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Micromorphological evaluation of the foliar trichomes of field grown and micropropagated *Stachys natalensis* Hochst. (Lamiaceae)

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ABSTRACT

Stachys natalensis Hochst is a perennial shrub with aromatic leaves that are covered with hairs. Micromorphological features of the leaf secretory structures were evaluated using scanning electron microscopy. Glandular and non-glandular trichomes were present on the adaxial and abaxial foliar surfaces of field grown and micropropagated plants. Greater trichome distribution was observed on the abaxial surface, decreasing as the leaf developed ($P < 0.05$). Micropropagated plants did not show gross morphological abnormalities compared to field-grown plants, apart from the presence of visibly longer non-glandular trichomes. Foliar peltate and capitate glandular trichomes identified across most developmental stages were morphologically similar between micropropagated and field grown plants. This study shows that the mass *in vitro* propagation of the species, as a means of mitigating declining numbers in the wild, does not compromise trichome morphology or distribution.

1. Introduction

Stachys is one of the largest genera in the flowering family Lamiaceae, comprised of more than 300 different species (Tundis et al. 2014). Many members of this genus have been used as ornamental plants, edible food sources, and as therapeutic agents in traditional medicine (Piozzi and Bruno, 2011). Many *Stachys* spp. are used in ethnomedicine in the form of extracts or decoctions made from the aerial parts of the plant and roots (Piozzi and Bruno 2011). However, removal of these parts requires whole plant destruction (Mander et al. 2007). The high demand for plant organs for traditional medicine and novel drug development merits the use of plant tissue culture techniques to produce preferred plant genotypes or specific plant organs. Furthermore, *in vitro* propagation may alleviate the pressure of over-harvesting medicinally important species (Legkobit and Khadeeva 2004).

Stachys natalensis Hochst. is a perennial, aromatic shrub that may be erect or straggling and grows up to 2 m in height. It is found in grassy and woody areas along the east coast of southern Africa, as well as in certain areas of Swaziland and Zimbabwe. *Stachys natalensis* retains familial traits, such as bilabiate flowers and thin, opposite leaves. The leaves are narrowly cordate with crenate margins and are covered with hairs on both foliar surfaces, as well as along the multiple, branched stems (Pooley 1998). Foliar hairs, known as trichomes, originate from epidermal cells and may vary in appearance, size, and distribution among different plant species (Werker 2000). Most glandular trichomes secrete biologically active compounds which may be responsible for the curative properties of a plant (Naidoo et al. 2012). There are many discrepancies in the taxonomic evaluation of the genus *Stachys* due to its variable morphology and wide habitat (Dinc and Ozturk 2008). Trichome morphology may be a useful preliminary classification tool to resolve complex phylogenetic relationships within the genus (Salmaki et al. 2009).

This study was undertaken to describe and compare key micromorphological features of the foliar structures between field grown and micropropagated plants.

2. Materials and methods

2.1. Plant collection

Stachys natalensis plants were collected from Durban, KwaZulu-Natal. A voucher specimen (Kalicharan 02) was prepared and deposited at the Ward Herbarium, University of KwaZulu-Natal, Westville Campus. Randomly selected leaf specimens at different developmental stages, *i.e.*, emergent (<10 mm leaf length), young (10–25 mm leaf

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length), and mature (>30 mm leaf length) were used for each sample preparation batch ($n = 10$).

2.2. Micropropagation

Field-grown plants were obtained from Durban, South Africa. Axillary bud segments were used as nodal explants (10–15 mm) and were initially surface decontaminated by intermittent washing with tap water for 20 min. Explants were further immersed in 1% NaOCl for 5 min followed by 20 min in 0.1% $HgCl_2$, and thereafter rinsed with sterile distilled water thrice. Nodal explants were cultured on MS salts (Murashige and Skoog 1962) supplemented with 20.0 g/l sucrose, 0.5 mg/l kinetin, 0.5 mg/l indole acetic acid (IAA), and 0.8% (w/v) agar. The pH was adjusted to 5.7 ± 0.1 by the addition of either 1 M HCl or 1 M NaOH before autoclaving at 121 °C (1 kPa) for 20 min. Cultures were incubated for 12 weeks in a growth room with a 16 h photoperiod, at 200 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD), and air temperature of 26 ± 0.1/23 ± 0.1 °C (day/night). Following shoot induction and multiplication, *in vitro* shoots of between 8 and 10 cm were transferred to 50 ml culture tubes with 10 ml of rooting medium which contained ½ strength MS salts supplemented with 2 mg/l IAA, for 5 weeks. Rooted plantlets were transplanted to plastic pots containing organic potting soil and kept in a mist tent (three second water spray per six-minute interval) for acclimatization. The soil was supplemented with ¼ strength MS salts for a week. After a month, acclimatized plants were transferred and maintained in a greenhouse till use. Leaves from these acclimatized plants, at different stages of development, were harvested for micromorphological evaluation using scanning electron microscopy.

