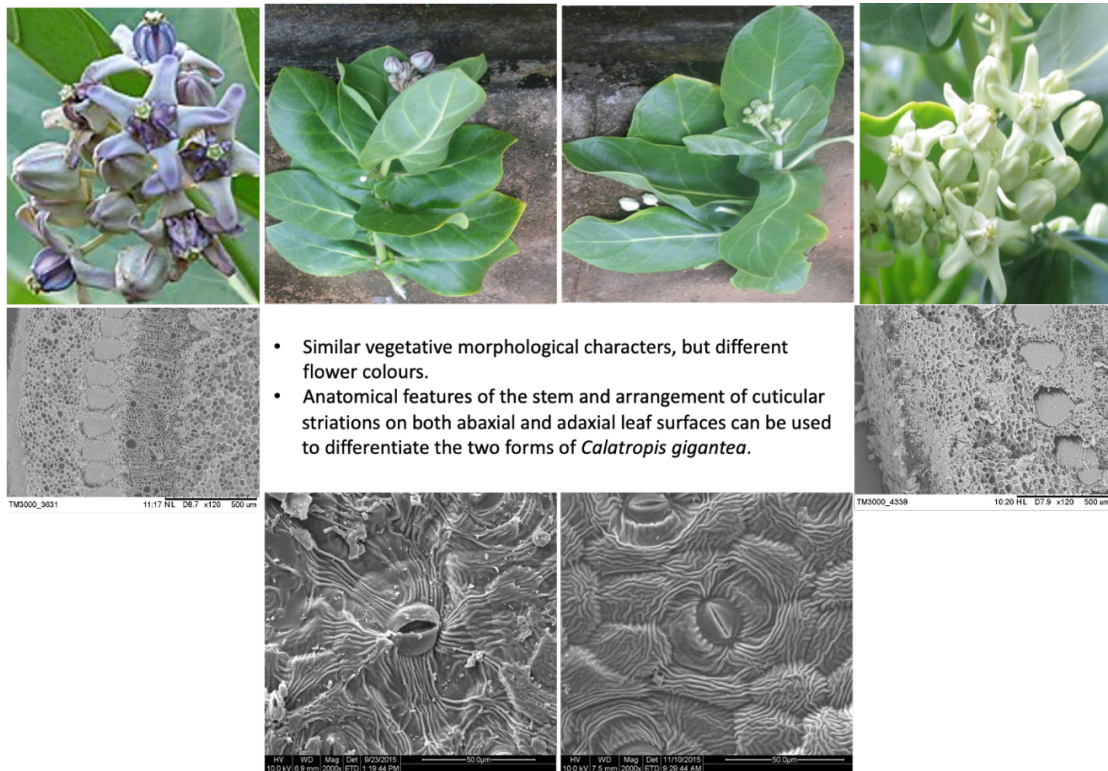


Comparative anatomy of two forms of Sri Lankan *Calotropis gigantea* (L.) R. Br. (Family Apocynaceae s.l. – Subfamily Asclepiadoideae) -Taxonomic implications

P.D. Abeysinghe* and T. Scharaschkin



Highlights

- Vegetative characters were examined to differentiate purple and white flower colour forms of *Calotropis gigantea* in the absence of flowers.
- Simple or branched, “Y”, and “H” shaped laticifers were observed in both forms throughout the vegetative parts.
- Stem and stomatal anatomy and the arrangement of cuticular materials can be used to differentiate the two forms, in addition to the flower colour.

RESEARCH ARTICLE

Comparative anatomy of two forms of Sri Lankan *Calotropis gigantea* (L.) R. Br. (Family Apocynaceae s.l. – Subfamily Asclepiadoideae) -Taxonomic implications

P. D. Abeysinghe^{1*} and T. Scharaschkin^{2,3}

¹Department of Botany, Faculty of Science, University of Ruhuna, Matara, Sri Lanka.

²School of Earth, Environment and Biological Sciences, Science and Engineering Faculty, Queensland University of Technology, Brisbane, QLD 4001, Australia.

³Botanical Research, Art and Training, 54 Mill Road, Collinsvale, TAS 7012, Australia.

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Abstract: *Calotropis gigantea* (L.) R. Br. is considered a versatile plant that has been described in Ayurvedic medicine. Two forms of *C. gigantea*, differing in flower colour (purple and white) occur in Sri Lanka. In the absence of flowers, it is difficult to differentiate the two forms due to the similarities in their vegetative morphology. Accurate botanical identification of the two forms of *C. gigantea* is essential in developing conservation strategies and establishing a proper management system. Anatomical studies of the vegetative structures of the two forms of *C. gigantea* were carried out using Light Microscopy and Scanning Electron Microscopy to study the anatomical features between the two colour forms. The adaxial and abaxial leaf surfaces of the same form, as well as the two forms, differ in terms of cuticular material deposition and cuticular material organization on the leaf surfaces. The amount of leaf cuticular material was higher on the abaxial surface than on the adaxial surface of the purple form, compared to the white form where both surfaces have more or less similar amounts of cuticular material based on the observation. Irregularly scattered and sunken amphistomatic, anomocytic, elliptical-shaped stomatal complexes, unbranched, and uni/multicellular trichomes in the leaves and stems were observed in both forms. Simple, branched, “Y”, “H” shaped laticifers were observed throughout the vegetative parts. Stem and stomatal anatomy and the arrangement of cuticular materials are useful to differentiate the two-colour forms of *C. gigantea* which is important taxonomically as well as in conservation.

Keywords: *Calotropis gigantea*; purple and white forms; anatomy; light microscopy; scanning electron microscopy.

INTRODUCTION


Apocynaceae s.l. (Endress and Bruyns, 2000), commonly known as the dogbane family, comprises 357 genera and about 5100 species (Nazar *et al.*, 2013) of flowering plants including herbaceous or shrubby climbers, trees, and stem succulents (Wong *et al.*, 2013; Lu *et al.*, 2014; Chan *et al.*, 2016:). Members of the family Apocynaceae are most diverse in tropical and subtropical regions and extend into temperate areas (Nazar *et al.*, 2013). The family Asclepiadaceae is included in Apocynaceae as a subfamily (Asclepiadoideae) (Angiosperm Phylogeny Group, 2016). In Sri Lanka, the family Apocynaceae is represented by 80 species in 49 genera (Senaratna, 2001) distributed throughout the country. *Calotropis* is a small

genus of laticiferous shrubs or succulent small trees consisting of two species globally, *Calotropis gigantea* Br. Dryand and *C. procera* (Aiton) Dryand, (Trimen, 1985; Endress and Bruyns, 2000; Sennblad and Singh *et al.*, 2014). *Calotropis* species, commonly known as “milkweed” or “crown flower” (Kumar *et al.*, 2010), are found in Bangladesh, Burma, China, India, Indonesia, Malaysia, Pakistan, Philippines, Sri Lanka, and Thailand (Kumar *et al.*, 2010; Singh *et al.*, 2014) and have been introduced in the Pacific Islands, Australia, Central and North, and South America and Africa as an ornamental tree (Kaur *et al.*, 2021). *C. gigantea* is considered a weed in India (Meena *et al.*, 2010), Australia, Brazil (Dhileepan, 2014), and Saudi Arabia (Hindi and Arabia, 2013). In Sri Lanka, *C. gigantea*, is the only species recorded as occurring on the island (wara, Silva *et al.*, 2017). *C. gigantea* has been described in Ayurvedic medicine (Krentkowski and Duarte, 2012; Frosi *et al.*, 2013; Poonam and Punia, 2013). *C. gigantea* is used in traditional medicine not only in Sri Lanka (Solohokara *et al.*, 2015) but also in other countries, like India (Kumar *et al.*, 2010; Meena *et al.*, 2010; Kaur *et al.*, 2013; Harsimran and Shikha, 2015;) and Bangladesh (Haque *et al.*, 2012) and Malaysia (Wong *et al.*, 2013). Reviews of phytochemistry, pharmacological activity, medicinal properties, and biological properties of this plant indicate that it is a versatile plant used for a variety of purposes in traditional medicine (Ranade and Acharya, 2014; Solohokara *et al.*, 2015).

