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ORIGINAL ARTICLE

Expanding the laticifer knowledge in Cannabaceae: distribution, morphology, origin, and latex composition

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Abstract

Cannabaceae is a known family because of the production of cannabinoids in laticifers and glandular trichomes of *Cannabis sativa*. Laticifers are latex-secreting structures, which in Cannabaceae were identified only in *C. sativa* and *Humulus lupulus*. This study aimed to expand the knowledge of laticifers in Cannabaceae by checking their structural type and distribution, and the main classes of substances in the latex of *Celtis pubescens*, *Pteroceltis tatarinowii*, and *Trema micrantha*. Such information is also updated for *C. sativa*. Samples of shoot apices, stems, leaves, and flowers were processed for anatomical, histochemical, ultrastructural, and cytochemical analyses. Laticifers are articulated unbranched in all species instead of non-articulated as previously described for the family. They occur in all sampled organs. They are thick-walled, multinucleate, with a large vacuole and a peripheral cytoplasm. The cytoplasm is rich in mitochondria, endoplasmic reticulum, dictyosomes, ribosomes, and plastids containing starch grains and oil drops. Pectinase and cellulase activities were detected in the laticifer wall and vacuole, confirming its articulated origin, described by first time in the family. These enzymes promote the complete dissolution of the laticifer terminal walls. The latex contains proteins, lipids, and polysaccharides in addition to phenolics (*C. sativa*) and terpenes (*C. pubescens*, *T. micrantha*). The presence of laticifers with similar distribution and morphology supports the recent insertion of *Celtis*, *Pteroceltis*, and *Trema* in Cannabaceae. The articulated type of laticifer found in Cannabaceae, Moraceae, and Urticaceae indicates that the separation of these families by having distinct laticifer types should be reviewed.

Keywords *Cannabis sativa* · Cellulase · Flowers · Latex · Pectinase · Urticalean rosids

Introduction

The secretory structures of Cannabaceae, especially of *Cannabis sativa* L., have aroused the interest of researchers since a long time ago because they are responsible for the production of a large amount of secondary metabolites of medicinal importance (Furr and Mahlberg 1981; Kim and Mahlberg 1991, 1997; Williamson and Evans 2000; Happyana et al. 2013). The latex of *C. sativa*, for example, is rich in cannabinoids and alkaloids (Furr and Mahlberg 1981), substances that have the medicinal potential to relieve symptoms related to the treatment of cancer, AIDS, and sclerosis (Ashton 2001; Honório et al. 2006; Hill et al. 2010). The latex is produced in laticifers, internal secretory structures that form organized systems (Fahn 1990), composed of one specialized cell (non-articulated type) or several cells forming a tube (articulated type) that produce an emulsion of many small particles dispersed in a liquid with a different refractive index (Fahn 1979).

Surprisingly, laticifers have been reported only in two species of Cannabaceae, *Cannabis sativa* (Furr and Mahlberg 1981; Mesquita and Dias 1984) and *Humulus lupulus* L. (Hagel et al. 2008), a small number of species if we consider that this family comprises ca. 109 species and 10 genera (Yang et al. 2013). The laticifers of *Cannabis sativa* (Furr and Mahlberg 1981; Mesquita and Dias 1984) and *Humulus*

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lupulus (Hagel et al. 2008) are currently classified as non-articulated and unbranched.

Cannabaceae belongs to the Urticalean Rosid clade that also comprises Moraceae, Ulmaceae, and Urticaceae (Sytsma et al. 2002). Differences in the distribution and morphology of laticifers have always been considered as important features for the diagnosis of the families of the Urticalean Rosid clade (Judd et al. 2009). For example, in Moraceae, the laticifers were described as non-articulated branched and distributed throughout the plant; in Urticaceae, the laticifers were described as non-articulated unbranched and present only in the bark; in Cannabaceae, the laticifers were also described as non-articulated unbranched and distributed throughout the plant vegetative body, and finally, Ulmaceae representatives do not present laticifers (Metcalfe 1966; Evert 2006; Fahn 1979). However, recent ontogenetic studies of laticifers have shown that their distribution and morphology in species of Moraceae and Urticaceae are similar, i.e., that species of these families present articulated branched (and anastomosed) laticifers with wide distribution in the plant (Marinho and Teixeira 2019a). The lack of studies on laticifer origin can be the cause of misinterpretations of the laticifer types (Demarco et al. 2006; Demarco and Castro 2008; Lopes et al. 2009; Canaveze and Machado 2016, Marinho and Teixeira 2019a), which means that other species of the Urticalean Rosid clade, including species of Cannabaceae, could also show articulated laticifers. Therefore, ontogenic studies are essential to a better laticifer classification in this clade. Even the latex exhibits different colors depending on the family, such as yellow-brown or colorless latex in Cannabaceae (Mahlberg 1993; Evert 2006), milky latex in Moraceae, and colorless latex in Urticaceae (Evert 2006). Thus, there appears to be a diversity of types of laticifers and of latex in the Urticalean rosids that can be confirmed by further studies.

Therefore, the objectives of the present study were to check the origin, detailed morphology, and distribution of laticifers, and the main classes of compounds of the latex in species of different genera of Cannabaceae (*Celtis pubescens* (Kunth) Spreng., *Pteroceltis tatarinowii* Maxim., and *Trema micrantha* (L.) Blume). Such data are also updated for *Cannabis sativa*. The cytochemical localization of cellulase and pectinase activities was also tested to better understand the formation of laticifers. We intend to contribute new data to help reviewing the synapomorphies established for the Urticalean Rosid clade.

Materials and methods

Samples of shoot and floral apices, stems, leaves, and flowers were obtained in the field or from herbarium specimens (Table 1). Vouchers were deposited in the SPFR herbarium (FFCLRP/USP) and in the CGMS herbarium (INBIO/UFMS).

Table 1 Information about the Cannabaceae species sampled

Species	Habit	Sexual expression	Sample	Voucher
Cannabis sativa	Herb	Dioecious	Herbarium ESA, Piracicaba, SP, Brazil	G.M Tenório n° 5 (123034), G. A Ogasawara n° 20 (119653), O. Marilia n° 13,433 (68853), J.A. Zandoval n° 102 (13268) (ESA)
			Herbarium IAC, Campinas, SP, Brazil	A.S. Lima s/n° (24827), A.P. Viégas s/n° (3881), C. Pacheco s/n° (18681) (IAC)
Celtis pubescens	Shrub or tree	Monoecious	USP, campus Ribeirão Preto, SP, Brazil	F.M. Leme n° 98 (16046) and 107 (16047) (SPFR)
Pteroceltis tatarinowii	Tree	Monoecious	Botanical Garden, University of Vienna, Vienna, Austria	F.M. Leme n°128 (CGMS)
Trema micrantha	Tree	Dioecious	USP, campus Ribeirão Preto, SP, Brazil	F.M. Leme n° 92 (15959), 93 (15960). 94 (15957), 97 (16306) and 101 (15958) (SPFR)

The samples collected in the field were fixed in buffered formalin (Lillie 1965) or in formalin-acetic acid-ethanol (70% FAA) for 48 h (Johansen 1940). The herbarium samples were rehydrated in heated distilled water and then treated overnight with 2% KOH (Smith and Smith 1942). Both types of samples were dehydrated through an ethanolic series, embedded in histological resin (Historesin, Leica), and sectioned in longitudinal planes (5 μm), using a rotary microtome (Leica RM 2245). The sections were stained with 0.1% Toluidine Blue in phosphate buffer, pH 6.8 (O'Brien et al. 1964), mounted in immersion oil, and analyzed under a light microscope.

Stem samples were also free-hand sectioned (in fresh material for *Trema micrantha* and *Celtis pubescens* and fixed material for *Cannabis sativa* and *Pteroceltis tatarinowii*), and the main compounds of the latex were investigated using the following reagents: Sudan IV for total lipids (Pearse 1985), Lugol for starch (Johansen

1940), ferric chloride for phenolic compounds (Johansen 1940), and Wagner's reagent for alkaloids (Furr and Mahlberg 1981). Material embedded in historesin was stained with Toluidine Blue O for detection of phenolic compounds (O'Brien et al. 1964), period acid-Schiff (PAS) for neutral polysaccharides (Jensen 1962), and xylidine Ponceau for proteins (Vidal 1970). The presence of terpenes and tannins was tested in fresh samples of *C. pubescens* and *T. micrantha* free hand cut. The Nadi reagent was employed for terpenes (David and Carde 1964), and vanillin hydrochloric acid was employed for tannins (Mace and Howell 1974). Photomicrographs were obtained using a Leica DFC 295 digital camera coupled to a Leica DM 5000 B light microscope.

For the ultrastructural analysis, small pieces of the shoot apex of *Celtis pubescens* and *Trema micrantha* were fixed in Karnovsky's solution (Karnovsky 1965) for 24 h, post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.2, washed in distilled water, dehydrated, and embedded in Araldite. Sections cut with a Leica Reichert Ultracut S ultramicrotome at 60–70 nm were collected on copper grids and contrasted with 2% uranyl acetate and lead citrate for 15 min. Transmission electron micrographs were obtained using a Jeol 100CXII instrument.