2.3. Microscopy

Scanning electron microscopy (SEM) was used to investigate trichome micromorphology and distribution on leaves of field- and *in vitro*-derived plants. Leaves were subjected to two different modes of preparation for SEM, *i.e.*, freeze-drying and cryo-SEM to overcome material distortion and obtain high quality images. Fresh leaf sections of approximately 2 mm^2 were quenched in liquid nitrogen and freeze-dried in an Edwards' Modulyo freeze-dryer for 72 h (−40 to −60 °C; 10^{-1} Torr). Samples were mounted on brass stubs using double-sided carbon adhesive tape and sputter-coated with gold in a Polaron SC500 sputtercoater. Samples were viewed with a Zeiss UltraPlus Field Emission microscope. Areas of interest were identified and images were captured using SmartSEM image software (v.0.5 Zeiss, Germany). For cryo-SEM, leaf segments (±1 mm^2) were mounted on brass stubs with 50:50 colloidal graphite & Tissue-Tek® O.C.T compound (Sakura® Finetek) mounting medium. Samples were quenched in liquid N_2, sublimed at −90 °C for 10 min, and coated with platinum (60 s; 10 mA) under vacuum. Samples were viewed and images were captured using a Zeiss UltraPlus Field Emission microscope with an additional Quorum PP3000T Cryo-SEM system.

2.4. Statistical analysis

Glandular and non-glandular trichome counts (per cm^2 leaf surface) were obtained from SEM micrographs using the iTEM imaging software (Soft Imaging System GmBH, Münster, Germany). Mean shoot

Fig. 1. (A) *In vitro* shoot multiplication of *Stachys natalensis* axillary nodal explants after six weeks in culture (Bar: 10 mm); (B) *In vitro* propagated plantlet after five weeks in culture before acclimatization (Bar: 80 mm); (C) Micropropagated plants in the greenhouse.