C. gigantea exhibits two morphological forms (Figure 1A and 1B) differing in flower colour (purple and white) that are otherwise similar in vegetative morphology (Figure 1C- 1D) (Huber, 1983). In Sri Lanka, the purple-flowered form is more prevalent in terms of abundance and distribution than the white-flowered form. Both forms are used in Ayurvedic medicine. However, due to road construction and other anthropogenic activities, the natural habitats of this important plant species are disappearing.

Accurate botanical identification of the two forms of *C. gigantea* in Sri Lanka is essential for proper management, germplasm collections, and the development

*Corresponding Author's Email: pushpa@bot.ruh.ac.lk

 <https://orcid.org/0000-0002-2026-3014>



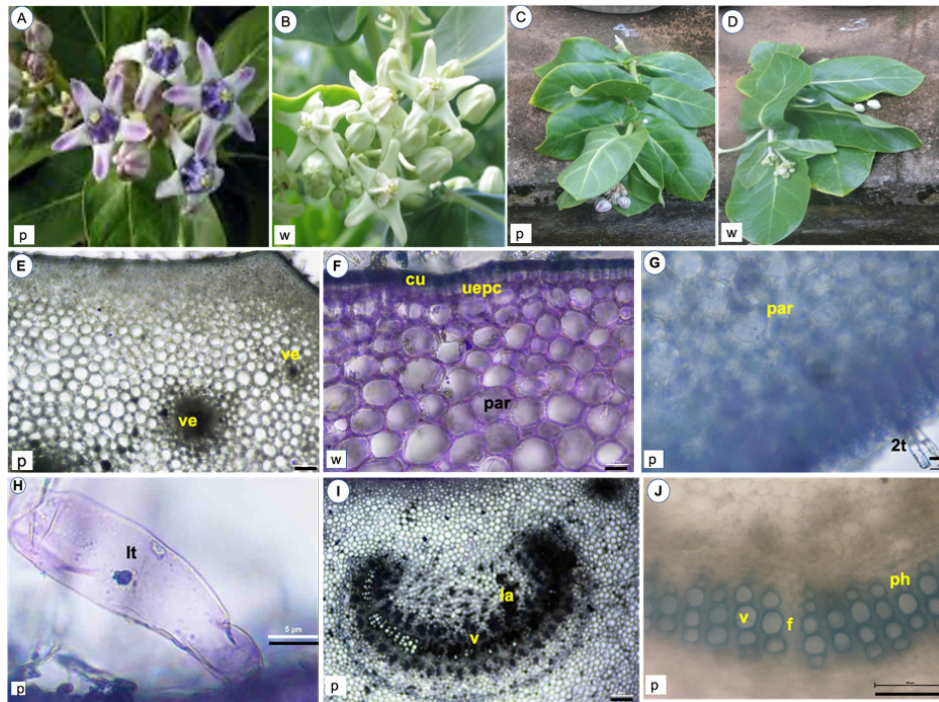


Figure 1: Gross morphological and petiole anatomical characters of purple and white-coloured forms of *Calotropis gigantea*. (A) flowers of purple-coloured form. (B) floral morphological characters of white-coloured form. (C) vegetative parts of the purple-coloured form. (D) vegetative parts of the white-coloured form. (E) TS of the petiole of purple form showing the two lateral bundles above the main vascular bundle. (F) the upper epidermis shows a clear thick cuticle with trichomes, the cuticle is rich with polyphenols. (G) the lower part of the purple petiole shows the two-celled two trichomes originating from the same place. (H) leaf-like trichome. (I) structure of the vascular system. (J) arrangement of the vessel elements. Sub-figure letters: p: purple. w: white. Abbreviations used: cu: cuticle. f: fiber. lt: leaf-like trichome. par: parenchyma. ph: phloem. uepc: upper epidermal pavement cells. v: vascular bundle. ve: vein. 2t: two trichomes originated from a single place. Scale bar: 5 µm: D, E, F, H; 100 µm: J.

of conservation strategies. As it is difficult to differentiate the two forms in the absence of flowers, anatomical characteristics may support distinguishing the two forms of *C. gigantea*. The objectives of this study were to undertake a comparative study of anatomical characteristics of leaves, stems, and roots using light microscopy (LM) and Scanning Electron Microscopy (SEM) to document and describe the anatomical features and to identify anatomical characteristics that can be used to differentiate the two colour forms of *C. gigantea*.

MATERIALS AND METHODS

Plant collection, leaf clearing

Young leaves, stem, and root samples were randomly sampled from sun-exposed branches from 3-5 typical and healthy individuals of *C. gigantea* purple and white-coloured flower plant from three different natural populations in the vicinity of the University of Ruhuna, Matara, Sri Lanka. Samples were rinsed in water, trimmed in to small pieces, and fixed in F.A.A. (Formalin acetic acid-alcohol, 1:1:18) for a week and transferred to 70% alcohol. Approximately 2 x 2 cm pieces of the leaf blade, excluding the midrib, were used for leaf clearings. The samples were immersed in 10% potassium hydroxide overnight, transferred to a fresh potassium hydroxide solution, and kept at room temperature. This was repeated until the samples became decolorized. Leaf samples were washed thoroughly with deionized water and treated with bleach (7% sodium

hypochlorite) until obtaining a clear and transparent appearance. Cleared leaf samples were stained with 1% safranin, observed under a Nikon Eclipse 50i compound microscope (low, medium, and high as appropriate), and images were captured using the Nikon NIS-Elements imaging software (Nikon Instruments Inc., Amsterdam, Netherlands).

Sectioning, staining and light microscopy (LM), and scanning electron microscopy (SEM)

Freehand sections of preserved leaves along the petiole, the midrib, the stem, and the roots were taken using a sharp blade. Transverse sections were obtained from leaves and petioles, while radial, tangential and transverse sections were taken from the stem and root samples. Sections were stained in TBO or phloroglucinol (20% HCl) (Ruzin, 1999; Retamales and Scharaschkin, 2014) and images were captured as stated above. SEM was used to study the surface micromorphology of leaves and anatomical features in roots and stems. Two 1x1 cm leaf samples and radial, tangential, and transverse freehand sections of root and stem materials were dehydrated using a graded ethanol series and then critical point dried (Anderson 1951) in an Autosamdri-815 automatic critical point dryer (Tousimis, Rockville, USA). Samples were mounted on stubs with self-adhesive double-sided carbon discs. Leaf samples were mounted to observe abaxial and adaxial surfaces. Samples were sputter-coated with gold-palladium for the 70s using a Leica EM SCD005 Gold Coater (Leica Microsystems, Macquarie Park, NSW,

Australia). Examination and documentation of images were conducted using an FEI Quanta 200 SEM/ESEM (FEI, Hillsboro, Oregon, USA) or TM 3000 Hitachi TM-3000 Tabletop operated at 10 kV.

