

Aerial organ anatomy of *Smilax syphilitica* (Smilacaceae)

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Abstract: *Smilax* L. in Brazil is represented by 32 taxa and it is a taxonomically difficult genus because the plants are dioecious and show wide phenotypic variation. The analysis and use of leaf anatomy characters is recognized as a frequently successful taxonomic method to distinguish between individual taxon, when floral material is absent or minute differences in flowers and foliage exist such as in *Smilax*. The aim of this study was to characterize the anatomical features of the aerial organs in *Smilax syphilitica* collected from the Atlantic Rainforest, in Santa Teresa-ES and the *Smilax aff. syphilitica* from the Amazon Rainforest, in Manaus, Brazil. For this, a total of three samples of *Smilax* were collected per site. Sample leaves and stems were fixed with FAA 50, embedded in historesin, sectioned on a rotary microtome, stained and mounted in synthetic resin. Additionally, histochemical tests were performed and cuticle ornamentation was analyzed with standard scanning electron microscopy. *S. syphilitica* and *S. aff. syphilitica* differed in cuticle ornamentation, epidermal cell arrangement and wall thickness, stomata type and orientation, calcium oxalate crystal type, and position of stem thorns. Leaf blades of *S. syphilitica* from the Amazon Rainforest have a network of rounded ridges on both sides, while in *S. aff. syphilitica*, these ridges are parallel and the spaces between them are filled with numerous membranous platelets. Viewed from the front, the epidermal cells of *S. syphilitica* have sinuous walls (even more pronounced in samples from the Amazon); while in *S. aff. syphilitica*, these cells are also sinuous but elongated in the cross-section of the blade and arranged in parallel. Stomata of *S. syphilitica* are paracytic, whereas in *S. aff. syphilitica*, are both paracytic and anisocytic, and their polar axes are directed towards the mid-vein. Calcium oxalate crystals in *S. syphilitica* are prisms, whereas in *S. aff. syphilitica*, crystal sand. Thorns occur in nodes and internodes in *S. syphilitica* but only in internodes in *S. aff. syphilitica*. These features have proven to be of diagnostic value and may support a separation into two species, but future studies are needed to confirm that *S. aff. syphilitica* is indeed a new taxon. Rev. Biol. Trop. 60 (3): 1137-1148. Epub 2012 September 01.

Key words: aerial stems, crystals, cuticle, leaves, greenbrier, Smilacaceae.

The genus *Smilax* L. (Smilacaceae Vent) contains the largest number of species within the Smilacaceae family, of which 32 species occur in Brazil (Andreato 1997, 2009). The medicinal importance of this genus has been globally recognized since antiquity, and extracts from its leaves and roots are used to treat diseases such as syphilis, gout, rheumatism, skin disorders, asthma, toothaches, wounds, and eye pain (Vandercolme 1947). In Brazil, medicinal use of the *Smilax* species popularly known as “sarsaparilla” dates back

to the 16th century (Medeiros *et al.* 2007). Sarsaparilla is often misidentified due to morphological similarities among *Smilax* species (Lorenzi & Matos 2002). Similarly, Martins & Appezzato-da-Glória (2006) showed that the descriptive features listed in the Brazilian Pharmacopoeia of 1929 are insufficient to differentiate the species.

Andreato (1997) recognized 100 synonyms for 60 *Smilax* L. species. The confusion of *Smilax* species with some of the *Herreria* ones (Lorenzi & Matos 2002) reinforces the

need for a conclusive taxonomic revision, as pointed out by Koyama (1960), Andreata (1980), and Guaglianone & Gattuso (1991).

According to Moore *et al.* (2010), identification of *Smilax* species from Thailand by floral features is complicated because the plants have similar flowers; the same problem occurs for Brazilian species (Andreata 1997). Anatomical studies in this group are important, since there are many external feature variations within populations and in the same individual of *Smilax* as verified by Mandarin-de-Lacerda *et al.* (1992) and Andreata (1997) in *S. rufescens* Grisebach and *S. japecanga* Grisebach respectively, among other species.