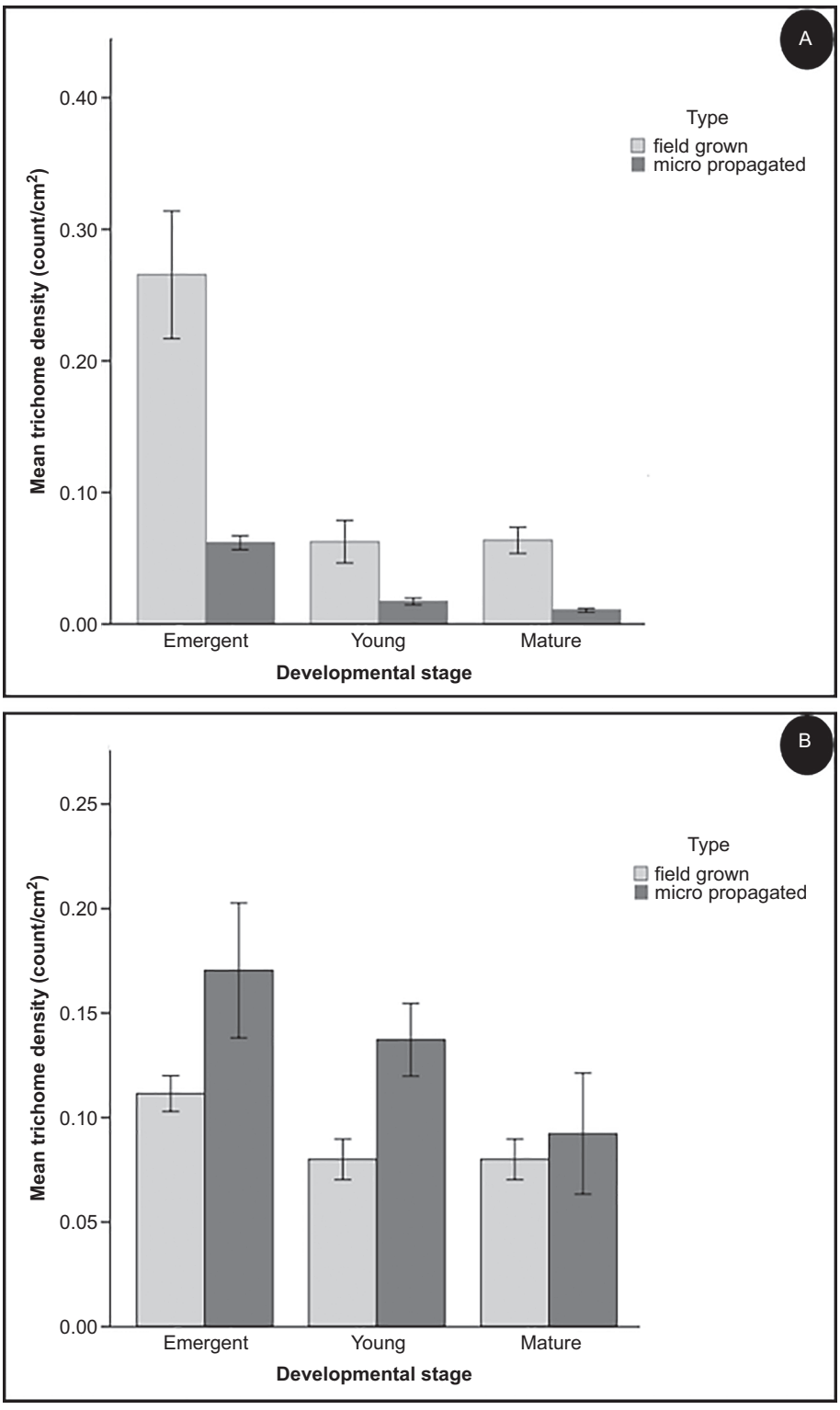

Fig. 2. Mean trichome density of field grown and micropropagated plants on abaxial surface. (A) Mean non-glandular trichome density at three stages of leaf development (emergent, young and mature); (B) Mean glandular trichome density at three stages of leaf development (emergent, young and mature). Values are mean ± SE.

production per explant and shoot height per explant was assessed using a one-way ANOVA. A two-way ANOVA statistical test was conducted in IBM SPSS Statistics (version 21.0, IBM®, USA) to determine trichome density across three developmental stages (emergent, young, and mature) of field grown and micropropagated plants as well as between the abaxial and adaxial foliar surfaces. Tukey's post-hoc test was implemented to obtain multiple comparisons of factors investigated.

3. Results

Micropropagated plants showed significant shoot proliferation (9.1 ± 3.6 shoots/explant) and elongation (50.2 ± 5 mm) after 6 weeks in culture (Fig. 1 A). Rooting occurred in 64% of plantlets after five weeks. The average number of roots formed per shoot was 5.54 ± 0.43 and the mean root length per shoot was

Table 1
Distribution of the different types of trichomes on the foliar surfaces of field grown and micropropagated *Stachys natalensis* across different developmental stages.

Plant	Leaf age	Foliar surface	Trichome type			
			Non-glandular	Peltate	Capitate type I	Capitate type II
Field grown	Emergent	Abaxial	++	++	++	++
		Adaxial	+++	++	−	+
	Young	Abaxial	++	++	++	++
		Adaxial	+++	++	−	−
	Mature	Abaxial	+	++	+	+
		Adaxial	+	+	−	−
Micropropagated	Emergent	Abaxial	++	+++	++	++
		Adaxial	++	++	−	++
	Young	Abaxial	++	++	+	+
		Adaxial	++	++	−	−
	Mature	Abaxial	+	++	+	+
		Adaxial	+	+	−	−

absent (−): not found on foliar surface.
scarce (+): occurs <15% of foliar surface.
present (++): occurs 15–60% of foliar surface.
abundant (+++): occurs >60% of foliar surface.

66.13 ± 3.55 mm. Plantlets showed 92 ± 4.2% survival after six weeks in the greenhouse (Fig. 1 B and C).