Cytochemical localization of cellulase and pectinase activities at the ultrastructural level was performed for *Celtis pubescens* and *Trema micrantha.* The shoot apices were collected, fixed in Karnovsky's solution (Karnovsky 1965) for 24 h, washed 10 times in 0.1 M phosphate buffer, pH 7.2, and stored overnight in the buffer at 4 °C. For testing the cellulase activity, the samples were incubated in 0.05 M citrate buffer, pH 4.8, with 0.02% carboxymethylcellulose for 10 min at room temperature (Bal 1974). For pectinase activity, the samples were incubated in 0.1 M sodium acetate buffer, pH 5.0, with 0.5% pectin for 20 min at room temperature (Allen and Nessler 1984). Control samples were incubated in buffer respective of each test but without carboxymethylcellulose and pectin. Both treated and control samples were transferred to Benedict's reagent heated to 80 °C for 10 min, washed in 0.1 M phosphate buffer, and post-fixed in 1% osmium tetroxide for 2 h. Then, the samples continued to be processed using the usual method for ultrastructural analysis.

Results

Laticifer distribution, origin, and morphology

Most of the species studied exhibit laticifers in all analised parts of the plant (Table 2, Fig. 1), such as leaf blade, petiole, stem, pedicel, sepal, filament, ovary, style, and stigma (Fig. 1a–h, Table 2). The exceptions are the sepals and filaments of *Cannabis sativa* flowers (Fig. 1a, b, Table 2).

Table 2 Distribution of laticifers in the vegetative organs and in the apetalous flower of Cannabaceae species

	Organ	*Cannabis sativa*	*Celtis pubescens*	*Pteroceltis tatarinowii*	*Trema micrantha*
	Stem	+	+	+	+
	Leaf	+	+	+	+
Pistillate flower	Pedicel	+	+	+	+
	Sepal	–	+	+	+
	Ovary	+	+	+	+
	Stigma	+	+	+	+
Staminate flower	Pedicel	+	+	+	+
	Sepal	+	+	+	+
	Filament	+	+	+	+
	Anther	–	–	–	–

Laticifers are originated from ground meristem cells and are located externally to the phloem (Fig. 2). They are organized into a set of three or more laticifers (Fig. 2). The laticifer is articulated, formed by the addition of cells in the apex (Fig. 3a, b) and the disintegration of the terminal cell walls (Figs. 3c, d and 4a, b). Then, it elongates with the growth of the plant between the phloem and cortical parenchyma cells (Fig. 3b), or among other laticifers. The laticifers have thickened pecto-cellulosic cell walls (Figs. 2 and 3b), and usually possess nuclei of fusiform shape (Fig. 2).

Ultrastructure

Subcellular characteristics of mature (Figs. 4a and 6a) and of developing laticifers (Figs. 4b–d, 5, 6c, d, and 7) present in the stems of *Celtis pubescens* (Figs. 4 and 5) and *Trema micrantha* (Figs. 6 and 7) were similar for these two species.

The laticifer walls are thicker when in contact with adjacent parenchymatic cells and thinner when in contact with another laticifer wall (Fig. 4a), mainly in the terminal wall which are degraded during the laticifer formation (Fig. 4b, black arrows).

The mature laticifer has a large central vacuole, a peripheral cytoplasm (Figs. 4a and 6a), and small vacuoles close to the large vacuole (Fig. 4a). Developing laticifers show cytoplasm with dictyosomes (Figs. 4b, 5a, 6d, and 7b), plastids (Fig. 5a, b), amyloplasts (Fig. 7c), and numerous mitochondria with conspicuous cristae (Figs. 4b, c, 6b–d, and 7a, b), free ribosomes and polyribosomes (Figs. 4b, 6d, and 7a, b) as well as rough endoplasmic reticulum (Figs. 4b, c and 7a). The dictyosomes are formed of few cisterns and are usually located close to cell wall (Figs. 4b, 5a, and 6d). They are active and produce vesicles from the *trans* face of the *trans*-Golgi network that are released into the small vacuole (arrow, Fig. 7b). Some dictyosomes were found close to the endoplasmic reticulum. Plastids contain some thylakoids, oil droplets (Fig. 5b),

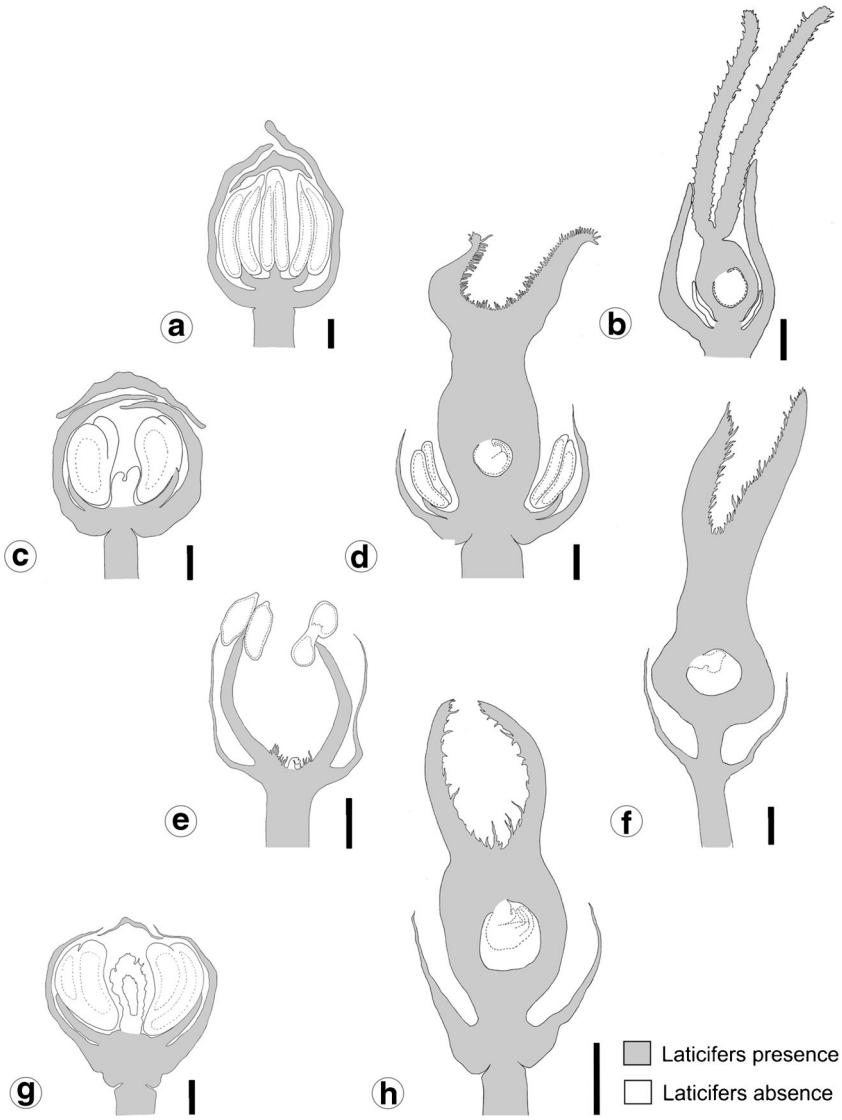


Fig. 1 Schematic drawings of longitudinal sections of flowers of Cannabaceae species showing the wide distribution of laticifers (hatched). **a**, **b** *Cannabis sativa*: staminate (**a**) and pistillate flowers (**b**). **c**, **d** *Celtis pubescens:* staminate (**c**) and pistillate flowers (**d**). **e**, **f** *Pteroceltis tatarinowii*: staminate (**e**) and pistillate flowers (**f**). **g**, **h** *Trema micrantha*: staminate (**g**) and pistillate flowers (**h**). Scale bars: **a** 1 mm; **b** 200 μm; **c**, **d** 500 μm; **e**, **f** 500 μm; **g**, **h** 500 μm

