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Planta (2015) 242:1107–1122
DOI 10.1007/s00425-015-2341-1

ORIGINAL ARTICLE

Micromorphology, histochemistry and ultrastructure of the foliar trichomes of *Withania somnifera* (L.) Dunal (Solanaceae)

Prelina Munien[1] · Yougasphree Naidoo[1] · Gonasageran Naidoo[1]

Received: 23 March 2015 / Accepted: 28 May 2015 / Published online: 11 June 2015

Abstract
Main conclusion **The leaves of *Withania somnifera* contained four morphologically distinct trichome types: glandular capitate, non-glandular dendritic (branched), non-glandular bicellular and non-glandular multicellular trichomes. Major phytochemical compounds present within glandular and non-glandular trichomes were alkaloids and phenolic compounds.**

The aim of this study was to characterize the micromorphology of the foliar trichomes of *Withania somnifera* as well as to elucidate the location and composition of the secretory products. Trichome density and length was also determined in three developmental stages of the leaves. Light microscopy and scanning electron microscopy showed the presence of four morphologically distinct trichome types: glandular capitate, non-glandular dendritic, non-glandular bicellular and non-glandular multicellular. The dendritic trichomes exhibited cuticular warts which are involved in the "Lotus-Effect". 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 also revealed the presence of two major classes of phytochemical compounds that are of medicinal importance, i.e. alkaloids and phenolic compounds. These compounds are used to treat a wide variety of ailments and also act as chemical deterrents in plants. The results of this study explain possible roles of four morphologically distinct trichome types based on their morphology, foliar distribution and content.

Keywords Alkaloids · Calcium oxalate crystals · Capitate trichomes · Cuticular warts · Dendritic trichomes · Phenolic compounds

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Introduction

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). 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). The increased acceptance of traditional medicine has also led to developed countries utilizing medicinal plants in the treatment of both old and emerging sicknesses.

Withania somnifera (L.) Dunal is an evergreen, perennial shrub found in the drier parts of South Africa, India, 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. The leaves and roots of this plant 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).

Plants have specialized structures that produce, secrete and store metabolites that are utilized for medicinal purposes (Pickard 2008). The great variety of important chemical compounds is produced by specialized secretory cells, on many plants in the form of trichomes, and has various functions in the plants themselves (Fahn 2000). Numerous studies have highlighted the structural diversity of glandular trichomes among different plant taxa and even between species (e.g. Gairola et al. 2008; Marin et al. 2012; Rusydi et al. 2013). These secretory compounds are utilized as pharmaceuticals, nutraceuticals, natural pesticides, flavourings, fragrances and for other non-food and fibre purposes (Duke 1994). Trichomes are unicellular or multicellular appendages that originate from the cells of the aerial epidermis (Werker 2000) and vary considerably in morphology, location, ability to secrete and mode of secretion, and different types of trichomes can be produced by the same plant. Trichomes are glandular and non-glandular type and have a range of functions. Morphology of both these trichome types, as found on different plants, varies greatly. Trichome morphology may assist as a useful diagnostic characteristic within the family. The functions of trichomes are dependent on the trichome type and its location (Wagner et al. 2004), and their density may evolve in response to a variation in several environmental factors. Trichomes also play an important role in maintaining plant function upon exposure to environmental stress (Yan et al. 2012).

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 aimed to describe the micromorphology of the trichomes present on the adaxial and abaxial surfaces of the leaves and determine the composition of phytochemicals in the secretions.

Materials and methods

Plant material

Leaves of *W. somnifera* were collected from a private residence in Umhlanga, KwaZulu-Natal (29°43′31″S, 31°5′9″E), South Africa. *W. 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.

Stereomicroscopy

Stereomicroscopy was used to obtain information on trichome type and density. Images of adaxial and abaxial leaf surfaces were obtained using a Nikon AZ100 stereomicroscope equipped with a Nikon Fibre Illuminator as well as the NIS-Elements Software.

Environmental scanning electron microscopy (ESEM)

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.

Scanning electron microscopy (SEM)

SEM preparations involved fixing segments of fresh material in 2.5 % glutaraldehyde for 24 h. The material was subsequently subjected to three 5-min washes with phosphate buffer, followed by a 1 h post-fixation with 0.5 % osmium tetroxide. Thereafter, the material was subjected to three 5-min washes with distilled water and dehydrated in 30, 50 and 70 % alcohol (two changes, each of 5 min); followed by two changes for 10 min in 100 % alcohol. The segments were then dried to their critical point in a Hitachi Critical Point Dryer, 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 min) using a Polaron SC 500 Sputter Coater (Kim et al. 2011). Samples were viewed and imaged 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.

Transmission electron microscopy (TEM)

TEM preparations involved fixing segments (approximately 2 mm^2) of fresh material in 2.5 % glutaraldehyde for 24 h. The material was subsequently subjected to three 5-min washes with phosphate buffer, followed by a 1-h 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-min phosphate buffer washes and dehydrated in 20, 30, 50 and 75 % acetone (two changes, each of 5 min), followed by two changes for 10 min in 75 % acetone and four changes for 10 min in 100 % acetone. The dehydrated leaf sections were infiltrated with equal parts of Spurr's resin (Spurr 1969) and acetone for 4 h, thereafter in whole resin for 24 h. The leaf sections were embedded in whole resin using

silicone moulds, and polymerized at 70 °C in an oven for 8 h.

Sections were cut using a Reichert Jung Ultracut-E ultramicrotome (Boix et al. 2011; Kim et al. 2011). Ultrathin sections (80 nm) were picked up onto copper grids and post-stained with 2.5 % uranyl acetate followed with lead citrate solution (Reynolds 1963). The sections were viewed (at 100 kV) and imaged using a Jeol 1010 TEM equipped with an Olympus MegaView III CCD camera.

Trichome density and length

Selected images obtained from ESEM and SEM were analysed using the iTEM software programme 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. The lengths and widths of all trichomes were also measured. 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.

The differences in trichome density in different developmental stages, as well as between adaxial and abaxial surfaces were analysed, such that there were five 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). The MANOVA was performed on ranked data as the data were not normally distributed ($p < 0.0005$).

A One-way Analysis of Variance (ANOVA) 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 $p < 0.05$ was recognized as being significant.