RESULTS AND DISCUSSION

The results and discussion part will be explained in three parts: (1) petiole and leaf characters, (2) root and stem characters, and (3) laticifers and other deposits. Considering the anatomical research on *Calotropis* species, only a very few references are available (Nasser *et al.*, 2012; Harsimran and Shikha, 2015). Harsimran and Shikha (2015) carried out the leaf and stem anatomy to identify *C. procera* and *C. gigantea*. However no research have been carried out towards gathering data in differentiating the two flower colour forms. The foliar and petiole (Martínez-Cabrera *et al.*, 2009), the epidermis (Breitwieser and Ward, 1998; Chen *et al.*, 2008), stomata (Baranova, 1992; Ahmad *et al.*, 2009), and other anatomical characters of the stem, root, etc. generally useful for the systematic classification and identification of species and genera of the plants (Baranova, 1992; Hernández-Ledesma *et al.*, 2011).

Petiolar and leaf anatomical characters

The anatomical characteristics of the petiole and leaves were quite homogeneous in both forms of *C. gigantea*. The petiole was a concave-convex shape in a transverse section with two pairs of lateral veins (Figure 1E). The epidermis was uniseriate and coated with a thick cuticle which indicates the presence of polyphenols due to bluish-green staining with TBO (Figure 1F). One to three layers of annular to angular collenchyma occur on both abaxial and adaxial sides (Figure 1F). Uniseriate and multicellular trichomes (Figure 1G) or leafy-shaped trichomes were present (Figure 1H). Leaves had non-glandular trichomes that were simple, curved, uniseriate, and multicellular.

These results obtained during the present study were similar to the results of the previous studies where unicellular and multicellular trichomes were found in *C. gigantea* leaves (Krentkowski and Duarte, 2012; Harsimran and Shikha, 2015). We observed that two trichomes originated from the middle of the rosette-like cell arrangement (Figure 1I). Trichomes were reported to be early caducous (Hassan *et al.*, 2015). This is probably the reason for observing a small number of trichomes. However, in *C. procera*, glandular trichomes with bicellular-uniseriate stalk and unicellular head had been observed (Gabr *et al.*, 2015). The present study recorded two trichomes originating from the middle of the rosette-like cell arrangement (Figure 2I) and a smaller number of trichomes on both sides of the leaf. The petiole of both forms showed a collateral vascular bundle arranged as a simple and open arc and the vascular system occupied a small area in the central region of the petiole (Figure 1I). The vascular bundle consisted of 15-20 columns of vessel elements embedded in the ground parenchyma (Figure 1J). The xylem consisted mainly of vessel elements and tracheids. These vessel elements were separated from each other by thick-walled fibers. Generally, four to five vessel elements were compactly

arranged vertically. The phloem is distributed in small groups, but not conspicuous. Although fiber sheathing was present around the bundle, it is not obvious (Figure 1J).

The leaves of both forms of *C. gigantea* were thick and leathery in appearance (Figure 1C and 1D) with similar anatomical structures. There was, however, a certain exception, e.g., the transverse section of the midrib of the purple form leaf showed a ribbon-like shape while the white form has a different shape e.g., a broadly convex lower surface (Figures 2A, 2B). The cellular arrangement of a transverse section of the midrib of both purple and white leaves was fairly similar. Both forms have more or less flat leaf blades with an undulated, slightly grooved surface above the vascular bundle (Figure 2A -2B). The results that we observed for the structure and cellular arrangements of the leaf were very much similar to the anatomical study of two species of *Calotropis* from Chandigarh, India (Harsimran and Shikha, 2015). In the transverse section of the lamina, the adaxial surface of the leaf was flat, smooth, and even on both surfaces. However, we observed an undulating structure of the epidermis of both surfaces. The epidermis was uni-seriated in both forms with a similar outline of the anticlinal walls (Figure 2C-2E). Epidermal pavement cells were rectangular, pentagonal, hexagonal, or octagonal in shape (Figures 2C-2F). Angular or lacunar collenchyma tissue, consisting of three to five cellular layers, was present adjacent to the abaxial epidermal layer (Figure 2G). Parenchyma tissue, consisting of four to eight layers of isodiametric to circular with intercellular space was present around the vascular bundle (Figure 2H). Both forms of *C. gigantea* had sparsely pubescent leaves on both adaxial and abaxial surfaces (Figures 2D – 2F) with simple and straight (Figure 2D), curved (Figure 2F), multicellular (Figure 2D), or leaf-like trichomes (Figure 2F). Two or three trichomes originated from a single point (Figure 2E).

Non-glandular trichomes were observed on both surfaces, consisting of five to seven epidermal pavement cells arranged to form a rosette with two hairs arising from the middle (Figure 2I, Figure 3E). Fewer layers of angular collenchyma occurred on both the abaxial and adaxial sides (Figure 2G). Similar vascular tissue arrangement was observed in the petiole and leaf (Figure 1I, Figures 2A-2B). No detailed anatomical studies carried out in *Calotropis* were found in the literature to compare our results of leaf anatomical structures.

Leaf surface micromorphology (Cuticular and epicuticular waxes)

The leaves of both forms have a waxy appearance. The single-layered epidermis was covered with an undulating, thick, striated waxy cuticle (Figure 3). A denser layer of epicuticular wax was observed on the abaxial surface than on the adaxial surface of the purple form. Similar amounts of epicuticular wax, although less dense than that seen in the purple form, were observed on both surfaces of the white form. Cuticular striations were observed on the epidermal cells on both leaf surfaces of both forms (Figure 3G - 3P). In the white form, bundles of cuticle materials were well arranged around the stomata (Figure 3F, 3H, 3N) while in

