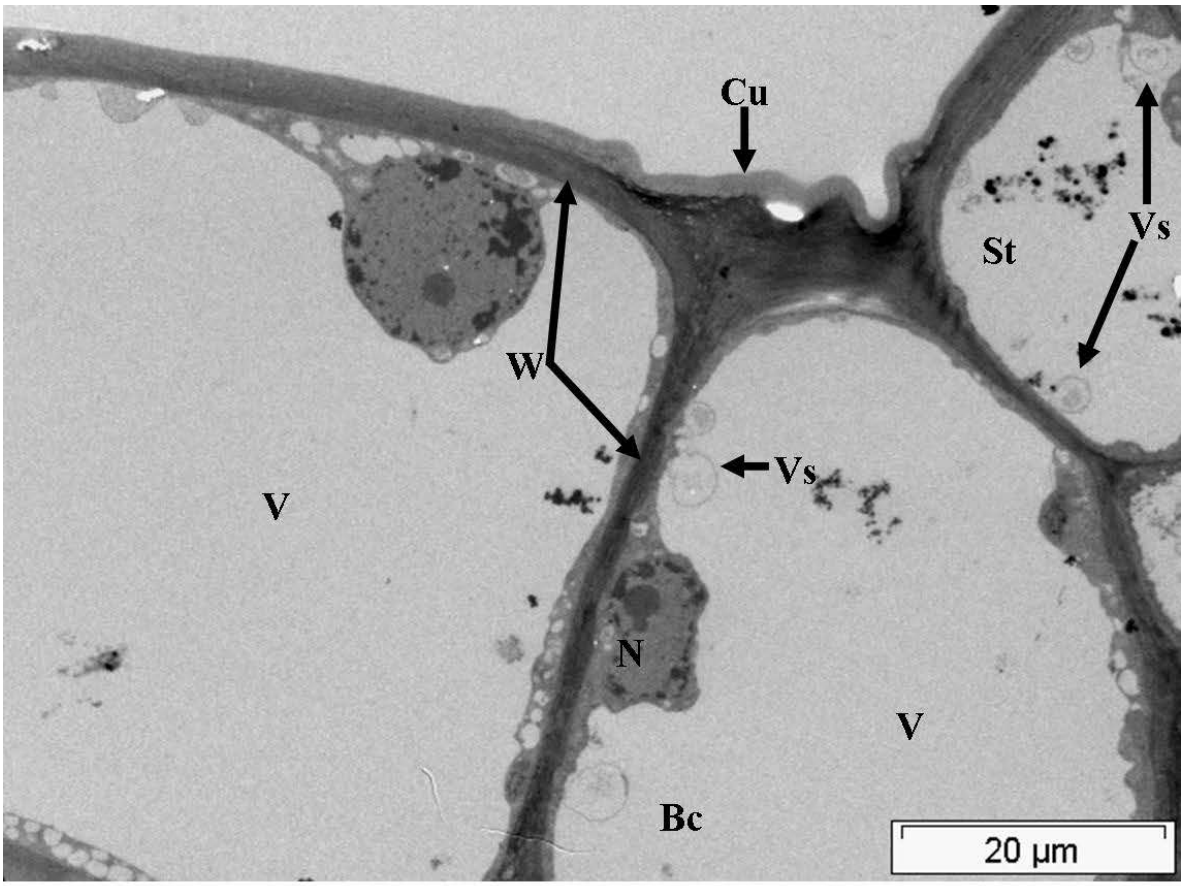
4.3. Trichome Ultrastructure

TEM of the non-glandular dendritic trichomes appeared to be highly vacuolated with the cytoplasm restricted to a narrow peripheral band around the interior of the cell walls (Fig. 18a). Plastids were also found to be closely appressed to the cell walls of dendritic trichomes (Fig. 19a). Basal and stalk cells of dendritic trichomes appeared highly vacuolated (Fig. 19a). Nuclei were prominent in basal and stalk cells. The nuclei of the basal and stalk cells and the cells of the branches were appressed to the cell wall. Numerous vesicles were seen associated with the plasma membrane (Fig. 18 a–b & 19). Multivesicular structures, probably artifactual in nature, were commonly observed within the vacuoles (Fig. 18b). Cuticular warts appeared as bumps or globular protrusions of the cells walls of dendritic trichomes, with cuticular thickening over raised surfaces (Fig. 19a). It was interesting to note the presence of cellulose microfibrils within the cell walls (Fig. 19a).

The heads of glandular trichomes are comprised of 6 cells. These cells appear densely cytoplasmic. Thin cell walls separated the cells of the glandular secretory head of capitate trichomes (Fig. 19b). These head cells contained large nuclei that were appressed to large vacuoles. Noticeable organelles in the cytoplasm include plastids, large nuclei, mitochondria and vacuoles of varying sizes (Fig. 19b). The secretory material within the glandular head appeared to be loosely fibrillar (Fig. 19b).

Figure 18. TEM micrographs of dendritic trichomes.

a) Highly vacuolated (V) basal (Bc) and stalk (St) cells of dendritic trichome. Cuticle (Cu), nucleoli (N) and vesicles (Vs) are also visible.
b) Vesicles (Vc) within stalk cell of dendritic trichome.


Cu
Vs
St
W
Vs
V
N
V
Bc
20 µm

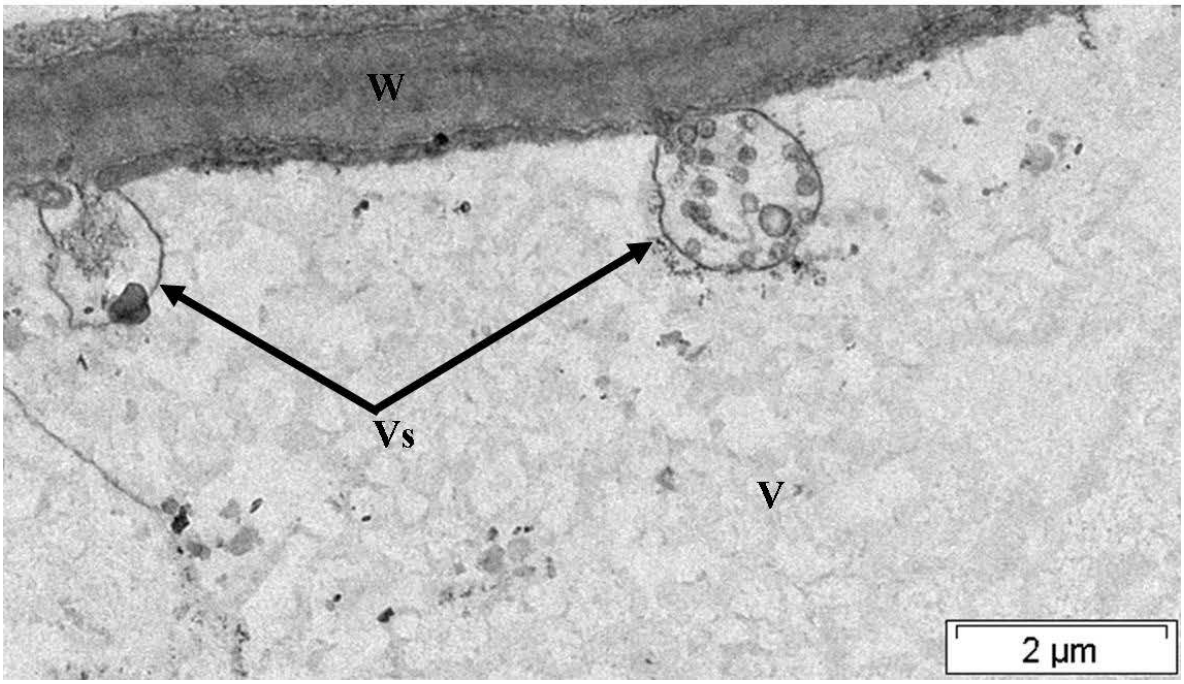
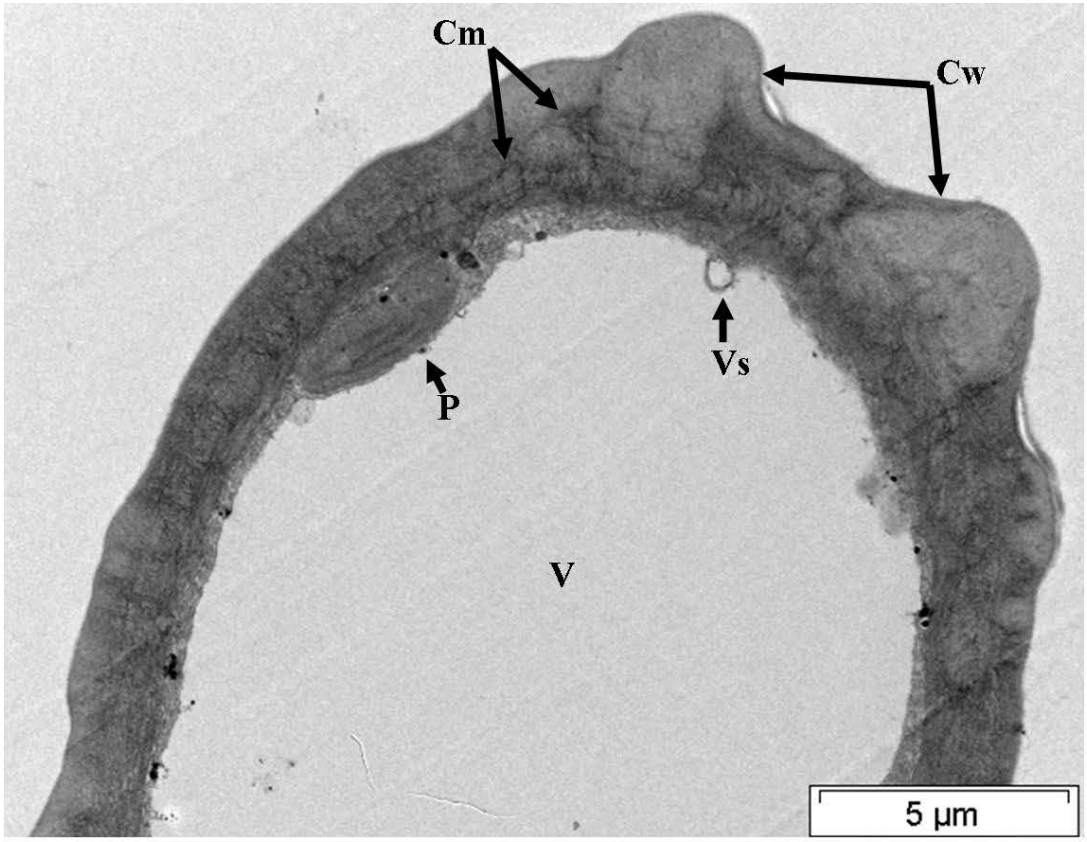
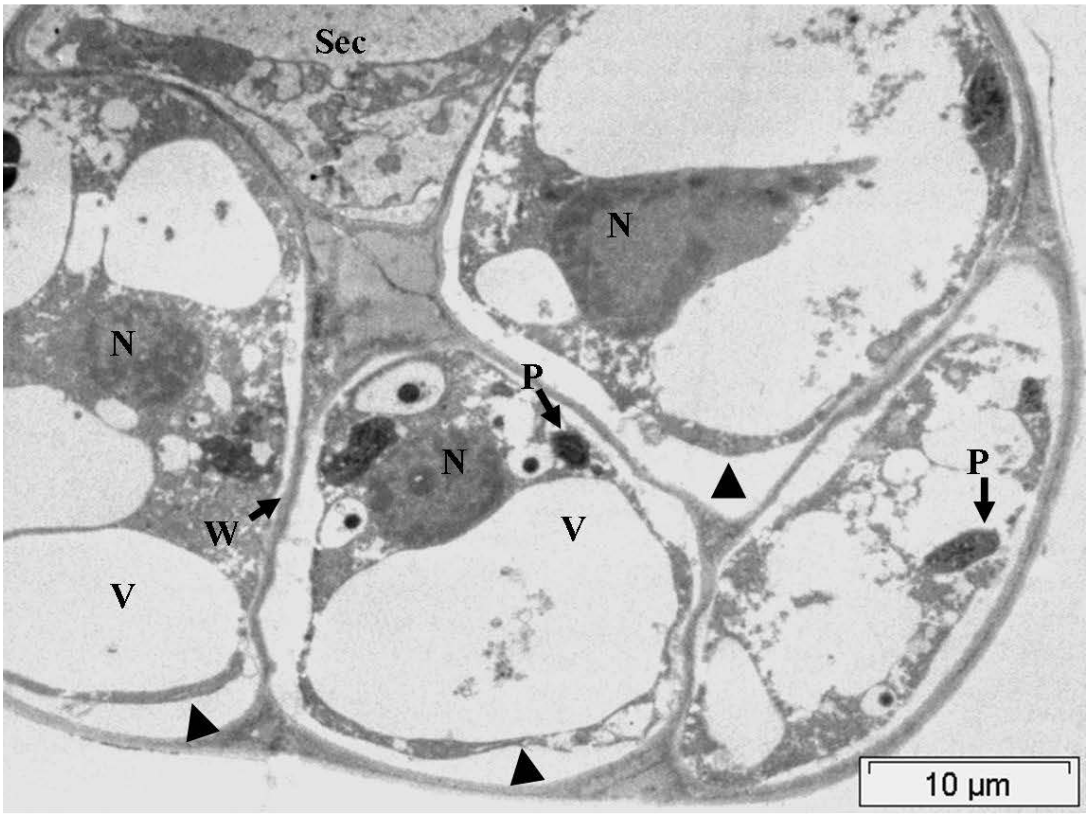