Caponetti & Quimby (1956) studied leaf, stem, and root anatomy and found differences between the epidermal and hypodermal cells in the following five species: *S. auriculata*, *S. hispida*, *S. glauca*, *S. bona-nox*, and *S. herba-cea*. Guaglianone & Gattuso (1991) proposed a comparative framework for distinguishing between four Argentine species of the genus (*S. fluminensis*, *S. cognata*, *S. pilcomayensis*, *S. assumptionis* and *S. campestris*) on the basis of mesophyll tissue, parenchyma cells, stomata location, venation patterns, vascular bundles, and leaf blade edges. Marquete & Pontes (1994) compared the anatomy of *S. spicata*, *S. rufescens*, and *S. fluminensis* and observed a difference only in the leaves; *S. fluminensis* has leaves that are amphistomatic, and the other two species have leaves that are hypostomatic. Moore *et al.* (2008) identified the species *S. petiolatumidus* from Thailand based only on epicuticular leaf architecture. Finally, Palhares *et al.* (2009) distinguished *S. goyazana* from other species of the genus based on the stomata; mesophyll; sclerenchyma sheaths shared by mid-vein vascular bundles; the occurrence of idioblasts and raphides; and the presence of starch and tannin in the leaf blade.

The current study presents anatomical characteristics of the aerial system in *Smilax syphilitica* Humboldt & Bonpland ex Willdenow and *S. aff. syphilitica* Humboldt & Bonpland ex Willdenow. The first species is found in seven Brazilian states, and its extracts

are used in the treatment of syphilis, menstrual cramps, and as an abortifacient (Andreata 1997). *Smilax aff. syphilitica* has not been described until now. Our objective was to increase the diagnostic value of plant features to support correct identification.

MATERIALS AND METHODS

We analyzed three individual *Smilax syphilitica* Humboldt & Bonpland ex Willdenow collected from the Atlantic Rainforest in Santa Teresa-ES and the Amazon Rainforest in Manaus, Brazil. The three samples of *Smilax aff. syphilitica* Humboldt & Bonpland ex Willdenow were collected exclusively from the Amazon Rainforest in Manaus, Brazil. In this study we chose to identify the material as *S. aff. syphilitica* until future studies, morphological and anatomical, let clarify the true identity of the taxon. Sampling from the Atlantic Rainforest occurred at Santa Teresa-ES (19°56'21.0" S - 0°35'55.2" W) in March 2009. Sampling from the Amazon Rainforest occurred at the Adolpho Ducke Forest Reserve in the municipality of Manaus-AM (03°00'00" S - 03°08'00" S - 59°52'40" - 59°58'00" W) in January 2010. The collected material was identified by Dr. Regina Helena Potsch Andreata, an expert on the genus *Smilax* in Brazil, and specimens were incorporated into the ESA herbarium under the numbers 107665 (*S. syphilitica*-Atlantic Rainforest), 112606 (*S. syphilitica*-Amazon Rainforest), and 111412 (*S. aff. syphilitica*-Amazon Rainforest).

The leaf blades were cut at the mid-vein, main vein, interveinal area and at the leaf edge. The stems were analyzed at the third internode (from the apex), the internode closest to the ground, and the underground internode.

For the anatomical study, aerial systems of three adult plants were fixed in FAA 50 (one part formaldehyde: one part glacial acetic acid: 18 parts 50% ethanol, v/v) for 48h (Johansen 1940), dehydrated in a graded ethylic series and infiltrated in glycol methacrylate resin (Leica Histo-resin-LeicaTM-Wetzlar, Germany). Serial sections (5-7µm thick) were performed

on a rotary microtome and stained with toluidine blue (Sakai 1973), astra blue and basic fuchsin (Roeser 1972). The chemical nature of the cellular content was determined using the following histochemical tests: Ferric trichloride solution for phenolic compounds (Johansen 1940), Lugol's iodine solution to identify starch (Berlyn & Miksche 1976), Methylene blue to identify pectins (Johansen 1940) and Aniline blue black to identify the total protein (Fisher 1968). For analysis of Calcofluor White MR2 for cellulose (Hughes & McCully 1975) the microscope was equipped for epillumination with an HBO 50 mercury lamp and a Leica® D filter, providing excitation (Bandpass filter 355-425nm) and suppression (Long-pass filter 470nm).

