

RESEARCH ARTICLE

Distribution, morphology, anatomy and histochemistry of *Crepidium acuminatum*

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Abstract

Crepidium acuminatum is largely confine to the Himalayan region although it has been reported in South India, where its presence distribution needs to be critically studied. This study describes the morphological, anatomical and histochemical aspects of the materials of the authenticated species collected from Himalayas. This study also highlights the features of histochemistry and anatomy that should be used for the correct identification and authentication of *C. acuminatum*, especially because of the therapeutic importance of the species and its possible adulteration by other orchids.

Keywords: *Malaxis acuminata*, *Microstylis wallichii*, orchid mycorrhizae, pelotons, pseudobulb

Introduction

Crepidium acuminatum (D. Don) Szlach. belongs to the family Orchidaceae. Commonly known as Jivaka, this Ashtavarga plant is mainly used for its cooling effect, febrifuge, spermopiotic and refrigerant activity; it acts against haematemesis, fever, semen-related weakness, burning sensation, dipsia, emaciation, tuberculosis,

general debility, bleeding diathesis and phthisis (Pushpa et al. 2011; Balkrishna 2012; Subedi et al. 2013; Adams et al. 2017). The main part of the plant applied in folk and Ayurvedic medicine is the pseudobulb, which in fact is the aerial stem of the plant. Its Ayurvedic dynamics are: sweet in taste, cold in potency, pacifies vata and aggravates kapha (Singh 2006). It is also proven that the ethanolic extract of pseudobulbs exhibits

antiproliferative activity (Singh et al. 2017). It also forms a useful therapy in combination with drugs from other ashtavarga plants. Astavarg Churna, Jevaniyo Dashko Mahakshay and Chyawanprash Linctus are some of the compound drugs from orchids, including this plant that are well-known in Ayurveda (Kaushik 1983; Bose et al. 1999; Chinmay et al. 2011). There are market problems in meeting the demands for this plant, as it is in very short supply giving scope for possible adulterations / substitute of other orchids like *Malaxis cylindrostachya* Kuntze and *M. mackinnoni* Duthie (Balkrishna et al. 2012). The trade value of this material is around 120 Rs/Kg in North-West Indian markets (Sultan & Singh 2006). Unsustainable harvest of wild populations of this orchid for pharmaceutical and nutraceuticals purpose has been a major threat to causing the rarity of this species (Hinsley et al. 2017). Hence, there is an urgent need for proper authentication of this species, as well as defining its distribution in India so as to enable the collection of genuine plants. This article describes the distribution, morphology and anatomy of *C. acuminatum*, all of which are very important in the proper authentication of this species.

Material and methods

Fresh plants were collected from Raisaar Devta, Tehri Garhwal District, Uttarakhand, India (30.34465, 78.415371) and identified by Dr. Amit Singh (G.B. Pant National Institute of Himalayan Environment and Sustainable Development, Mohal-Kullu, Himachal Pradesh), Dr. T.N. Manohara (Scientist, Rain Forest Research Institution, Jorhat, Assam), and verified and authenticated by Dr. K. Ravikumar

(Foundation for Revitalization of Local Health Traditions FRLHT, Bangalore) and Prof. K.V. Krishnamurthy (Research and Development Consultant, Sami Labs Ltd, Bangalore). The vouchers were deposited in FRLHT herbarium, Bangalore. The voucher raw drug material is also deposited there. Some pseudobulbs are being cultivated in the medicinal plant garden of that institute.

Fresh samples of leaf, pseudobulb, protocorm and root were used for taking transverse sections (about 10–20 μm in thickness), using razor blade and cryomicrotome Medi Meas MCM-ST. The sections were stained using various staining procedures (Tab. 1) and observed under a microscope Nikon Eclipse Ci. Photographic images were captured using Nikon DS Ri2 attached to a microscope. The images were processed on Nikon Basic Essential software.

Autofluorescence and induced fluorescence images were captured in the above mentioned microscope fitted with epifluorescence unit and these results were correlated with data obtained under normal light. Calcium oxalate crystals were localized using polarizing optics.

Results and discussion

Distribution and taxonomic identity

Crepidium acuminatum is reported to be mainly distributed in the temperate to subtropical Himalayas (Clarke 1885) at an altitude of 1200–2100 m a.s.l. It is found in Himachal Pradesh, Uttarakhand, Arunachal Pradesh, Assam, Nagaland, Manipur, Mizoram and Tripura states of India (Samant et al. 1998; Chauhan 1999; Singh 2005, 2006; Dhyani et al. 2010; Balkrishna et al. 2012). Ved et al. (2003) and Lohani et al. (2013) have reported that it is a rare and

Table 1. Localization of chemicals components and applied procedures.

Chemical components	Method	References
Alkaloid	Dragendorff's Reagent	Yoder & Mahlberg 1976; Ferreira et al. 1998
Acidic polysaccharide	Toluidine Blue O (TBO)	Krishnamurthy 1988
Anthocyanin	Vanillin-Perchloric Acid	Abraham et al. 1988; Narayana et al. 2002
Lignin	Phloroglucinol-HCl; TBO	Krishnamurthy 1988
Polyphenols	TBO	Krishnamurthy 1988
Lipids	Oil Red O, Sudan Black	Krishnamurthy 1988
Cutin, Suberin	Auramin O, Azure B	Krishnamurthy 1988
Starch	Iodine-Potassium Iodide (Lugol's Iodine)	Krishnamurthy 1988

vulnerable species of the Himalayan region. *C. acuminatum* has also been reported from South India especially in the South Western Ghats and recently in Andhra Pradesh (Gamble & Fischer 1915–1936; Abraham & Vatsala 1981; Henry et al. 1989; Reddy et al. 2001, 2006; Murugesan & Balasubramaniam 2008; Karuppusamy et al. 2009; Cheruvathur et al. 2010; Dutt et al. 2010; Aravindhhan et al. 2011; Chinmay et al. 2011; Manikandan & Lakshminarasimhan 2012; Gupta 2016; Ved et al. 2016). According to Matthew (1983), this species (recorded as *Malaxis acuminata* D. Don) is found in Servarayans Hills (Yercaud) of Tamil Nadu Eastern Ghats at an altitude of 1500 m, on the mountain slopes. He distinguished this species from *Malaxis rheedii* B. Heyne ex Wallace on the basis of that there are large auricles and a mid lobe with a split at apex of the perianth lip in *M. acuminata*, while the lip is without distinct auricles in *M. rheedii*. However, he mentioned that *M. acuminata*, although is a different species from *M. rheedii* in Yercaud hills, he still was not satisfied with his identification and requested for some more work in order to confirm the presence of this species in this region. Ridley (1887) distinguished the species *Microstylis rheedii* Wt. from *Crepidium* Blume by the colour and structural characters of