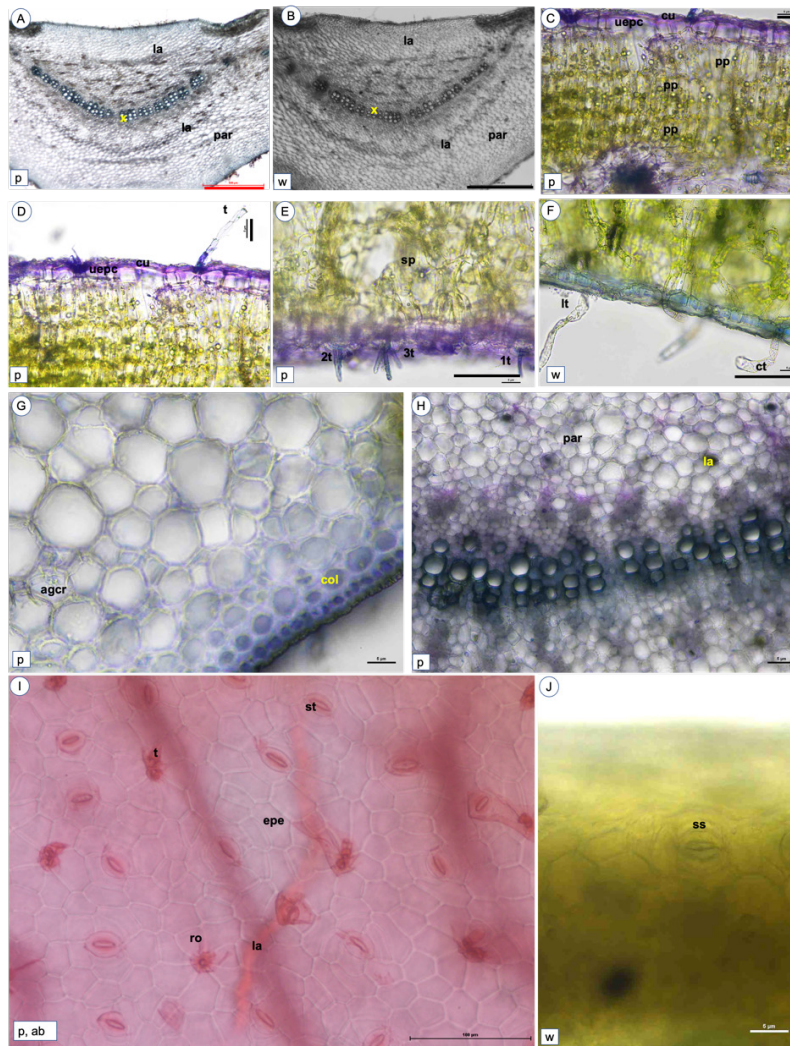


Figure 2: LM and SEM of leaf anatomy of the two flower colour forms of *Calotropis gigantea*. (A) TS of a leaf of purple form. (B) white form along with the leaf bundle, similar structure to purple form. (c) three-storied palisade parenchyma. (D) thick cuticle with multicellular trichomes. (E) abaxial surface showing different types of trichomes. (F) abaxial surface showing leafy shape, multicellular or curved trichomes. (G) the lower surface of the leaf showing a thick cuticle and a few layers of collenchyma. (H) vascular tissues of purple form. (I) after leaf clearing and stained with safranin, stomata arrangement of the abaxial surface of the purple leaf and different shapes of epidermal pavement cells and stomata. (J) sunken stomata on the adaxial surface. Sub-figure letters: p: purple. w: white. Abbreviations used: p, ab: purple, abaxial. cu: cuticle. epe: epidermal pavement cells. la: latex. par: parenchyma. la; latex. lt: leaf-like trichome. par: parenchyma. ph: phloem. pp: palisade parenchyma. ro: rosette-like structure. sp: spongy parenchyma. ss: sunken stomata. st: stomata. lt: one trichome originated from a single place. t: trichome. 2t: two trichomes originated from a single place. 3t: three trichomes originated from a single place. lt: leafy shape trichome. t: trichome. uepc: upper epidermal pavement cells. x: xylem. Scale bar: 5 μ m: C, D, E, F, H, I, J; 100 μ m: A, B, G.

the purple form, bundles of cuticle materials originated around the stomata and radiated outward not only over the guard cells but also subsidiary cells and normal epidermal cells (Figure 3G, 3M). The organization of the thick and straight with favulariate ornamentation around the stomata was variable not only on the two surfaces of the same leaf (purple form Figure 3E - 3G) but also between the two forms (purple form Figures 3E - 3G and white form Figures 3F - 3H). A striated cuticle has been observed in two members of the Apocynaceae, *Aspidosperma carapanauba* and *A. excelsum* (Krentkowski and Duarte 2012) but no studies on *Calotropis* were found in order to compare our observations of *C. gigantea*. The cuticle had ornamentations of epicuticular waxes (Figure 3E-3L). Cuticular striations were frequently observed around the stomata on both surfaces of both forms (Figures 3E-3L). The organization

of the striae around the stomata was variable not only on the two surfaces of the same leaf (purple - Figures 3E- 3G) but also between the two forms (purple - Figures 3G-3H and white - Figure 3F, Figure 3H). However, no data is available to compare the wax deposition of leaves of *Calotropis* species with related taxa. A striated cuticle has been observed in two members of the Apocynaceae, *A. carapanauba* and *A. excelsum* (Krentkowski and Duarte 2012). In our study, a diverse arrangement of cuticle material was observed in both forms of *C. gigantea*. The arrangement of cuticle material on the leaf surfaces of both forms can be used to identify them accurately. In the white form, the bundles of cuticle materials were well arranged around the stomata while in the purple form, bundles of cuticle materials originate around the stomata and radiate outward not only over the guard cells but also subsidiary