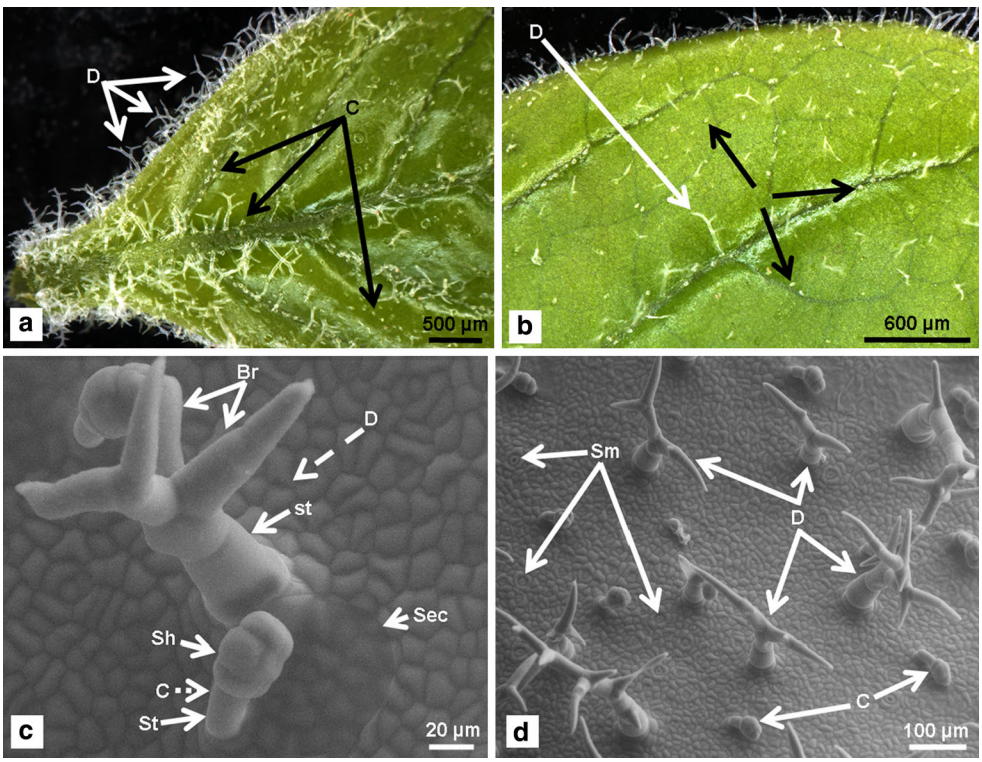

Fig. 1 Dendritic and glandular capitate trichomes on fresh leaves of *W. somnifera*. **a** Non-glandular dendritic (*D*) and glandular capitate (*C*) trichomes on emergent adaxial surface. **b** Non-glandular dendritic (*D*) and glandular capitate (*C*) trichomes on young adaxial surface. **c** Dendritic trichome with four branches (*Br*) and glandular capitate trichome with a single-celled stalk (*St*) and six-celled secretory head (*Sh*) on emergent leaf surface. The secretion (sec) of the capitate trichome has been released from the secretory head. **d** Glandular capitate trichomes adjacent to non-glandular dendritic trichomes. Branches of dendritic trichomes tower over capitate trichomes; on young leaf surface. Stomata (*Sm*) also visible on leaf surface. **a**, **b** Stereomicroscopy, **c**, **d** SEM

Histochemistry

Fresh emergent (80 µm) and young (100 µm) leaf sections were cut using an Oxford vibratome sectioning system. Leaf sections were stained with Wagner's and Dittmar reagents for alkaloids (Furr and Mahlberg 1981; Ascensão and Pais 1987); Nile blue for lipids (Cain 1947; Ascensão and Pais 1987); Sudan III and IV, and Sudan Black for lipids and cutin/suberin (Furr and Mahlberg 1981; Ascensão and Pais 1987); phloroglucinol for lignin aldehydes; Hydroxylamine hydrochloride solution for esterified pectins; ferric trichloride for phenolic compounds; bromophenol blue for total proteins; Ruthenium Red for polysaccharides and unesterified pectins; and Toluidine Blue for carboxylated polysaccharides and polyphenols. Histochemically stained sections were viewed and images captured with the Nikon Eclipse 80i light microscope.