flower. According to the checklist prepared based on the earlier reports and literature, Nayar et al. (2014) concluded that only two species of *Crepidium* are distributed in Western Ghats, namely *C. mackinnonii* (Duthie) Szlach. [syn. *Microstylis mackinnonii* Duthie, *Malaxis mackinnonii* (Duthie) Ames] and *C. purpureum* (Lindl.) Szlach. [syn. *Microstylis purpurea* Lindl., *Microstylis wallichii* auct. non. Lindl., *Malaxis purpurea* (Lindl.) Kuntze, *Malaxis acuminata* auct. non. D. Don]. Thus, according to these authors *Crepidium acuminatum* (D. Don) Szlach. [syn. *Malaxis acuminata* D. Don, *Microstylis wallichii* Lindl.] does not occur in Western Ghats. We also consulted Dr. C. Sathish Kumar, former Scientist of Jawaharlal Nehru Tropical Botanic Garden & Research Institute, Trivandrum, and a leading orchidologist of India. He has categorically stated that *C. acuminatum* is restricted to the Himalaya region and that all reports of this species in South India are based on wrong identification. Indian species of *Malaxis* Sol. ex Sw. were merged with *Crepidium* Blume, *Liparis* Rich., *Oberonia* Lindl. and *Seidenfia* Szlach., and it was suggested that the genus *Malaxis* s.l. (including the species *M. acuminata*) probably is not represented in South India. It was also suggested that further studies may be made to verify

whether the South India samples reported as *M. acuminata* represent natural hybrid between *M. acuminata* and *M. rheedii* (both of them now are known under the new names). Such possibilities for natural hybridization in *Crepidium* have been reported by Nuammee et al. (2016). However, we feel that the reports of this species in South India by earlier authors cited above should not be dismissed without making a critical study on the distribution of this taxon in India.

Macromorphology

The plants of *C. acuminatum* arise from a swollen protocorm often mistaken with a rhizome (Uma et al. 2015) that is produced during embryogenesis. Protocorm sooner or later loses its distinct identity due to the formation of several roots and more than one pseudobulb (aerial stems), but it still seen until the death of the plant. Plants have an erect short stem bearing 3–5, alternate, broadly lance-like, acuminate, leaves with undulating margins, parallel venation and sheathing leaf bases (Fig. 1 A). Leaves when young are green, but on maturity become yellow. The leaf sheaths are green to start with, yellow when old, but become violet-pink at maturity (Fig. 1 B). Flowering occurs in June to August, the terminal raceme inflorescence is erect, 10–30 cm long, many-flowered and with lanceolate acute floral bracts. Flowers are pale-yellowish green, tinged with purple; sides of the lip produced upwards into auricles, apex notched. Perianth in two whorls, with 3 outer and 3 inner segments. The outer dorsal tepal is linear to oblong, sub-acute, while lateral outer tepals are oblong. The inner tepals are linear, obtuse, with recurved margins, lips slightly convex, bases with auricles (Fig. 1 C, D). The stem is conical with a tapering end. Young stems are green and

smooth, shinning outside and fleshy inside. Mature stem is purple in colour at least on the side bearing the leafy sheath. Stem is 2 to 12 cm long and 1 to 4 cm in diameter, mucilaginous when in contact with water. The dry stem is covered with the shiny translucent grayish pink coloured sheathing leaf bases (Fig. 1 E–G). Dried stems give a pleasant smell and astringent in taste.

Earlier anatomical description of this species as well as of morphology of flower and pseudobulb made by Sharma et al. (2014) collaborates our observations. The illustration of *M. acuminata* found in Uma et al. (2015) differs from typical specimens of this species collected from Himalayas in a number of respects: (a) they do not show the typical protocorm which persists almost throughout the life of the plant; (b) they lack additional pseudobulbs; (c) they possess leaves with long petioles and acute leaf tip. The authenticated species has persistent protocorm till the death of the plant, has more than one pseudobulb, leaves almost sessile with acuminate apex. Perhaps this species shows very great phenotypic plasticity that is especially different from specimen collected from Himalayas.

Micromorphology of the leaf

The transverse section of the leaf shows a layer of wavy cuticle on both sides of the lamina. Both adaxial and abaxial epidermises have rectangular cells (Fig. 2 A, B). The mesophyll region is homogenous spongy, and all cells are loosely arranged with prominent intercellular spaces. Some mesophyll cells are with raphide crystals (Fig. 2 C, D). Most mesophyll cells contain many chloroplasts, which fluoresce to a red colour under fluorescence optics (Fig. 2 B, E). A number of collateral vascular bundles is present in the lamina besides a prominent 'midrib' bundle, which is

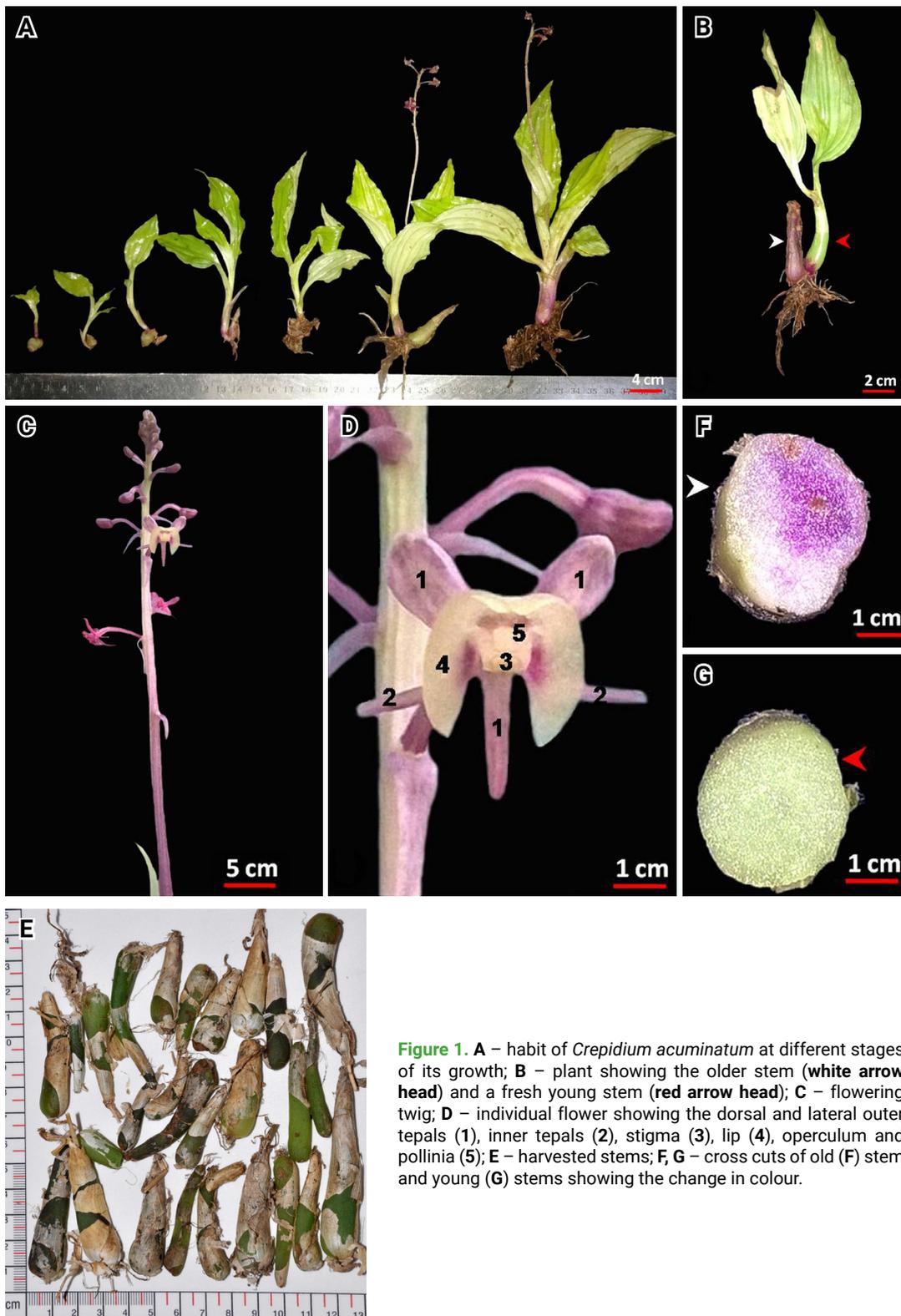
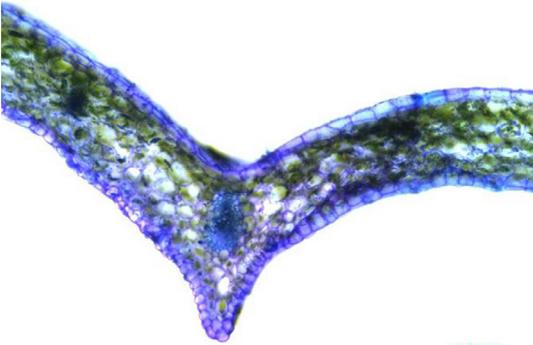
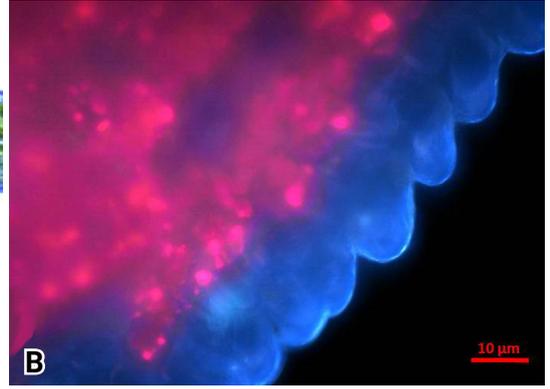