The leaf surfaces of field grown and micropropagated plants were visibly hispid and bore numerous non-glandular and glandular trichomes of different morphologies. In the present study, glandular and non-glandular trichomes were more densely distributed on the abaxial and adaxial surface, respectively. A greater glandular trichome density was detected on micropropagated plants compared to field grown plants ($F_{(1,54)} = 7.707$; $P < 0.05$; $\text{Mean}_{(\text{field grown})} = 0.235$; $\text{Mean}_{(\text{micropropagated})} = 0.387$). Non-glandular trichome density was higher in field grown plants as compared to micropropagated plants ($F_{(1,54)} = 33.243$; $P < 0.05$; $\text{Mean}_{(\text{field grown})} = 0.131$; $\text{Mean}_{(\text{micropropagated})} = 0.030$). In both cases, trichome density was significantly higher on emergent leaves, decreasing as the leaf matured (Fig. 2). Non-glandular trichomes were especially abundant on the adaxial foliar surface of both field grown and *in vitro* micropropagated emergent and young plants (Table 1).

The non-glandular trichomes of micropropagated plants, similar to their field grown counterparts, appeared uniseriate and unbranched. These trichomes consisted of an elongated, septate stalk attached to the leaf epidermis *via* a multicellular basal pedestal which was composed of between 2 and 10 epidermal cells. Interestingly, the length of these non-glandular trichomes appeared to be greater than those found on the surface of field grown plants (Fig. 3). Glandular trichomes, observed on the foliar surfaces of field grown and micropropagated plants across most developmental stages, were divided into two types: peltate and capitate trichomes. Peltate trichomes were attached to the epidermis *via* a small stalk (Fig. 4 A) with a large, multicellular head which appeared to contain between two and eight head cells (Fig. 4 B and C). These trichomes were present on both foliar surfaces across all developmental stages of field grown and micropropagated plants (Table 1). Two subtypes of capitate glandular trichomes were observed: Type I and Type II (Fig. 4 D and E). Type I was characterized by an elongated stalk and a spherical shaped head that may be bi-cellular or unicellular, according to Werker (2000) (Fig. 4 D). Type II capitate trichomes were composed of a bulbous base, long, tapering septate stalk, a distinctive neck cell, and a multicellular head cell (Fig. 4 E). Capitate trichomes were either completely absent or scarce on older leaves. Type I and II was observed primarily on the abaxial surfaces of emergent and young leaves (Table 1).

Glandular trichomes were observed in both pre- and post-secretory phases. The former was characterized by the accumulation of secretion in the subcuticular space in peltate head cells (Fig. 5 A). Glandular trichomes in the post-secretory phase showed a distinct contraction of the secretory head cells upon loss of secretion, possibly *via* a porose cuticle (Fig. 5 B). Two possible mechanisms of secretion were observed in Type I and Type II capitate trichomes. Loss of secretion *via* cuticular rupture was noted in Type I capitate trichomes (Fig. 5 D), while Type II capitate trichomes were shown to secrete substances *via* pore-like openings on each of the multicellular head cells (Fig. 5 C). Morphological characteristics of each type, such as stalk septation, number of secretory head cells and mode of secretion, were maintained in both field grown and micropropagated plants.

4. Discussion

The indumentum of *S. natalensis* Hochst contains a spread of both glandular and non-glandular trichomes on both foliar surfaces, as seen in most species belonging to the Lamiaceae family (Werker et al. 1993; Bhatt et al. 2010a; Rusydi et al. 2013). In the present study, trichome density progressively decreased with leaf maturation. The phenomenon of leaf expansion has been used in the literature to account for the gradual decrease in trichome density from young leaves to older specimens (Ascensao et al. 1995). In some plants, trichomes may be produced in young leaves only resulting in a decline in trichome density as the leaf grows and increases in surface area (Valkama et al. 2004). In other plants, trichomes are produced throughout leaf development and expansion (Maffei et al. 1989) which maintains the dense indumentum across all growth stages. The change in trichome density is thus subject to the rate of trichome production during leaf growth (Oosthuizen and Coetzee 1983). The greater number of non-glandular trichomes on the younger leaves of field grown plants suggests that these trichomes serve a protective function over their glandular counterparts during early development, which may or may not be required at later stages. Conversely, micropropagated plants had a lower non-glandular trichome density. Many studies indicate that significant increases in trichome density are due to exposure to adverse conditions, such as mechanical damage or herbivory (Jaime et al. 2012; Loughner et al. 2012), water loss (Abdulrahaman and Oladele 2011) and environmental stresses (Bhatt et al. 2010b; Adebooye et al. 2012), most of which are lacking in a controlled *in vitro* environment.