W
Vs
V
2 µm

Figure 19. TEM micrographs of the glandular secretory head of a capitate trichome and cuticular warts of a dendritic trichome.

a) Cuticular warts (Cw) of highly vacuolated (V) dendritic trichome. A plastid (P), vesicle (Vs) and cellulose microfibrils (Cm) are also visible.
b) Glandular secretory head of capitate trichome with thin cell walls (Cw), numerous vacuoles (V), large nuclei (N) appressed to vacuoles and secretory material (Sec) appearing loosely fibrillar. Plasmolysis (indicated by arrowheads) appears to be taking place.


Cm
Cw
Vs
P
V
5 µm


Sec
N
N
P
N
P
W
V
V
10 µm

4.4. Histochemistry

Various histochemical stains (Table 2) were used to detect the presence and localisation of major chemical compound groups in glandular and non-glandular trichomes. Due to the orientation of branched trichomes, some trichomes in the images obtained appeared to be out of focus or damaged.

Sections stained with toluidine blue showed that all cells of the non-glandular trichome types were lignified (Fig. 20b & 21a–b). Staining with ruthenium red also showed that the cell walls of the secretory heads of glandular capitate trichomes and the basal cells of all non-glandular trichome types contained unesterified pectins (Fig. 25a). The stalk cells of capitate trichomes and all cells of non-glandular trichomes were cutinised/suberised, as shown with Sudan black (Fig. 25b) and Sudan III and IV (not represented in figures). Lignin aldehydes (not represented in figures) were present only in glandular capitate trichomes. Lignified and cutinised walls of both glandular and non-glandular trichome types were verified by sections stained with KI/I_2 (not represented in figures).

The secretory head of capitate trichomes stained orange-brown with Wagner's and Dittmar reagents, indicating the presence of alkaloids (Fig 22a–b). All cells of non-glandular bicellular and multicellular, as well as the branches of dendritic trichomes contained alkaloids. The secretory head of capitate trichomes also contained phenolic compounds (Fig. 23b), indicated by the dark greenish-black staining by ferric trichloride. Phenolic compounds were also present in all 3 non-glandular trichome types (Fig. 23a & 23c). Glandular capitate trichomes of sections stained with hydroxylamine hydrochloride solution stained reddish-brown, indicating the presence of esterified pectins (Fig. 24a). The non-glandular trichome types in these sections remained unchanged (unstained). All cells of glandular capitate trichomes and non-glandular dendritic, bicellular and multicellular trichomes contained lipids, as indicated by the blue stain of Nile blue (Fig. 24b). Staining with Sudan black (Fig. 25b), Sudan III and IV (not represented in figures) confirmed the presence of lipids in the secretory heads of capitate trichomes and all cells of the non-glandular trichome types. Polysaccharides were present in the stalk cells of dendritic trichomes, as well as in the lower cells of bicellular and multicellular trichomes (Fig. 25a). Total proteins were present in all cells of the glandular capitate and non-glandular trichome types (Fig. 25c–d).

Calcium oxalate (CaOx) crystals were also present in leaf sections. These crystals occurred in two forms, prismatic (Pc) (Fig. 13a & 20b) and druse (Dc) (Fig. 25a).

Table 1. Observations of histochemical tests on fresh, emergent and young leaf sections of *W. somnifera*.

Compound Group	Stain/s	Glandular trichomes	Non-glandular trichomes	Reaction observed
Alkaloids	Wagner's and Dittmar	+	+	Head cells of capitate trichomes stained orange-brown; non-glandular trichomes stained orange-brown.
Control: Cutin/Lipids	Pre-treatment with chloroform & methanol; Sudan Black			Head cells of capitate trichomes stained brown; non-glandular trichomes stained brown.
	Pre-treatment with chloroform & methanol; Sudan III and IV			Glandular trichomes stained brown; non-glandular trichomes stained brown.
Cutin/Suberin/Lipids	Sudan Black	+	+	Head cells of glandular trichomes stained dark blue-black; non-glandular trichomes stained blue.
	Sudan III and IV	+	+	Glandular trichomes stained orange-red; non-glandular trichomes stained orange-red.
Esterified pectins	Hydroxylamine hydrochloride solution	+	-	Glandular trichomes stained reddish-brown; non-glandular trichomes remained unstained.
Lignin aldehydes	Phloroglucinol	+	-	Head cells of capitate trichomes stained reddish brown; non-glandular trichomes remained unstained.
Control: lipids	Pre-treatment with chloroform & methanol; Nile Blue			Contents of glandular head stained blue, stalk and cells walls of capitate trichomes remained unstained; non-glandular trichomes stained light blue.
Lipids	Nile blue	+	+	Glandular trichomes stained blue; non-glandular trichomes stained blue.
Phenolic compounds	Ferric trichloride	+	+	Head cells of capitate trichomes stained dark greenish- black; non-glandular trichomes stained dark-green.
Polysaccharides/ Unesterified pectins	Ruthenium red	+	+	Head cells of glandular trichomes stained dark pink-red; basal and stalk cells of non-glandular trichomes stained dark pink-red.

+/- indicates presence/absence of compound groups.

Table 1 (Continued). Observations of histochemical tests on fresh, emergent and young leaf sections of *W. somnifera*.

Total proteins	Bromophenol blue	+	+	Capitate trichomes stained blue; non-glandular trichomes stained blue.
Carboxylated polysaccharides/Lignin/ polyphenols	Toluidine blue	-	+	Glandular trichomes remained unstained; non-glandular trichomes stained blue
Starch and cellulose	KI/I_2	-	-	Head cells of capitate trichomes stained orange-brown; non-glandular trichomes stained orange-brown.

+/- indicates presence/absence of compound groups.

Figure 20. Unstained and toluidine stained emergent leaf sections of *W. somnifera*.

a) Dendritic (D) trichomes on an unstained emergent leaf section.
b) Lignified cells (stained blue) of dendritic trichomes on emergent leaf section stained with toluidine blue. Section also contains prismatic crystals (Pc).