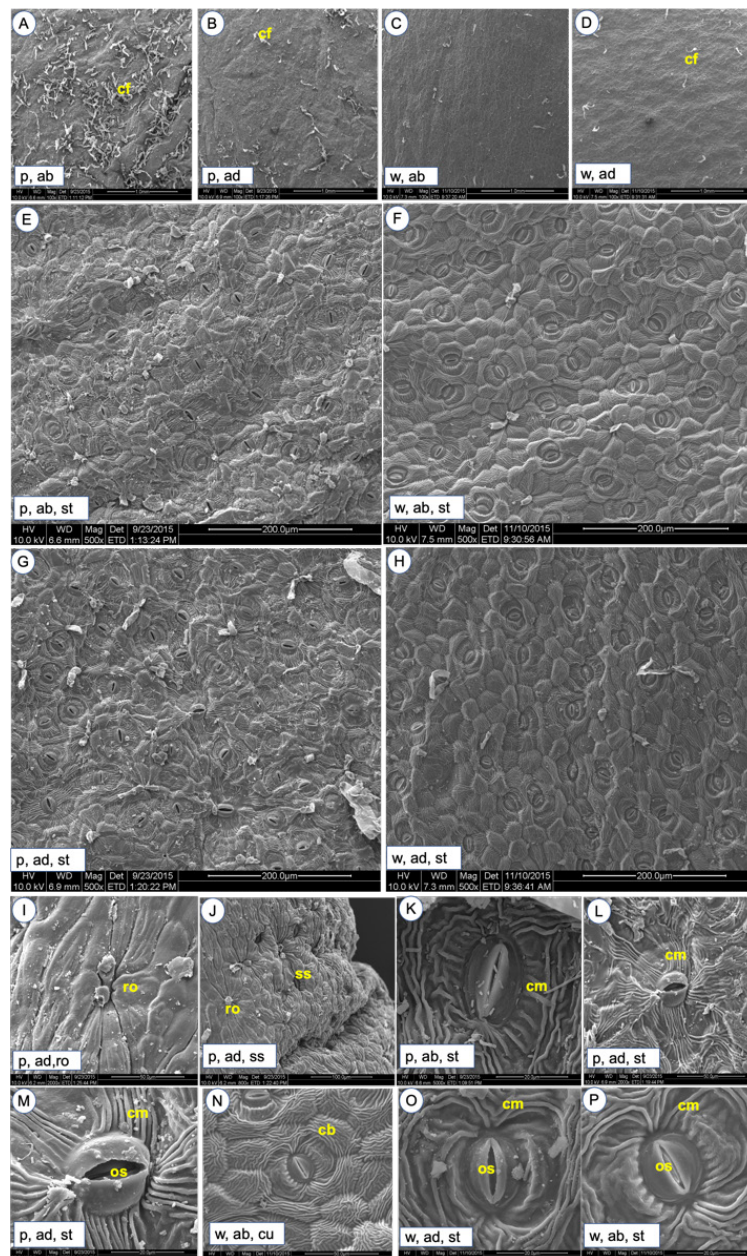


Figure 3: SEM images of leaf surfaces showing the different arrangement of cuticle striae of *Calotropis gigantea*. A-E: differences in surface waxes. (A) a high density of cuticular wax flakes on the abaxial surface.; (B) less wax deposition on the adaxial surface; (C) abaxial surface; (D) adaxial surface; (E) cuticular material arrangement of the purple form on the abaxial surface; (F) Cuticular material arrangement of the white form on abaxial surface; (G) cuticular material arrangement of the purple form on the adaxial surface; (H) cuticular material arrangement of the white form on the adaxial surface; (I) rosette-like structure on the adaxial surface; (J) sunken stomata; (K), (L), (M) different cuticular striae arrangement around stomata in purple form leaf abaxial and adaxial surfaces; (N), (O) and (P) different cuticular striae arrangement around stomata in white form leaf abaxial and adaxial surfaces. Sub-figure letters: p, ab: purple, abaxial. p, ad: purple, adaxial. p, ad, r: purple, adaxial, rosette. p, ab, st: purple, abaxial, stomata. p, ad, st: purple, adaxial, stomata. p, ad, ss: purple, adaxial, sunken stomata. w, ab: white, abaxial. w, ab, cu - white, abaxial, cuticle material arrangement. w, ad, st: white, adaxial, stomata. w, ad: white, adaxial. Abbreviations used: cm: a cluster of cuticular material; cf: cuticular flakes; cs: cuticular striations; os: ostiole; ro: rosette-like arrangement of cuticular material; ss - sunken stomata. Scale bar: 20 μ m: K, M, O, P, M; 50 μ m: I, L, N; 100 μ m: F, 100 μ m: J; 200 μ m: E, F, G, H; 1 mm: A, B, C, D.

cells and normal epidermal cells. The arrangement of cuticle surface ornaments of both forms of *Calotropis* has not been previously recorded.

Along with epicuticular wax structures, the striae may be effective in preventing the formation of a film of water on the leaf surface, enhancing the self-cleaning effect of the leaf, or mechanically stabilizing the leaf (more

resistant to tearing), reducing leaf wettability. The amount and arrangement of deposition of cuticular materials on the leaf surfaces may be a distinguishing feature between the two forms.

Stomata and striations.

The leaves were amphistomatous with conspicuous

and irregularly scattered stomata Figure 2I). Amphistomatic stomata arrangement of the cells in the epidermis has been observed in *C. procera* by Paliwal *et al.*, 1980. This is in agreement with the results of this study. *C. gigantea* is a xeromorphic plant mainly found as a creeping or erect shrub, sub-shrub, or shallow tree that grows in many arid and semi-arid environments. Amphistomatic leaves, on the other hand, are commonly found in hydrophytes and creeping species from wet habitats (Evert, 2006). In this study, the irregular orientation of anomocytic-type stomata was observed on both adaxial and abaxial leaf surfaces of the purple and white forms. The stomatal density of the adaxial and abaxial surfaces of both forms was more or less similar. Stomata were sunken and not superficial (Figure 2J and Figure 3J), elliptical in shape (Figures 3K – 3P). Ostioles were typically slit-like and their orientation was irregular (Figure 2I, Figure 3F, Figure 3H). Sunken stomata have been observed in *C. procera* (Shirsat *et al.*, 2011). Anomocytic (Figure 2I) arrangement of subsidiary cells was observed on both surfaces of both forms. The guard cells were surrounded by 4-5 subsidiary cells, which were not regular in shape and size (Figure 2I). In *C. procera*, diacytic (Ahmad *et al.*, 2009) and paracytic, cyclocytic, anisocytic, or tetracytic and tetracytic type subsidiary cell arrangement of stomata (Paliwal *et al.*, 1980) have been observed. Moreover, anomocytic stomata have been observed in the *Aspidosperma olivaceum*, *A. polyneuron*, *Cryptostegia grandiflora*, *Catharanthus roseus*, and *Mascarenhasia elastica* which are members of the family Apocynaceae (Krentkowski and Duarte, 2012; Gabr *et al.*, 2015).

Therefore, according to our results and the results of other research (Ahmad *et al.*, 2009; Shirsat *et al.*, 2011), different types of stomatal structures occur in the genus *Calotropis*. In the present study, SEM was used out to study the structure, and arrangement of the subsidiary cells of the stomata and the cuticular materials around the stomata. According to the literature, there is no research has been carried out to study the stomata structure of *Calotropis* species using the SEM. The organization of the striae around the stomata varies between the two surfaces, ab and adaxial and the two forms under study as described below.

***C. gigantea* purple form**

The arrangement, structure, and cuticular deposition/striations of abaxial stomatal complexes (Figure 3E) were different from those seen on the adaxial side (Figures 3G). Adjacent stomatal complexes were usually interconnected by a few to several heavily cutinized threads of ornamentation radiating around the stomata (Figure 3G). Each bundle of ornamentation consists of 5 - 8 parallel striations (Figures 3K). The striations were typically thick and remarkable on both abaxial and adaxial surfaces and not confined to areas around the stomata (Figure 3G) but distributed unevenly over different cell types such as stomatal subsidiary and epidermal pavement cells.

***C. gigantea* white form**

In the white form, wax was deposited in regular patches

around guard cells (Figure 3F and Figure 3H). The individual stomata on both surfaces were separated from one another by 5-6 bundles or bundles of cuticular materials (Figure 3F and Figure 3H). On the adaxial surface, the striations originate from the opposite ends of the stomata, curve after a short distance, and run parallel to the ostioles of the stomata, as a result, striae become orientated parallel to the stomata (Figure 3K). On the abaxial surface, striae originating from the middle part of the stomata were raised and made ledges around the stomata (Figure 3O). These striations originate at regular intervals around the stomata and from opposite ends of guard cells radiating in an outward direction across subsidiary cells (Figure 3P).