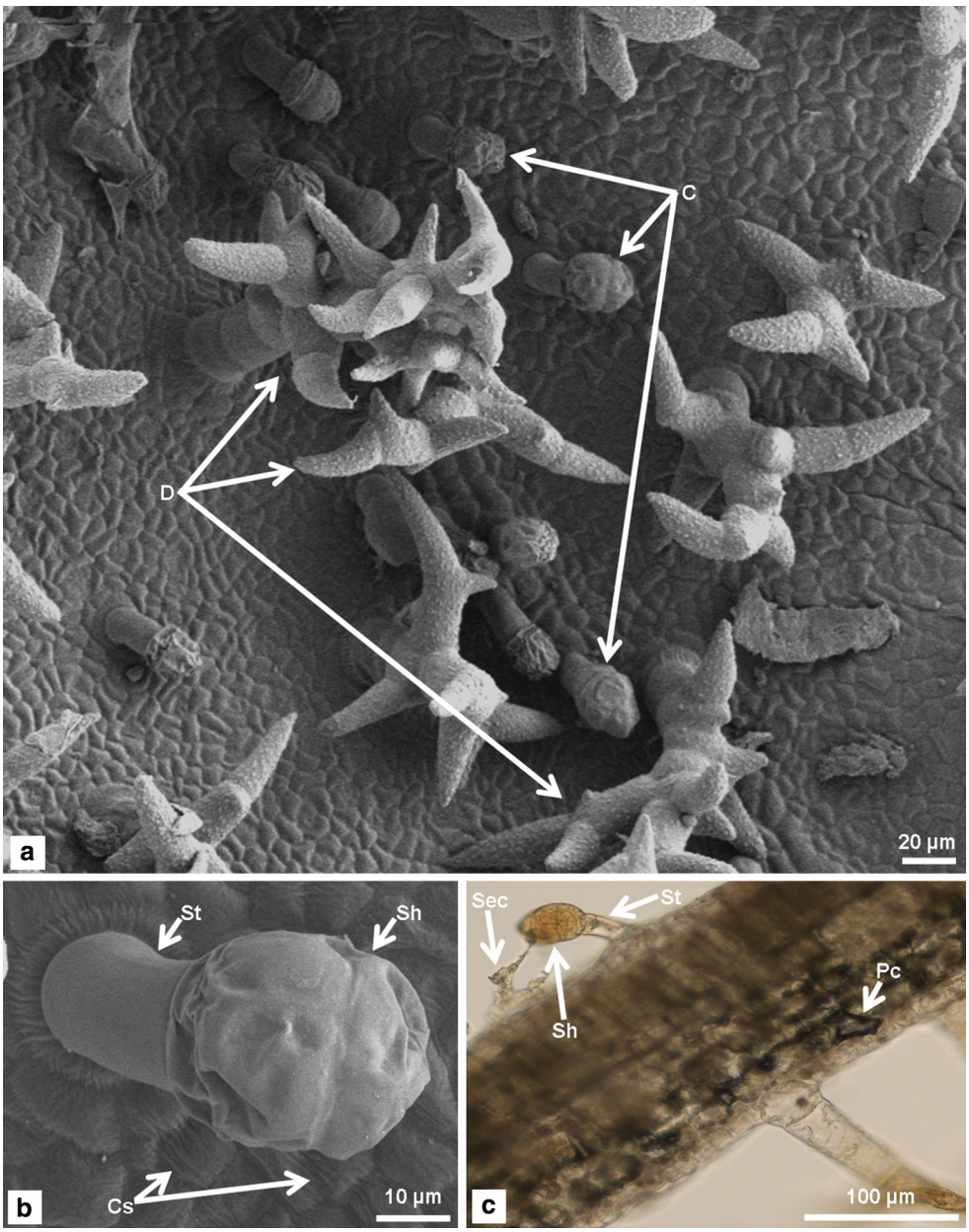

Fig. 2 Glandular capitate trichomes. **a** Six-celled glandular capitate (*C*) trichomes in close proximity to non-glandular dendritic (*D*) trichomes on emergent adaxial leaf surface. **b** Glandular capitate trichome with a single-celled stalk (*St*) and six-celled secretory head (*Sh*) on emergent leaf surface. Cuticular striations (*Cs*) are also visible on leaf surface. **c** Glandular capitate trichome with single-celled stalk (*St*) and six-celled secretory head (*Sh*) on emergent leaf section. A prismatic crystal (*Pc*) is also present within the leaf section. **a**, **b** SEM, **c** Light microscopy

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.

Fresh hand-cut sections were also stained with 2 % acridine orange for 2 min 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, indicating viability of cells (Mirrett 1982; Winter et al. 2007).

Results

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 (Figs. 1, 2, 3, 4). Cuticular striations were visible on both foliar surfaces (Figs. 2b, 3a). 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. 1d).

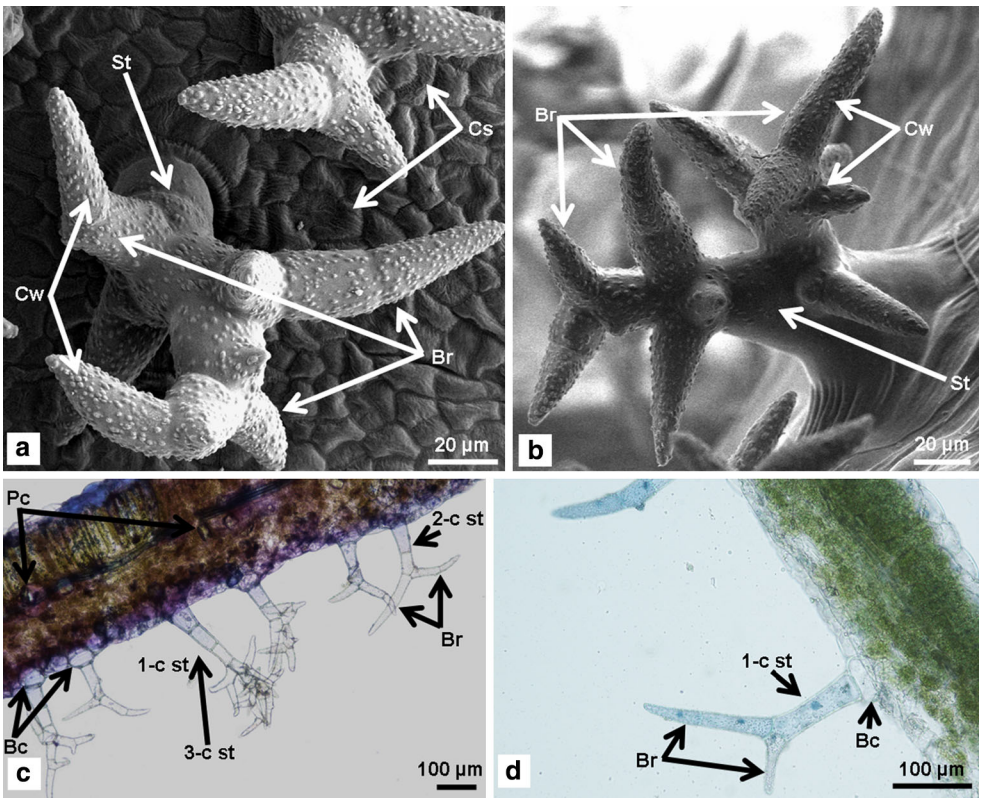

Fig. 3 Non-glandular dendritic trichomes with cuticular warts. **a** Aerial view of a non-glandular dendritic trichome on emergent adaxial leaf surface. Cuticular warts (*Cw*) are present on the surface of the trichome and cuticular striations (*Cs*) are visible on the leaf surface. **b** Side view of a non-glandular dendritic trichome on mature abaxial leaf surface. Cuticular warts (*Cw*) are present on the surface. **c** Toluidine blue stained section containing non-glandular dendritic trichomes with various stalk cell numbers (1-, 2- and 3-c st) and prismatic crystals (*Pc*). **d** Section stained with Sudan III and IV containing a non-glandular trichome with a single-celled stalk, emanating from a single basal cell (*Bc*). **a**, **b** SEM, **c**, **d** Light microscopy

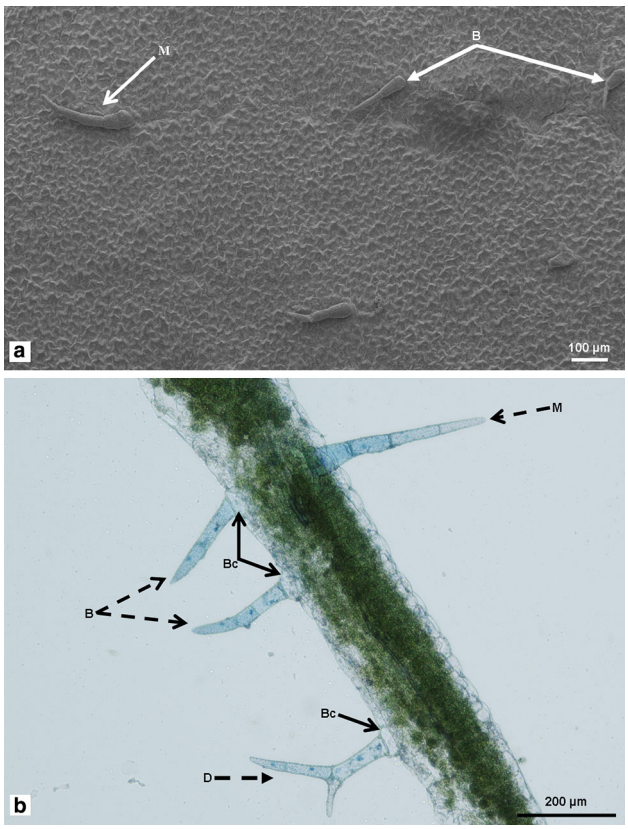

Fig. 4 Non-glandular bicellular and multicellular trichomes. **a** Bicellular (*B*) and multicellular (*M*) trichomes on adaxial surface of mature leaf. **b** Section stained with Sudan black containing a 4-celled uniseriate multicellular (*M*) trichome, bicellular trichomes emanating from single basal cells (*Bc*) and a non-glandular dendritic trichome (D) with a single-celled stalk, emanating from a single basal cell (*Bc*). Figure 3**a** SEM, **b** Light microscopy

Glandular secretory trichomes

Glandular capitate trichomes contained a single-celled stalk and a six-celled secretory head (Fig. 2). The secretions of glandular trichomes were also observed (Figs. 1c, 2b). Post-secretory trichomes did not appear to have a definitive rupture in the secretory head. Glandular capitate trichomes 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 and were occasionally orientated parallel to the leaf surface.