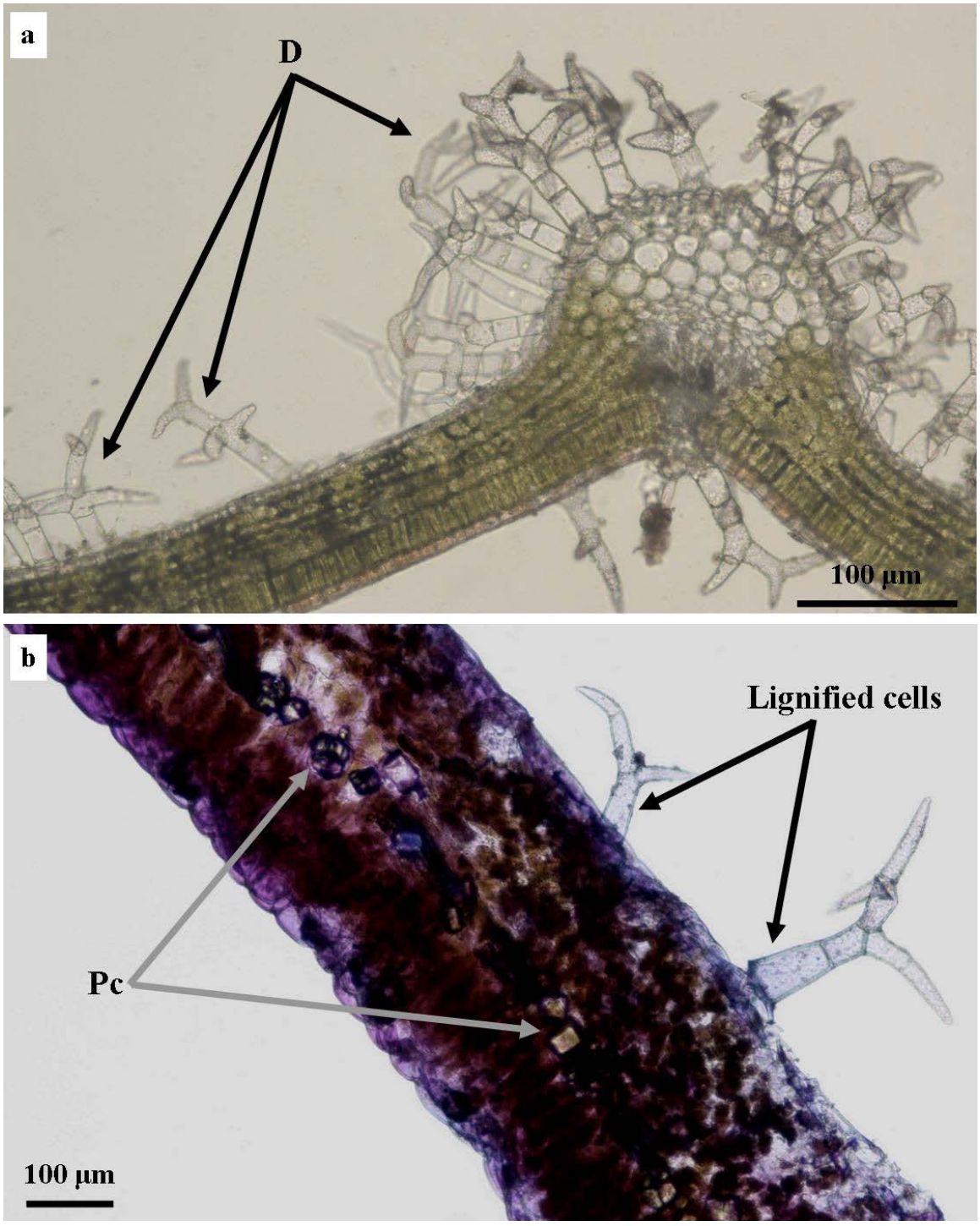
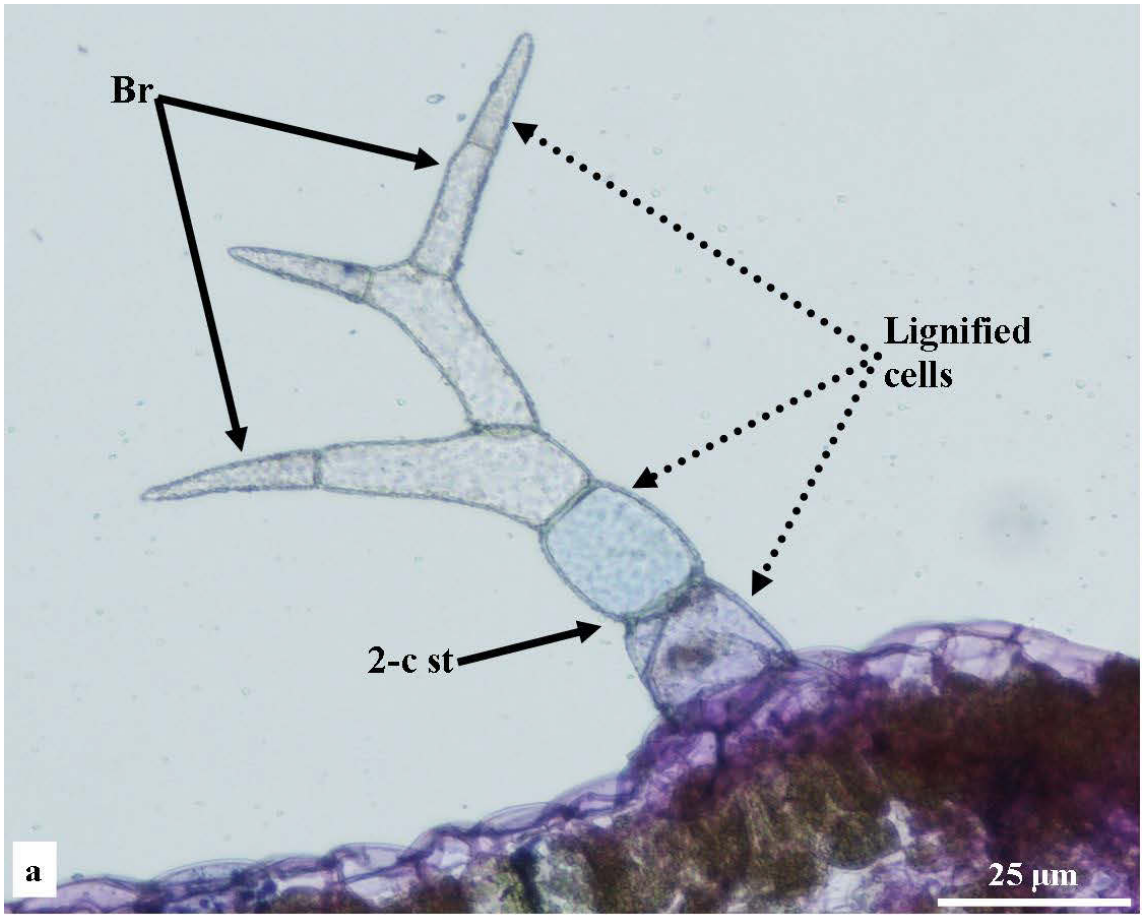

a
D
100 µm
b
Lignified cells
Pc
100 µm

Figure 21. Lignified cells of young leaf sections stained with toluidine blue.

a) Lignified cells (stained blue) of dendritic trichome.
b) Lignified cells (stained blue) of multicellular (M) and bicellular (B) trichomes.


Br
Lignified cells
2-c st
a
25 µm

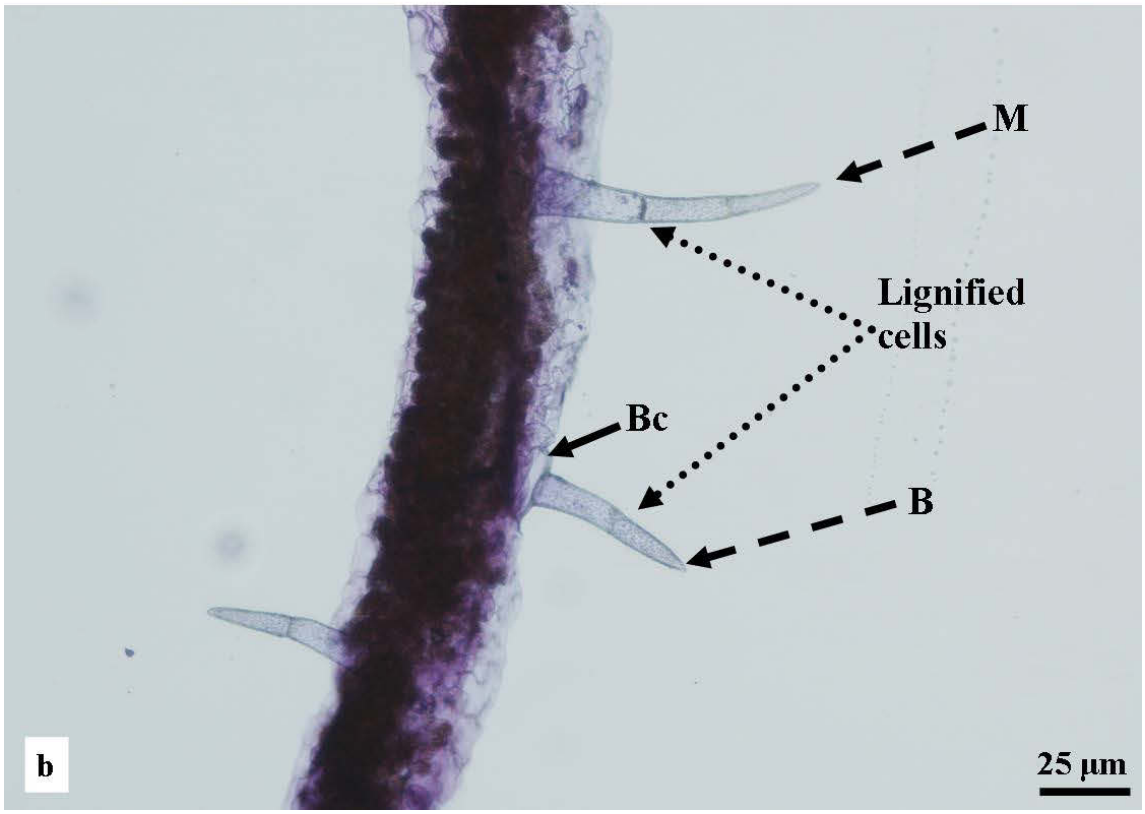
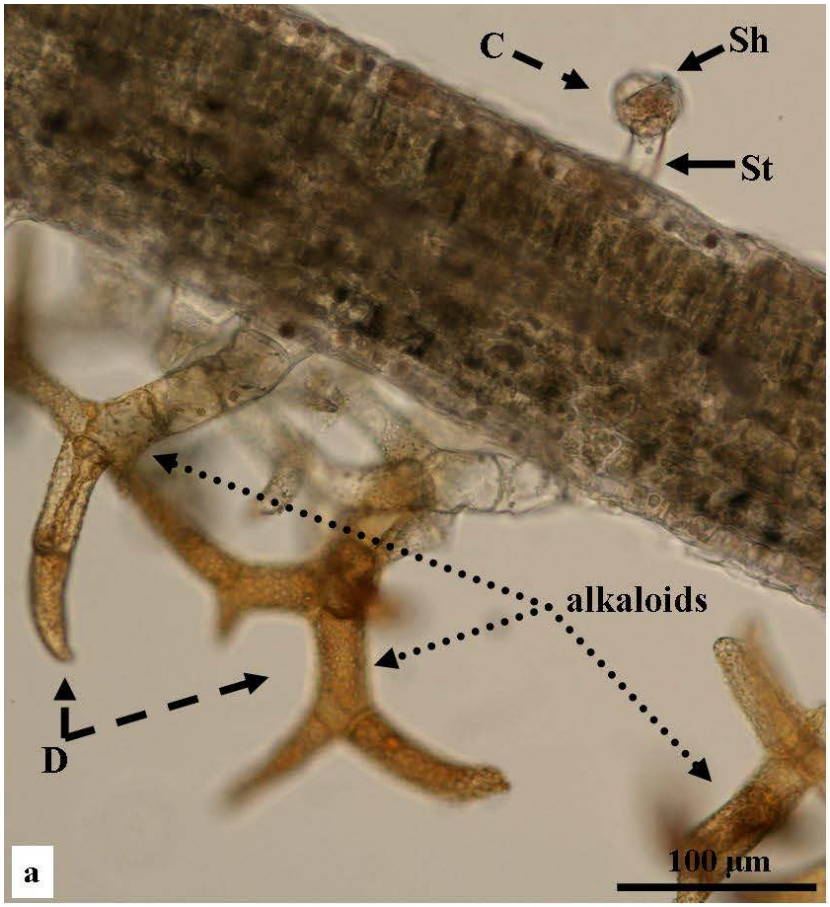

M
Lignified cells
Bc
B
b
25 µm

Figure 22. Alkaloids in emergent and young leaf sections stained with Wagner's and Dittmar reagents.

a) Secretory head (Sh) of glandular capitate (C) trichome and branches (Br) of dendritic (D) trichomes containing alkaloids (stained orange-brown).
b) Branches (Br) of dendritic trichome containing alkaloids (stained orange-brown).


C
Sh
St
alkaloids
D
a
100 µm

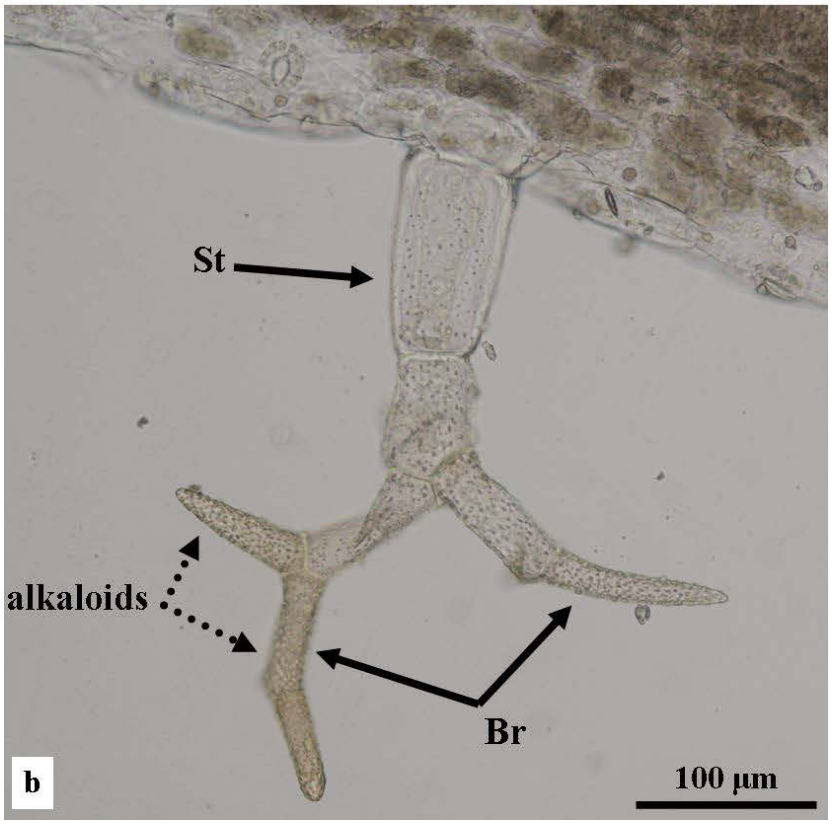

St
alkaloids
Br
b
100 µm

Figure 23. Phenolic compounds in emergent and young leaf sections stained with ferric trichloride.

a) Basal cells (Bc) and lower cells of multicellular trichomes containing phenolic compounds (stained green).
b) Secretory head (Sh) of glandular capitate trichome containing phenolic compounds (stained dark-green).
c) Lower cells of dendritic trichome containing phenolic compounds (stained green).