The non-glandular trichomes of *S. natalensis* were uniseriate and unbranched, appearing jointed in form. These were typical of the non-glandular trichomes of other *Stachys* spp. found worldwide, including *Stachys cretica L. subsp. vacillans*, *S. ehrenbergii* and *S. distans* from Lebanon (El-Beyrouthy et al. 2009), *S. ballotiformis* and *S. megalodonta* from Iran (Salmaki et al. 2009) and *S. iva* from Macedonia (Kremer et al. 2016). Non-glandular trichomes of *S. natalensis* contained a multicellular basal pedestal which contained up to 10 epidermal cells, depending on the stage of differentiation. These raised cells serve to

support the trichome and to provide a point of attachment that "anchors" the trichome to the epidermal surface (Bhatt et al. 2010a). It is a characteristic trichome feature in plants such as *Plectranthus tomentosa* (Kim 2013) and *Stachys alopecuros* (Venditti et al. 2013).

An examination of the trichomes of *in vitro* micropropagated plants showed no gross anatomical differences compared to their field-grown counterparts, apart from observably longer non-glandular trichomes on the micropropagated plant epidermis. Similarly, Abbaspour et al. (2012) determined that the average length of leaf trichomes in transgenic tobacco plants was significantly higher than those in the non-transgenic plants. That study suggested that phytohormones in the culture medium were responsible for the observed differences in trichome length. Further optimization of the *in vitro* protocol of *S. natalensis* is required to determine the effect, if any, of different plant growth regulators (PGRs) on trichome development.

The structural and functional aspects of glandular trichomes within the Lamiaceae are well documented (Ascensao et al. 1999; Giuliani and Maleci-Bini 2008: Marin et al. 2012; Rusydi et al. 2013; Venditti et al. 2014). The peltate trichomes of *S. natalensis* were morphologically similar to other *Stachys* spp., differing in terms of the number of secretory head cells (4–16 cells in *S. officinalis*) and the development of a fairly elongated stalk cell (40–60 μm length), as seen in *S. germanica* and related taxa *Prasium majus, Sideritis romana*, and *Scutellaria galericulata* (Giuliani and Maleci-Bini 2008). The Type I capitate trichomes of *S. natalensis*, widespread within the family Lamiaceae and genus *Stachys*, occurred on both foliar surfaces across all developmental stages of field grown and micropropagated plants. The Type II capitate trichome with an elongated stalk and distinct neck cell bearing a variable number of secretory cells identified in this study shares similarities with the capitate trichomes described in many species of *Stachys, Sideritis, Prasium*, and *Scutellaria* (Giuliani and Maleci-Bini 2008). However, this trichome type was generally found only on the plant reproductive organs of the Lamiaceae (Giuliani and Maleci-Bini 2008). Giuliani et al. (2011) later identified the exception in the multicellular capitate trichomes of *Sideritis italica*, which occurred on the leaves. In the present study, *S. natalensis* was found to also possess this capitate trichome on the foliar surfaces. Further investigations of *Stachys*, *Sideritis*, and other related species are required to re-assess the occurrence and distribution of this capitate trichome as a potential taxonomic marker.

Both peltate and capitate glandular trichomes have been shown to accumulate secretory material in a subcuticular space of the glandular secretory head in the family Lamiaceae (Ventrella and Marinho 2008). The subcuticular space is formed by the detachment of the cuticle from the cell wall (Ascensao et al. 1999; Tissier 2012). The release of secretions in glandular trichomes can occur *via* cuticular rupture at fragile regions of the secretory head (Naidoo et al. 2013), through a singular apical pore or multiple micropores (Ascensao et al. 1999), or even by means of diffusion through the cuticle (Caissard et al. 2012). The release of secretions in *S. natalensis* seems to occur *via* different mechanisms, depending on the type of glandular trichome. The peltate and