Non-glandular trichomes

The non-glandular dendritic trichomes consisted of 2–4 stalk cells and varying branch numbers (Fig. 3). 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. 4). 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. 3a, b). All three non-glandular trichome types originated from single protodermal cells and were highly vacuolated (Fig. 8a).

Trichome distribution and density

On initial inspection of the images obtained, trichome density appeared to decrease with progressive leaf development (Fig. 5). 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 (Fig. 5), with non-glandular dendritic trichomes appearing to 'arch over' glandular capitate trichomes. Trichomes on emergent leaves were so dense that it was difficult to view the leaf surface. 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 ($df = 4$, $F = 47.496$, $p < 0.0005$).

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 ($p < 0.0005$) and mature leaves ($p < 0.0005$). Figure 6 shows these differences in dendritic trichome densities between adaxial and abaxial leaf surfaces; however, there was no direct pattern across emergent, young and mature leaves. There were also significant differences in the densities of non-glandular bi-/multi-cellular trichomes across the five different locations ($p < 0.0005$). However, Fig. 6 shows bi-/multi-cellular non-glandular trichomes to be present only on mature adaxial ($1 \pm 1/mm^2$) leaf 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 (Fig. 7). Due to a significantly low number of glandular trichomes on the abaxial surface of mature leaves, the

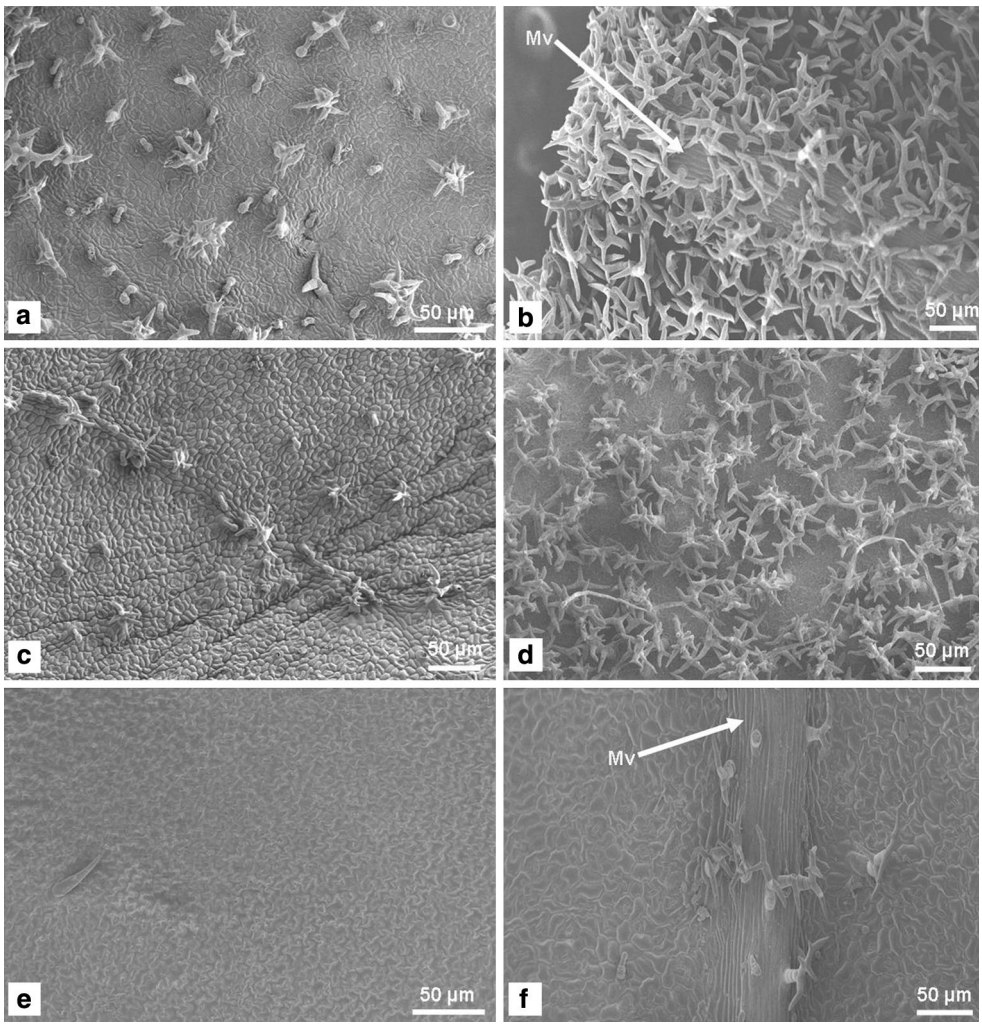

Fig. 5 SEM micrographs showing the decrease in trichome density with increasing leaf development. **a** Adaxial surface and **b** abaxial surface of emergent leaf; **c** adaxial surface and **d** Abaxial surface of young leaf; **e** adaxial surface and **f** abaxial surface of mature leaf

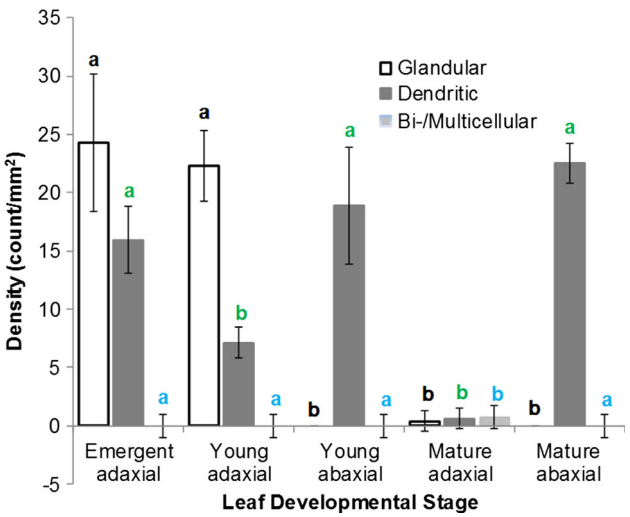

Fig. 6 Frequency of glandular capitate (capitate), non-glandular dendritic (dendritic) and non-glandular bicellular and multicellular (bi-/multi-cellular) trichomes at different leaf developmental stages ($n = 5$)

Tukey's test did not take into account the comparisons between young abaxial and mature abaxial surfaces. Figure 7, however, shows 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 ($df = 3$, $F = 2.640$, $p = 0.093$). According to Fig. 7, 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. 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 Fig. 7, dendritic trichomes on adaxial surfaces were greater in length on emergent leaves (181 ± 78 µm) as compared to young leaves (124 ± 54 µm).