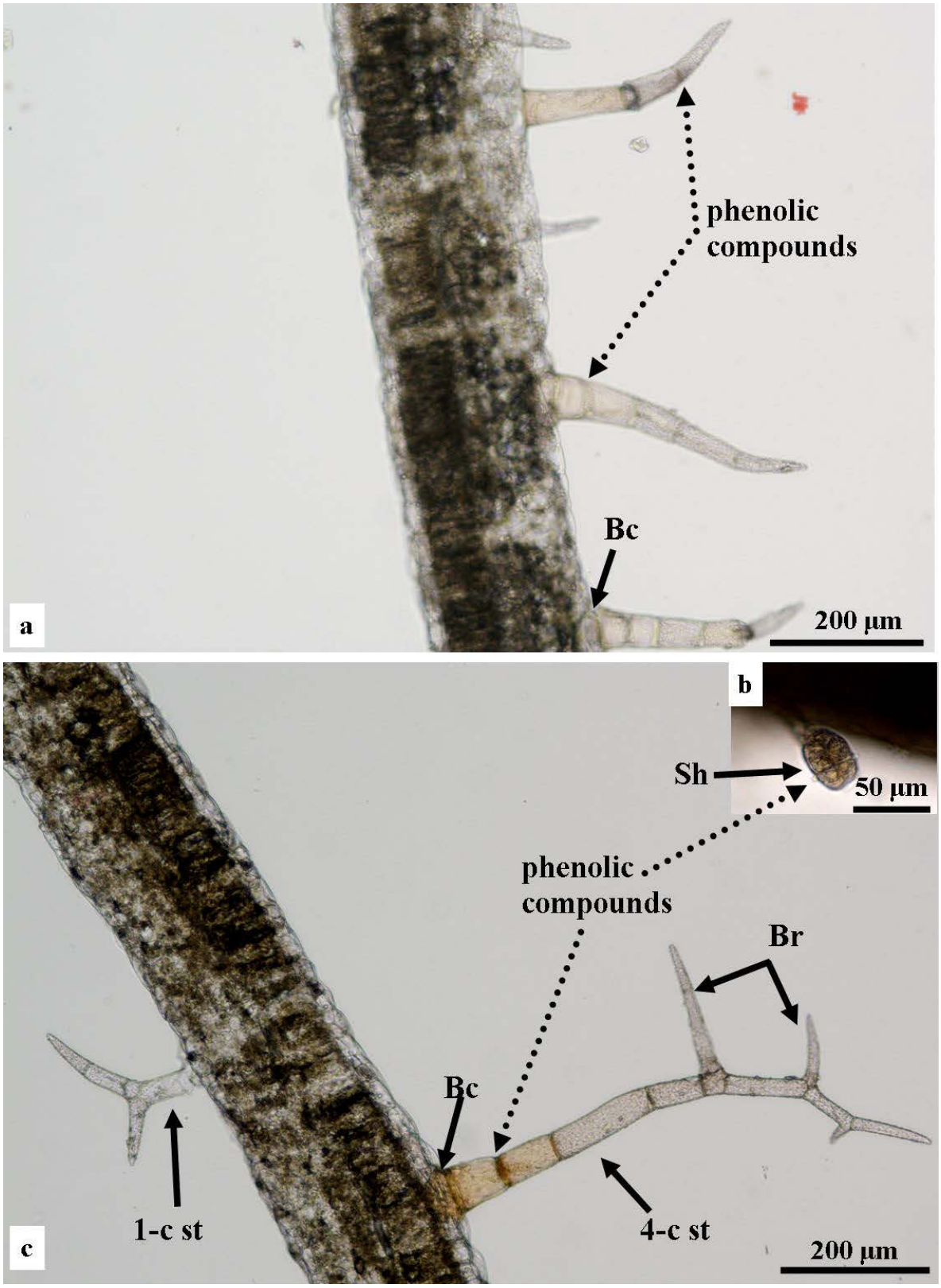

phenolic
compounds
Bc
a
200 µm
b
Sh
50 µm
phenolic
compounds
Br
Bc
1-c st
4-c st
c
200 µm

Figure 24. Emergent and young leaf sections showing the presence of esterified pectins and lipids.

a) Secretory head (Sh) of glandular capitate trichome containing esterified pectins (stained reddish-brown with hydroxylamine hydrochloride solution).
b) Non-glandular multicellular (M) and glandular capitate (C) trichomes containing lipids (stained blue with Nile blue)

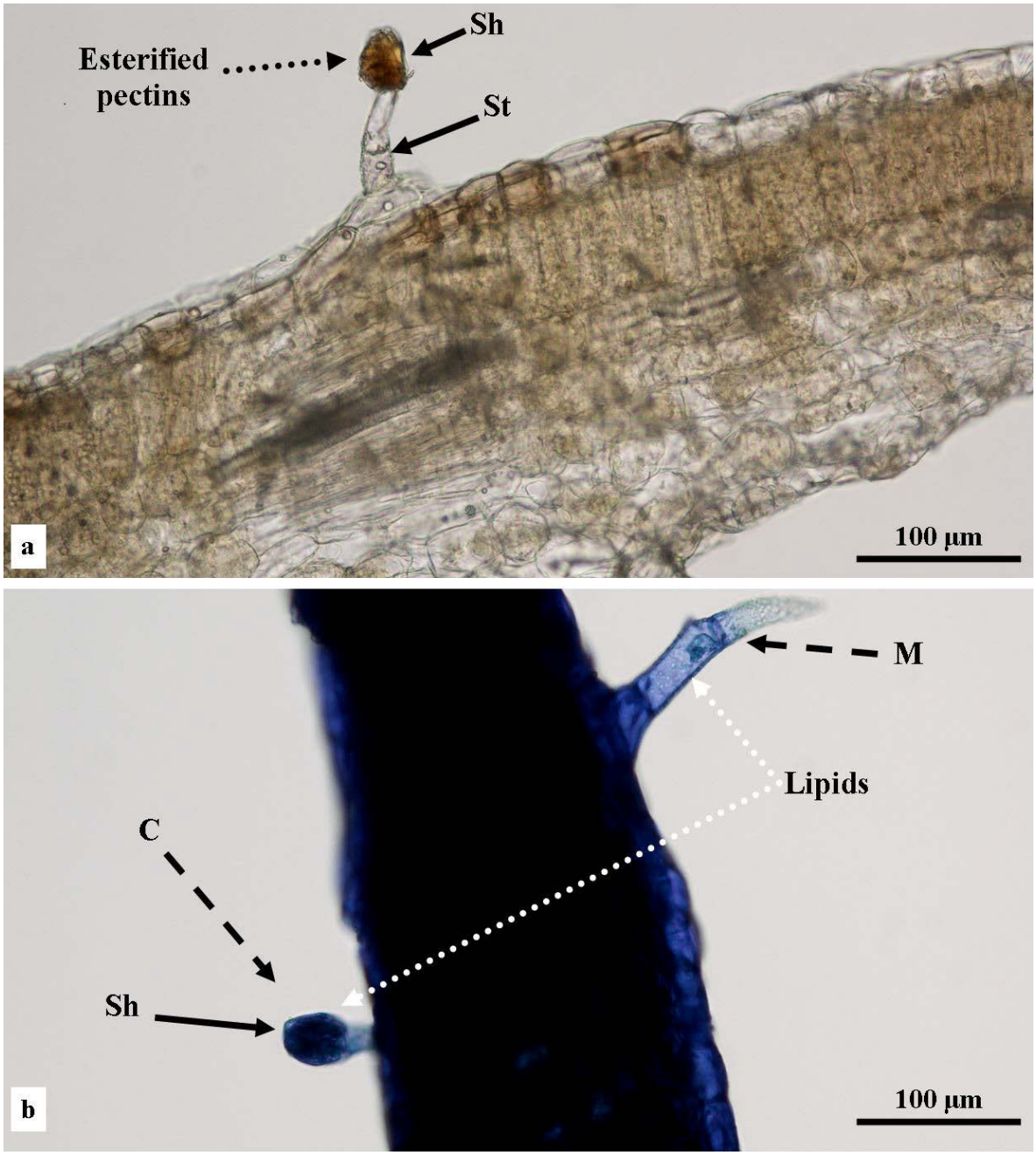
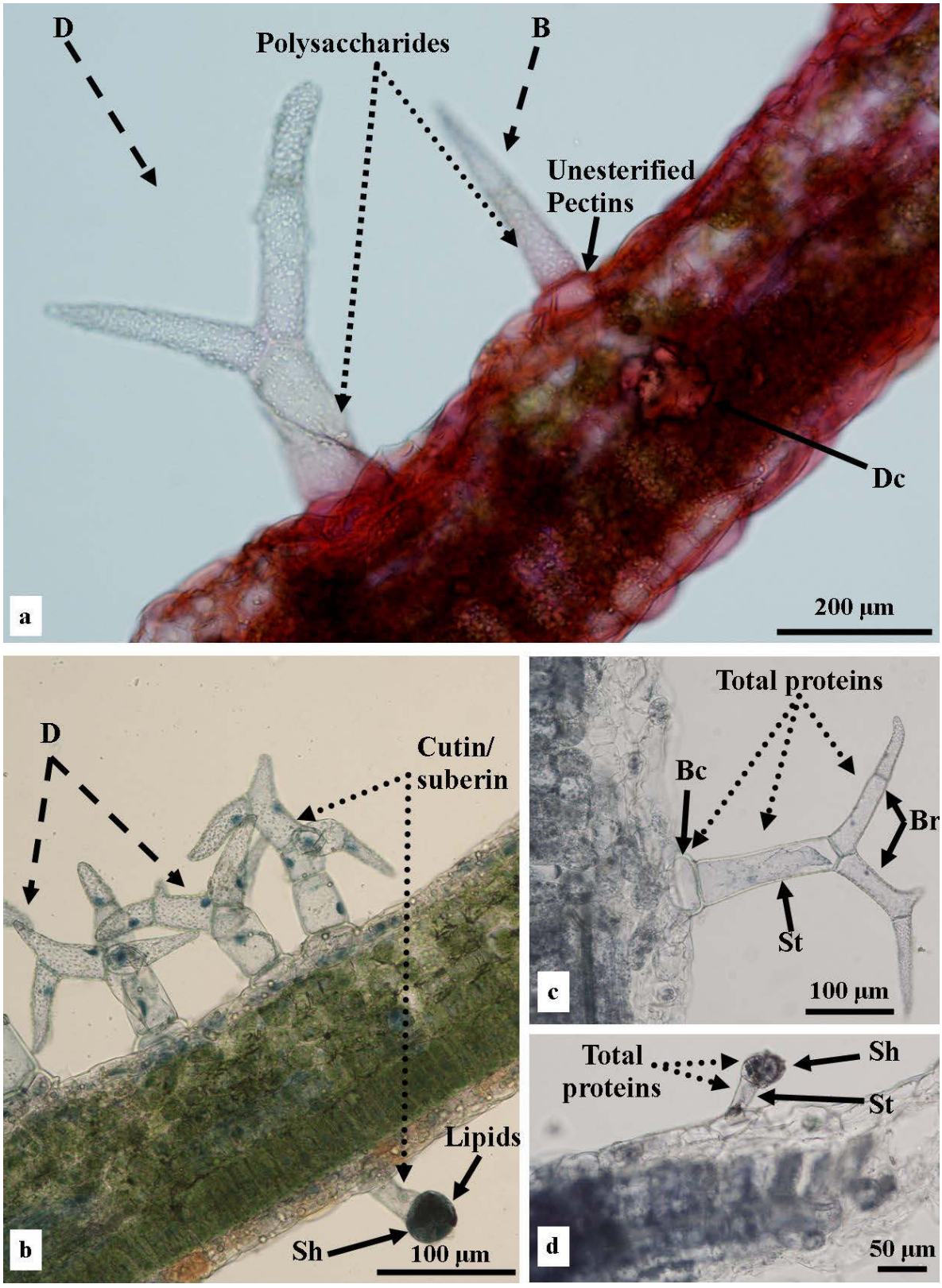