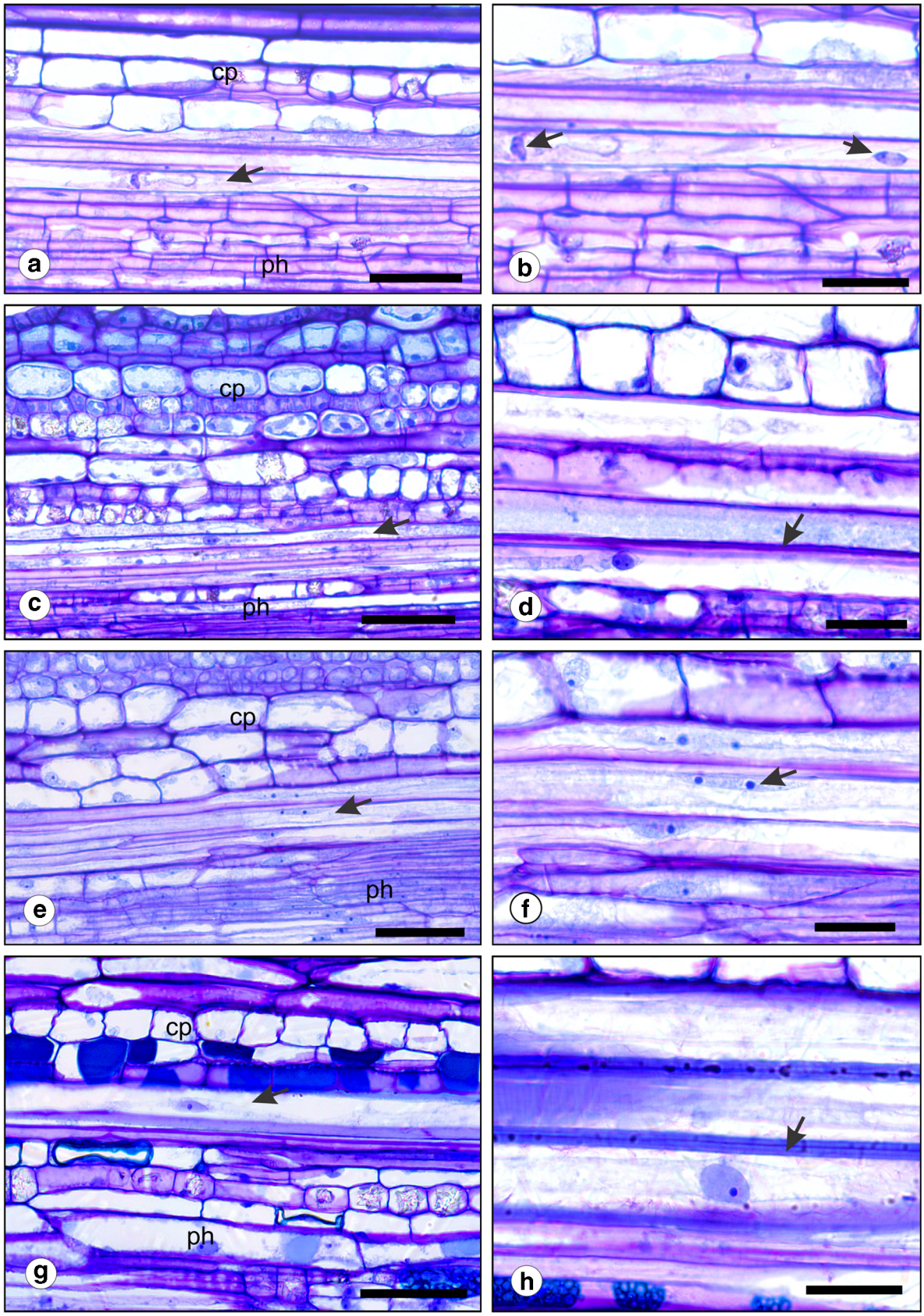
and starch grains (Figs. 5a, b and 7c), which are partially consumed (electron-dense material, Fig. 5b). Plastids can also disintegrate and are found inside the small vacuole (Fig. 5c) with oil droplets, electron-dense material, and starch (asterisk, Figs. 5c, 6b, and 7b). The small vacuoles formed contain latex substances (Figs. 4a, c, 5c, 6a, b, and 7b, d) and join to the large central vacuole in the end of the laticifer development (Fig. 6a). Osmiophilic material is also present in the cytoplasm (asterisk, Figs. 4c, 5d, and 7b).

The nucleus has one or two nucleoli (Fig. 5d).

Cytochemical localization of cellulases and pectinases

Positive reactions for cellulase (Fig. 8) and pectinase (Fig. 9) activities were found in the cell wall close to the middle lamella (Figs. 8a–c and 9a, c, d), vacuole (Figs. 8a, b and 9b), and endoplasmatic reticulum (Fig. 8a, b) by electron-dense crystalline inclusions. These electron-dense inclusions are reducing sugars, products of pectinase, and cellulase activities in the laticifers that react with Benedict's reagent. The reaction products appear widespread (Figs. 8a, b and 9a, b), or densely accumulated, forming groups in the vacuole (Figs. 8a, b and 9b) or in the cell wall (Figs. 8c and 9c, d). In the adjacent cells to the laticifers, positive reaction was also observed but less

Fig. 2 Articulated laticifers of Cannabaceae species (longitudinal sections of the stem stained with Toluidine Blue). **a** Laticifers of *Cannabis sativa* (arrow) located between the cortical parenchyma and phloem. **b** Detail showing two laticifers; the arrow shows a nucleus. **c** Laticifers of *Celtis pubescens* (arrow) arranged into a set and located between the cortical parenchyma and phloem. **d** Detail of the laticifers showing a thick wall (arrow). **e** Multinucleate laticifers (arrow) of *Pteroceltis tatarinowii* located between the cortical parenchyma and phloem. **f** Detail showing laticifers with a nucleus and its nucleoli (arrow). **g** Laticifers of *Trema micrantha* (arrow) located between the cortical parenchyma and the phloem. **h** Detail showing laticifers with a thick wall (arrow). cp cortical parenchyma, ph phloem. Scale bars: **a**, **c**, **e**, **g** 50 μm, **b**, **d**, **f**, **h** 20 μm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intense. In the control samples that were boiled without pectin or carboxymethylcellulose, the activity of cellulase was positive but less intense than in the treated samples (Fig. 8d). However, the positive reaction of the pectinase activity was similar between the control and the treated samples for the enzyme activity in the cell wall (Fig. 9d).

Latex composition

The natural color of the latex is colorless in most species (Fig. 10a) except *Cannabis sativa* in which it is yellowish (Fig. 10b).

The latex of the four species is similar in chemical composition, except for the presence of phenolic compounds, terpenes, and large starch grains (Table 3). It reacted positively for proteins with xylidine Ponceau (Fig. 10c, d), for neutral polysaccharides with PAS (Fig. 10e, f), and for total lipids with Sudan IV (Fig. 10g, h). Large starch grains were detected with Lugol (Fig. 10a, b) in the latex of *C. pubescens*, *P. tatarinowii*, and *Trema micrantha*, but not in *C. sativa.* Terpenes were detected with Nadi reagent in the latex of fresh samples of *C. pubescens* and *T. micrantha* (Fig. 11c, d). No tannins were found for these species with vanillin hydrochloric acid (Table 3). Phenolic compounds were only detected in the latex of *C. sativa* using ferric chloride (Fig. 11e) and Toluidine Blue (Fig. 11f). No alkaloids were detected in all studied species by using Wagner's reagent (Table 3).

Discussion

The present report of laticifers for *Celtis*, *Pteroceltis*, and *Trema* is a great novelty for the family because in a previous

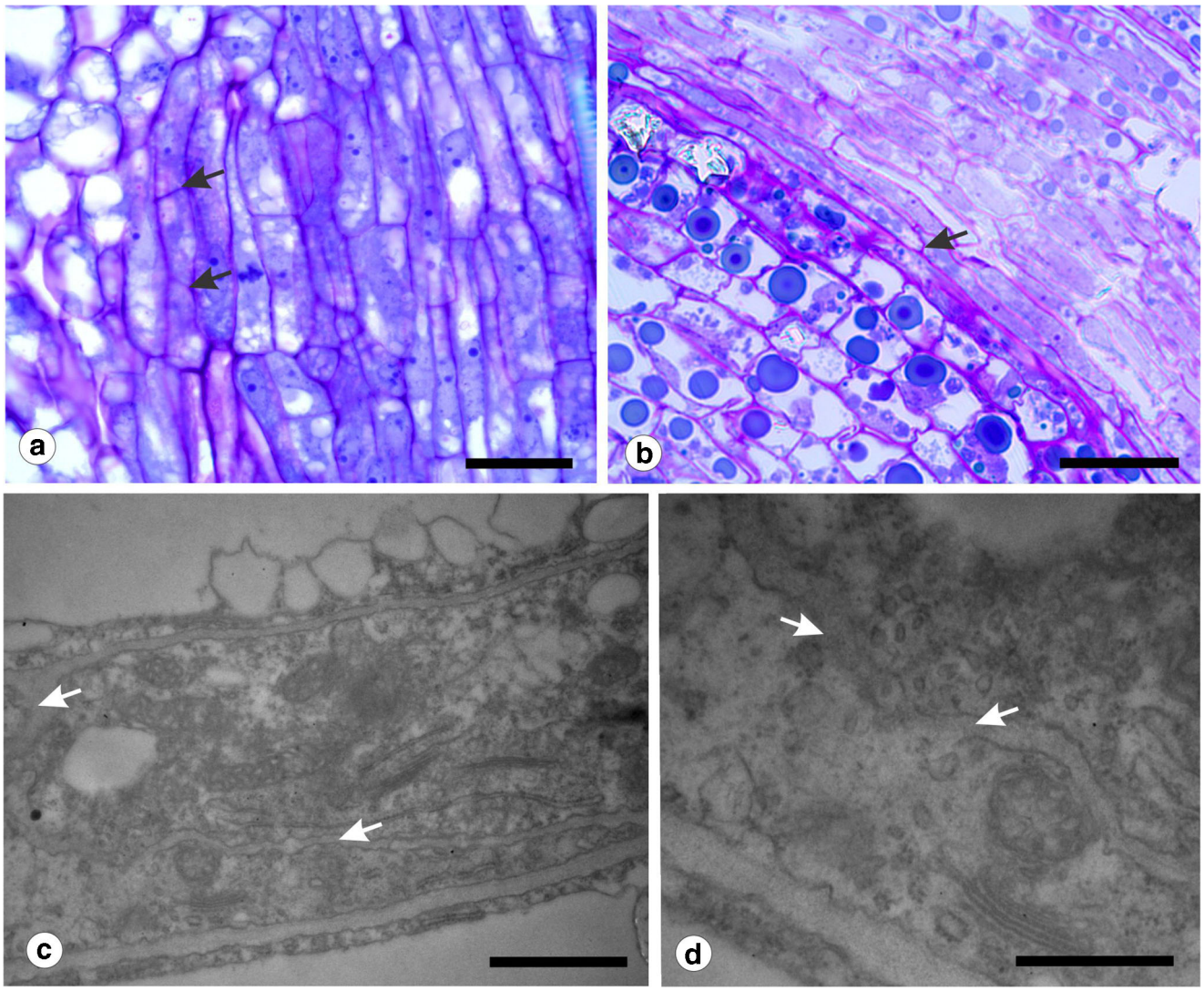

Fig. 3 Origin of the laticifers of Cannabaceae species (longitudinal sections; **a**, **b** stained with Toluidine Blue; **c**, **d** transmission electron microscope). **a** Vegetative meristem of *Cannabis sativa*. Note a series of laticifers cells with the terminal cell walls still present (arrows). **b** Laticifers of *Celtis pubescens* that elongate with the growth of the plant. **c** Laticifers of *Celtis pubescens* showing the thin terminal cell wall (arrow). **d** Disintegration process of the cell wall to form the articulated laticifer (arrows) in *Celtis pubescens*. Scale bars: **a**, **b** 20 μm, **c** 2 μm, **d** 1 μm