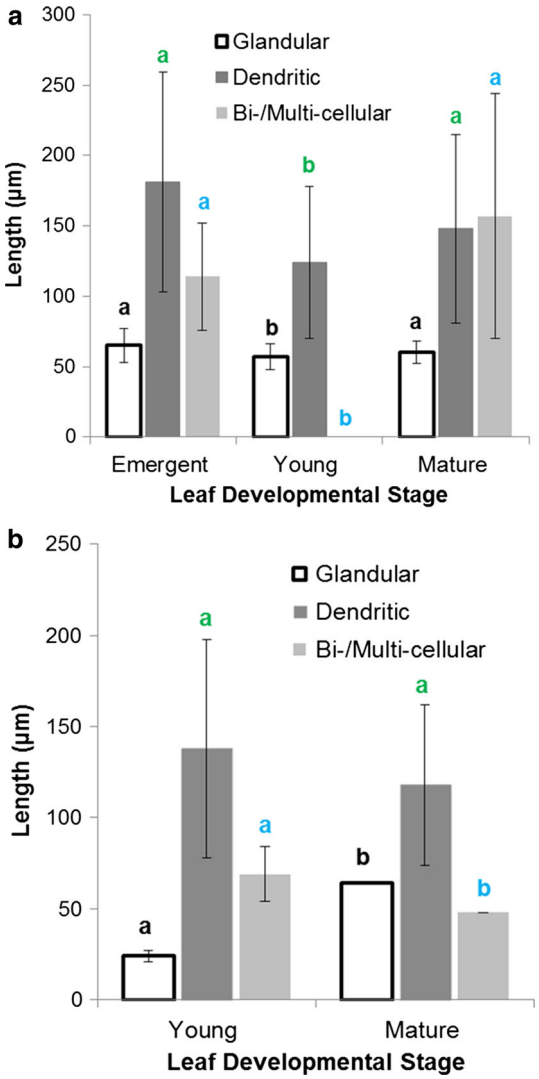

Fig. 7 Average lengths of glandular capitate (capitate), non-glandular dendritic (dendritic) and non-glandular bicellular and multicellular (bi-/multi-cellular) trichomes at different leaf developmental stages. **a** Trichome lengths on adaxial surfaces. **b** Trichome lengths on abaxial surfaces

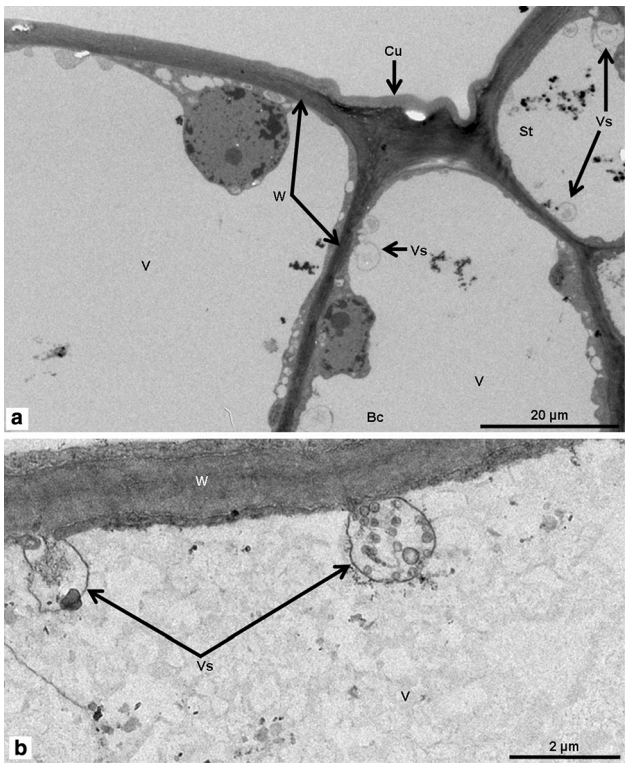

Fig. 8 TEM micrographs of dendritic trichomes. **a** Highly vacuolated (*V*) basal (*Bc*) and stalk (*St*) cells of dendritic trichome. Cuticle (*Cu*), nucleoli (*N*) and vesicles (*Vs*) are also visible. **b** Vesicles (*Vc*) within stalk cell of dendritic trichome

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. 8a). Plastids were also found to be closely appressed to the cell walls of dendritic trichomes (Fig. 9a). Basal and stalk cells of dendritic trichomes appeared highly vacuolated (Figs. 8a, 9a). 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. 8b). Multivesicular structures, probably artifactual in nature, were commonly observed within the vacuoles (Fig. 8b). Cuticular warts appeared as bumps or globular protrusions of the cells walls of dendritic trichomes, with cuticular thickening over raised surfaces (Fig. 9a). It was interesting to note the presence of cellulose microfibrils within the cell walls (Fig. 9a).

The heads of glandular trichomes comprised of six cells. These cells appeared densely cytoplasmic. Thin cell walls separated the cells of the glandular secretory head of capitate trichomes (Fig. 9b). 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. 9b). The secretory material within the glandular head appeared to be loosely fibrillar (Fig. 9b).

Histochemistry

Various histochemical stains (Table 1) were used to detect the presence and localization of major chemical compound groups in glandular and non-glandular trichomes. Staining with ruthenium red 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. 10a). Polysaccharides were