Figure 1. A – habit of *Crepidium acuminatum* at different stages of its growth; B – plant showing the older stem (**white arrow head**) and a fresh young stem (**red arrow head**); C – flowering twig; D – individual flower showing the dorsal and lateral outer tepals (1), inner tepals (2), stigma (3), lip (4), operculum and pollinia (5); E – harvested stems; F, G – cross cuts of old (F) stem and young (G) stems showing the change in colour.



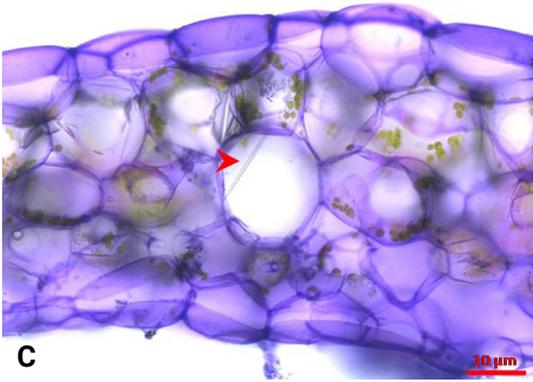
A

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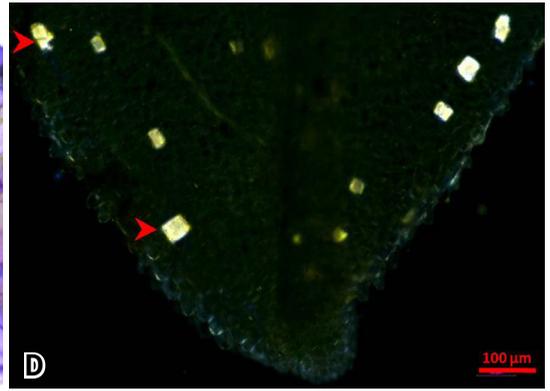
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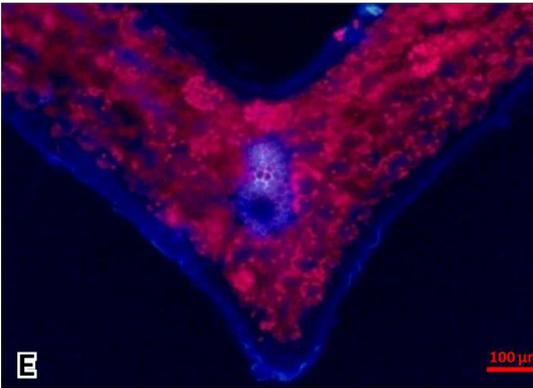
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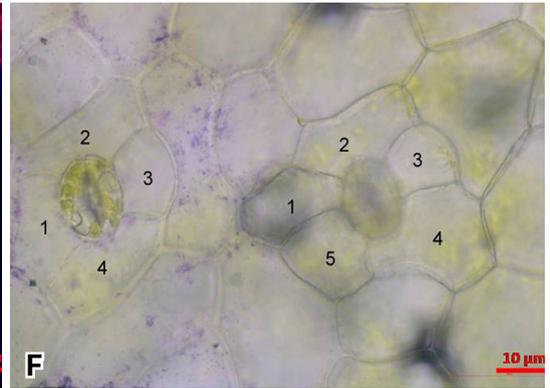
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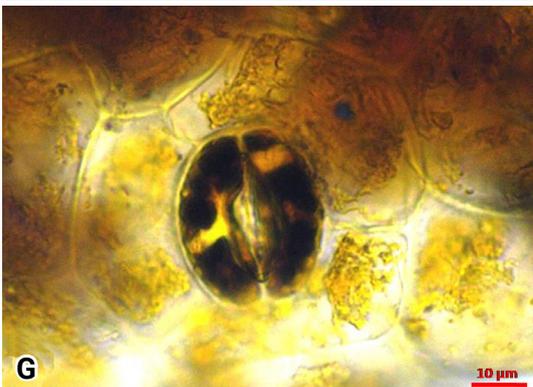
E

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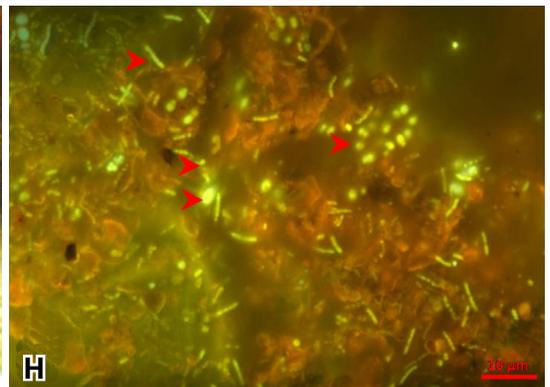
F