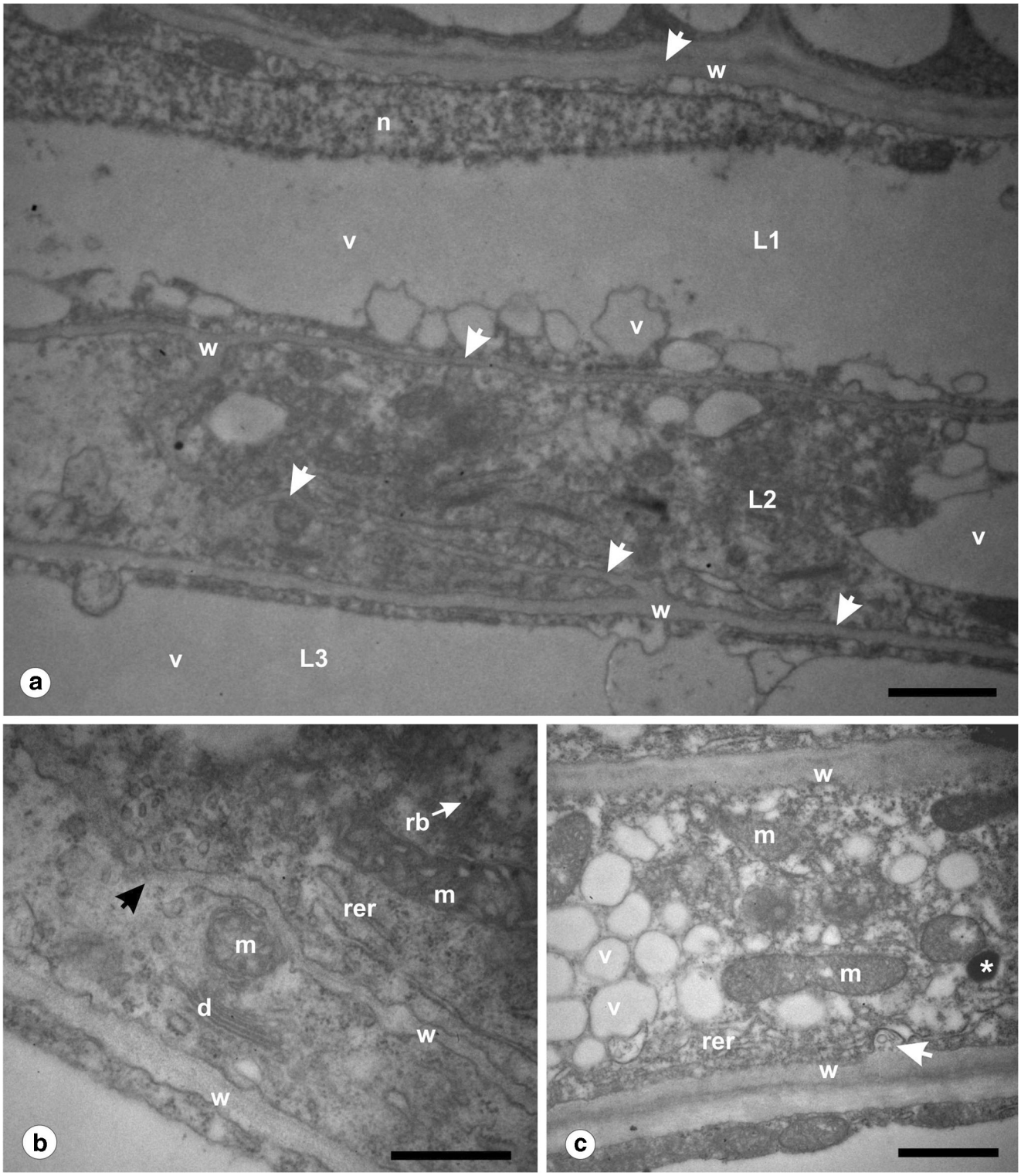

Fig. 4 Ultrastructure of articulated laticifers of *Celtis pubescens* (TEM). **a** Three laticifers. Two mature laticifer (L1, L3) with peripheral cytoplasm showing a large vacuole and other small vacuoles near the wall, and part of the nucleus in the first. Developing laticifer; note two cells with a thin terminal wall (arrow) in degradation process (L2). **b** Detail of a disintegrating terminal wall (black arrow); note that the cytoplasm is rich in mitochondria, rough endoplasmic reticulum, and dictyosomes (close to the cell wall). **c** Cytoplasm with mitochondria, small vacuoles, rough endoplasmic reticulum, and osmiophilic bodies (*); note the thick wall and vesicles being added to it (arrow). d dictyosome, m mitochondria, n nucleus, rer rough endoplasmic reticulum, v vacuole, w wall. Scale bars: **a**, **c** 2 μm, **b** 1 μm

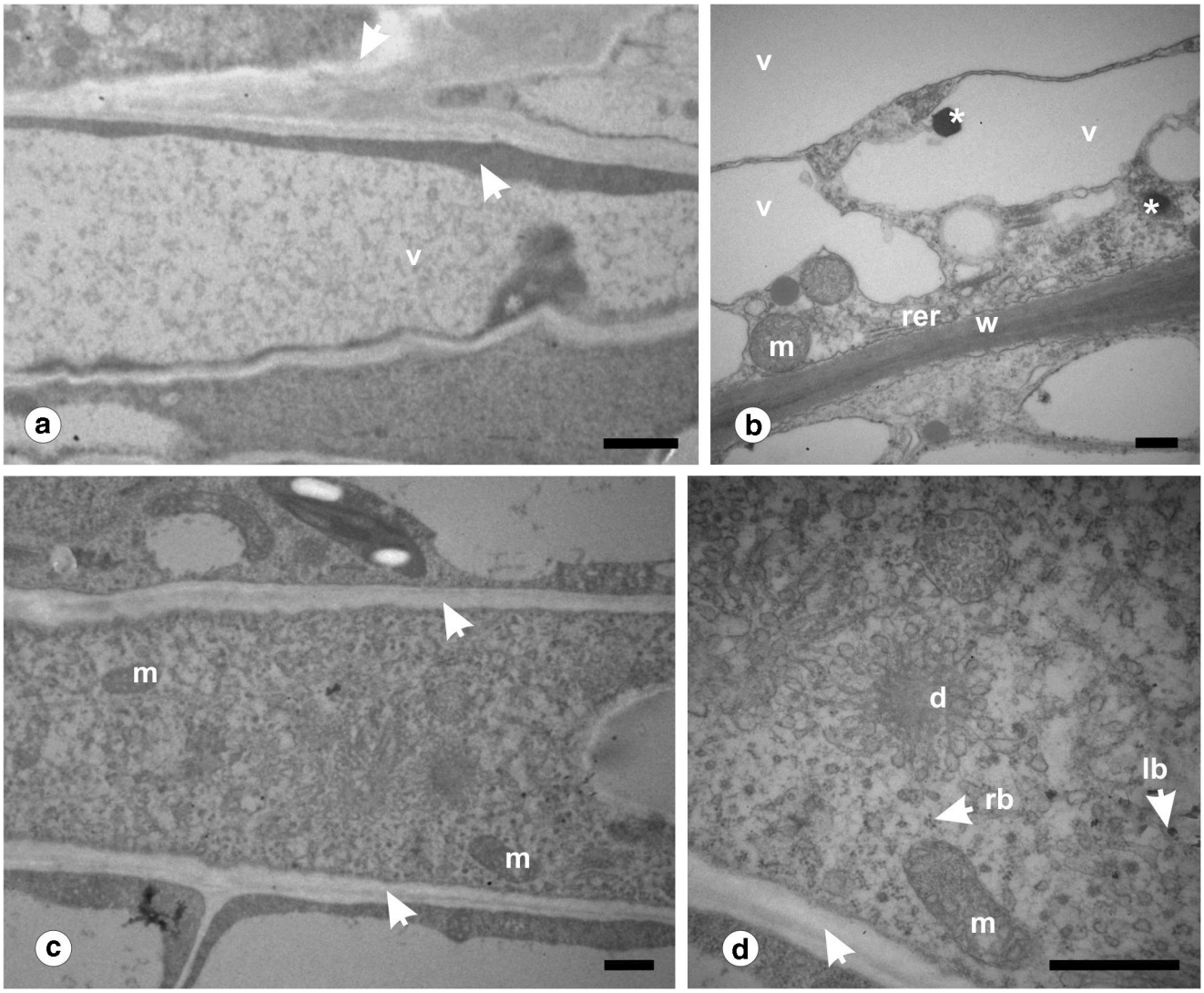

Fig. 6 Ultrastructure of articulated laticifers of *Trema micrantha* (TEM). **a** Peripheral cytoplasm of a mature laticifer (arrows) showing a large vacuole containing latex substances. **b** Peripheral cytoplasm rich in mitochondria, rough endoplasmic reticulum, vacuoles formed by the endoplasmic reticulum, and osmiophilic bodies (*). **c**, **d** Laticifer in differentiation with active organelles. **d** Cytoplasm with mitochondria, dictyosomes, ribosomes, and lipophilic bodies; note the laticifer thick walls (arrow). d dictyosomes, lb lipophilic bodies, m mitochondria, rb ribosomes, rer rough endoplasmic reticulum, v vacuole. Scale bars: **a** 3 μm, **b**–**d** 1 μm

study of Sytsma et al. (2002), laticifers were considered to be absent in these genera that were recently inserted into Cannabaceae. Therefore, our results corroborate the insertion of these genera into Cannabaceae together with *Cannabis* and *Humulus* in which the presence of laticifers throughout the plant has been previously described (Meeuse 1942; Furr and Mahlberg 1981; Mesquita and Dias 1984; Hagel et al. 2008).