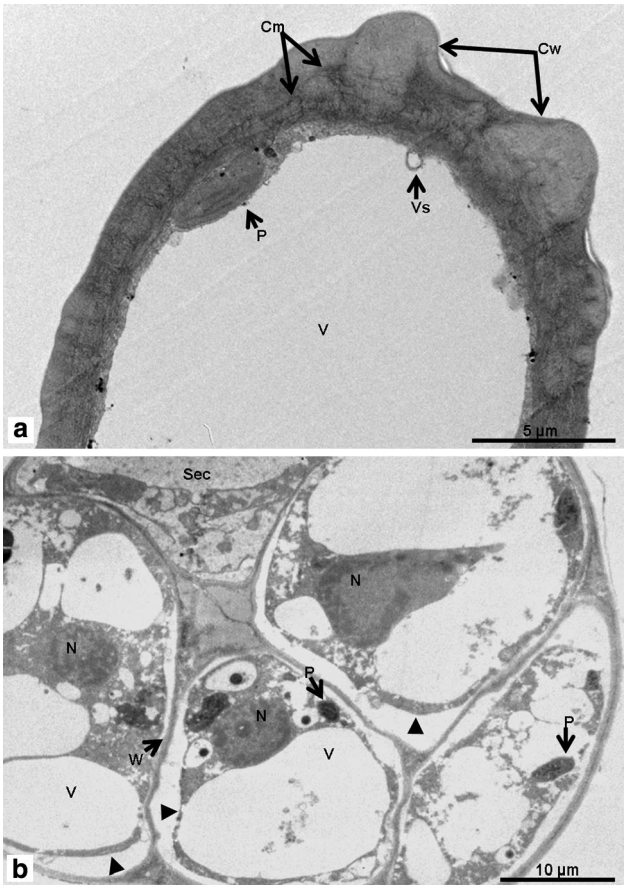

Fig. 9 TEM micrographs of the glandular secretory head of a capitate trichome and cuticular warts of a dendritic trichome. **a** Cuticular warts (*Cw*) of highly vacuolated (*V*) dendritic trichome. A plastid (*P*), vesicle (*Vs*) and cellulose microfibrils (*Cm*) are also visible. **b** Glandular secretory 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

present in the stalk cells of dendritic trichomes, as well as in the lower cells of bicellular and multicellular trichomes (Fig. 10a). The stalk cells of capitate trichomes and all cells of non-glandular trichomes were cutinized/suberized, as shown with Sudan black (Fig. 10b) and Sudan III and IV. Lignin aldehydes were present only in glandular capitate trichomes. Total proteins were present in all cells of the glandular capitate and non-glandular trichome types (Fig. 10c, d).

The secretory head of capitate trichomes stained orange-brown with Wagner's and Dittmar reagents, indicating the presence of alkaloids (Fig. 11a, 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. 11d), indicated by the dark greenish-black staining with ferric trichloride. Phenolic compounds were also present in all three non-glandular trichome types (Fig. 11c). Sections stained with toluidine blue showed that all cells of the non-glandular trichome types were lignified (Fig. 12a, b). Glandular capitate trichomes of sections stained with hydroxylamine hydrochloride solution stained reddish-brown, indicating the presence of esterified pectins (Fig. 12c). 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. Calcium oxalate (CaOx) crystals were also present in leaf sections. These crystals occurred in two forms, prismatic (Pc) (Fig. 12a) and druse (Dc) (Fig. 10a).

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. 13a, b). The branches of the non-glandular trichomes did not appear to be viable or metabolizing 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. 13c). 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. 13d) indicative of the presence of phenolic compounds (Ascensão and Pais 1987).

Discussion

Four morphologically distinct trichome types were observed on the foliar surfaces of *W. somnifera*; glandular capitate, non-glandular dendritic, non-glandular bicellular and non-glandular multicellular trichomes. All four trichome types have been reported previously within the family *Solanaceae* (Harisha and Switu 2013; Adedeji et al. 2007). 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*. Non-glandular trichomes might also reduce transpiration rates and limit leaf surface exposure to extreme temperatures (Werker 2000; Dai et al. 2010). Since *W. somnifera* might occur in dry regions, dendritic trichomes are likely to play a major role in water conservation (Hameed and Hussain 2011).

Glandular capitate and non-glandular dendritic trichomes were found to be aggregated on the mid-veins (Fig. 5) of leaves. Within Solanaceae, secondary metabolites such as alkaloids and phenolic compounds are

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 and methanol; Sudan Black			Head cells of capitate trichomes stained brown; non-glandular trichomes stained brown
	Pre-treatment with chloroform and 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 and 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

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.

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 et al. 1993; Duke 1994; Gairola et al. 2008). This was true for the glandular capitate trichomes. 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). Dendritic trichome density is much higher on adaxial surfaces of emergent leaves as compared to young and mature leaf surfaces. Emergent leaves might require more protection by trichomes as 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. 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 have not reached maturity at the emergent leaf stage. The development of non-glandular dendritic trichomes was also evident from the cellulose microfibrils observed in TEM micrographs. Randomly organized cellulose microfibrils are generally located 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).

Even though non-glandular trichomes are considered to be non-secretory, autofluorescence microscopy of leaf