10 μm



G

10 μm



H

10 μm

capped with abaxial sclerenchymatous cap cells (Fig. 2 E). Centrally located vascular bundles are larger than those on margin. Tetracytic stomata with four subsidiary cells or, occasionally, anomocytic stomata with three or five subsidiary cells are present on the abaxial side of the leaf and are randomly distributed on the lamina (Fig. 2 F, G). The guard cells contain chloroplasts as well as starch (Fig. 2 F, G). Some of the normal epidermal cells contain highly dispersed acidic polysaccharide materials (probably mucilage). The leaf tissue stained with auramine O showed the presence of rod-shaped bacterial colonies, since auramine O stain binds with mycolic acid (fatty acid) found in the cell wall of this bacterium and emit a greenish yellow fluorescence. This bacterium is likely to be a species of *Corynebacterium* since it has mycolic acid in its wall, and since it is common in the rhizosphere area of plant roots, causing root diseases as well as leading a sporophytic mode of life (Lelliott 1966). The presence of bacterial colonies in the leaf tissue is the first report for this species (Fig. 2 H).

The structure of the leaf as described above is similar to those of the tribe Malaxideae Lindl. in most respects: presence of anomocytic and tetracytic stomata, moderately thick cuticle, homogenous mesophyll, presence of calcium oxalate crystals, presence of chloroplast and starch in guard cells (Stern 2014). Epidermal hairs, sunken stomata, parenchymatous bundle sheath with chloroplasts, reticulately

thickened water storage cells described in some other *Crepidium* species are absent here. The presence of mucilaginous material in the epidermal cells is not reported so far in any other species of *Crepidium*. Another important feature is the presence of rod-shaped bacterial cells inside the mesophyll cells. Such bacteria are common in the soil and when they are found in plants, they are usually parasitic (Lelliott 1966). In the present species, there is no structural anomaly in the leaves containing this bacterium and probably it may be a normal endophyte. The significance of bacterial presence in the leaves of this species is not clear. To our knowledge, there is no report of bacterial association in any part of *C. acuminatum*. Uma et al. (2015) did not mention bacterial presence in any part in the material (*Malaxis acuminata*) studied by them; perhaps South Indian specimens are not associated with this bacterium. However it is interesting to note that there are reports of bacterial presence in orchids like *Cattleya walkeriana* Gardner (Júnior et al. 2011) and *Dactylorhiza maculata* (L.) Soó (Shekhovtsova et al. 2013).

Micromorphology of the pseudobulb

The young pseudobulb has a single layered epidermis and an inner ground tissue of parenchyma cells in which is scattered a number of collateral vascular bundles that are devoid of sclerenchymatous cap (Fig. 3 A). Almost all parenchyma cells are filled with chloroplasts. The epidermis is

- ◀ **Figure 2.** *Crepidium acuminatum* leaf: **A** – transverse section of lamina stained with toluidine blue O; **B** – lamina under fluorescence optics showing the wavy and cutinized epidermal cells (**blue fluorescence**) and mesophyll cells filled with chloroplasts (**red fluorescence**); **C** – portion of lamina stained with toluidine blue O to show the presence of mucilage canals and raphide crystals (**arrow head**); **D** – lamina under polarized light showing the presence of bundles of raphide calcium oxalate crystals (**arrow heads**); **E** – midrib region of lamina with vascular bundle capped at adaxial end with sclerenchymatous cells, observed under UV light; **F** – surface view of leaf epidermis showing the tetracytic stomata on the abaxial side, note also the presence of chloroplasts; **G** – peel of the leaf stained with Lugol's iodine to show the presence of starch in the guard cells of a stoma; **H** – transverse section of lamina stained with auramin O and viewed under UV to show the presence of greenish-yellow rod shaped bacterial cells inside the parenchymatous cells (**red arrow heads**).

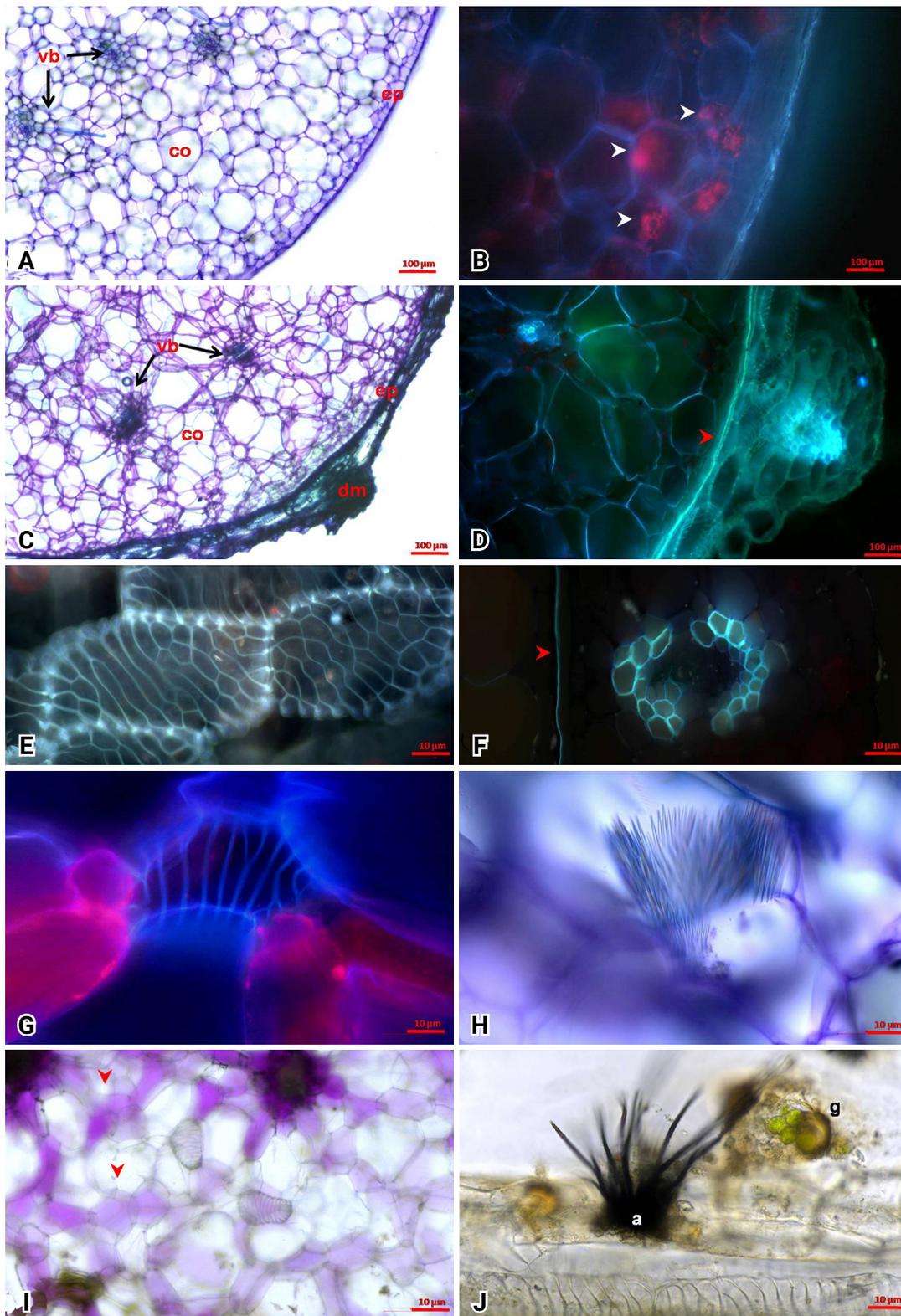
covered by a thin cuticle and is smooth throughout (Fig. 3 B). There are very few mucilage cells. Full grown pseudobulbs retain the same structure but show six important features. (i) The number of mucilage cells increases and chloroplast are lost (Fig. 3 C, D). (ii) Some of ground tissue cells demonstrate reticulate cell wall thickenings; these cells are probably involved in storage of water (Fig. 3 G). (iii) Peculiar dome shaped structures are sparsely found over the epidermis of the pseudobulb. These domes often contain vascular bundles (Fig. 3 D, F) and represent the remnants of the leaf sheath, where the features of adaxial and abaxial epidermal cells cannot be easily studied. However they also show the presence of some reticulately thickened cells, which are likely to be involved in water storage (Fig. 3 E). (iv) Raphides of calcium oxalate are fully developed and form an important component of the ground tissue (Fig. 3 H). (v) A number of peripheral cortical cells contains amorphous anthocyanin pigments (Fig. 3 I). (vi) Decaying leaf sheath remnants at the base of the pseudobulbs also have some associated fungal and algal thalli; the fungus (probably a species of soil fungus) shows a number of acervuli attached to the leaf sheath and partially embedded in it, while the alga, taking into account its four-celled coenobium, is probably represented by a species of *Gonium* (Fig. 3 J). The pseudobulbs are totally devoid of mycorrhizae fungi and bacteria cells as seen in root and protocorm.