The laticifer structure of the Cannabaceae species studied so far is very similar. Our data of laticifer origin showed that they are articulated, contradicting previous studies that described non-articulated laticifers for *Cannabis sativa* and *Humulus lupulus* (Metcalfe 1966; Fahn 1979; Furr and Mahlberg 1981; Hagel et al. 2008). Likely, the lack of studies on the laticifer ontogeny has led to a misinterpretation of the laticifer types. Therefore, the classification of the laticifer types in the Urticalean rosid clade needs more attention, since in Moraceae and Urticaceae, there are also descriptions of both types of laticifers (non-articulated—Van Veenendaal and Den Outer 1990; Machado and Santos 2004; Quintanar et al. 2004; Jacomassi et al. 2007, 2010; Kitajima et al. 2012; and articulated—Milanez 1954; Topper and Koek-Noorman 1980; Marinho and Teixeira 2019a).

The laticifer distribution along the plant body is also similar because they are widely distributed in the vegetative and floral organs; the exception were the sepals of the pistillate apetalous flower of *Cannabis sativa*, which are structurally reduced with only three layers of cells. A counter-proposal is that laticifers were not observed because they can be extremely narrow and inconspicuous in such reduced organs.

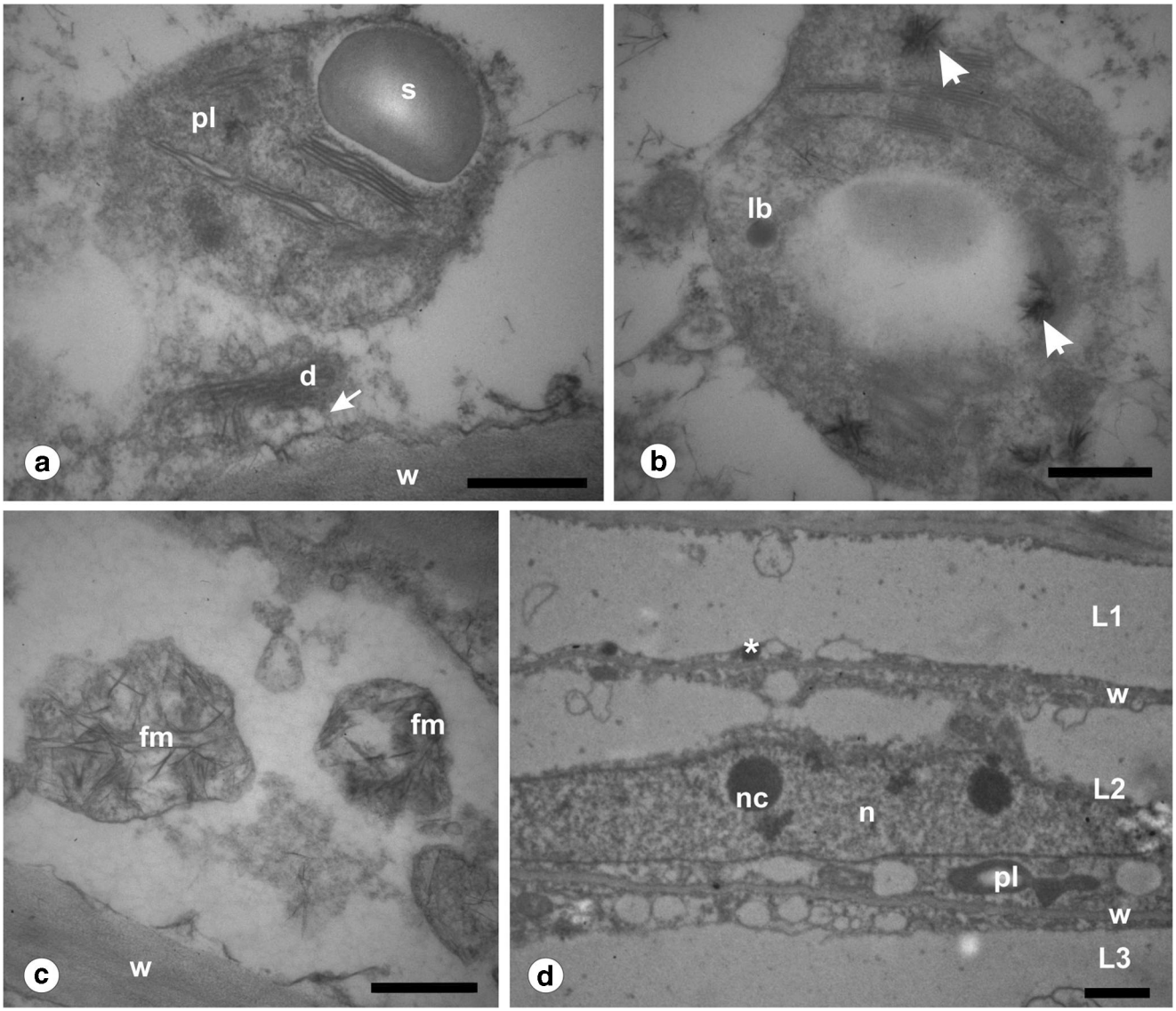

Fig. 5 Ultrastructure of articulated laticifers of *Celtis pubescens* (TEM). **a** Plastid with starch grains and evident stacked thylakoids, and dictyosomes near the wall. **b** Plastid with stacked thylakoids containing lipid bodies and starch in degradation evidenced by electron-dense material (arrows). **c** Detail of plastids partially digested inside the vacuole. **d** Three laticifers arranged in parallel (L1, L2, L3) showing their thin walls; note the peripheral cytoplasm with osmiophilic bodies (*), a plastid, and the nucleus with two nucleoli. d dictyosomes, em electron-dense material, lb lipid bodies, pl plastid, n nucleus, nc nucleoli, s starch grains, w wall. Scale bars: **a**, **c** 1 μm, **b** 2 μm, **d** 3 μm

The main compounds of the latex of Cannabaceae species are polysaccharides, proteins, and lipids (Furr and Mahlberg 1981; present study). Compounds such as starch grains (*Pteroceltis tatarinowii*, *Celtis pubescens*, and *Trema micrantha*) and terpenes (*C. pubescens* and *T. micrantha*) are reported here for the first time for the family. Alkaloids, previously found in *Cannabis sativa* by other researches (Furr and Mahlberg 1981), were not found in this study, even when using fresh samples of *C. pubescens* and *T. micrantha*. Terpenes and phenols were found in the cytoplasm of glandular trichomes (Mahlberg and Kim 2004) and in the latex (Furr and Mahlberg 1981, present study) of *C. sativa.* It is likely that such terpenes and phenols constitute the cannabinoids that are defined as a group of terpenophenolic compounds and are exclusively found in *C. sativa* (Mechoulam and Gaoni 1967; Croteau et al. 2000; Andre et al. 2016). Recent studies showed two biosynthetic pathways that form the precursors of the cannabinoids: one is the plastid pathway (produce—methylerythritol 4-phosphate (MEP)), and the other is the polyketide pathway (produce—olivetolic acid (OLA)) (Andre et al. 2016; Sirikantaramas and Taura 2017). It is noteworthy that the polyketide pathway is still uncertain in terms of location; preliminary analyses indicate that it occurs in the cytoplasm (Gagne et al. 2012). The presence of lipid bodies in the plastids of *Trema micrantha* and *Celtis pubescens* indicates the occurrence of the plastid pathway for the synthesis