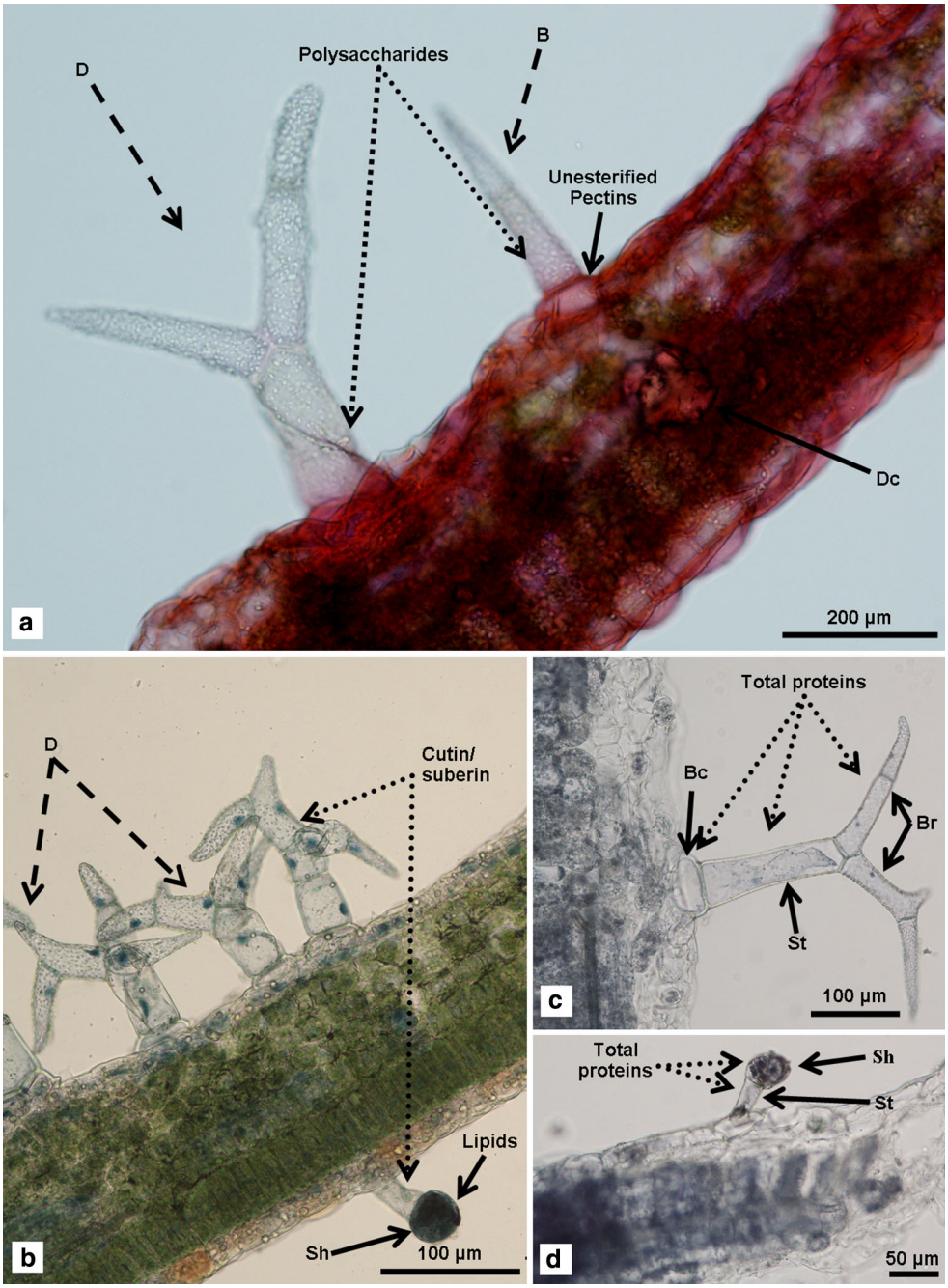

Fig. 10 Emergent and young leaf sections showing the presence of polysaccharides, total proteins druse crystals and cutinized cells. **a** Basal (*Bc*) and lower cells of dendritic (*D*) and bicellular (*B*) trichomes containing polysaccharides (stained *dark pink-red* with Ruthenium red). Section also contains a druse crystal (*Dc*). **b** Cutinized 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). **a**–**d** Light microscopy

sections stained with acridine orange showed that the basal and stalk cells of these trichomes are viable (Fig. 13a, b). 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. The chemical compound groups of medicinal importance found in both glandular and non-glandular trichomes were alkaloids and phenolic compounds. Although poisonous, alkaloids are used in low concentrations in the medicinal industry to

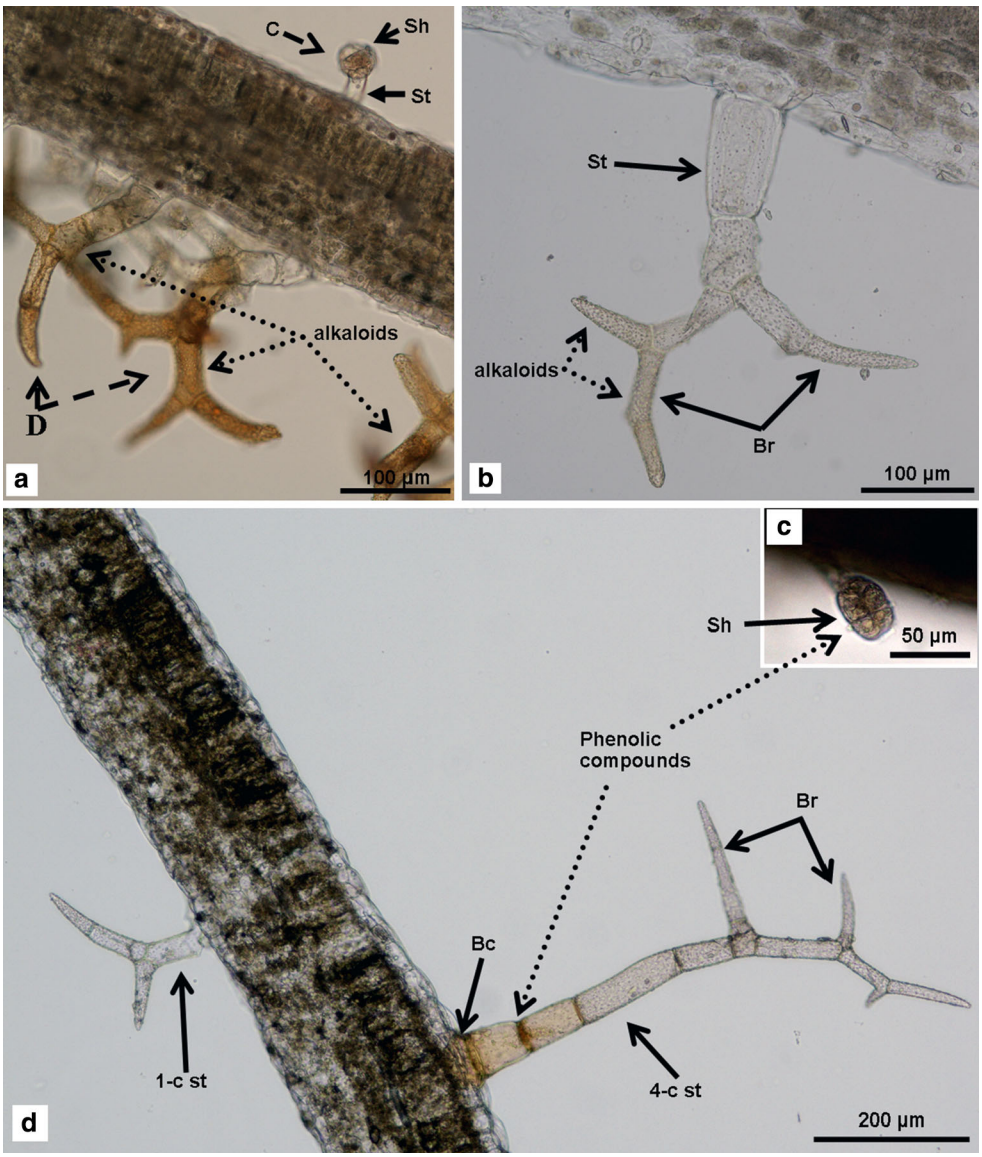

Fig. 11 Emergent and young leaf sections showing the presence of alkaloids and phenolic compounds. **a** Secretory head (*Sh*) of glandular capitate (*C*) trichome and branches (*Br*) of dendritic (*D*) trichomes containing alkaloids (stained *orange-brown*). **b** Branches (*Br*) of dendritic trichome containing alkaloids (stained *orange-brown*). **c** Secretory head (*Sh*) of glandular capitate trichome containing phenolic compounds (stained *dark-green*). **d** Lower cells of dendritic trichome containing phenolic compounds (stained *dark-green*). **a**–**d** Light microscopy

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 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 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 inhibit the oxidation of specific proteins (Negro et al. 2003). Exudates 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