Root and stem anatomy

To our knowledge, no wood anatomical studies have been carried out on the two flower colour forms of *C. gigantea*. The anatomy of the roots of both purple and white types was similar. Root wood was soft and light. The well-developed periderm consisted of about 10-12 layers of different shapes (rectangular, circular, irregular) cells (Figure 4A, 4B). A few layers of the rectangular, barrel or elongated-shaped endodermis cells were visible at the periphery of the cortex (Figure 4B). Growth rings were not distinct. Vessels were clustered in the center of the root as seen in the transverse section (Figure 4A). In the transverse section of the root, both the xylem vessels and the tracheid of the vascular system were well developed and scattered (diffusive). Vessels were solitary, round, or irregular (Figure 4C), or multiples of 2-3 rounds or irregular (Figures 4D- 4E) and semi-ring-porous (Figure 4C), mainly in radial and/or diagonal (Figure 4D), radial-oblique. Thickenings were alternate (Figure 4F, 4G), oval-shaped, and with slit-like apertures (Figure 4H). The epidermis of the stem was multicellular (Figure 5A) consisting of highly packed barrel to rectangular-shaped cells with a thick cuticle (Figure 5B) and covered with a thick coat of trichomes (Figures 5D-5F). Large, elongated trichomes were present in the purple form (Figure 5E) while the white form had uniseriate, elongated, and uniseriate multicellular trichomes (Figure 5F). The cortex consists of 2-3 layers of collenchyma cells adjacent to the epidermis and several layers of parenchyma cells further in (Figure 5D). The small patches of sclerenchyma fibers form a discontinuous pericycle (Figure 5B, 5C, 5D). The vascular cambium forms a wavy ring between the xylem and phloem tissues (Figure 5B). A comparison of the detailed structure of stems is listed in Table 1.

Although there are few reference data on the wood anatomy of *Calotropis* species, available results on the stem anatomy agree with the observations made in other species of *Calotropis* (Harsimran and Shikha, 2015; Hassan *et al.*, 2015; İlçm *et al.*, 2010; Krentkowski and Duarte, 2012; Nasser *et al.*, 2012; Yaman and Tumen, 2012;). In *C. gigantea*, from Sri Lanka, multiple layers of the epidermis in the stem were observed. Stem trichomes were unicellular, uniseriate, or multicellular and were simple, leafy, or short (Figure 5D, 5E, 5F). The presence of cork, thick cuticle, trichomes, and sub-epidermal layer constitutes an additional barrier against water loss from plants, and these features could be considered important xeromorphic characters.

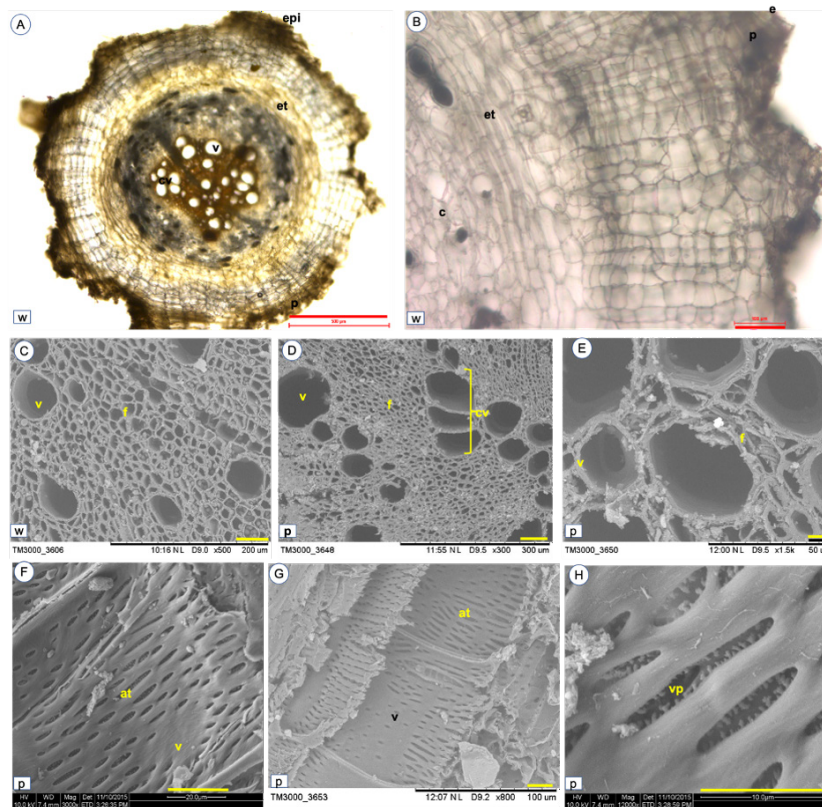


Figure 4: Root anatomy of the two flower colour types of *Calotropis gigantea* as seen by LM (A and B) and SEM (C - H). (A) cross-section of root stained with TBO. (B) cellular arrangement of ground tissue and endodermal layer. (C) xylem tissue showing semi-ring-porous vessels (D) xylem tissue showing clusters of 2–3 round or irregular vessel elements (E) barrel-shaped vessels. (F) alternate thickenings. (G) slit-like apertures of vessel element. (H) vessels and fibers. Sub-figure letters: p - purple. w - white. All images are of the purple form other than A, B, and C which was from the white form. Abbreviations used: at: alternate thickenings. co: cortex. cv: a cluster of vessels. e: epidermis. et: endodermal tissues. f: fibers. p: phellem. v: vascular tissues. vp: vascular projections. Scale bar 10 µm; H; 20 µm; F; 50 µm; E, H; 100 µm; B, G; 200 µm; C; 300 µm; 500 µm: A.

Table 1: Comparison of stem anatomy of the purple and white forms of *Calotropis gigantea*.

<i>C. gigantea</i> purple form	<i>C. gigantea</i> white form
Stem characterized by the presence of large number of bunches of sclerenchyma in the cortex (pericycle - Figure 5C). Adjacent to the outer phloem, there is a zone of sclerenchyma fibers arranged in separate bundles with one or two intermittent parenchyma (Figure 5C).	Stem was characterized by the presence of bundles of sclerenchyma fiber belt in the cortex (pericycle - Figure 5D). This was conspicuous and prominent near to the cortex. Each bundle was separated by three to four parenchyma cells (Figure 5D).
The vascular tissue forms a ring around the periphery of the pith (Figure 5C, Figure 5G). The xylem tissue forms a broad and extensive region of the stem. Vessels were thin walled, diffused, solitary, circular, or oval in cross-section (Figure 5G). Vessels were arranged as long chains of radial multiples or in groups (Figure 5G). Semi-ring porous was distinct. Vessels in the earlywood were distinctly larger than those in the latewood of the previous growth ring (Figure 5G) although growth rings were not conspicuous. Fiber cells with thin walls and wide lumens were found to occupy most of the area of the vascular tissues of the transverse section (Figure 5G). Phloem was around the xylem tissue	The vascular tissue forms a ring around the periphery of the pith (Figure 5D). This consists of a multiseriate column of vessels that form a ring around the pith. Secondary growth occurs but was not prominent. Semi-ring porous vessels were present. Tracheids are mixed with the vessels; ray and axial parenchyma were not obvious. As in the stem of the purple form, vessel elements from the early wood had wide pores. The arrangement of the phloem was similar to the purple stem. (Figure 5H)
The secondary walls of most tracheary elements contain opposite thickening (Figure 5K). Vessels were vested (Figure 5J). Perforations were simple and located in the middle of the cells (Figure 5L).	Annular thickenings are dominant (Figure 5I). Perforations were simple and located in the middle of the parenchyma cells.

