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What are tilosomes? An update and new perspectives[1]

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ABSTRACT

Characterization of tilosomes and study of their development revealed that they are similar to the cell wall ingrowths of transfer cells. Roots from *Anathallis sclerophylla* were used to examine the development, histochemistry and ultrastructure of tilosomes using light, confocal and electron microscopy (both SEM and TEM). Tilosomes initiate as cellulosic papillae in the root elongation zone, increasing the apo/symplast surface throughout the wall-membrane apparatus. Both the development and structure of tilosomes and velamen strips appear similar to the reticulated and flange cell wall ingrowths of *Vicia faba* transfer cells. We suggest two functional phases for tilosomes: a) in young tissues they increase the symplast connection and thus improve outside-inside transport; and b) in mature tissues they direct solutes to passage cells. Both the physiology and the genetic mechanisms controlling the ontogeny of tilosomes remain obscure.

Keywords: autofluorescence, callose, cell wall biochemistry, ontogeny, oriented wall deposition, waxes

Introduction

A tilosome historic and current view

The tilosomes known as protrusions or excrescences (Pridgeon *et al.* 1983; 1999) actually are wall ingrowths (WI) on the inner periclinal wall of the endovelamen cells that lay adjacent to the exodermis passage cells (Fig. 1). They occur in the aerial roots of specific epiphytic orchids. These branched lignified structures (Porembski & Barthlott 1988; Pridgeon *et al.* 1999; Stern 2014) were first described in *Bulbophyllum careyanum* (Chatin 1856) and *Sobralia liliastrum* (Oudemans 1861) and interpreted as adaptations that developed in association with the epiphytic habit in the Orchidaceae (Pridgeon 1983; Benzing 1990). Their function seems to be directly associated with water balance in the plant, reducing the evapotranspiration or promoting the condensation of atmospheric moisture (Benzing *et al.* 1982). Besides their ecophysiological importance (Zotz 2016), they are important taxonomic characters in the groups in which they occur and define seven morphological types (Pridgeon *et al.* 1983), although others can also occur. Tilosomes predominate in the Polystachyeae tribe, subtribes Sobraliinae, Coelogyninae, Laeliinae, Pleurothallidinae, Bulbophyllinae, Lycastinae, and Maxillariinae (Pridgeon 1983; Pridgeon *et al.* 1983). Within the Pleurothallidinae, the largest orchid subtribe (Karremans 2016), the presence of tilosomes can be considered a plesiomorphic characteristic however frequent and showing a high morphological diversity (HR Kedrovski, unpubl. res.).

The ultrastructure of developed tilosome was first shown for *Sobralia macrantha* (Benzing *et al.* 1982). Subsequent anatomical studies pointed out their taxonomic importance (Stern & Judd 2001; 2002; Stern *et al.* 2004; Carlsward *et al.* 2006; Figueroa *et al.* 2008; Stern & Carlswards 2009; Silva *et al.* 2010; 2015; Pedroso-de-Moraes *et al.* 2012; Andreota *et al.* 2015) but did not show when or how the tilosomes are formed and neither correlate their morphology with the

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wall-membrane apparatus of transfer cells (TC) (Talbot *et al.* 2002; McCurdy *et al*. 2008). Unlike other types of structure where WI are observed such as in seeds (Monjardino *et al.* 2013; Rocha *et al.* 2014; Arun-Chinnappa & McCurdy 2016), phloem parenchyma (Arun-Chinnapa *et al.* 2013; Nguyen & McCurdy 2017; Nguyen *et al*. 2017), giant-cells in nematode feeding tubes (Cabrera *et al.* 2014; Miyashita & Koga 2017), phi cells (Fernandez-Garcia 2009; Melo 2011; Idris & Collings 2015), tracheoidal idioblasts (Burr & Barthlott 1991; Leroux *et al.* 2011), haustorium flange cells (Fineran 1998; Fineran & Calvin 2000) and glands (Gama *et al*. 2016; Tozin & Rodrigues 2017; Wilson *et al*. 2017), the development, ultrastructure and wall composition of the tilosomes have not yet been described. According to Pridgeon (Stern 2014), the wall deposition that forms the tilosome in the endovelamen cells, is oriented early in development by plasmodesmos between an exodermis passage cell and an endovelamen cell. This author concluded that: "much more study is needed to determine the function and ontogeny of tilosomes and whether or not the environment plays a role in the extent of their development".