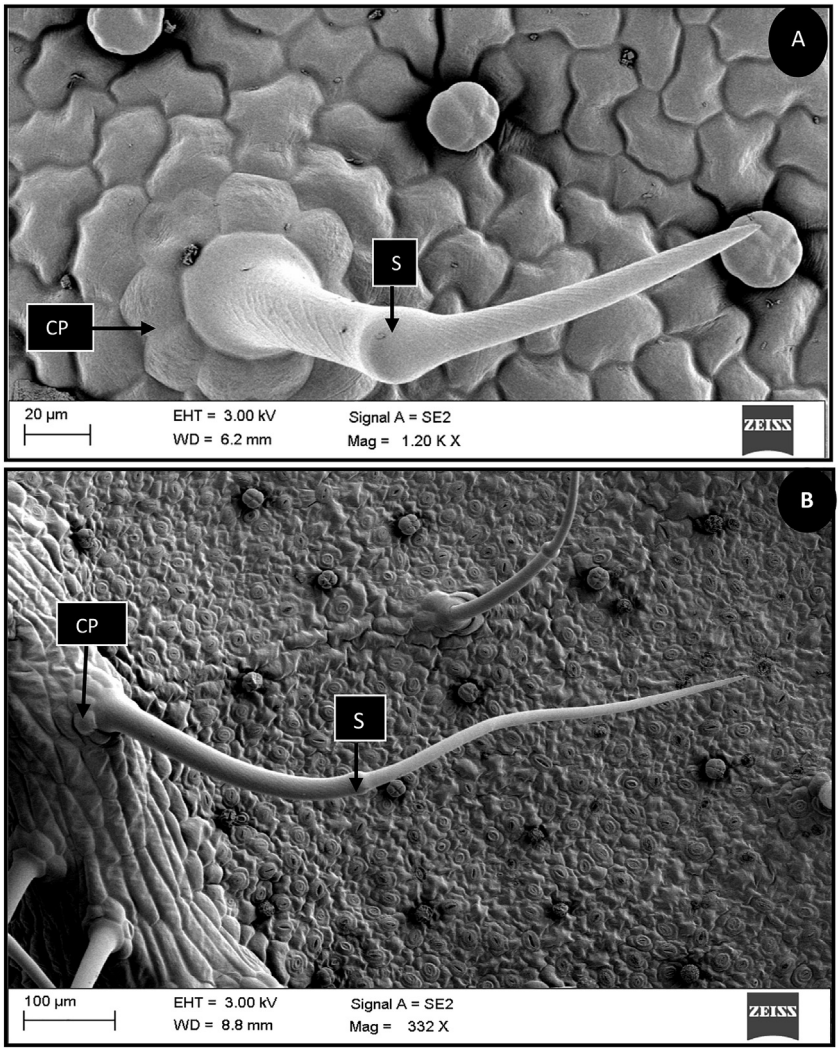

Fig. 3. SEM micrographs of the non-glandular trichomes on the foliar surface of field grown (A) and micropropagated (B) plants. Note the multicellular epidermal cellular pedestal (CP) and the septation of the trichome stalk (S).