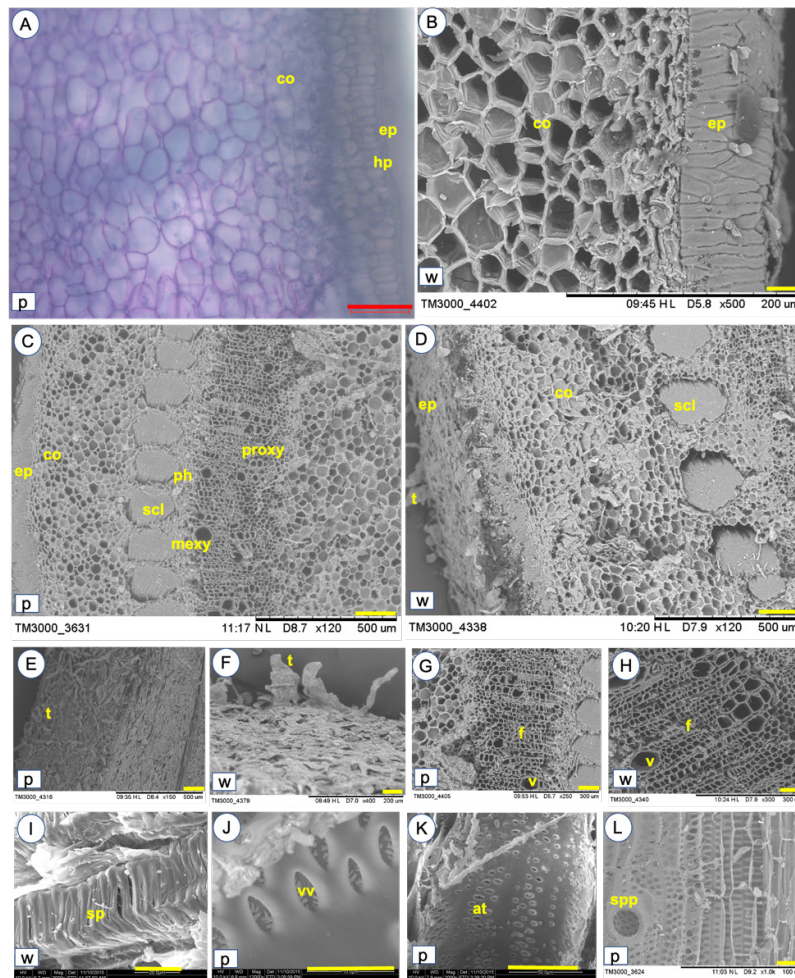


Figure 5: SEM and LM of stem anatomy of the two flower colour forms of *Calotropis gigantea*. Except for B, D, F, H, and I, all figures are taken from the purple form. (A) soft and corky bark. (B) TS of stem epidermis showing the wax deposition. (C) TS of stem showing multi-seriate hairs. (D) TS of stem highly packed barrel to rectangular shaped cells. (E) thick coat of epidermis of stem consists of different shapes (leafy, elongated) hairs. (F) leaf-like trichomes of white form. (G) a vascular system of purple form. (H) a vascular system of white form: (I) annular thickenings of the white stem. (J) vestured inter-vessel pits. (K) alternate thickenings (L) LS showing simple perforation plate with a wide and prominent rim. Sub-figure letters: p - purple. w- white. Abbreviations used: op: opposite pitting in the surface. at: annular thickenings. co: cortex. ep: epidermis. f: fiber. mexy: metaxylem. op: opposite thickenings. ph: phloem. proxy protoxylem. scl: sclerenchyma. spp: simple perforation plate. v: vessel. vv: vestured vessel pits. x: xylem. Scale bar: 10 μ m; J; 20 μ m; I; 50 μ m; K; 100 μ m: A, L; 200 μ m: B, F; 300 μ m: G, H; 500 μ m: C, D, E.

Stems of both forms were characterized by the presence of bundles of sclerenchyma fiber belt (pericycle) in the cortex (Figure 5C, 5D). In the purple form, between two sclerenchyma bundles one or two intermittent parenchyma cells were present (Figure 5C), while in the white form, these sclerenchyma bundles were separated by more than three parenchyma cells (Figure 5D). Growth rings were indistinct but observable. Wood had solitary or semi-ring porous in radial and/or diagonal multiples of 2–4 or many rounded, solitary vessels (Figure 5G, 5H). The wide range of vessel diameters in both directions has been observed in this study as well as by previous researchers (Hassan *et al.*, 2015). This may be attributed to the environmental conditions and the nature of plant growth conditions (Nasser *et al.*, 2012). However, in Hindi and Arabia, (2013) and Nasser *et al.*, (2012), diffuse-porous wood with even-sized vessels or pores of *C. procera* has been observed in the Arabian Peninsula, so the water-conducting capability was scattered throughout the ring. Fibers occupy the bulk of the area in the cross-section and have thin walls with

wide lumens. These characters had been observed by other authors as well (Hindi and Arabia, 2013; Nasser *et al.*, 2012). Simple perforations were observed in the middle of the cell (Figure 5L). Intervessel pits were alternate and vestured (Figure 5J) rounded with slit-like apertures (Figure 5K) and annular thickenings (Figure 5I) and alternate thickenings (Figure 5K) were observed in this study. Ingle and Dadswe (1953) also found the general characteristics (scanty axial parenchyma scattered and intermixed with fibers) of Apocynaceae. Similar features were observed in *C. procera* (Hindi and Arabia, 2013; Nasser *et al.*, 2012). As we observed, relatively thin walls, less lignified fibers, ray parenchyma, long chains of radial multiples of vessels, and scalariform border pits have been observed by Hindi and Arabia, (2013). Lens *et al.*, (2008) have observed the striking differences in vessel grouping patterns (radial multiples vs. large clusters) among the mainly non-climbing apocynoid tribes and the climbing lineages.

The knowledge of the root anatomy of *Calotropis* is scanty. According to a study done by Lopes *et al.*, (2009), Hassan *et al.*, (2015) a similar arrangement of the tissues (well-developed periderm, scattered/diffusive xylem, large vessels, and tracheids) of *C. procera* root have been observed. Common features in Apocynaceae such as collateral vascular bundles and the distinctive arrangement of the external and internal phloem as well as the tracheary elements aligned in rows were observed during the present study.

Laticifers and other depositions

The laticifers established an entire laticifer network in the petiole, leaf, stem, and root of both forms of *C. gigantea* found in Sri Lanka as individual long tubular, thin-walled, and unbranched or branched latex-containing structures in both the underground and aerial parts of the plant. In the petiole (Figure 1I) and leaf blade along the main vascular bundle region (Figures 2A, 2B), laticifers occur below and above the vascular bundle (Figure 1H, Figures 2A, 2B). Long tubular, unbranched latex-containing structures

were observed in the leaf blade (Figure 2I). In longitudinal sections of root and stem, laticifers appeared as thin-walled tubular-shaped (Figure 6A), and simple or branched structures (Figures 6A-6D). Bifurcate laticifers, branching into a “Y” or an “H” was observed in the cortex of purple form (Figures 6C-6D). Laticifers were distributed predominantly near the secondary phloem (Figure 4A), in the ground cortical parenchyma (dark-stained bodies in the cortex), pith, and vascular region of the root tissues (Figure 4A). The presence of laticifers in leaves near the vascular tissue and the cortex of the stem and root of *Calotropis* was observed. Similar results have been recorded by Harsimran and Shikha (2015). Moreover, the different shapes of the laticifers in different parts (pith, vascular region, and ground parenchyma and palisade tissues) of the two trees of *Calotropis* were observed in this study. Articulated laticifers were observed in a member of the Apocynaceae, *Mandevilla atrovioleacea*, in different tissues, i. e. ground meristem, root apex, tuberous roots, leaf, and stem. Moreover, as we observed, Y-shaped laticifers and secretory nature by the granularity of different tissues were also observed by Lopes *et al.*, (2009). Laticifer distribution