Materials and methods

Light microscopy

Root apices of *Anathallis sclerophylla* (Lindl.) Pridgeon & M.W.Chase were fixed in FAA 70 (Johansen 1940), stored in alcohol 70 %, dehydrated in terc-butanol series and imbedded in Paraplast. Longitudinal and transversal serial sections were cut at 20 µm using a rotary microtome. The permanent slides were stained with 0.05 % toluidine blue (Feder & O'Brien 1968) and safrablue (Bukatsch 1972; Kraus & Arduin 1997), mounted in Entellan. Photomicrographs were taken under a light microscope (DM 4000B, Leica Microsystem, Wetzlar, Alemanha) with attached camera (DFC 450, Leica Microsystem), through LAS 4.0 software (Leica Aplication Suite, Leica) in the Photomicrograph Laboratory at the Botany Department, UNESP - Rio Claro.

Scanning electron microscopy

The fixed samples were dehydrated in alcoholic series, and then in a critical point dryer (EM CDP 300 Leica Microsystem, Wetzlar, Gemany), sputter-coated with gold (EM SCD 500 Leica Microsystem, Wetzlar, Germany) and observed in SEM (JSM 6390 LV Jeol, Tokio, Japan) in the Electron Microscopy Central Laboratory of the Federal University of Santa Catarina – UFSC.

Transmission electron microscopy

The root tips were fixed in 2.5 % glutaraldehyde in 0.1M sodium phosphate buffer at pH 7.2 for 24 hours (Kraus & Arduim 1997). The material was post-fixed in osmium tetroxide (OsO 4) 1 % in 0.1M phosphate buffer, pH 7.2 for 4 hours at room temperature (Bozzola & Russel 1999). After being dehydrated with increasing series of acetone, the material was infiltrated and embedded in a polymer mixture (Araudite, and Epon DDSA). The ultra-thin transverse sections were taken by an ultramicrotome, stretched and attached to the grids, counterstained with uranyl acetate and lead citrate, according to Reynolds (1963) procedures, observed and photographed in a TEM (Tecnai Spirit - FEI Company) in the Electron Microscopy Center of the Biosciences Institute, UNESP – Botucatu.

Confocal microscopy

Roots previously fixed and stored were included in polyethylene glycol, 20 µm transversely and longitudinally sectioned using a rotary microtome in the Laboratory of Morphology of the Botany Department of UNESP-RC. Sections were stained separately with calcofluor to mark cellulose and aniline blue for callose, according to Kraus & Arduin (1997). The slides were observed in the Leica SP5 AOBS Point Scanning confocal Microscope from the UNESP-RC Electronic Microscopy Laboratory. The fluorophores were excited at 405 nm with near UV diode and the emission wavelengths were collected between 415 and 448 nm (blue). Lignin and waxes could be visualized by autofluorescence when respectively excited at 488 nm by Argon laser collected between 491 and 563 nm (green), and at 633 nm by HeNe laser collected between 522-622 nm (red). The laser intensity was set to the minimum necessary for imaging (~ 20 %) in order to increase the specificity and definition of the wall components localization. The images were obtained in the 3 channels (blue, green and red) in z-stacking with 10 steps for three-dimensional reconstruction.

Samples

All fresh samples were obtained from vegetative clones under cultivation in UNESP-RC Botany Department greenhouses.

Results and discussion

Tilosome development, morphology and wall biochemistry

At the elongation zone of *Anathallis sclerophylla* root apex (Fig. 2), tilosomes develop as cellulosic inconspicuous papillae; on the inner primary cell wall of the endovelamen, adjacent to the exodermis passage cells (Figs. 2G, 3A-D). Papillae synthesis is associated with the presence of mitochondria and RER (Fig. 3). During cell differentiation, the tilosomes undergo lignin impregnations (Fig. 2G-I) to assume their final structure (Figs. 1, 4). Within the