Esterified pectins
Sh
St
a
100 µm
M
Lipids
C
Sh
b
100 µm

Figure 25. Emergent and young leaf sections showing the presence of polysaccharides, total proteins druse crystals and cutinised cells.

a) Basal (Bc) and lower cells of dendritic (D) and bicellular (B) trichomes containing polysaccharides (stained dark pink-red with Ruthenium red. Section also contains a druse crystal (Dc).
b) Cutinised cell of dendritic (D) trichomes and stalk cell of a glandular capitate trichome (stained dark blue-black with Sudan Black). Secretory head (Sh) of capitate trichome contains lipids.
c) Basal cell (Bc), stalk cells (St) and branches (Br) of dendritic trichome containing total proteins (stained blue with bromophenol blue).
d) Secretory head (Sh) and stalk cell (St) of glandular capitate trichome containing total proteins (stained blue with bromophenol blue).


D
Polysaccharides
B
Unesterified
Pectins
Dc
a
200 µm
D
Cutin/
suberin
Lipids
Sh
b
100 µm
Total proteins
Bc
Br
St
c
100 µm
Total
proteins
Sh
St
d
50 µm

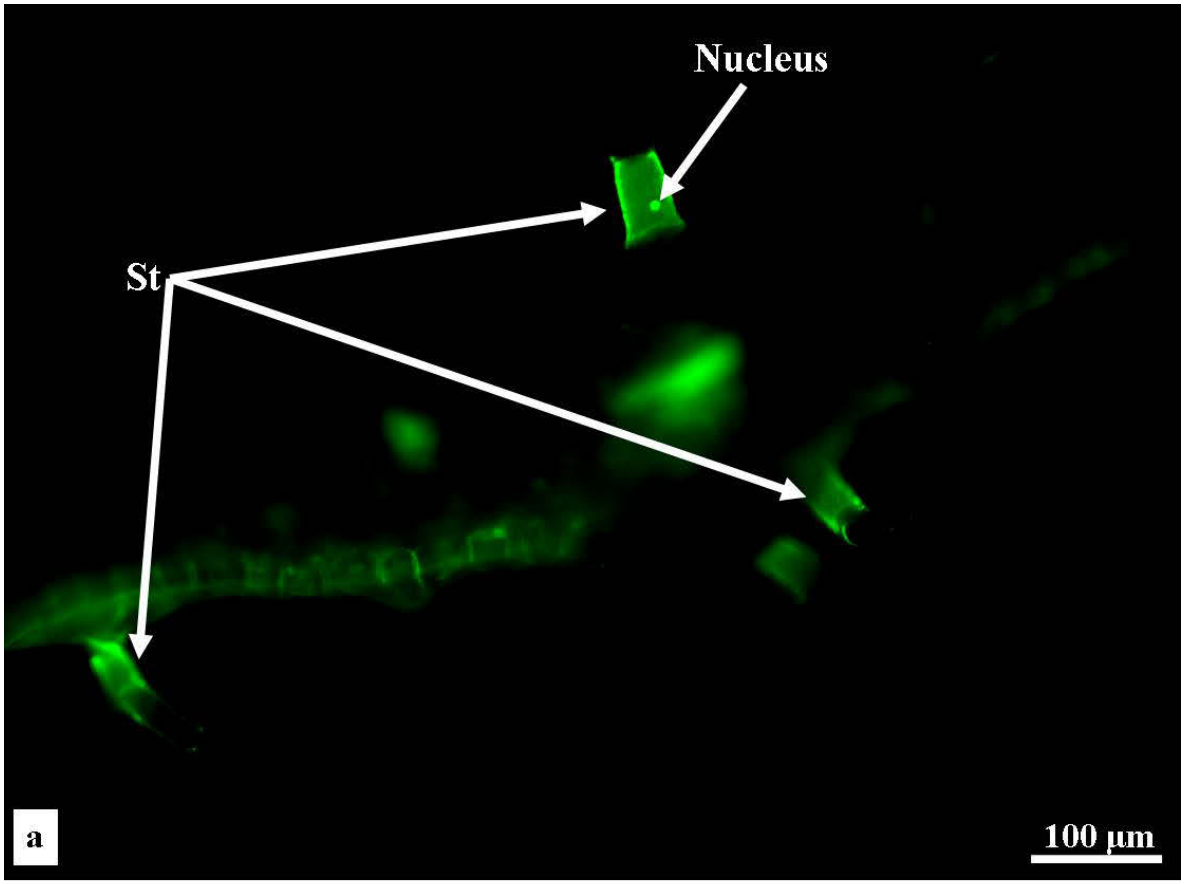
4.5. Fluorescence microscopy

Sections stained with 2 % acridine orange and viewed using fluorescence microscopy depicted the viability of epidermal and mesophyll cells as well as the basal and stalk (St) cells of dendritic trichomes (Fig. 26a–b). The branches of the non-glandular trichomes did not appear to be viable or metabolising as they did not autofluoresce.

The epidermal cells, palisade mesophyll cells as well as the basal and stalk cells of dendritic trichomes exhibited red autofluorescence (Fig. 27a). The red autofluorescence indicated the presence of plastids within these cells (Ascensão and Pais, 1987; Köhler *et al*., 1997). Epidermal cells, as well as the stalk cells (St) and branches (Br) of dendritic trichomes exhibited blue autofluorescence (Fig. 27b) indicative of the presence of phenolic compounds (Ascensão and Pais, 1987).

Figure 26. Autofluorescence of young leaf sections stained with acridine orange and viewed at a wavelength of 488 nm.

a) Viable stalk (st) cells of dendritic trichomes autofluoresce green. Autofluorescent nucleus is also visible in stalk cells.
b) Stalk cells (st) of dendritic trichomes and nuclei of epidermal emitting a green autofluorescence.