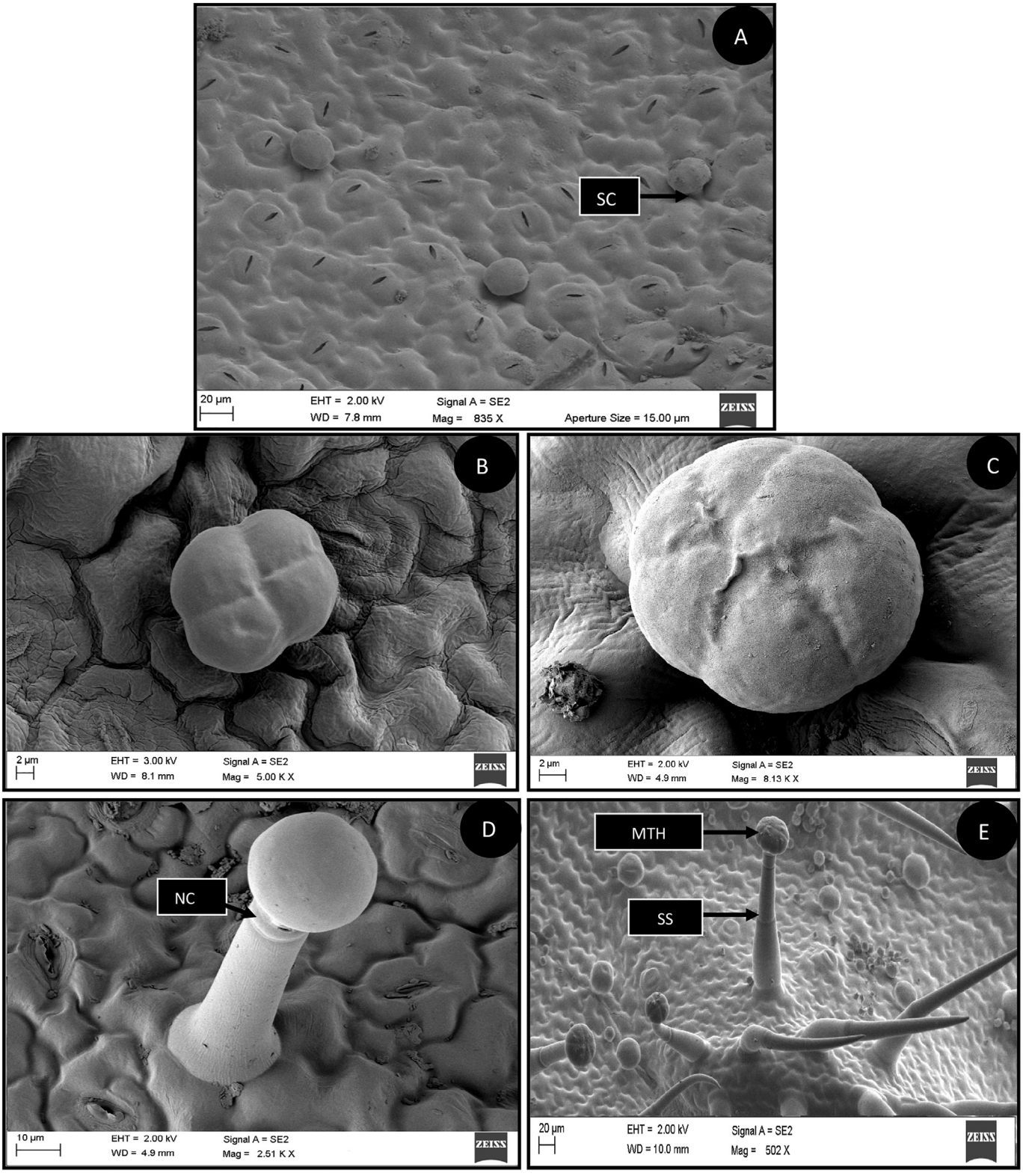

Fig. 4. SEM micrographs showing the morphology of glandular trichomes of *S. natalensis* (A) Cryo-SEM image of peltate trichomes in epidermal depression with single stalk cell (SC) found on the abaxial surface of a mature leaf. (B) Peltate trichome with multicellular (four-celled) head found on the abaxial surface of a young leaf. (C) Multicellular (eight or more cells) head of peltate trichome on the abaxial surface of an emergent leaf specimen. (D) Glandular capitate trichome Type I with bulbous head cell, distinct neck cell (NC) and a shorter, non-septate stalk on the abaxial foliar surface. (E) Glandular capitate trichome Type II on the adaxial surface of micro propagated leaf characterized by disc-shaped multicellular head (MTH) and elongated, septate stalk (SS) which appears bulbous at the base and narrow toward the apex.

Type II capitate trichomes appear to have a porose cuticle which facilitates the release of droplets of secretions to the exterior. This has also been observed in *Scutellaria galericulata*, *Stachys germanica* (Giuliani and Maleci-Bini 2008), and *Salvia smyrnea* (Baran et al. 2010). In the present study, cuticle elevation resulting from the build-up of secretions in the sub-cuticular space, followed by cuticular rupture at weak points at the equatorial plane of the secretory head cell was observed in Type I capitate trichomes. Cuticle rupture may also be due to external factors