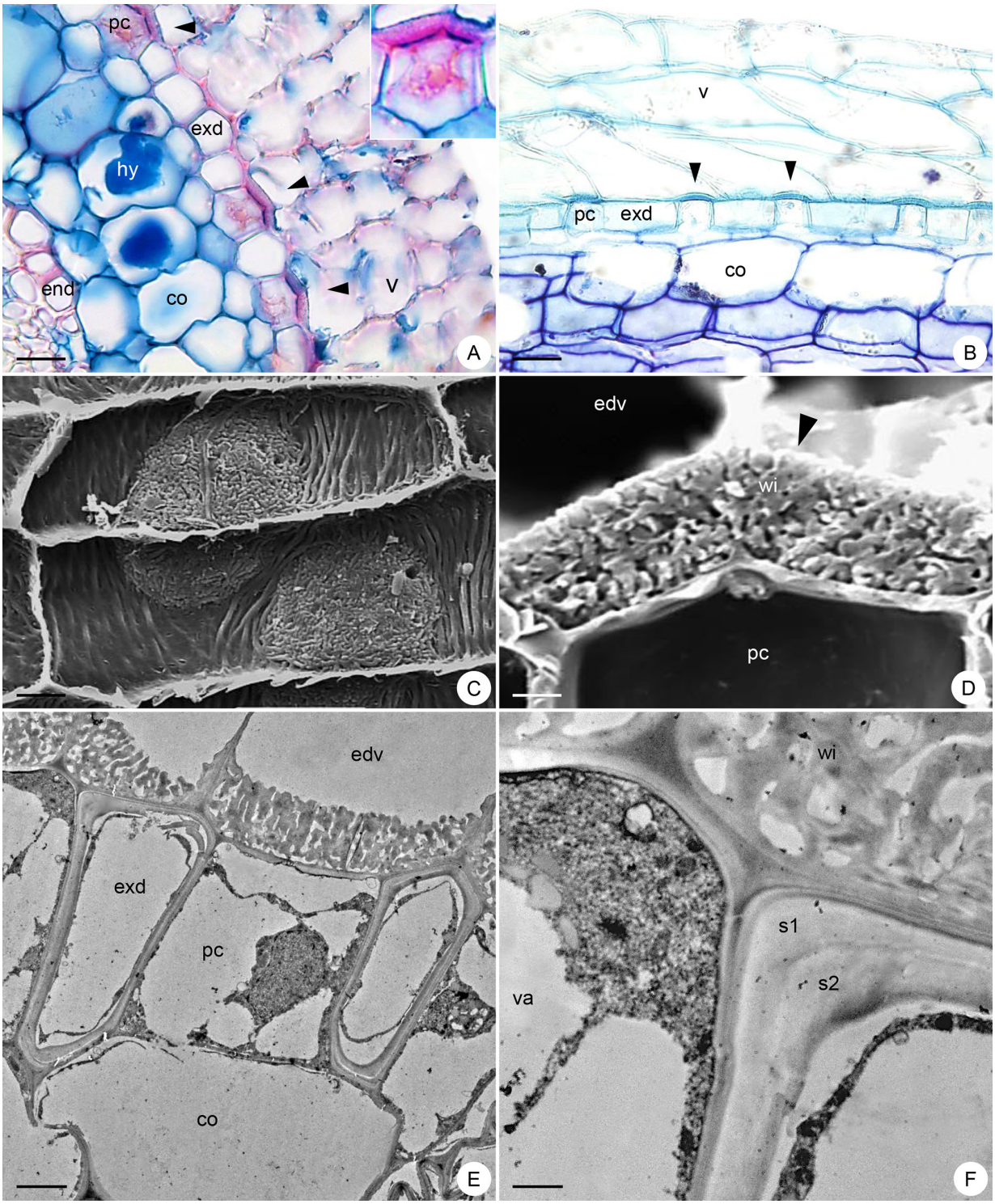

Figure 1. Tilosome morphology in *Anathallis sclerophylla* root. **A-B** Light Microscopy. **C-D** SEM. **E-F** TEM. **A.** Cross section of differentiated roots stained with safrablue, showing "O" thickened endodermis cells (end), cortical cells (co) with pelotons (hy), dead exodermis cells (exd), passage cells of dense cytoplasm (pc), tilosomes (arrowhead) with a thin blue primary wall and the stratified velamen (v). **B.** Longitudinal section stained with toluidine blue, showing by metachromasy the lignified walls (green) in velamen (v), tilosome (arrowhead) and exodermis (exd); note the cortex cellulosic walls (anil blue) (co) and the difference between the common and the passage cells of exodermis. **C-D**. Paradermic (C) and cross section (D) showing the tilosomes (arrowhead) adjacent to the exodermis passage cells (pc) and the reticulated and flange patterns of the endovelamen (edv) WIs. **E-F**. Velamen-exodermis complex showing dead cells in the endovelamen (edv), WIs lumen directed to environment, common exodermis cell (exd) with two layers of secondary wall (s1, s2), vacuolated passage cells (pc) with conspicuous nucleus, mitochondria, ribosomes on an electron-dense matrix and vesicles adjacent to the tonoplast,. Scale bars: 25µm(A), 50 µm(B), 10 µm(C), 3 µm(D), 5 µm(E).