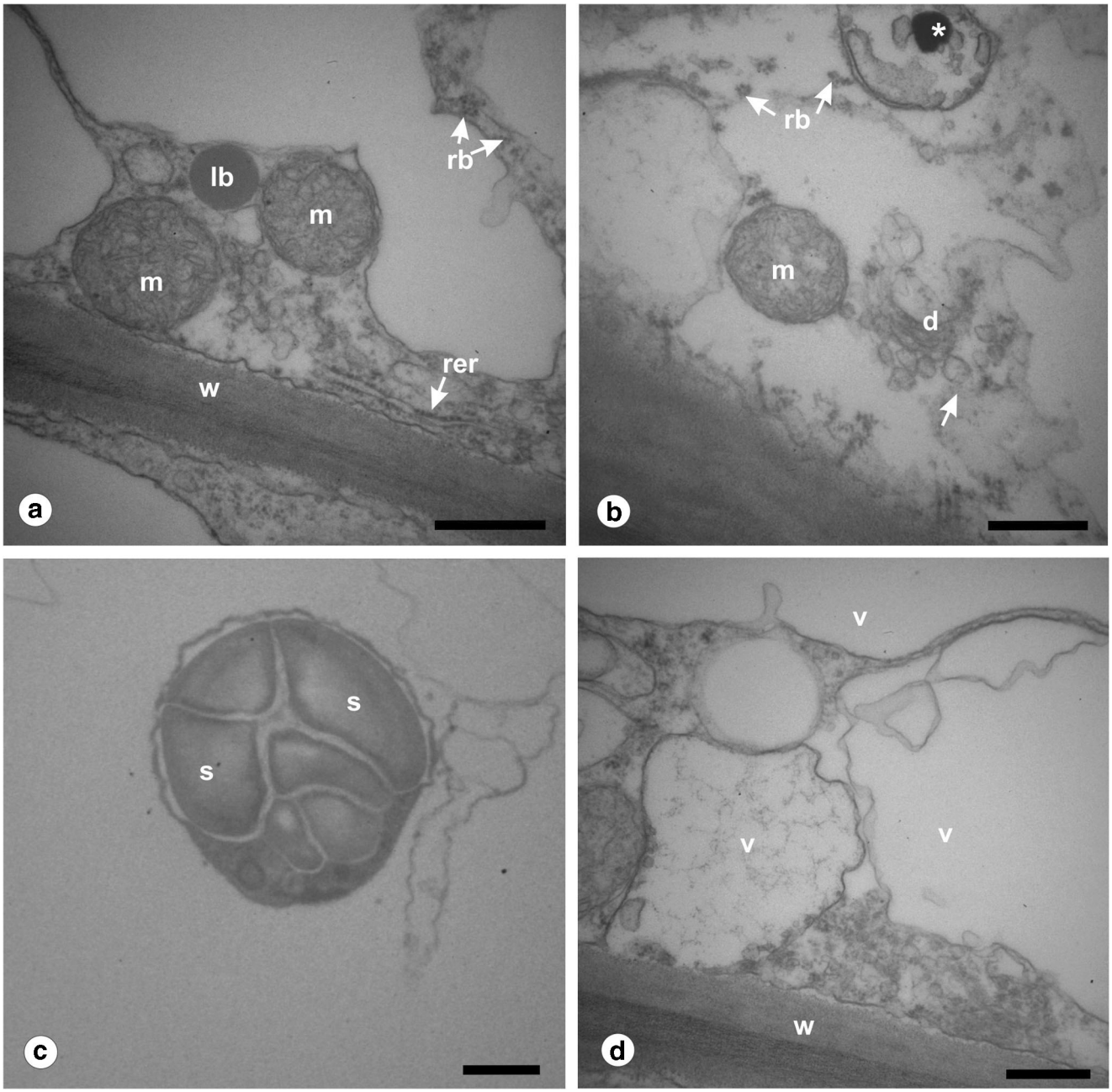

Fig. 7 Ultrastructure of articulated laticifers of *Trema micrantha* (TEM). **a** Cytoplasm with mitochondria, rough endoplasmic reticulum, ribosomes, and lipophilic bodies; note the laticifer thick walls. **b** Cytoplasm with a mitochondria, dictyosomes releasing vesicles in the trans-Golgi face (arrow), ribosomes, and an osmiophilic body (*). **c** Plastid with starch grains (s). **d** Small vacuoles with latex substances. d dictyosomes, lb lipophilic bodies, m mitochondria, rer rough endoplasmic reticulum, rb ribosomes, v vacuole. Scale bars: **a**–**c** 1 μm, **d** 3 μm

of terpenes in both species. Phenolic compounds may actually be absent or occur in an amount that it is not detectable by histochemical techniques and TEM analyses. Thus, it is difficult to identify a potential cannabinoid production in the latex of *Celtis pubescens* and *Trema micrantha*.

Cannabis sativa (Mesquita and Dias 1984), *Celtis pubescens*, and *Trema micrantha* (present study) show similar laticifer ultrastructure, while in Moraceae species, it is different (see Heinrich 1970; Rachmilevitz and Fahn 1982; Marinho and Teixeira 2019b). The Cannabaceae species differ only in the amount of osmiophilic material that is larger in *C. sativa* (Mesquita and Dias 1984) and lower in *C. pubescens* and *T. micrantha* (present study), because of their different latex composition. Therefore, not only the distribution of the laticifers is a conserved character in the family but also the subcellular morphology of the laticifers.

The abundant mitochondria with conspicuous cristae detected in the analyzed species are related to the energy supply

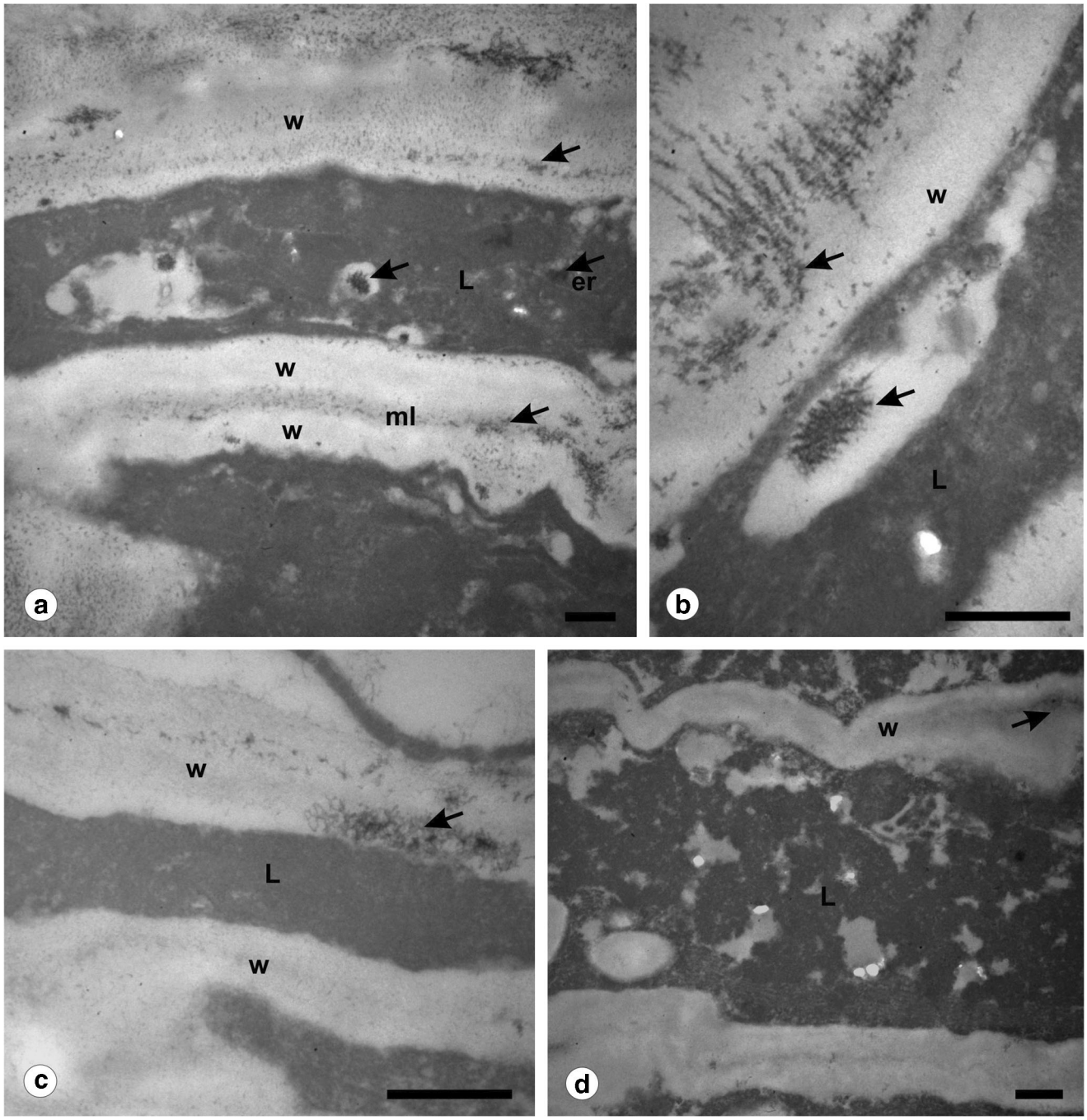

Fig. 8 Cytochemical localization of cellulase activity in the laticifers of *Celtis pubescens* (**a**, **b**, **d**) and *Trema micrantha* (**c**). **a**–**c** Laticifers incubated with carboxymethylcellulose. **a**, **b** Note the electron-dense reaction products in the middle lamella, vacuole, and endoplasmic reticulum (positive reaction—arrows). **c** Reaction product of the cellulase in the laticifer cell wall (positive reaction—arrow). **d** Laticifer of control specimen incubated without carboxymethylcellulose. Note the absence of reaction product. There are only small electron-dense products in the middle lamella (arrow). L laticifer, ml middle lamella, w cell wall. Scale bars: **a**–**d** 1 μm

for the synthesis of compounds in the secretory structures (Wilson and Mahlberg 1978; Evert 2006). Dictyosomes act on the secretion of polysaccharides (Fahn 1979, 1990; Dickison 2000; Evert 2006), and the plastids are involved in the production of terpenes and starch grains (Heinrich 1970; Wilson and Mahlberg 1978; Evert 2006). Beyond producing proteins and ribosomes (Evert 2006), the endoplasmic reticulum also participates in the formation of small vacuoles in the laticifers (Mesquita 1969; Nessler and Mahlberg 1977; Wilson and Mahlberg 1978; Mesquita and Dias 1984; Cai et al. 2009; present study).