The structure of pseudobulb, as described above for *Crepidium acuminatum*, differs from the anatomy of *Malaxis acuminata* described earlier by others in some respects. There are no sclerenchymatous sheaths around the vascular bundles in the specimen collected by us from Himalayas, although Uma et al. (2015) and Chinmay et al. (2011) mentioned them. It is noteworthy that such sheaths are also not reported by Stern (2014).

Micromorphology of the protocorm

The protocorm is formed from the basal part of the developing embryo. This is a swollen structure from which roots are produced towards the soil (all roots arise from a pericycle region that surrounds the ring of vascular bundles) and pseudobulbs or stems are produced aerielly (Vinogradova & Andronova 2013; Yeung 2017). Later it forms a structure intermediate between the root and the pseudobulb (Fig. 4 A). The root features include the presence of inner cortex harbouring the mycorrhizal fungus in the form of pelotons. It also has a rhizodermal-like structure producing root hairs, which perhaps form the channels for the entry of mycorrhizal fungi. The outer cortex is devoid of fungal pelotons and is made up of parenchymatous tissue (Fig. 4 B). The stem features include the presence of scattered vascular bundles in the ground tissue in addition to an almost circular ring of vascular bundles, from which region the roots are produced (Fig. 4 C). Vascular bundles are collateral, with xylem on the inside,

Figure 3. *Crepidium acuminatum* pseudobulb: **A, C** – transverse sections of young (**A**) and mature (**C**) pseudobulbs stained with toluidine blue O; **B** – transverse section of young pseudobulb under UV, showing the presence of chloroplasts (**arrow heads**) in cortex region; **D** – transverse section of old pseudobulb under UV, showing thick cuticle (**arrow head**) and leaf sheath dome with vascular bundle; **E** – reticulate thickened cells in the outer leafy sheath, examined under UV; **F** – section of leafy sheath showing vascular bundle and outer thin suberized layer of pseudobulb (**arrow head**); **G** – cortical cells with the secondary wall thickening, examined under UV; **H** – raphides of calcium oxalate; **I** – histochemical localization of the anthocyanin, stained using vanillin-perchloric acid method; **J** – matured outer leaf sheath, observed under the bright field, showing the presence of an acervuli and an algal coenobium vegetative body. **a** – acervulus; **co** – cortex; **dm** – dome structure; **ep** – epidermis; **g** – gonium; **vb** – vascular bundle.