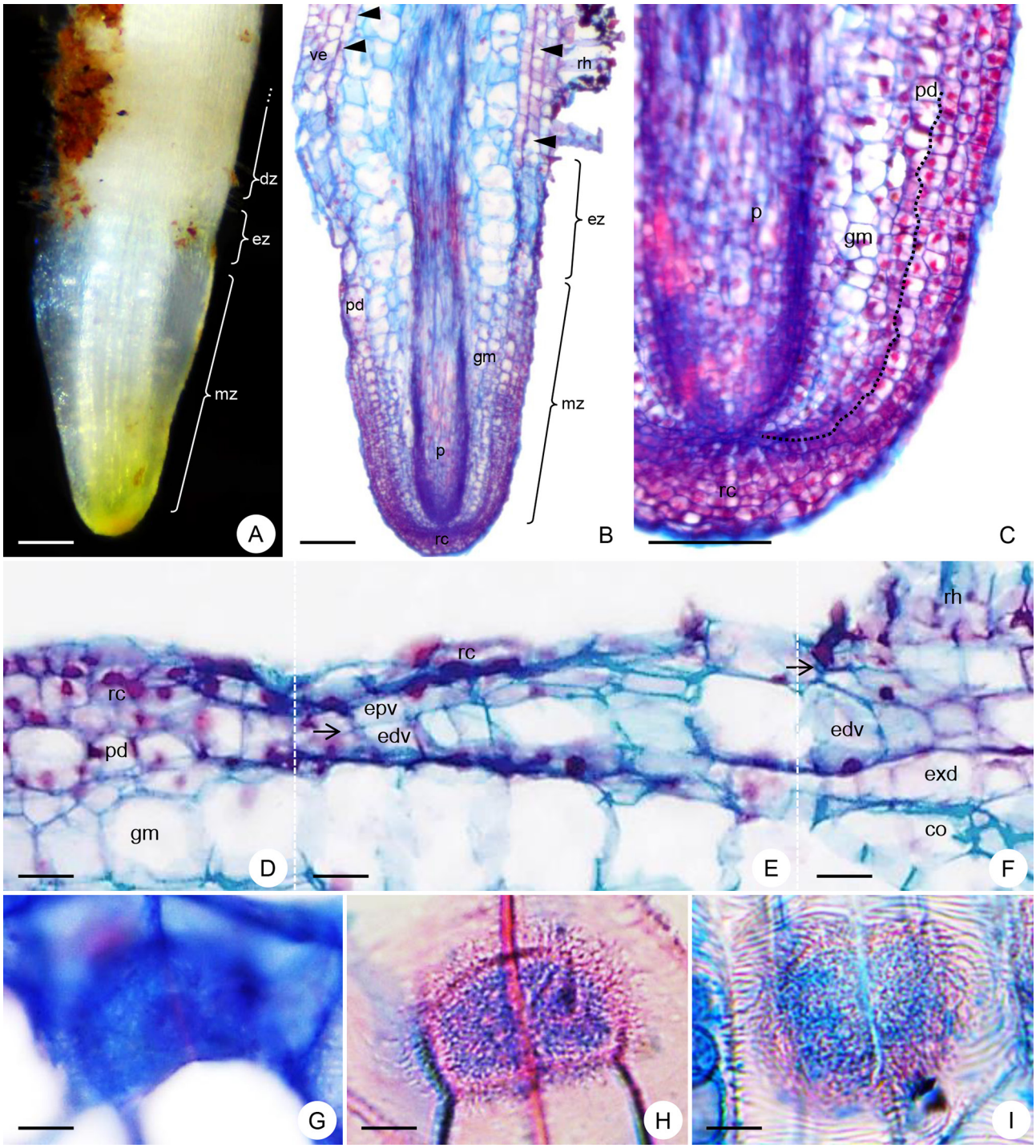

Figure 2. Macro (**A**) and micromorphology (**B-I**) of *Anathallis sclerophylla* roots. Longitudinal (**B-I**) sections of the apex, showing the epidermis and cortex (**D-F**) and the tilosomes (**G-I**) development. There are three zones in the apex (**A-B**): **1.** Meristematic (mz) (**C**) with isodiametric protodermis cells (pd), ground meristem (gm) of elliptical cells and procambium (p) of fusiform cells (**B-C**); **2.** Short elongation zone (ez) at the boundary of the root cap (**D**) with primary walled cells (**B**); it is characterized by the increase of the cellular volume in the median region of the ground meristem (causing a compression in the exodermis layer (**E**)), and by the first division of the protodermis originating the endo and epivelamen (edv, epv); **3.** Differentiation zone (dz) (**D-F**), with a second division in the epivelamen, the development of root hairs (rh) and the progressive tissue specialization (**A-B**). The tilosome (**G**) begin as small, papillose-like regions in the inner wall of some endovelamen cells laying adjacent the exodermis passage cells. The secondary wall deposition in these cells follow the orientation of the primary WI (**G**), leading to the tilosomes or velamen stripes formation (**H-I**). The dotted line indicates the boundary between protoderm and root cap, and the arrows indicate the velamen cell divisions. Scale bars: 120μm (A-B), 50μm (C), 25μm (D-F), 10μm (G-I).

elongation zone of the root apex (Fig. 2A-B, E), we can see that: **1.** the protodermis is exposed to the external environment, once no root cap is observed; **2.** periclinal divisions on the protodermis cells give rise to a multilayred velamen, composed by an epi and endovelamen; **3.** cells of the outer layer of the ground meristem, which give rise to the exodermis, are collapsed due to the rapid grow of the cortical cells. The protodermal cells that give rise to WI possess a larger nuclear region and fewer vacuoles in the cytoplasm if compared to the ground meristem that gives rise to the root cortex (Fig. 3A-C).