The autophagy, the formation of a large vacuole from small vacuoles with participation of the endoplasmic reticulum followed by cytoplasm lysis, is usual in laticifers (i.e.,

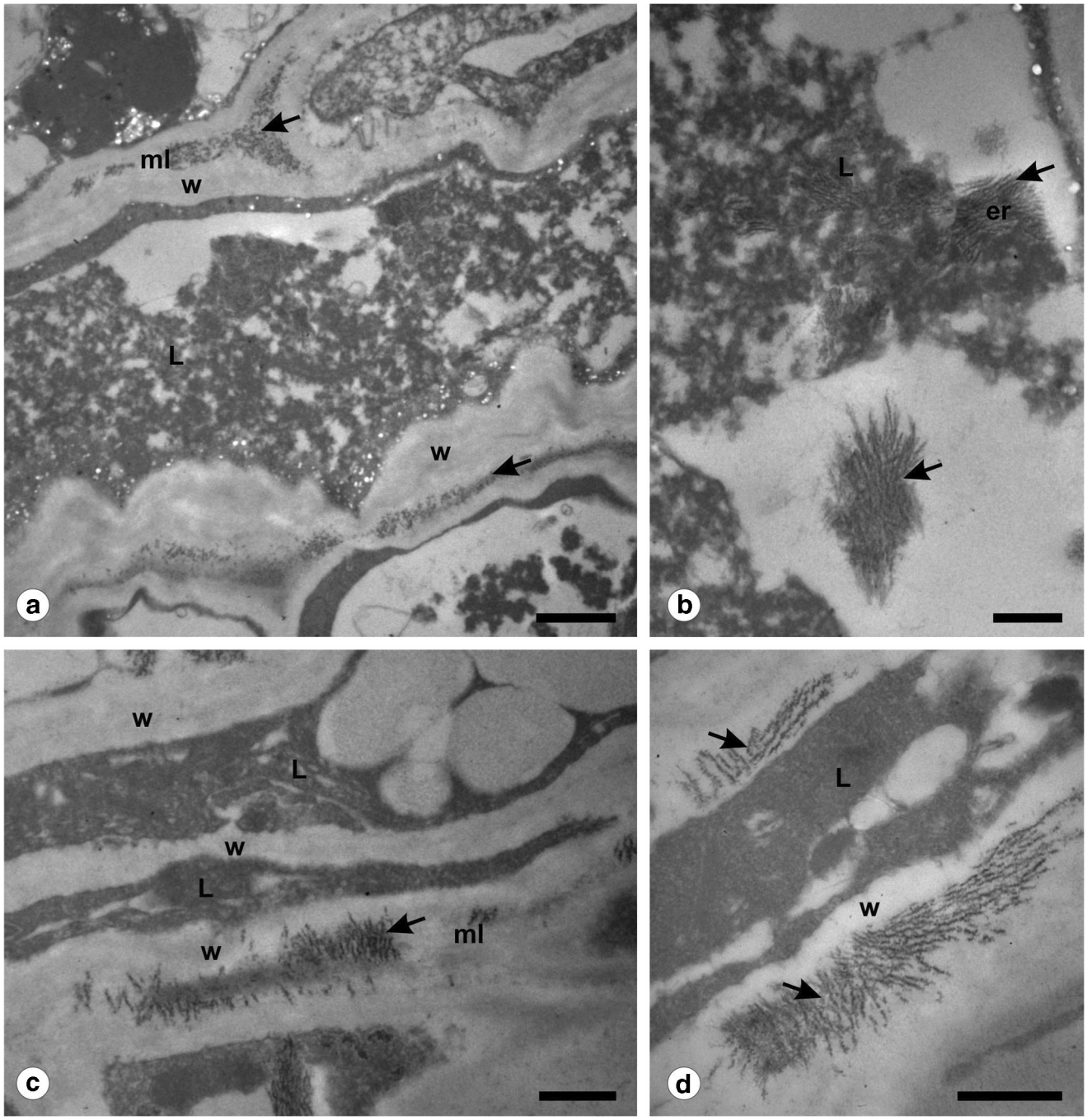

Fig. 9 Cytochemical localization of pectinase activity in the laticifers of *Celtis pubescens* (**a**, **b**) and *Trema micrantha* (**c**, **d**). **a**–**c** Laticifers incubated with pectin. **a** Electron-dense reaction products in the middle lamella (positive reaction—arrow). **b** Product reaction in the vacuole (positive reaction—arrow). **c** Reaction product of pectinase in the middle lamella (positive reaction—arrow). **d** Laticifer of control specimen incubated without pectin. Note that there is reaction product in the cell wall (positive reaction—arrow). L laticifer, ml middle lamella, er endoplasmic reticulum, w cell wall. Scale bars: **a** 2 μm, **b**–**d** 1 μm

Lupinus albus L., Mesquita 1969; *Papaver soniferum* L., Nessler and Mahlberg 1977; and *Asclepia syriaca* L., Wilson and Mahlberg 1978; *Ficus carica*, Rachmilevitz and Fahn 1982; *Cannabis sativa*, Mesquita and Dias 1984; *Euphorbia kansui* Liou, Cai et al. 2009; Zhang et al. 2018) and evident in the laticifers of Cannabaceae and Moraceae species (Heinrich 1970; Rachmilevitz and Fahn 1982; Mesquita and Dias 1984; present study), being considered an important process in the latex production and development of non-articulated and articulated laticifers (Evert 2006; Zhang et al. 2018). The hydrolysis renders the cytoplasm more transparent and forms small particles (Cai et al. 2009). Such particles, together with other compounds produced by the organelles such as starch, oil droplets, proteins, and

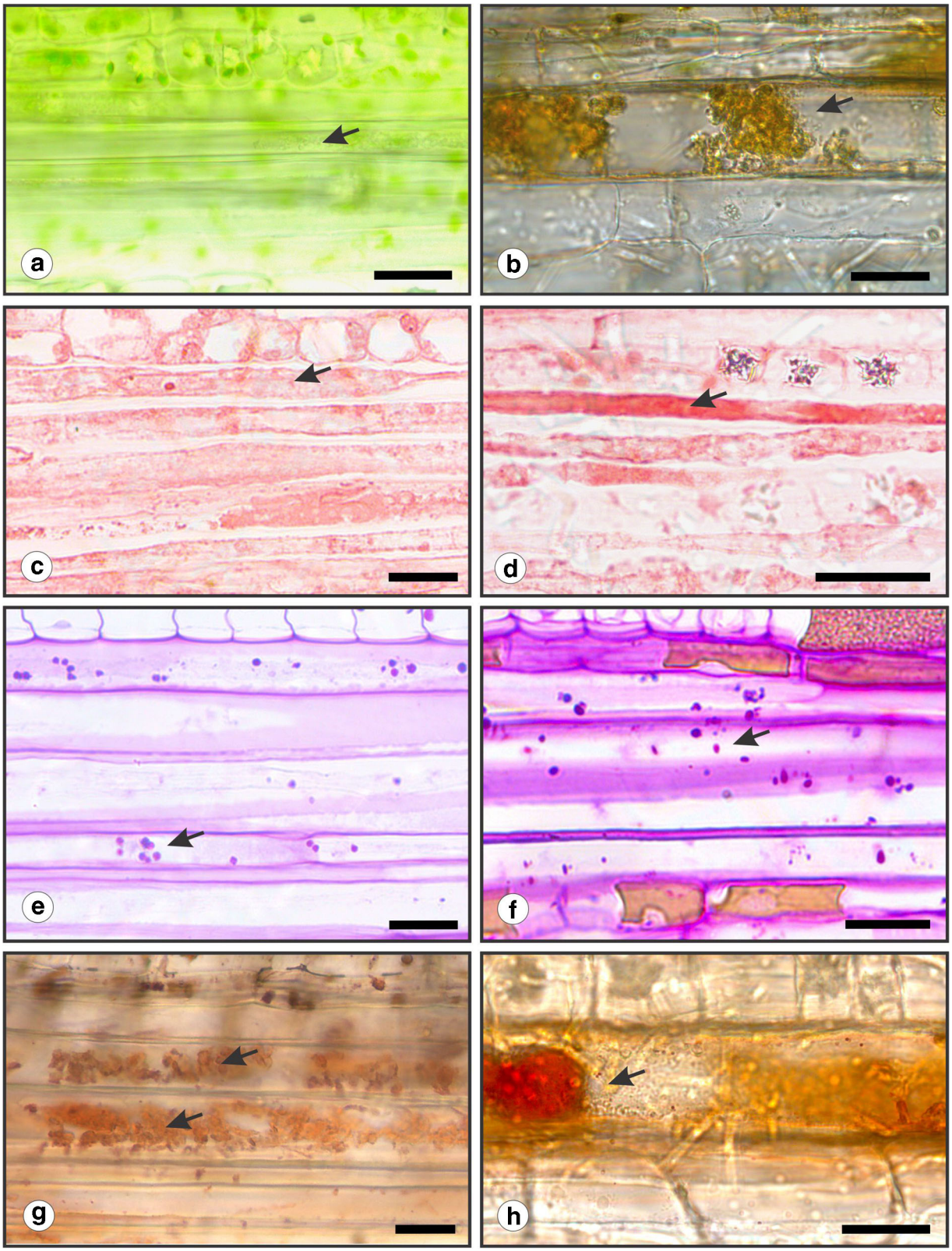

Fig. 10 Histochemical analyses of the latex of Cannabaceae species (longitudinal sections). **a** Laticifers of *Celtis pubescens* without staining; note the colorless latex. **b** Laticifers of *Cannabis sativa* without staining; note the yellowish latex. **c** Positive reaction of the latex of *Pteroceltis tatarinowii* for proteins (stain: xylidine Ponceau). **d** Positive reaction of the latex of *Celtis pubescens* for proteins (stain: xylidine Ponceau). **e** Positive reaction of the latex of *Trema micrantha* for neutral polysaccharides (stain: PAS). **f** Positive reaction of the latex of *Cannabis sativa* for neutral polysaccharides (stain: PAS). **g** Positive reaction of the latex of *Trema micrantha* for total lipids (stain: Sudan IV). **h** Positive reaction of the latex of *Cannabis sativa* for total lipids (stain: Sudan IV). Scale bars 20 µm

Table 3 Histochemical data obtained for the stem latex of Cannabaceae species

Reagents	Target compound	*Cannabis sativa*	*Celtis pubescens*	*Pteroceltis tatarinowii*	*Trema micrantha*
PAS	Neutral polysaccharides	+	+	+	+
Lugol	Starch	–	+	+	+
Sudan IV	Total lipids	+	+	+	+
Xylidine Ponceau	Protein	+	+	+	+
Wagner's reagent	Alkaloids	?	–	?	–
Toluidine Blue	Phenolic compounds	+	–	–	–
Ferric chloride	Phenolic compounds	+	–	–	–
Nadi reagent	Terpenes	?	+	?	+
Vanillin hydrochloric acid	Tannins	?	–	?	–

Symbols: (+) presence; (−) absence; (?) no analyzed

phenolics before cytoplasm hydrolysis, compose the latex (Cai et al. 2009; present study).