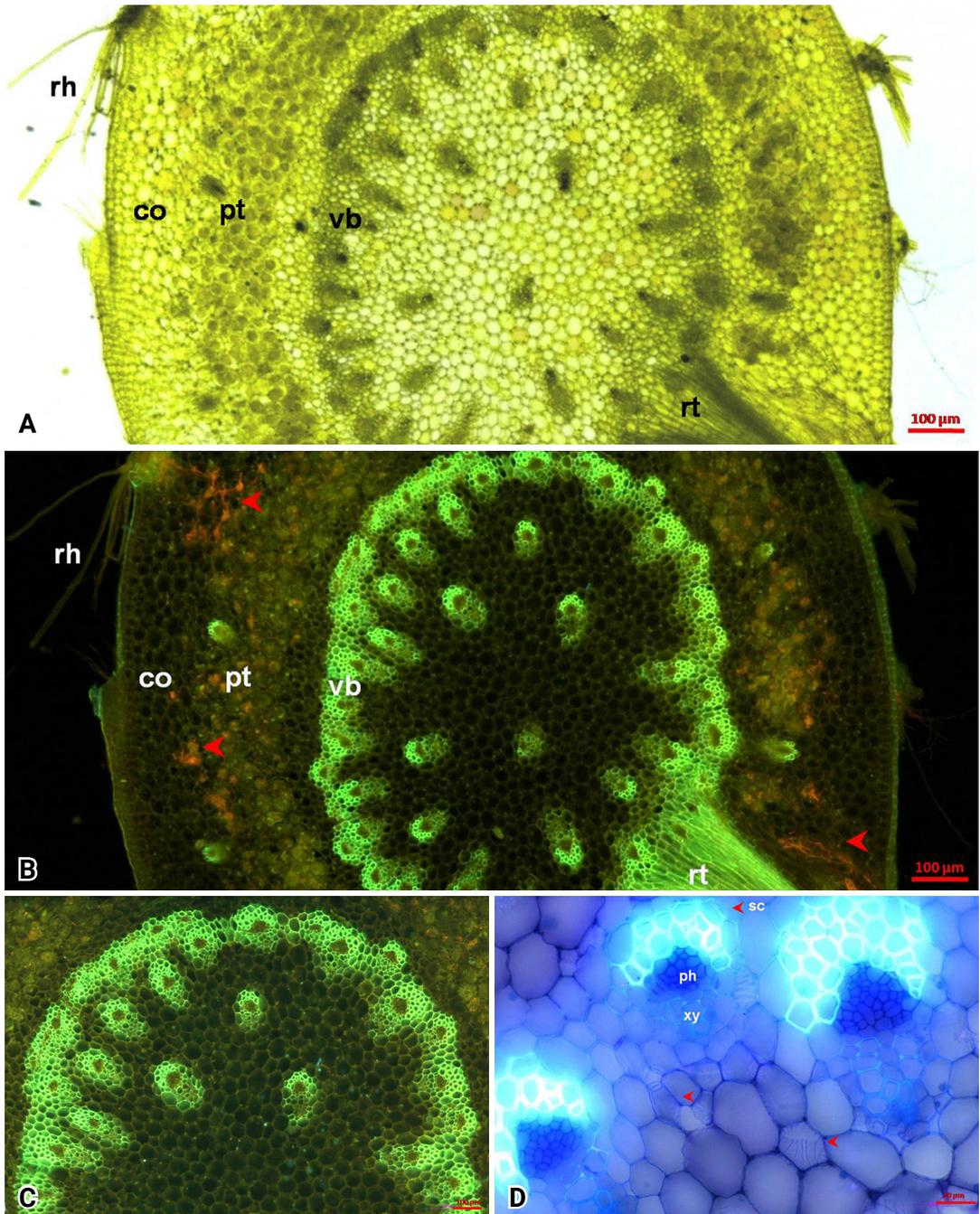


Figure 4. Transverse sections of *Crepidium acuminatum* protocorm: **A** – stained with auramine O and viewed under bright field; **B** – the same under UV, exhibiting circular vascular ring with scattered vascular bundles inside the ring distinctly visible in greenish yellow colour, and fungal pelotons (**red arrow head**) in the cortex region; **C** – magnified view of circular vascular ring and internal scattered vascular bundles; **D** – unstained section under UV, showing the bright sclerenchymatous cap (**sc**) towards the periphery region with phloem on the outside and xylem on the inside. Also reticulate secondary wall thickening (**red arrow heads**) is visible. **co** – cortex; **ph** – phloem; **pt** – pelotons; **rh** – root hair; **rt** – root trace; **sc** – sclerenchyma; **vb** – vascular bundle; **xy** – xylem.

phloem on the outside, and supported by sclerenchymatous cap on the outside of the phloem (Fig. 4 D). The pelotons are similar to that observed in the roots. Bacterial cells are also seen but are not abundant as in the roots. Typical endodermis is absent. Some of the ground parenchyma cells have reticulate thickening in their cell walls and devoid of living contents, as described for similar cells in the root.

The structure described as a 'rhizome' for *Malaxis acuminata* by Uma et al. (2015) is not really a rhizome, which is invariably underground (in some orchids aerial rhizomes occur) and invariably grows horizontally. It is, in fact, the protocorm region, that persists in the mature plant. Although some people considered the protocorm as the structure persisting only in early stages of morphogenesis (Chang et al. 2005; Yeung 2017), in this plant the protocorm has an extended presence from the embryo stage onwards and is involved in the production of additional pseudobulbs (aerial stems) above the ground and roots below the ground (Peterson et al. 2004: p. 130). Hence, it is no wonder that it combines anatomical features of both the pseudobulb and the root. True rhizomes of other species produce aerial shoots from the axils of scale leaves, but in *C. acuminatum* the so-called rhizome has neither scale leaves nor nodes and internodes. Here the production of new shoots appears from a group of meristematic cells de-differentiated from the mature protocorm. Therefore the authors of this paper have considered this structure found in *C. acuminatum* as an extended protocorm and not a rhizome. At the best, it can be considered as intermediate structure between true protocorm and true rhizome. The structural details of so-called rhizome described by Uma et al. (2015) differs in the

following respects: (a) the outer scattered vascular bundles are really the root traces and are not belonging to the protocorm; (b) the sclerenchymatous sheath does not surround the entire vascular bundle, but is located only around the phloem as a cap (Fig. 4 D). Stern (2014) does not mention about sclerenchymatous sheath of vascular bundles. As far as we are aware, such sclerenchymatous sheaths are not known for the so-called rhizome of any terrestrial orchid.

Micromorphology of the root

Almost all roots of the plant are endomycorrhizal, of orchid type, with numerous root hairs. Transverse sections of root are more or less circular in outline (Fig. 5 A; Fig. 6 A). Cells of outer layer of the root, excepting root hairs, form the so-called velamen. These cells do not have reticulate or spiral thickenings, which are characteristic for velamen, but still possess a cell wall that is mildly suberized (along with phenolic materials), as in the subjacent hypodermal (exodermal) cells (Fig. 6 A, C). Thus, these plants have a single velamen layer, which also serves as the rhizodermis. There are passage hypodermal cells below each root hair (Fig. 6 A, C). The hypodermal cells emit a strong greenish yellow fluorescence indicating the presence of suberin along with some phenolic materials (Fig. 6 A, C). There is a parenchymatous cortex inside from hypodermis (Fig. 5 A, B; Fig. 6 A, C). The cortex is 7–8 cells thick, and most of the inner cortical cells are filled with fungal hyphal balls or pelotons, while the outer cortical cells show only few fungal hyphae (Fig. 5 A–C; Fig. 6 A). The endodermis follows the cortex and consists of two types of cells: (a) thin-walled passage cells, which are present opposite to protoxylem poles; (b) cells lying opposite to