Nucleus
St
a
100 µm

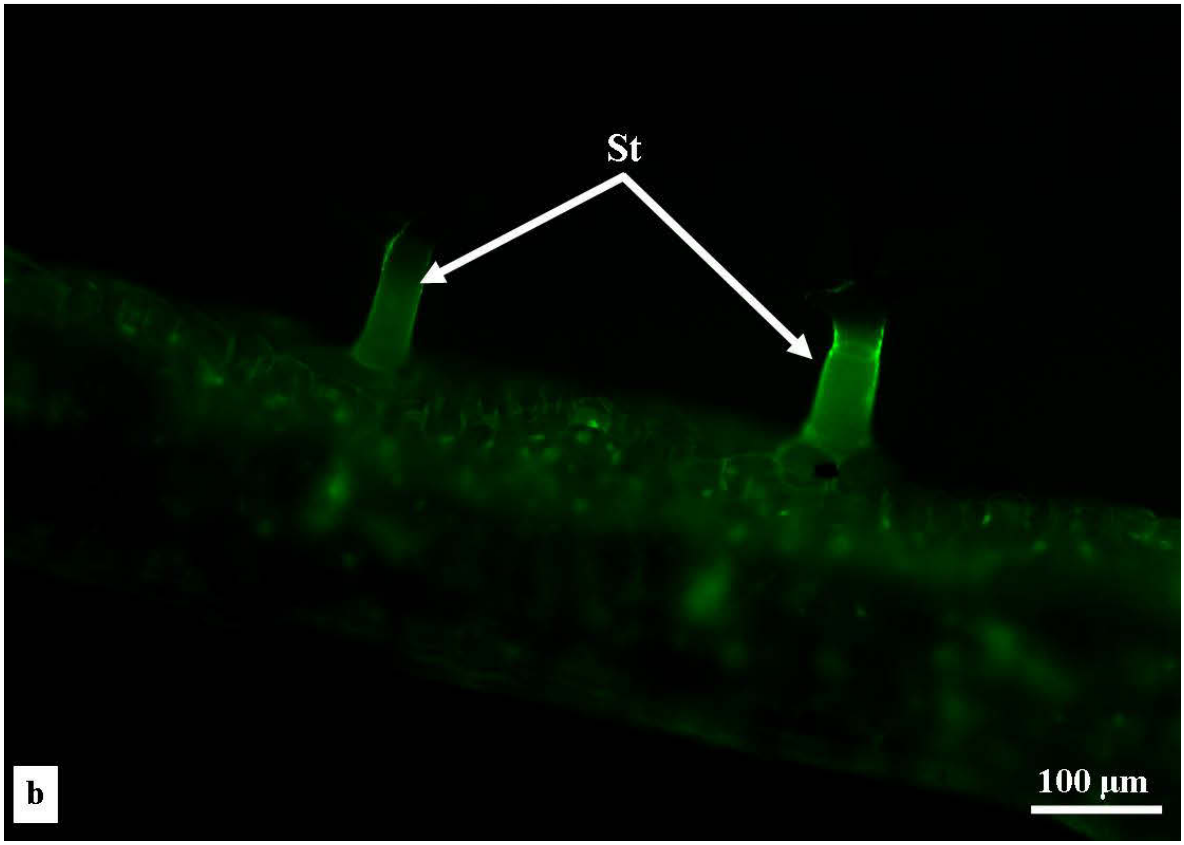
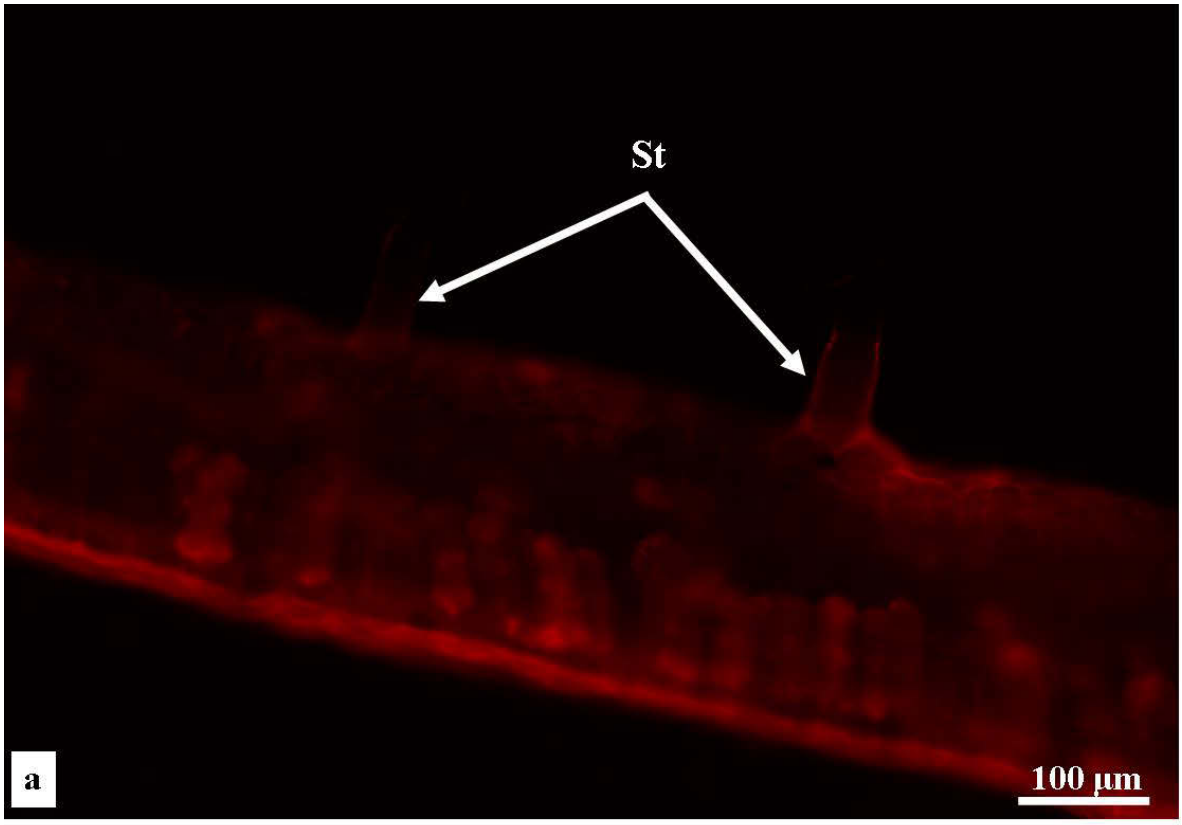
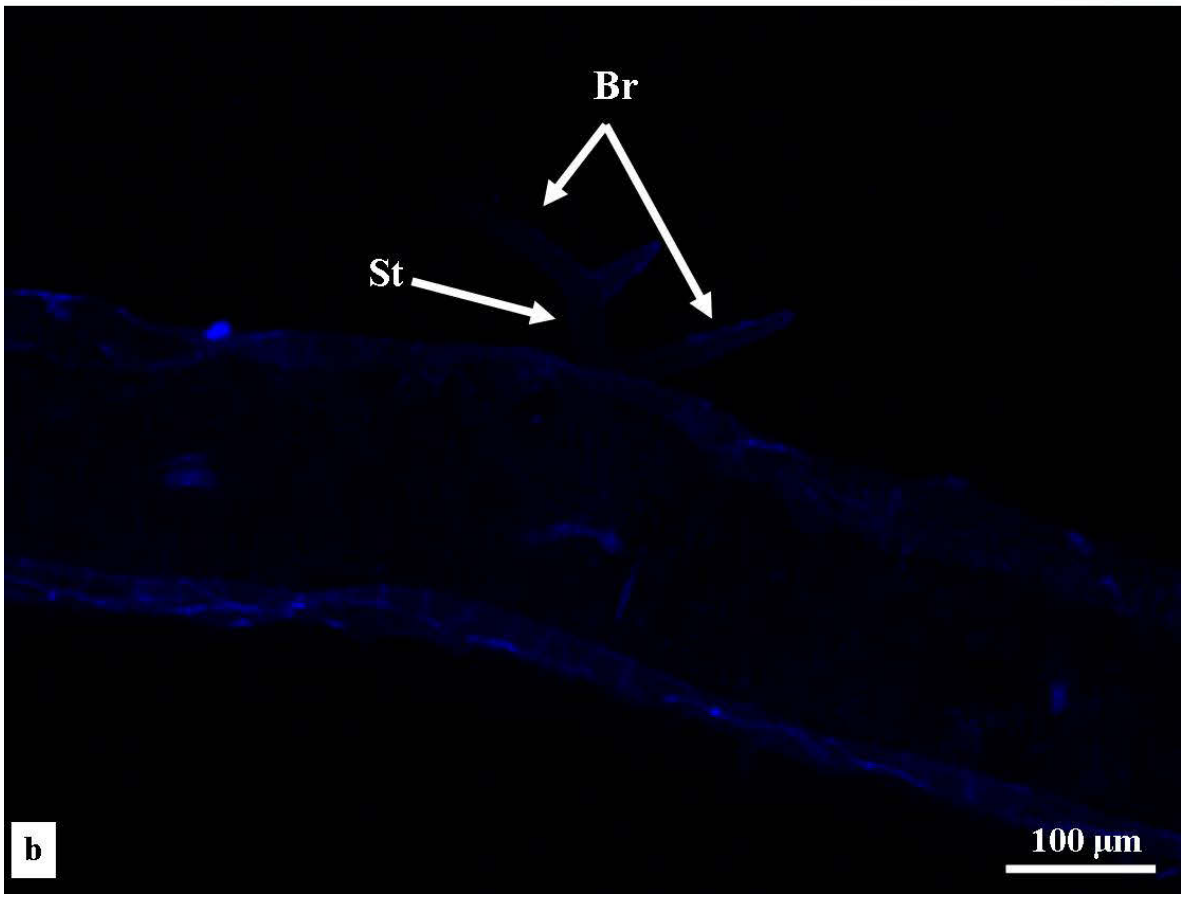

St
b
100 µm

Figure 27. Autofluorescence of fresh sections of young *W. somnifera* leaves showing the presence of plastids and phenolic compounds.

a) Red autofluorescence exhibited by cells of leaf section as well as stalk cells (St) of dendritic trichomes indicating the presence of plastids.
b) Blue autofluorescence exhibited by epidermal layers as well as stalk cells (St) and branches (Br) of dendritic trichomes indicating the presence of phenolic compounds.


St
a
100 µm


Br
St
b
100 µm

4.6. Preliminary phytochemistry

Major phytochemical groups detected in leaves of *W. somnifera* are summarised in Table 1. The two major groups present within methanol, chloroform and hexane extracts were alkaloids and phenolic compounds. Cardiac glycosides were also present in all three extracts. Methanol, chloroform and hexane extracts did not contain carbohydrates, flavones and flavonones, proteins and amino acids. Figure 24 showed the separation of the different compound groups on a TLC plate viewed at 254 and 366 nm, as well as after exposure to vanillin-boric acid-sulphuric acid-methanol.

Results of the phytochemical test reactions observed, for the methanol, chloroform and hexane extracts are shown in appendix 1–3.

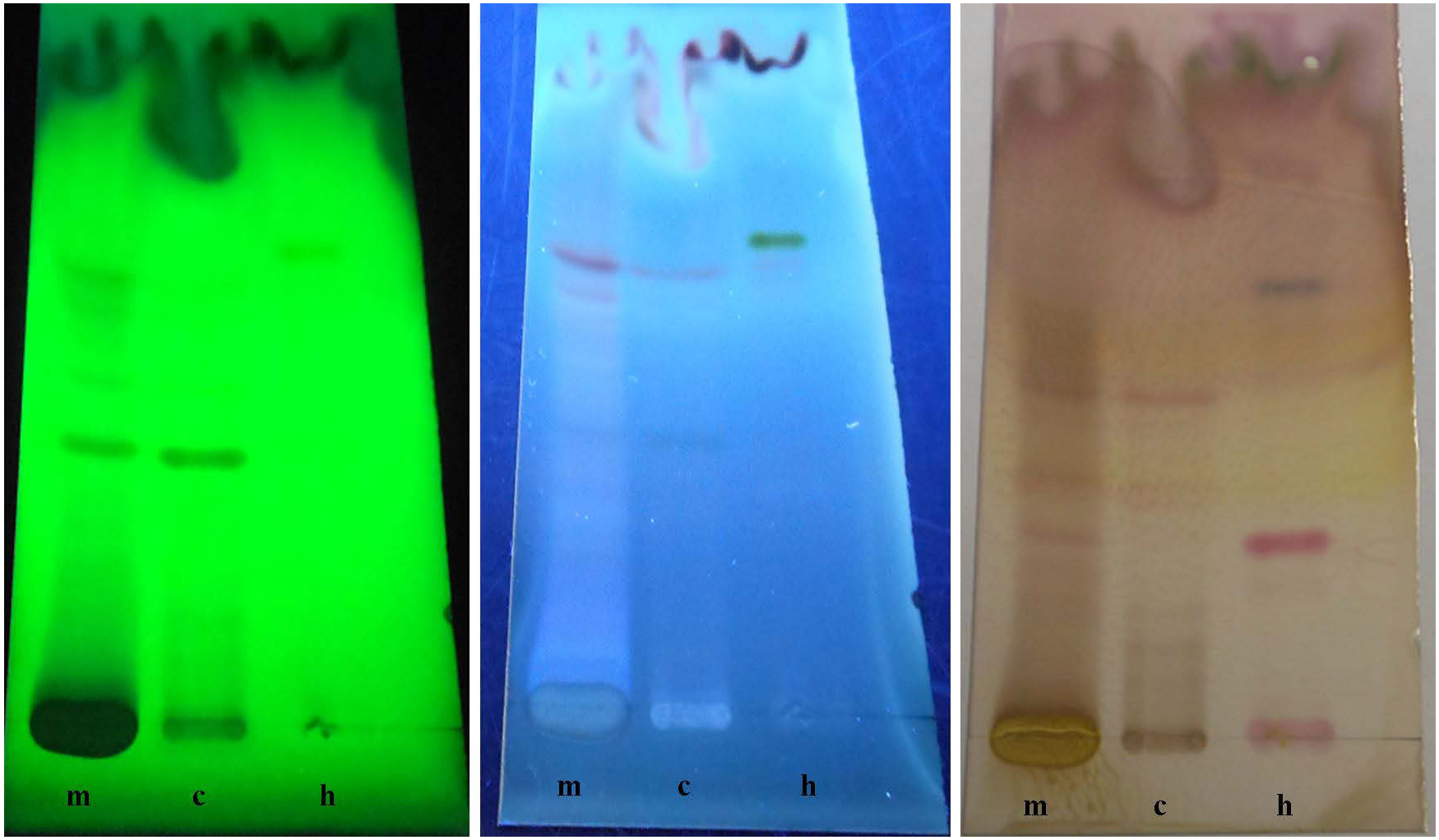
Table 2. Summary of phytochemical groups present/absent in chloroform, methanol and hexane extracts of *W. somnifera* (n=3).

Compound group	Phytochemical test	Chloroform	Methanol	Hexane
Alkaloids	Dragendroff's	+	+	+
	Hager's	+	+	+
	Wagner's	+	+	+
Phenolic compounds	Ferric chloride	+	+	+
	Lead acetate	+	+	+
Sterols	Salkowski's	-	+	+
Cardiac glycosides	Extract was mixed with glacial acetic acid and concentrated sulphuric acid was slowly added	+	+	+
Carbohydrates	Benedict's	-	-	-
	Fehlings A and B	-	-	-
	Molisch's	-	-	-
Flavones and flavonones	Concentrated sulphuric acid	-	-	-
Proteins and amino acids	Ninhydrin	-	-	-

+/- indicates presence/absence of compound groups.