The latex composition of Cannabaceae species (Furr and Mahlberg 1981; present study) indicates that laticifers act in plant defense against herbivores. This defense includes preventing the insect from feeding on the plant and the accumulation of gums, gel, or phenols that form tyloses, suggesting an increased resistance, as observed in elm trees (Ulmaceae, Dickison 2000). The laticifer distribution on the plant body is another criterion that cannot be neglected in the inference of functions for such an interesting and complex secretory structure. In *Ficus* species, laticifers have been considered to act in promoting the pollination by protecting the galled flowers (flowers where the wasp offspring emerges) against attack by non-pollinating wasps (Marinho et al. 2018). Cannabaceae consist of exclusively wind-pollinated species (Miller 1970; Barth et al. 1975; Arruda and Sazima 1988; Culley et al. 2002); thus, different selective pressures should act on laticifer distribution along the flower. Differently from *Ficus*, the flowers of Cannabaceae are exposed favoring wind pollination but also exposed to UV radiation, insects, or other animals. Thus, protection appears to be the main function of laticifers in Cannabaceae. This can be illustrated by the finding of laticifers in the stigmatic region of the species studied, which is an important part of the flower for the reproductive success of wind-pollinated species (Culley et al. 2002; Friedman and Barrett 2009) with rare reports of laticifers.

Pectinase and cellulase activities were found in the wall and vacuole of *Celtis pubescens* and *Trema micrantha*; thus, these enzymes participate in the formation of its articulated laticifers, by the complete dissolution of cellulose and pectin in the terminal walls (Nessler and Mahlberg 1981; Allen and Nessler 1984). Pectinase and cellulase activities were reported for articulated laticifers (Sheldrake 1969; Nessler and Mahlberg 1981; Pilatzke-Wunderlich and Nessler 2001; Marinho and Teixeira 2019b), while there are only reports of pectinases for non-articulated laticifers (Wilson et al. 1976; Allen and Nessler 1984). Therefore, the presence of these enzymes is an important tool to confirm the occurrence of articulated laticifers in Cannabaceae.

The reaction product also shows pectinase and cellulase activities in the lateral region of the laticifer wall, suggesting therefore that these enzymes can also be important in the wall lateral expansion (Allen and Nessler 1984; Marinho and Teixeira 2019b, present study). Interestingly, pectinase activity is found inclusive in the control test, but only in the cell wall. Similar results were obtained for nonarticulated laticifers of *Nerium oleander* (Allen and Nessler 1984) in that the saturation of the pectinase by endogenous pectin of the middle lamella and the addition of exogenous pectin do not alter the density of the reaction product in this region.

The results suggest that the cellulase and pectinase enzymes are synthesized in the endoplasmic reticulum and then are released to the cell wall through exocytosis (Liang et al. 2009; Yu et al. 2004; Wang et al. 1998; Marinho and Teixeira 2019b). Likely, the reaction product presence in the vacuole indicates the occurrence of endocytosis as a result of the translocation of the products of the degraded cell wall to the vacuole (Giordani 1980; Demarco and Castro 2008).

In conclusion, we suggest that the presence of articulated anastomosing laticifers (sensu Ramos et al. 2019) can be a synapomorphy for Cannabaceae. In addition, we believe that the vast majority of the laticiferous species of the Urticalean rosid clade are likely to have articulated laticifers, and thus, the separation of these families

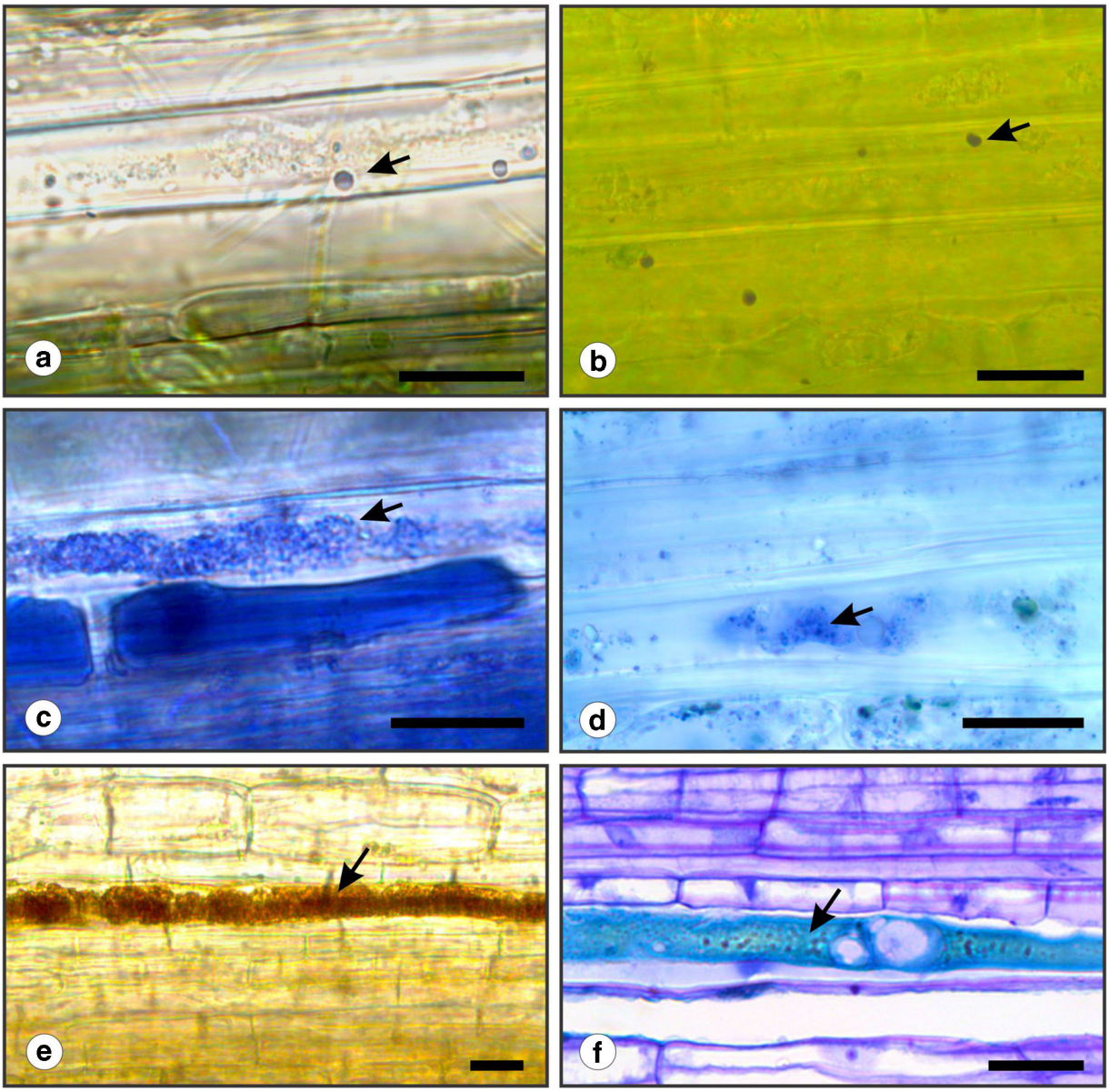

Fig. 11 Histochemical analyses of the latex of Cannabaceae species (longitudinal sections). **a** Positive reaction of the latex of *Trema micrantha* for starch (stain: lugol). **b** Positive reaction of the latex of *Celtis pubescens* for starch (stain: lugol). **c** Positive reaction of the latex of *Trema micrantha* for terpenes (stain: Nadi reagent). **d** Positive reaction of the latex of *Celtis pubescens* for terpenes (stain: Nadi reagent). **e**, **f** Positive reactions of the latex of *Cannabis sativa* for phenolic compounds with ferric chloride (**e**) and Toluidine Blue (**f**). Scale bars 20 μm

by having distinct laticifer types should be reviewed. The wide distribution of laticifers in vegetative and floral organs is reported here for the first time for the family, as well as the occurrence of large starch grains and terpenes in the latex. The similar laticifer ultrastructure of *C. sativa*, *C. pubescens*, and *T. micrantha* should indicate that these species produce the same chemical classes of compounds but in different amounts. The wide distribution of laticifers in the floral organs of Cannabaceae expands our knowledge about this secretory structure and suggests that they have an important function in the protection of floral organs in this family. Therefore, we emphasize the importance of more ecological studies to better understand the role of laticifers in flowers.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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