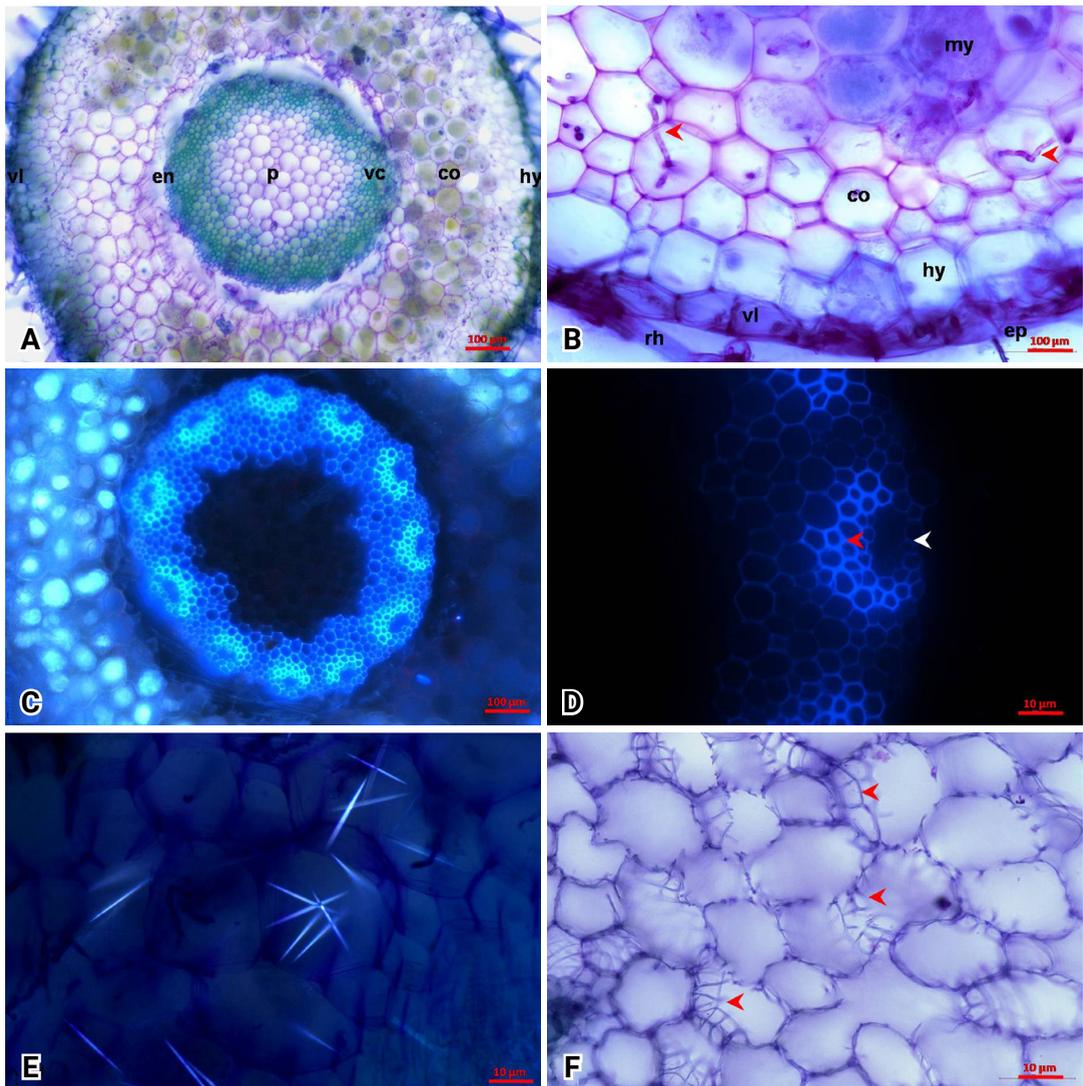


Figure 5. Transverse sections of *Crepidium acuminatum* root: **A** – stained with toluidine blue O showing outer thin and single layer of velamen cells, followed by single layer of hypodermal, broad cortex composed of parenchymatous cells, and inner endodermal layer surrounding the vascular cylinder with parenchymatous pith; **B** – the same, portion enlarged, outer cortical cells are traversed by fungal hyphae (**red arrow head**), while inner cortical cells are filled with fungal pelotons; **C** – transverse section under UV, showing inner cortical cells and stele; **D** – stele portion magnified to show the crescent-shaped xylem (**red arrow head**) facing towards exterior and phloem (**white arrow head**) located between the two arches of the crescent, viewed under UV; **E** – parenchymatous cells showing the presence of raphide calcium oxalate crystals, viewed under polarized light; **F** – cells with reticulate secondary wall thickening (**red arrow heads**). **co** – cortex; **en** – endodermis; **hy** – hypodermis; **my** – mycorrhizal fungus; **p** – pith; **rh** – root hair; **vc** – vascular cylinder; **vl** – velamen.

phloem with suberized thickening limited to outer tangential wall and two radial walls (not illustrated). The innermost region of the root is the stele (Fig. 5 A, C; Fig. 6 A). The

pericycle region is often one-layered above the protoxylem and three- to four-layered below the phloem region. The walls of these cells are slightly lignified, but have living

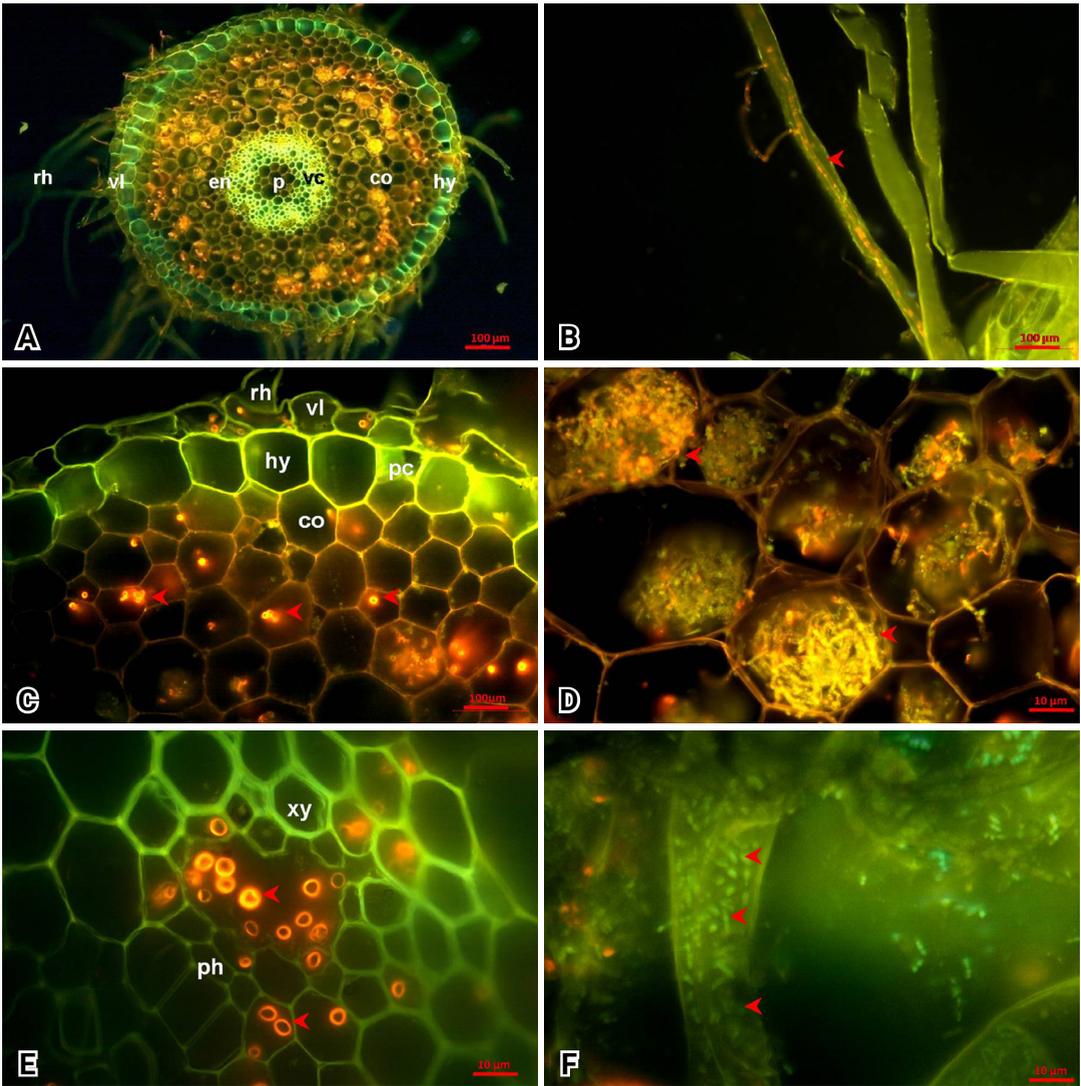


Figure 6. Transverse sections of a mycorrhizal root of *Crepidium acuminatum* stained with auramin O and observed under UV: **A** – general view of whole transverse section; **B** – fungal hyphae (**arrow head**) showing orange fluorescence, entering through the root hair; **C** – magnified portion of transverse section showing the entry and spread of fungal mycelium (**arrow heads**) through the passage cells in hypodermal layer, and formation of pelotons in the inner cortical region of the root; **D** – magnified view showing the presence of pelotons in cortical cells; **E** – magnified region of vascular bundle with fungal mycelium (**arrow head**); **F** – presence of bacterial colonies in the root hair cells (**arrow heads**) emitting bluish green fluorescence under UV, after the stain binds of bacterial cell wall with mycolic acid. **co** – cortex; **en** – endodermis; **hy** – hypodermis; **p** – pith; **pc** – passage cell; **ph** – phloem; **rh** – root hair; **vc** – vascular cylinder; **vl** – velamen (rhizodermis); **xy** – xylem.