Figure 28. TLC plates showing the separation of major compound groups in methanol (m) chloroform (c) and hexane (h) extracts.

a) Viewed at 254 nm.
b) Viewed at 366 nm.
c) Subsequent to spraying with vanillin-boric acid-sulphuric acid-methanol.


m
c
h
m
c
h
m
c
h

CHAPTER 5

Discussion

This study was undertaken to describe the morphology of the foliar trichomes of *W. somnifera*, as well as to determine the nature and localisation of the secretory products. Four morphologically distinct trichome types were observed on the foliar surfaces of *W. somnifera*; glandular capitate (Fig. 9, 10 & 11), non-glandular dendritic (Fig. 12 & 13), non-glandular bicellular and non-glandular multicellular trichomes (Fig. 14). All four trichome types have been reported previously within the family *Solanaceae* (Harisha and Switu, 2013; Adedji *et al*., 2007).

Examination of the morphology, distribution and the phytochemistry of the secretion of the trichomes could assist in elucidating possible functions of the trichomes of *W. somnifera* (Werker, 2000; Dai *et al*., 2010). Physical properties, such as size, density and arrangement of trichomes on the leaf surface may allow for the protection against insect pathogens and herbivores, such as the broad mites observed on the leaves of *W. somnifera* (Fig. 8b–c). Non-glandular trichomes might also reduce transpiration rates and limit surface leaf exposure to extreme temperatures (Werker, 2000; Dai *et al*., 2010). Non-glandular trichomes, especially the dendritic type, observed on *W. somnifera* leaves would assume these roles as these trichomes were so dense on emergent leaves that it is difficult to view the leaf surface. The dense distribution of dendritic trichomes on *W. somnifera* leaves was also observed by Kumar *et al*. (2010b). Since *W. somnifera* might occur in dry regions, dendritic trichomes were likely to play a major role in water conservation (Hameed and Hussain, 2011).

Even though non-glandular trichomes are considered to be non-secretory, autofluorescence microscopy of leaf sections stained with acridine orange showed that the basal and stalk cells of these trichomes are viable (Fig. 26). Histochemical analysis showed that the non-glandular trichome types accumulate phytochemical compounds, thus these trichomes also play a role in the chemical defence against insect herbivores and pathogens.

Glandular capitate and non-glandular dendritic trichomes were found to be aggregated on the mid–veins of leaves. This arrangement is consistent with that in *Solanum aculeastrum* (Koduru *et al*, 2006). Within Solanaceae, secondary metabolites such as alkaloids and phenolic compounds are synthesized in the roots and transported to the leaves (Sangwan *et al*., 2008, Senthil *et al*., 2009). Glandular trichomes are therefore located on mid-veins for easy sequestration of these

compounds. The aggregated distribution of non-glandular dendritic trichomes on mid-veins will protect vascular tissue against UV damage (Bhatt *et al*., 2010). Due to the close proximity and much longer length of the dendritic compared to glandular trichomes, it is possible that the dendritic trichomes act as a form of physical protection to the underlying glandular trichomes.

The density of glandular and non-glandular trichomes on leaves is also affected by insect herbivory or pathogen attack (Levin, 1973; Glas *et al*., 2012). The increase in density is activated by the octadecanoid pathway which results in increased jasmonic acid production. Jasmonic acid activates trichome formation, the expression of defense genes and the accumulation of secondary metabolites, such as those found in glandular trichomes (Glas *et al*., 2012). The high density of trichomes on leaf surfaces serves as a physical barrier to both movement and feeding by pests (Peiffer *et al*., 2009; Glas *et al*., 2012). Apart from herbivory, defoliation may also lead to increased glandular and non-glandular trichome density on newly-emerging leaves (Duke, 1994; Peiffer *et al*., 2009). As mentioned previously, leaves sampled in February 2013 appeared to contain a higher density of trichomes as compared to leaves sampled during May-July 2012. Individual plants of *W. somnifera* in early 2013 contained a substantial amount of leaves that turned yellow-brown and eventually became detached from the plants. Upon further investigation of the leaf surfaces, scale insects, broad mites and mite eggs were found (Fig. 8). According to a study conducted by Peña and Bullock (1994), some of the injuries induced upon *S. tuberosum* (potato) by broad mites included leaf curling, necrosis, bronzing, wilting as well as death of terminal and lateral apices. Despite the injury caused and the reduced heights of infected plants, it was also observed that *S. tuberosum* plants had an increased amount of leaves after exposure to the mites.

Trichome density may be determined in early leaf development, and as leaves mature, the density across the leaf area decreases (Ascensão and Pais, 1987; Werker, 1993; Duke, 1994; Gairola *et al*., 2008). This was only true for the glandular capitate trichomes (Fig. 16). Another possible reason for the decrease in glandular trichome density is that once these trichomes have reached the post-secretory phase, they senesce, and are shed (Werker, 2000; Gairola *et al*., 2008). With regard to the non-glandular trichomes, there was no definite pattern seen between trichome density and level of leaf maturity. However, dendritic trichome density is much higher on adaxial surfaces of emergent leaves as compared to young and mature leaf surfaces. A possible reason for emergent leaves needing more protection by trichomes is that these leaves are more susceptible to

attack by insects and pathogens due to their higher nutritional value (Duke, 1994; Chaurasiya *et al*., 2007).

The glandular trichomes of *W. somnifera* also appeared to be fully developed on emergent leaves (Fig. 17). Although, there were no statistical differences in length of the non-glandular bicellular and multicellular trichomes, the absence of these trichome types suggests that these trichomes developed later in leaf development. The length of non-glandular dendritic trichomes varied among the different leaf developmental stages, suggesting that these trichomes are not fully developed on emergent leaves. Both trichome development and density are variable between the different types of organs, such as leaf and sepals, as well as the different surfaces of these organs, such as the adaxial and abaxial leaf surfaces (Johnson, 1975; Werker, 1993; Gairola *et al*., 2008). The development of non-glandular dendritic trichomes was also evident from the cellulose microfibrils observed in TEM micrographs (Fig. 19a). Randomly organized cellulose microfibrils are generally located at in the walls of the tips or uppermost cells of growing trichomes (Werker, 2000). These "inextensible" microfibrils are surrounded by matrix polymers that rearrange and result in cell-wall expansion and cell growth (Cosgrove, 2000).

The cuticular warts or bumps observed on non-glandular dendritic trichomes (Fig. 19a–b) arise from the cell wall, cuticle or subcuticular inclusions (Werker, 2000; Gairola *et al*., 2008). Cuticular warts may be involved in a phenomenon known as the 'Lotus-Effect', in which the self-cleaning properties of these structures ensure that trichomes and leaf surfaces remain clear of dust particles that may result from environmental conditions (Bhatt *et al*, 2010). This mechanism may also assist in defending leaves against pathogen attack (Barthlott and Neinhuis, 1997). Cuticular warts may sometimes be a sign of trichome maturity (Gairola *et al*., 2008).

The stalk cells of capitate trichomes and all cells of non-glandular dendritic, bicellular and multicellular trichomes of *W. somnifera* were cutinized/suberised (Fig. 25b). All cells of the non-glandular types were also lignified (Fig. 21). These substances in the cell walls of trichomes protect against water loss, extreme light and temperature, herbivory and pathogen attack by acting as a mechanical barrier of the surface of trichomes (Werker, 2000). The cutin or suberin within the trichome walls act as "casparian strips" and prevent exudates from flowing back into trichome once secreted (Fahn, 1986; Werker, 2000). Lignin also serves as a mechanical barrier to pests (Fordyce and Agrawal, 2001).

The major phytochemicals of medicinal importance found in both glandular and non-glandular trichomes were alkaloids and phenolic compounds (Fig. 22 & 23). Alkaloids are synthesized in vacuoles, cytoplasm or chloroplasts and are translocated via the xylem or phloem and stored in the central vacuole of glandular trichomes (Wink, 1998). Although poisonous, alkaloids are used in low concentrations in the medicinal industry to treat a wide range of ailments such as fevers, dysentery, asthma and inflammation. In plants, alkaloids act as natural repellants against pests and even competitors (Robinson, 1974; Fordyce and Agrawal, 2001).

Phenolic compounds are stored within glandular and non-glandular trichomes and are released when the trichomes are damaged by insects. Once released, the phenolic compounds are oxidized to quinines by polyphenol oxidase (released from plastids of the trichome) and 'glue' insects to the leaf surface so that they are unable to feed (Duke, 1994; Werker, 2000). The antioxidant properties of this compound also protect plants against bacterial or viral attack by counteracting excessive reactive oxygen species (ROS) production (Grassmann *et al*., 2002). These antioxidant properties have also been exploited in the cosmetic industry for use in skin care applications (Negro *et al*., 2003). In the medicinal industry, phenolic compounds are used in the treatment of heart diseases as these compounds appeared to inhibit the oxidation of specific proteins (Negro *et al*., 2003). These compounds have also been reported to have anti-inflammatory and anti-carcinogenic properties.

Exudates that are produced or released by the trichomes may also be toxic or act as "gustatory repellents" to insect attackers (Levin, 1973; Peiffer *et al*., 2009). Aphids such as *Myzus persicae* and *Macrosiphum euphorbiae* are common pests of wild potatoes, *S. polyadenium, S. berthaultii* and *S. tarijense*. When the cell walls of glandular trichomes are ruptured by these aphids, the clear, water-soluble phenols that are released are converted to a black water-insoluble compound upon contact with oxygen. This black compound accumulates on the legs of the aphids, resulting in immobilization and starvation (Levin, 1973; Yu *et al*., 1992; Werker, 2000; Peiffer *et al*., 2009, Glas *et al*., 2012).

Fluorescence microscopy and TEM (Fig. 19a) showed the presence of plastids in the basal and stalk cells of dendritic trichomes (Ascensão and Pais, 1987; Köhler *et al*., 1997). Plastids were also present in the head cells of glandular capitate trichomes (Fig. 19b). Plastids have been reported to carry out a wide range of cellular functions, depending on the type of plastid and the cells they are contained within (Pyke and Howells, 2002). Plastids within trichomes play a role in

the biosynthesis, accumulation and secretory processes of various compounds (Turner *et al*., 2000; Pyke and Howells, 2002). Some plastids, such as those located in the glandular trichomes of *S. berthaultti*, contain PPOs. These copper metalloproteins are involved in the reduction of phenols to quinines, resulting in the entrapment of insect herbivores (Yu *et al*., 1992).

Apart from alkaloids and phenolic compounds, the glandular and non-glandular trichomes were found to accumulate lipophillic material, polysaccharides, protein, cardiac glycosides and sterols (Tables 1 & 2). Cardiac glycosides, a group of steroid-like compounds are used in the treatment of heart disorders such as congestive heart failure and arrhythmia (Newman *et* al., 2008; Prassas and Diamandis, 2008).

Due to the accumulation of alkaloids and phenolic compounds, the trichomes of *W. somnifera* also play a role in chemical defence against attack by insect herbivores and pathogens. Trichomes are ideal structures for the storage of alkaloids and phenolic compounds, ensuring the rapid release of these compounds at the time of attack (Wink, 1998). The storage and release of these exudates by trichomes are more beneficial to plant species rather than having the phytochemicals sequestered within leaf tissue. This is because insects and other herbivores can be deterred before they feed on and cause physical damage to plant parts (Levin, 1973).

Cuticular ruptures were not visible on post-secretory glandular capitate trichomes during ESEM. This suggests that the glandular capitate trichomes of *W. somnifera* may release the secretory material through cuticular pores, as seen in the Lamiaceae (Fahn 1988; Ascensão *et al*., 1995). These glandular trichomes however, also release the secretory material once the cuticle has been ruptured by insects and herbivores (Fahn, 1988; Duke, 1994; Ascensão *et al*., 1995). This was evident when the glandular trichomes were abraded during histochemical sectioning.