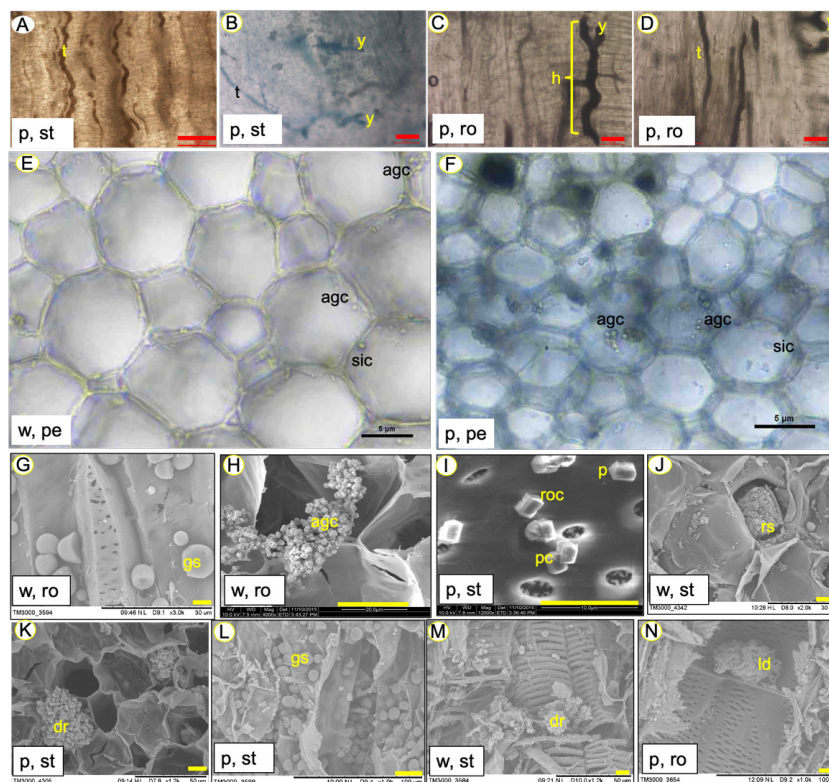


Figure 6: Latex and crystal forms found in different parts of the two forms of *Calotropis gigantea*. Top two rows: Light microscopy, last two rows, SEM. (A) longitudinal tangential section of wood showing latex traces and tubular-shaped laticifers in the stem. (B) bifurcate or Y- Y-shaped and tubular-shaped laticifers in stem in transverse section (TS), stained-with TBO. (C) bifurcate and tubular shape, H shape laticifers in root. (D) Y and H-shape laticifers in the root. (E) aggregated and single crystals in the petiole of white form. (F) aggregated and single crystals in the petiole of purple form. (G) heavily deposited, different shapes of crystals gathered to form compound crystals in pith parenchyma of the root of white form. (H) heavily aggregated a form of crystals in the root of white form. (I) different shapes (prismatic shaped, rhomboid) of crystals inside the stem vessels. (J) rhomboid shape crystals. (K) druse inside the stem parenchyma cells. (L) globular shape crystals inside parenchyma cells. (M) druse outside parenchyma cells. (N) irregularly shaped deposits of latex in root vessels. Sub-figure letters: pp - purple, petiole. ps - purple stem. pr - purple, root. wp - white, petiole. ws - white, stem. wr - white, root. Abbreviations used: agc - aggregated crystals. cc - compound crystals. dr - druse. gs: globular shape crystals. h - H-shaped laticifers. is: irregular shape crystals. ld: latex deposits in vessels. p: prismatic shape crystals. r: rhomboid shape crystals. sic - single crystal. t: tubular-shape laticifers. Y: Y- Y-shaped laticifers. Sub-figure letters - pr: purple root. ps: purple stem. wr - white root. ws - white stem. Scale bar: 5 μ m: E, F; 10 μ m: I; 20 μ m: H; 30 μ m: G, J; 50 μ m: K, M; 100 μ m: B, C, D, N; 500 μ m: A.

in both forms of *C. gigantea* showed the same pattern as recorded in the family Apocynaceae (Lopes *et al.*, 2009).

Single or aggregated crystals (Figures 6E, 6F) were observed in different parts of the plant under the LM. According to the LM and SEM images, globular-shaped bodies were observed in the cortex parenchyma cells (Figures 6F, 6G). Different shapes of latex deposits, crystals, or secretions were observed in different places of both forms (Figure 6G – 6N). Hassan *et al.*, (2015) observed the groups of sclereids, a few crystal druses, and the phloem of the stem. But in this study, we did not observe the sclereids in any tissues. As we observed the presence of different shapes of crystals in *C. gigantea*, Lens *et al.*, (2008) observed crystals in three species of the Rauvolfioideae, a subfamily within the Apocynaceae. Prismatic crystals in the cortex of *Periploca laevigata*, and crystal druses in the hemicryptophytes *Vincetoxicum*, *Asclepias*, and *Gomphocarpus* were observed (Evert, 2006). Although *C. gigantea* is an economically and ecologically very important plant species throughout the world, not much research work has been done to discriminate between the white and purple colour forms of the plant. The present study was carried out aiming to gather evidence from anatomical data; petiole, leaf, root and stem, using LM and SEM studies. Although the anatomical characteristics of leaves, petioles, stems, and roots were quite homogeneous and similar in both forms under study, the two flower forms of *C. gigantea* can be distinguished based on the amount of deposition of cuticular materials and the structure of striations of the leaf surfaces. The SEM data obtained during the present study would be useful for further studies to elaborate the anatomical studies with more samples of both forms from different geographical areas. Since there are clear differences observed in relation to few anatomical characters between the two flower colour types, these characters would facilitate the identification of the two forms in the absence of flowers. The distinct characteristics observed under each flower colour form of *C. gigantea* are summarized below;

***C. gigantea* purple form:** The amount of leaf cuticular material was higher on the abaxial surface than on the adaxial surface of the purple form. Radially arranged cuticle materials around the stomata were observed. In the stem, between the cortex and the vascular bundle, a discontinuous pericycle ring of sclerenchyma fibers was present. Between the two sclerenchyma patches, one or two intermittent parenchyma cells were present.

***C. gigantea* white form:** Both leaf surfaces have more or less similar amounts of cuticular material. Cuticle materials were arranged as blocks around the stomata. As in purple form, a discontinuous pericycle ring of sclerenchyma fibers was present. However, between the two sclerenchyma patches, three to four intermittent parenchyma cells were present.

CONCLUSION

The study has identified stem and stomatal anatomy and the arrangement of cuticular materials as useful characters to differentiate the two-colour forms of *Calotropis gigantea*

in Sri Lanka. As morphologically the two forms resemble each other, the results would facilitate the identification of the two flower colour forms in the absence of flowers.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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