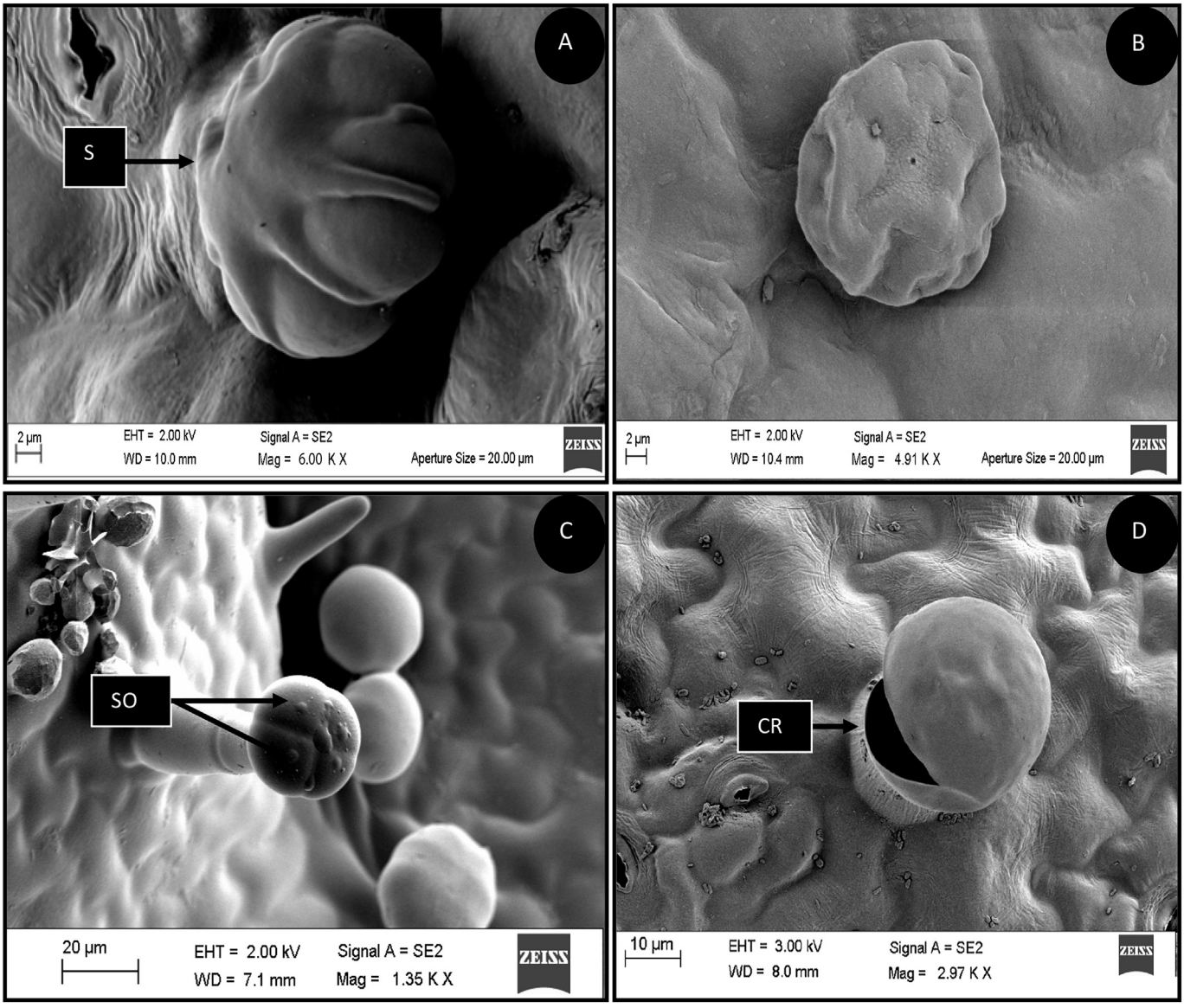

Fig. 5. SEM micrographs showing aspects of the secretory process of the glandular trichomes of *S. natalensis*. (A) Peltate trichome in the pre-secretory phase showing accumulation of secretion within the sub-cuticular space (S). (B) Peltate trichome in the post-secretory phase. Note the collapse of the peltate trichome (PT) after the release of exudates. (C) Type II capitate trichome in the process of secretion *via* openings (SO) on the surface of each of the multicellular head cells. It appears that the glandular cuticle may be highly porose. (D) Cellular rupture (CR) occurring at fragile regions in the head cell of Type I capitate trichome.

such as high temperature, low humidity, or mechanical damage (Ascensao et al. 1995; Ventrella and Marinho 2008; Baran et al. 2010). A similar phenomenon of cuticular rupture was noted in *Plectranthus grandidentatus* (Mota et al. 2013).

In the present study, the morphological fidelity was maintained between field grown and acclimatized plants. The observations in this study including the number of glandular head cells, mechanism and accumulation of secretions and location of capitate trichomes may serve as basic distinguishing features in the taxonomic evaluation of *S. natalensis* in comparison to other species and other closely related genera, such as *Sideritis*.

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Conflict of interest

The authors declare that they have no conflict of interest.

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