Study of the changes in morphology and wall composition during tilosome development, using light, confocal and transmission microscopy has revealed that

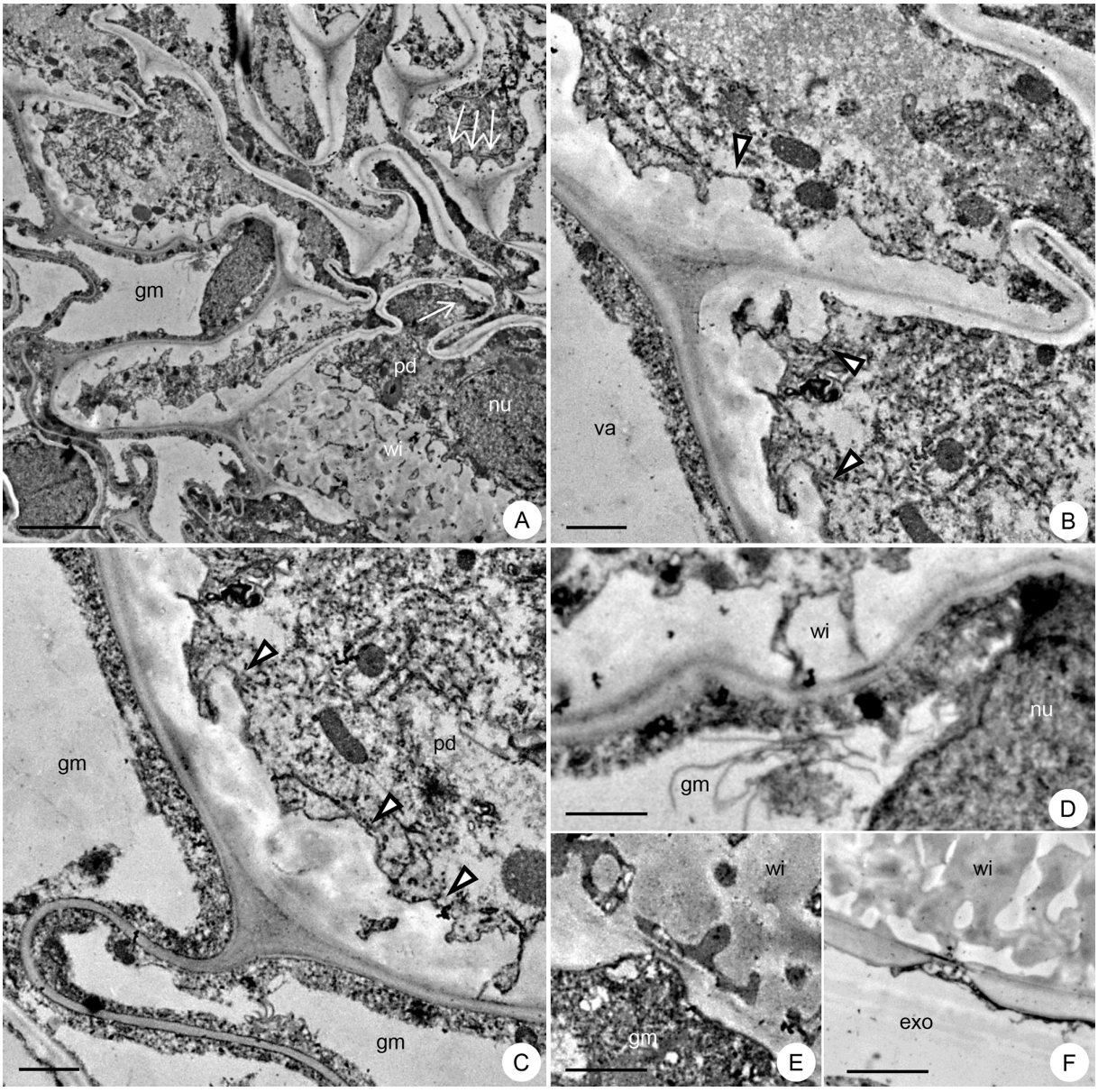

Figure 3. Ultrastructural details of the protodermis and ground meristem during the tilosomes formation in *Anathallis sclerophylla* root. The asynchronous WIs deposition (**A**) in the inner protodermis layer (dp) is noted by small papillae (arrowhead) of fibrillar material in the inner cell wall adjacent to the ground meristem (gm) (**A-C**). Each papillae is a precursor of the wall system that characterize the reticulate tilosome. In the outer protodermis layers (**A**), the cells are poorly vacuolated and vesicles/organels rich; they possess flange-like projections (arrows) on the walls, that will give rise to the characteristic velamen strips. The protodermis cells that develop walls ingrowth had increased nuclear region (nu) and few vacuoles in the cytoplasm compared to those which give rise to the cortex in ground meristem. The papillae wall synthesis is closely related to the presence of mitochondria and RER (**A-C**) and to the increase of cell surface. The ground meristem cells possess large vacuolated (va) and thin walls. The limit between the velamen cell walls and tilosome is not visible. Velamen-exodermis are continuous through the tilosome- exodermis (exo) passage cell; these cells possess many plasmodesmata both before (**D-E**) and after cell differentiation (**F**) that can or not be occluded by callose. Scale bars: 5µm(A), 2um(F), 1µm(B, C, D, E).