Histochemical sectioning and staining revealed the presence of prismatic (Fig. 13a & 20b) and druse (Fig. 25a) crystals in leaves of *W. somnifera*. These crystals are composed of calcium oxalate (CaOx) and are present in many plant families including Araceae, Rosaceae, Boraginaceae, Lauraceae, Leguminosae, Myrtaceae, Pinaceae and Orchidaceae (Franceschi and Horner, 1980). Calcium oxalate, as crystal deposits, occur in specialized vacuoles, called idioblasts, and have a range of functions depending on the amount, distribution, morphology and production (Franceschi and Nakata, 2005). Functions of crystals include plant protection, calcium regulation and metal detoxification. Plant protection by calcium oxalate crystals can take place in

two ways: passive or active. Active protection involves the accumulation of needle-shaped crystals on outer plant parts so that physical damage occurs when touched or brushed upon by insects and herbivores (Arnott and Webb, 2000; Franceschi and Nakata, 2005). The accumulation of crystals within plant parts (passive protection), as seen in *W. somnifera*, protect the plant against herbivore attack by causing irritations or burning sensations in the mouth and throat. A common example of this type of protection is seen when collard peccaries, pig-like mammals, avoid feeding on populations of prickly pears that contain calcium oxalate crystals (Franceschi and Nakata, 2005). Many of our plant foods such as spinach, peppers, beans, berries, chocolate, *Colocasia esculenta* (commonly known as amadumbies) and some types of nuts also contain calcium oxalate crystals. The calcium oxalate contained within these foods can negatively affect human health by acting as toxins and increasing kidney stone formation (Franceschi and Horner, 1980; Arnott and Webb, 2000; Franceschi and Nakata, 2005).

Therefore, with the theory of insect-induced increase in trichome density and the phytochemical constituents of the trichomes, it is possible that the non-glandular trichomes of *W. somnifera* provide both mechanical and chemical protection to leaves, while the glandular trichomes serve to accumulate and secrete phytochemicals containing alkaloids and phenolic compounds. It is also interesting to note that the dendritic trichomes of *W. somnifera* are considerably longer than the glandular capitate trichomes. Since the dendritic trichomes are lignified and contain phytochemicals, it is possible that these trichomes provide a first line of defence against insect herbivores (Levin, 1973; Werker, 2000). However, if this line of defence fails, the accumulation of the phytochemicals within the glandular trichomes would form the second line of defence.

The stomatal type classified from leaves used in this study was anisocytic. Although this contradicted the animocytic stomatal arrangement discussed by Kumar *et al*. (2010a), Palei and Harisha (2012) confirm the anisocytic arrangement. However, Hameed and Hussain (2011) discuss another stomatal type, anomotetracytic (guard cells surrounded by four irregularly shaped subsidiary cells), on both surfaces of *W. somnifera* leaves, with stomatal density being relatively higher on abaxial leaf surfaces. These studies also confirmed the closed stomata of adaxial surfaces and opened stomata of abaxial surfaces. This could correlate with the increased density of dendritic trichomes on abaxial surfaces. The higher density of dendritic trichomes would provide a ‘shading affect’ and reduce transpiration rates. Cuticular striations were also observed

on leaves of *W. somnifera*. As with stomatal arrangement, trichome type and cuticular warts, cuticular striations serve as diagnostic features of plant species (Ahmad, 1962; Shahbaz, 2008).

The study of the chemical constituents of trichome exudates is important as these chemicals act as natural deterrents against herbivores and other pests. A common example is citronella oil, a mosquito repellent, extracted from *Andropogon nardus* (Levin, 1973). Information from such studies will allow plant breeding programmes to "incorporate trichome-based resistance" into species (Duke, 1994; Peiffer *et al*., 2009). This would result in the natural enhancement of pest resistance, as well as the enhanced production of medicinally important phytochemicals such as phenolic compounds and alkaloids.

Characterising the morphology and the chemical constituents of the leaves of *W. somnifera* has not only highlighted the importance of studies of this nature, but has also emphasized the need for future research on this species. This research will allow for the optimization of phytochemical yields for medicinal uses by means of biotechnological approaches. Biotechnological applications depend on knowledge that is based on exploratory research. It is, therefore, vital to determine the constituents or chemical groups of exudates and the ultrastructure of the cells they are contained within, in order to advance research capabilities and improve yields of medicinally important compounds (Duke, 1994).

An important point to consider for future research is that the same species from different geographic regions may differ in compound composition and concentration (Mehboob, 2012). Therefore, there is a need for comparative phytochemical, as well as multidisciplinary studies. Understanding the contents of glandular and non-glandular trichomes on a cellular, biological and biochemical level together with plant biotechnology will most likely boost the production of the medicinally important compounds localized within trichomes (Duke, 1994).

CHAPTER 6

Conclusion

This study described the micromorphology and trichome density of the leaves of *Withania somnifera*. The major phytochemical constituents of the exudates produced that were claimed to be responsible for the extensive healing properties of this species were also determined.

Stereomicroscopy, SEM and histochemical staining of leaves showed the presence of four distinct trichome types on *W. somnifera*: glandular capitate, non-glandular dendritic, non-glandular bicellular and non-glandular multicellular trichomes. The capitate trichomes were uniseriate and consisted of a single basal cell, single-celled stalk and a six-celled secretory head. Non-glandular dendritic trichomes consisted of a single basal cell and 2–4 stalk cells with varying branch numbers. Non-glandular bicellular and multicellular (3–6 cells) trichomes were uniseriate and also appeared to emanate from a single basal cell. Capitate trichomes on fully developed leaves were considerably shorter than the non-glandular trichome types, with capitate trichomes having an average length of 60.52 ± 7.64 μm while the average length of dendritic trichomes was 125.96 ± 52.11μm. The average length of bicellular and multicellular trichomes was 138.85 ± 89.55 μm.

Glandular capitate and non-glandular dendritic trichomes occurred on both adaxial and abaxial leaf surfaces of emergent, young and mature leaves. Glandular capitate and non-glandular dendritic trichomes were aggregated on the mid-veins of leaves. Non-glandular bicellular and multicellular trichomes were present at all stages of development, however at a significantly lower density in comparison to dendritic and glandular capitate trichomes. Non-glandular trichome density varied between emergent, young and mature leaves, suggesting that trichome number is not established at the onset of leaf development. Glandular trichome density was higher on emergent leaves in comparison to mature leaves showing that the number of these trichomes is determined early in leaf development.

Phytochemical and histochemical tests were used to identify and locate major compound groups that were of medicinal importance within trichomes. Alkaloids and phenolic compounds were present in all four trichome types. These compounds have been reported to be used in the treatment of a range of illnesses such as arthritis, inflammation, paralysis, TB and asthma (Atal and Schwarting, 1961; Chaurasiya *et* al., 2007; Kumar *et al*., 2010a). Histochemical staining of

leaves also showed the presence of calcium oxalate in the form of prismatic and druse crystals. These crystals are believed to act as a deterrent to insect herbivores, causing irritations in the mouth and throat (Franceschi and Horner, 1980).

TEM of leaves showed the presence of cellulose microfibrils within the cell walls of the non-glandular dendritic trichomes. These microfibrils are involved in cell-wall expansion and growth. The presence of plastids within glandular trichomes might play a role in the accumulation and secretion processes of the phytochemical compounds. Plastids present in non-glandular dendritic trichomes is also claimed to play a role in the accumulation of phytochemical compounds (Turner *et al*., 2000; Pyke and Howells, 2002).

Due to the presence of lignin in the non-glandular trichomes and phytochemicals in both glandular and non-glandular trichomes, the foliar trichomes of *W. somnifera* appeared to provide both mechanical and chemical protection against insect herbivores. Glandular trichomes are assumed to secrete the protective chemicals when the secretory heads are ruptured by pests. In this manner, it is suspected that when these trichomes are damaged, insect herbivores are exposed to the phytochemicals stored within glandular trichomes.

Although this study has identified the major classes of compounds and the site at which these compounds were present within trichomes, it is still unclear whether these compounds are produced by cells of the trichomes or elsewhere in the plant and transported to the trichomes. Future studies should therefore be focussed on identifying the specific cells that may contribute to the production of exudates. This could be achieved by employing ultracytochemical techniques as described by Naidoo *et al*. (2011).

CHAPTER 7

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Appendix

Appendix 1. Phytochemical groups present/absent in the methanol extract of *W. somnifera*.

Compound Group	**Phytochemical Test**	**Present (+) / Absent (-)**	**Observation**
Alkaloids	Dragendroff's	+	Turned reddish-brown
	Hager's	+	Turned brown
	Wagner's	+	Turned yellow-brown
Phenolic compounds	Ferric chloride	+	Turned dark green
	Lead acetate	+	Turned milky
Sterols	Salkowski's	-	Turned light green
Cardiac glycosides	Extract was mixed with glacial acetic acid and concentrated sulphuric acid was added	+	Formation of red ring between the acid and extract
Carbohydrates	Benedicts	-	Remained dark green
	Fehlings A and B	-	Turned milky-green
	Molisch's	-	Remained dark-green
Flavones and flavonones	Concentrated sulphuric acid	-	Turned dark-green to black
Proteins and amino acids	Ninhydrin	-	Turned light green

Appendix 2. Phytochemical groups present/absent in the chloroform extract of *W. somnifera*.

Compound Group	**Phytochemical Test**	**Present (+) / Absent (-)**	**Observation**
Alkaloids	Dragendroff's	+	Reddish-brown precipitate at the top
	Hager's	+	Yellow precipitate at the top
	Wagner's	+	Pinkish-brown oily precipitate at the top
Phenolic compounds	Ferric chloride	+	Orange precipitate at the top
	Lead acetate	+	Clear precipitate at the top
Sterols	Salkowski's	+	Formation of a red ring
Cardiac glycosides	Extract was mixed with glacial acetic acid and concentrated sulphuric acid was added	+	Red ring between the acid and extract
Carbohydrates	Benedict's	-	Blue precipitate at the top
	Fehlings A and B	-	Blue precipitate at the top
	Molisch's	-	Purple precipitate at the top
Flavones and flavonones	Concentrated sulphuric acid		Milky-oily precipitate at the top
Proteins and amino acids	Ninhydrin	-	Turned milky

Appendix 3. Phytochemical groups present/absent in the hexane extract of *W. somnifera*.

Compound Group	**Phytochemical Test**	**Present (+) / Absent (-)**	**Observation**
Alkaloids	Dragendroff's	+	Reddish-brown precipitate at the bottom
	Hager's	+	Yellow precipitate at the bottom
	Wagner's	+	Dark pink-brown oily precipitate at the bottom
Phenolic compounds	Ferric chloride	+	Orange precipitate at the bottom
	Lead acetate	+	Clear precipitate at the bottom
Sterols	Salkowski's	+	Formation of a red ring
Cardiac glycosides	Extract was mixed with glacial acetic acid and concentrated sulphuric acid was added	+	Formation of red ring between the acid and extract
Carbohydrates	Benedict's	-	Blue precipitate at the bottom
	Fehlings A and B	-	Blue precipitate at the bottom
	Molisch's	-	Light purple precipitate at the bottom
Flavones and flavonones	Concentrated sulphuric acid	-	Clear-oily precipitate at the bottom
Proteins and amino acids	Ninhydrin	-	Turned clear-light yellow