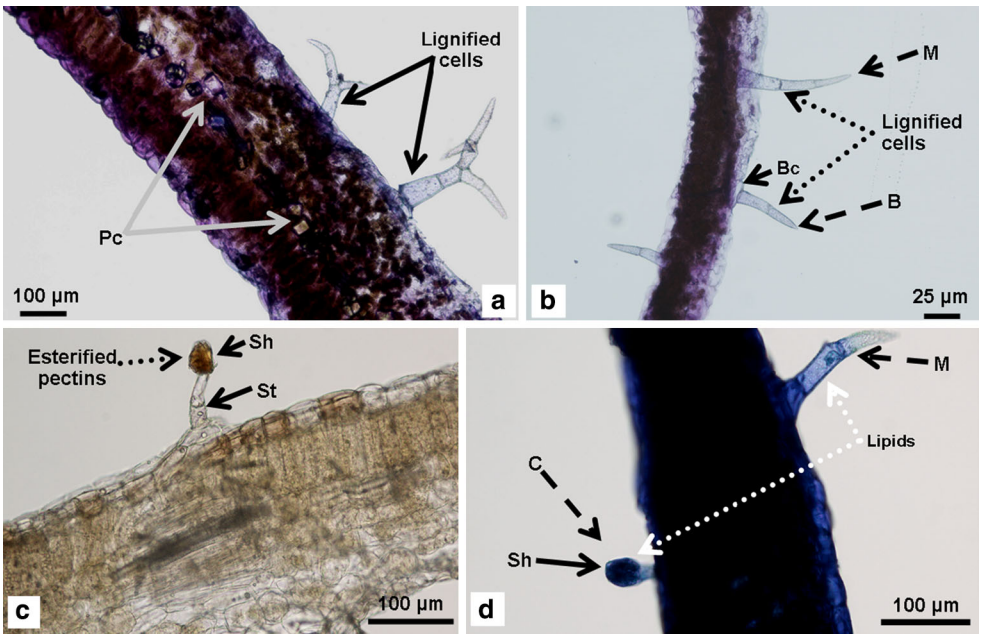

Fig. 12 Emergent and young leaf sections showing the presence of lignin, esterified pectins and lipids. **a** Lignified cells (stained *blue*) of dendritic trichomes on emergent leaf section stained with toluidine blue. Section also contains prismatic crystals (*Pc*). **b** Lignified cells (stained *blue*) of multicellular (*M*) and bicellular (*B*) trichomes. **c** Secretory head (*Sh*) of glandular capitate trichome containing esterified pectins (stained *reddish-brown* with hydroxylamine hydrochloride solution). **d** Non-glandular multicellular (*M*) and glandular capitate (*C*) trichomes containing lipids (stained *blue* with Nile blue). **a**–**d** Light microscopy

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).

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).

Fluorescence microscopy (Fig. 13) and TEM (Figs. 8; 9a) 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. 9). 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).

Prismatic (Fig. 12a) and druse (Fig. 10a) crystals in the leaves of *W. somnifera* 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). Functions of crystals include plant protection, calcium regulation and metal detoxification (Arnott and Webb 2000; Franceschi and Nakata 2005). 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*, protects the plant against herbivore attack by causing irritations or burning sensations in the mouth and throat.

Cuticular warts are 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 result from environmental conditions (Bhatt et al. 2010). This mechanism may

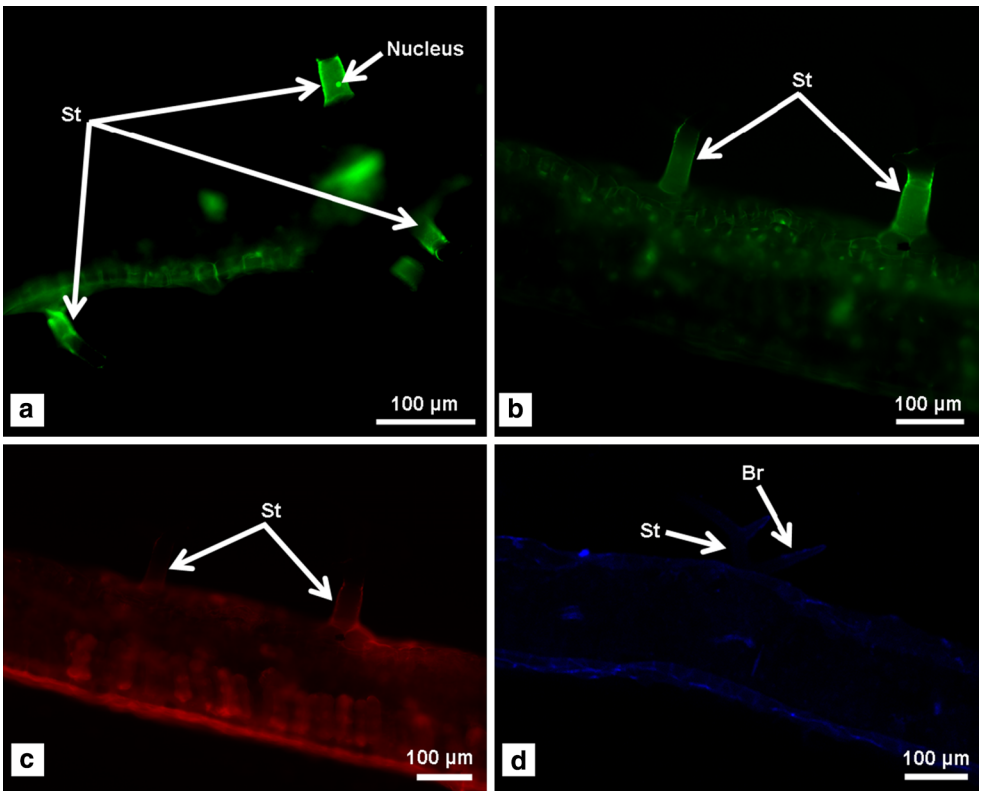

Fig. 13 Autofluorescence of young leaf sections. **a** Viable stalk (*st*) cells of dendritic trichomes autofluoresce green. Autofluorescent nucleus is also visible in stalk cells (sections stained with acridine *orange*). **b** Stalk cells (*st*) of dendritic trichomes and nuclei of epidermal emitting a *green* autofluorescence (sections stained with acridine *orange*). **c** *Red* autofluorescence exhibited by cells of leaf section as well as stalk cells (St) of dendritic trichomes indicating the presence of plastids. **d** *Blue* autofluorescence exhibited by epidermal layers as well as stalk cells (*St*) and branches (*Br*) of dendritic trichomes indicating the presence of phenolic compounds. **a–d** Confocal microscopy

also assist in defending leaves against pathogen attack (Barthlott and Neinhuis 1997).

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 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.

Author contribution statement PM and YN conceived and designed the research and conducted experiments. PM and YN analysed data. PM, YN and GN wrote the manuscript. All authors read and approved the manuscript.

Acknowledgments The authors would like to thank Preyan Arumugam, Kashmira Raghu and Ashlin Munsamy for research assistance and aiding with histochemical preparation. Drs S. Gairola and C.T. Sadashiva are thanked for editing the manuscript and assistance with phytochemical and TLC studies, respectively. Funding from the National Research Foundation (NRF) is also gratefully appreciated.

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