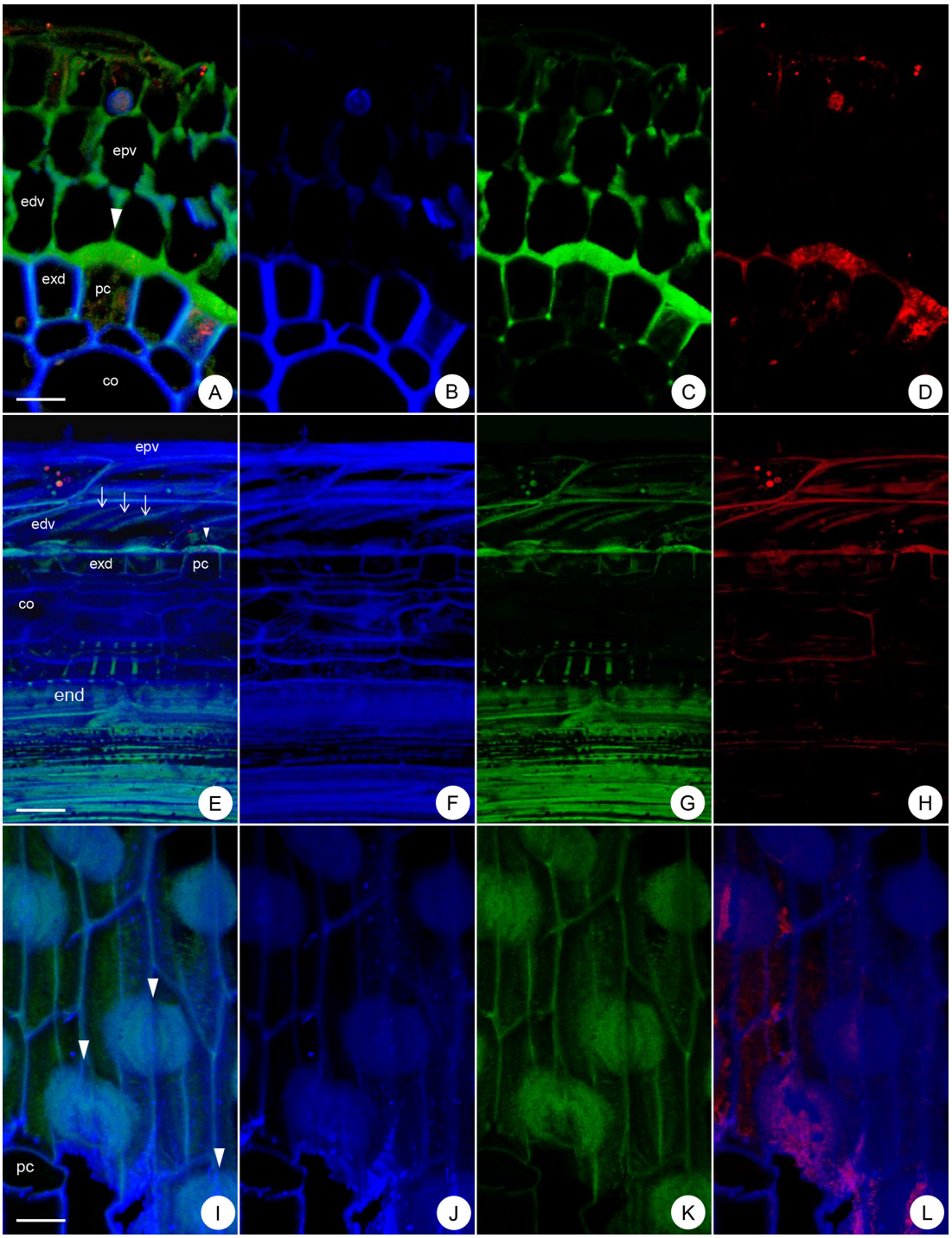

Figure 4. Three-dimensional z-stack reconstructions of cross (**A-D**), and longitudinal (**E -L**) sections of *Anathallis sclerophylla* roots stained with calcofluor (**A-D**) and aniline blue (**E-L**) fluorophores, in order to demonstrate the wall compositions. Cellulose (**A-B**) and callose (**F, G**) shown in blue and lignin in green (**C, G, K**) are the main walls constituents, although waxes could also be evidenced by autofluorescence when excited by low intensity red light in argon diode (**D, H, L**). By intense fluorescence, cellulose can be observed in cortical parenchyma (co) and in "O"-thickened cells of exodermis (ex), except for the outer periclinal wall of the passage cells (**A-B**). Lignin is found in endovelamen (edv), velamen stripes (arrow) (**A**), tilosomes (arrowhead) (**C**), "∩"- thickened exodermis cells (**C, G**), intercellular spaces of outer cortical parenchyma (**C**), spiral thickenings of internal cortical parenchyma (**G**), endodermis (en) and vascular cylinder (**E, G**). Callose occurs in all cell walls, with the highest concentration in the epivelamen (epv) (**E-F**), in the plasmodesmata around the tilosomes (**J**) and in the inner layer of the cortical parenchyma including endodermis (**F**). Waxes (suberin and cutin) occur in a smaller quantity when compared to the fluorescence of other polymers; it is concentrated in the outer layers of the velamen (**D, H**) but it is diffuse in the tilosomes (**D**), in the "∩"-thickened exodermis cells, intercellular spaces of the cortical parenchyma, endodermis and phloem (**H**). Note the occurrence of infections by algae and fungi. Scale bars: 30μm (E-H), 15μm(A-D), 10μm(I-L).

the papillary shape of the tilosomes at early development stages, are similar to those of the wall-membrane-apparatus of cotyledon TCs in *Vicia faba* (Gunning & Pate 1969; Offler *et al.* 2002). Its cellulosic composition may contribute to the out/inward transport over the root peripheral region that is in contact with the external medium and spatially distant from the vascular cylinder. Offler *et al.* (2002) argues that TC differentiation is strongly related both to the organ development stage and to the availability of solutes. When the tilosome WI is deposited, in the roots of *A. sclerophylla*, both the xylem and phloem are differentiated and thus able to perform transport in the elongation zone. As the cells differentiate the papillae (Fig. 2G-I), the velamen cell stripes and the exodermis cells undergo a progressive lignification (Fig. 4C, G, K), assisting the epidermal and exodermis cells to maintain their shape. The physiological properties of lignin (mechanical support, compressive strength and plant defense) (Zeier *et al.