protoplast. The stele has 10–12 crescent-shaped xylem groups. Phloem is present between the two arches of the crescents (Fig. 5 C, D). There is central pith, cells of

which have slightly lignified walls. Raphide calcium oxalate crystals are present in the pith cells (Fig. 5 E). Some scattered cells in the cortex and pith are without contents or

pelotons, have velamen-cell-like secondary reticulate thickenings and store water (Fig. 5 F).

Special attention should be made about the mycorrhizal association of the root. To clearly study the mycorrhizal fungus, the young root tip region was sectioned and stained with the auramine O and viewed under fluorescence optics. The root tips show the presence of the fungus (orange-yellow fluorescence) (Fig. 6 A). Root hairs is the only path for the fungus to enter into the root (Fig. 6 B); the rhizodermal cells bearing the root hairs have the fungal presence, whereas, and the nearby velamen cells show the absence of fungal hyphae. Each root hair is entered by a single fungal hypha only and no instance of multiple entries was seen. From the root hair the fungus enters into the non-suberized passage cells of the hypodermis, which is located just below. None of the suberized hypodermal cells is colonized by the mycelium (Fig. 6 C). From the passage cells the fungal mycelium branches and ramifies intracellularly in the peripheral cells of the cortex before entering into inner cells of the cortex (Fig. 5 B). None of the peripheral cortical cells showed more than two fungal mycelia (most of them represented only one mycelium) (Fig. 6 C). Invariably in the interior cells of the cortex, the fungal hyphae form loose ball like pelotons (Fig. 5 A, B; Fig. 6 A, D). The young pelotons are orange in colour, while slightly older pelotons fluoresce to yellow colour in preparations stained with auramine O (Fig. 6 C, D). The pelotons are digested by the host cell for their nutritional requirements, and as a result the peloton balls shrink in size, become more and more compact and finally amorphous (Fig. 6 D). Normally mycorrhizal fungi never enter the stelar region of the roots (Smith & Read 2008). In our case, it is very interestingly,

the fungus enters into the stelar region and occupies some of the phloem parenchyma cells of active mycorrhizal roots (Fig. 6 E).

A feature of special interest is the presence of rod-shaped bacterial colonies (probably *Corynebacterium*) inside root hairs, cortical cells and in stele. The root sections stained in auramine O and viewed under UV light, show the bacterial colonies with greenish-yellow colour (Fig. 6 F). The stain binds with mycolic acid, which is present in the bacterial cell wall. This is also a feature of *C. acuminatum* leaves, as mentioned before.

All the roots of *C. acuminatum* are mycorrhizal and hence have a very short span of apical growth. Similarly to *C. rheedii*, *Oberonia imbricata* (Blume) Lindl., *O. pumilio* Rchb. f., *O. wightiana* Lindl., and *Stichorkis latifolia* (Lindl.) Pfitze of the tribe Malaxideae (Stern 2014), the root epidermis in *C. acuminatum* is uniseriate. This layer is considered as the *Malaxis*-type of velamen (Porembski & Barthlott 1988) However, this layer fails to develop additional wall thickening (Stern 2014), probably because this species distributed in shady and moist habitats. Perhaps, since the cell walls are suberized and cutinized (as evident from their autofluorescence and bluish green staining with toluidine blue O), this layer may function like velamen. The cells of root hairs are, however, thin-walled and non-suberized as in other orchids. Tilosomes reported in some other members of Malaxideae (Pridgeon et al. 1983; Stern et al. 2004; Stern 2014) are absent in *C. acuminatum*. The hypodermis (exodermis) is also similar to other members of the tribe Malaxideae, in that the cells do not show any extra thickening in any of their walls, and all the walls are suberized to some extent. There are distinct passage cells in the hypodermis below the root hairs. The ground tissue in the cortex and the pith are

made up of thin-walled parenchyma cells with distinct intercellular spaces. A number of idoblastic, reticulately thickened water-storing dead cells, raphide bundles enclosed in a mucilaginous sheath, and single layered endodermis are present in roots of *C. acuminatum*, similarly to other orchids (Stern et al. 2004; Stern 2014). The vascular bundles are radial as in other orchid roots. There are 10 to 13 vascular groups, each containing a crescent-shaped xylem (with exarch protoxylem and described in earlier literature as xylem arches) and phloem located between the crescent arms. Surprisingly, this feature has not been reported for *M. acuminata* by Uma et al. (2015), while so-called sclerenchymatous cap (indicated as SC on figures in above mentioned paper) seems to be a phloem, which normally stains to pink colour with toluidine blue O. However, in their illustrations, there is a single layer of sclerenchyma cells (stained blue with toluidine blue O) bordering the phloem on outside.

The mycorrhizal association of *C. acuminatum* is broadly similar to such described for other ground orchids (Senthilkumar et al. 2000; Stern 2014; Uma et al. 2015). The common features of this association include the following: (i) entry of the fungus through the root hairs; (ii) entry into the cortex through passage cells, intracellular passage of fungal hyphae in the outer cortex; (iii) formation of fungal balls or pelotons in the inner cortical cells, and the gradual digestion of the fungus by the host cells. The major difference from the mycorrhizae of other orchids is that in this species the fungus colonizes even the stelar region and occupies cells of the phloem (clearly indicated in Fig. 6 E).

Conclusions

This study is made on the orchid *C. acuminatum*, one of the eight plants that make up the Ashtavarga group of Ayurvedic system of medicine. The plant is a threatened taxon that is probably restricted to the Himalayan region, although also reported from South India. The reports of its presence in South India should not be easily dismissed without making detailed and critical studies, although some investigators emphasizes that it has limited range of distribution. This study provides several characters of the species that would help in its morphological, anatomical and histochemical authentication, supported by many illustrations. These characters would help in correct identification of the plant material and would help to prevent adulteration, which is very possible due to limited knowledge on distribution, taxonomy and structure of *C. acuminatum*. Attention is also given to the endophytic mycorrhizal fungus observed in the root and protocorm, and the endophytic bacterium (probably *Corynebacterium*) observed in the leaves and root hairs.

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