* 1999) support this interpretation. If so, the lignin is synthesized as a response to the potential collapse of the exodermis cells, in order to maintain the shape and volume of both the cortex and velamen cells. Benzing (1986) also pointed out that the lignified velamen and cortex cells are essential to prevent collapse and maintain the shape of aerial roots exposed to desiccation.

Callose is usually deposited in specialized walls (Chen & Kim 2009) during the division, growth and differentiation of plant cells (Vatén *et al.* 2011) and can also appear in the plasmodesmata to regulate the cell-to-cell transport (Xie *et al.* 2012; Cui & Lee 2016). In the root of *A. sclerophylla*, callose occurs in the cell walls of the outer velamen layers and tilosomes, in the plasmodesmata between exodermis-velamen and endodermis-cortical parenchyma cells (Fig. 4E-F, I-J). Callose deposition can be induced by injury, pathogen infections, presence of chitin and chitosan, presence of aluminum, ABA and salicylic acid (Luna *et al.* 2011; Nedukha 2015). There is an antagonism between the presence of callose and symplastic transport as this transport considerably decreases after callose is deposited (Lee & Lu 2011). However, callose can be degraded, thereby reestablishing normal transport via the plasmodesmata opening. There is less or no callose deposition on the tilosomes plasmodesmata when compared to the adjacent flange walls (Fig. 4I-J). This indicates that these regions are capable of transport via the symplast.

Waxes, such as suberin and cutin (Nawrath 2002), are deposited on the cell wall of both aerial and underground plant organs. They reduce water loss and the penetration of incident UV, impose a barrier to apoplastic transport (Schreiber 2010) and protecting tissues against extreme temperatures, pathogens and herbivores (Ingram & Nawrath 2011). The presence of a suberized lamella in the exodermis and endodermis cells, as evidenced in *A. sclerophylla* by autofluorescence under red laser (Fig. 4D, H, L), is very common (Enstone *et al.* 2003; Schreiber & Franke 2011). The occurrence of hydrophobic compounds in the tilosomes and velamen cell walls may be related to an absence of root cap, to a protection against photooxidation (Chomicki *et al.* 2015) and to the retention of water inside the organ. Cutin is also involved in the recognition of symbiotic hyphae (Murray *et al.* 2013), required for seed germination and for the establishment of the protocorm and seedling. In tangential view, we noticed gaps in the distribution of fat compounds in the inner periclinal walls of the endovelamen, specifically inside the tilosome (Fig. 2L), indicating water permeable regions.

After the complete root differentiation and expansion (Fig. 1), the velamen cells degenerate remaining leaving only the secondary walls and tilosomes connecting the thin walled exodermis passage cells to the environment. The velamen cells lack protoplasts, the lumen of tilosomes is reduced (Fig. 3F) due to their labyrinthine wall systems (Fig. 1C-F) and they are connected to the exodermis passage cells via plasmodesmata (Fig. 4J). The passage cells are small (Fig. 1B) and covered by primary walls at their anticlinal and inner periclinal sides (Fig. 1A), with a thin layer of irregular secondary wall on the outer periclinal side (Fig. 1E). They possess a conspicuous nucleus and vacuole that together limit the cytoplasm to the cell margin (Fig. 1E-F). The remaining exodermal cells, dead at maturity, are completely covered by secondary walls (Fig. 1F). The tilosome walls are seen to be impregnated with lignin as revealed by a positive reaction to toluidine blue (Fig. 1B) and to acidified phloroglucinol.

Updating the interpretation of tilosomes

Tilosome originate as cellulosic papillae in cells of the inner layer of the stratified protodermis, adjacent to the exodermis passage cells. The exodermis does not participate in the WI deposition. Tilosomes probably have two functional phases: in young tissues, they increase the symplast connection, facilitating transport over the outer root regions, like the wall-membrane apparatus of TC; while, in mature tissues, they protect, direct solutes to the passage cells, imposing a physicochemical barrier for pathogens and recognizing symbionts. The structure of tilosomes is related to biotic and abiotic factors: cellulose forms the thin primary wall, lignin gives support, waxes acts in water retention and organ defence and callose regulates transport in the symplastic sites. Although the function of water supply and balance is widely accepted for the tilosomes (Engard 1944; Dycus & Knudson 1957; Sandford & Adanlawo 1973; Pridgeon *et al.* 1983; Zotz & Winkler 2013), the velamen cells are not interpreted as TC. It seems reasonable, since epidermal cells are alive when young and thus able to transport solutes; only when mature, the velamen cells form a saturated microclimate providing condensation and retention of atmospheric water through the WI hygroscopic and contact surfaces.

The morphology of the reticulate and flange TC WIs are identical to those of the tilosomes (Pridgeon *et al.* 1983) and velamen stripes (Sandford & Adanlawo 1973), corroborating the hypothesis of a dual physiological function. Two patterns of TC WIs are recognized (Talbot *et al.* 2002; McCurdy *et al.* 2008): the flange pattern, with curvilinear, rib-shaped non lignified projections that are more or less parallel, often bifurcated and fused that give rise to secondary wall ridges; and the reticulate pattern, that is a labyrinthine network of tubular projections, that start as small papillae emerging from the underlying wall at discrete loci (Talbot *et al.* 2001). These differences are related to the cytoskeleton. In the reticulate type, the WI coincides with the accumulation of actin filaments amongst the disordered microtubules arrangement; the rosettes concentration at specific points determines its cylindrical shape. The flange pattern originates by a cellulose deposition in parallel with the cytoplasm microtubules (Talbot *et al.* 2001; 2007a; b; Thompson *et al.* 2001). What distinguishes these two types of WIs in TC (flange or reticulate) is the presence or not of direct contact of the cell wall along its entire length, instead of the tilosomes architecture (Pridgeon 1983; Pridgeon *et al.* 1983). The lignification and, more importantly, the death of the velamen cells caused by aerial environment make this structure unique among those that possess WI keeping cells always alive.

Conclusion

New perspectives

Within the "Next-Generation Sequencing blast", the lack of information on the genetic mechanisms controlling tilosome formation should encourage comparison of the genomics of orchids with and without tilosomes, in order to clarify which gene(s) are being expressed in different root zones or tissues and to investigate how this control occurs. In this context, some questions remain unanswered such as: is the tilosome a phenotypic feature always expressed? When did this genetic expression appear, during subtribe history? Are there differences in the transport rate between root zones, tissues and roots with and without tilosomes? Studies on wall development and composition are of interest especially in tilosomes and other structures which depend on the oriented wall deposition. Disregarding easy serial sectioning, orchid roots are excellent models for cellular morphological and developmental studies, since they are easy to grow, to obtain, and to maintain under different stress conditions. Aseptic media, water supply, light or ionic stress and inoculations could delimit experiments designed to evaluate which factors interfere on the tilosomes development and to describe the occurrence of morphohistochemical and cytological alterations of roots under treatments. Essays using cellulose-synthase / callose-synthase / lignin-inhibiting drugs, hydrogen peroxide, chitosan, phytohormones, gene inactivation and cytoskeleton label may also be used to increase understanding of the mechanisms underlying wall structuring. Researches with labeled isotopes could reveal the physiology of transport (in or out) in young root tissues. Analyzes using light, transmission, scanning and confocal microscopy are indispensable to monitor the development, histochemical composition and the three-dimensional morphology of wall ingrowths occurring in orchid roots.

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