The tissue dissociation technique was used for analysis of epidermal and lignified mesophyll cells. This technique involves treating the leaf with chromic and nitric acids both at a concentration of 5%, applying dye, and mounting the blade with glycerin (Johansen 1940).

For SEM analyses, leaf blades were fixed in Karnovsky (Karnovsky 1965) for 24h, dehydrated in a graded acetone series and critical point-dried with CO₂ (Horridge & Tamm 1969). Samples were attached to aluminium stubs and coated with gold (30-40nm). Then, the samples were examined under a LEO VP 435 scanning electron microscope at 20kV. The ornamental patterns of epicuticular wax were identified following the classification of Metcalfe & Chalk (1979) and Barthlott *et al.* (1998).

The images were digitally captured with a Leica DMLB microscope (Leica™-Wetzlar, Germany) by using a video camera plugged to a computer utilizing the IM50 (Leica™-Wetzlar, Germany) software for image analysis.

RESULTS

Leaf blades of *Smilax sypilitica* and *S. aff. sypilitica* are leathery, lanceolate-shaped, apex apiculate, obtuse at the base, and fully margined. The venation is acrodromous, with three principle and two inconspicuous veins.

The leaf is complete and features a blade and petiole with a pair of tendrils and a sheath.

Epicuticular wax structure varies with leaf surface and species (Fig. 1-7). On the adaxial side, leaves of *S. sypilitica* from the Atlantic Rainforest have granules and platelets (Fig. 1), whereas on the abaxial surface, there is a reticulum with rounded ridges (Fig. 2). Leaf blades of *S. sypilitica* from the Amazon Rainforest have a network of rounded ridges on both sides (Fig. 3-4), and in *S. aff. sypilitica*, these ridges are parallel and the spaces between them are filled with numerous membranous platelets (Fig. 5-7).

In *Smilax sypilitica*, the leaves are hypostomatic with randomly oriented stomata at the same level as the adjacent cells (Fig. 8-11); the polar axes are directed towards the mid-vein in *S. aff. sypilitica* (Fig. 6). However, stomata of *S. sypilitica* are paracytic (Fig. 9, 11), whereas in *S. aff. sypilitica*, the stomata are both are paracytic and anisocytic (Fig. 13). In all of the samples, a uniseriate epidermis covered by a thick cuticle, which forms flanges. Viewed from the front, the epidermal cells of *S. sypilitica* have sinuous walls (Fig. 8-9) that are even more pronounced in samples from the Amazon (Fig. 10-11). In *S. aff. sypilitica*, these cells are also sinuous but elongated in the cross-section of the blade and arranged in parallel (Fig. 12-13).

Epidermal cell walls are thickened in the inner periclinal and anticlinal walls, as seen in the cross-sections of *S. sypilitica* leaves from the Amazon (Fig. 17-18). In *S. aff. sypilitica*, this thickening occurs in all epidermal cell walls (Fig. 20-21). Staining with methylene blue and Calcofluor White MR2 has shown that the thickened wall is composed of pectin and cellulose.

In all of the samples, the mesophyll is homogeneous (Fig. 14-15, 17-18, 20-21) and consists of five-seven layers of brachiform cells (Fig. 23). Idioblasts containing raphides along the leaf margin region are common (Fig. 14, 17, 20). Calcium oxalate crystals in *S. sypilitica* are prisms (Fig. 15), whereas in *S. aff. sypilitica*, they are crystal sand (Fig. 21).

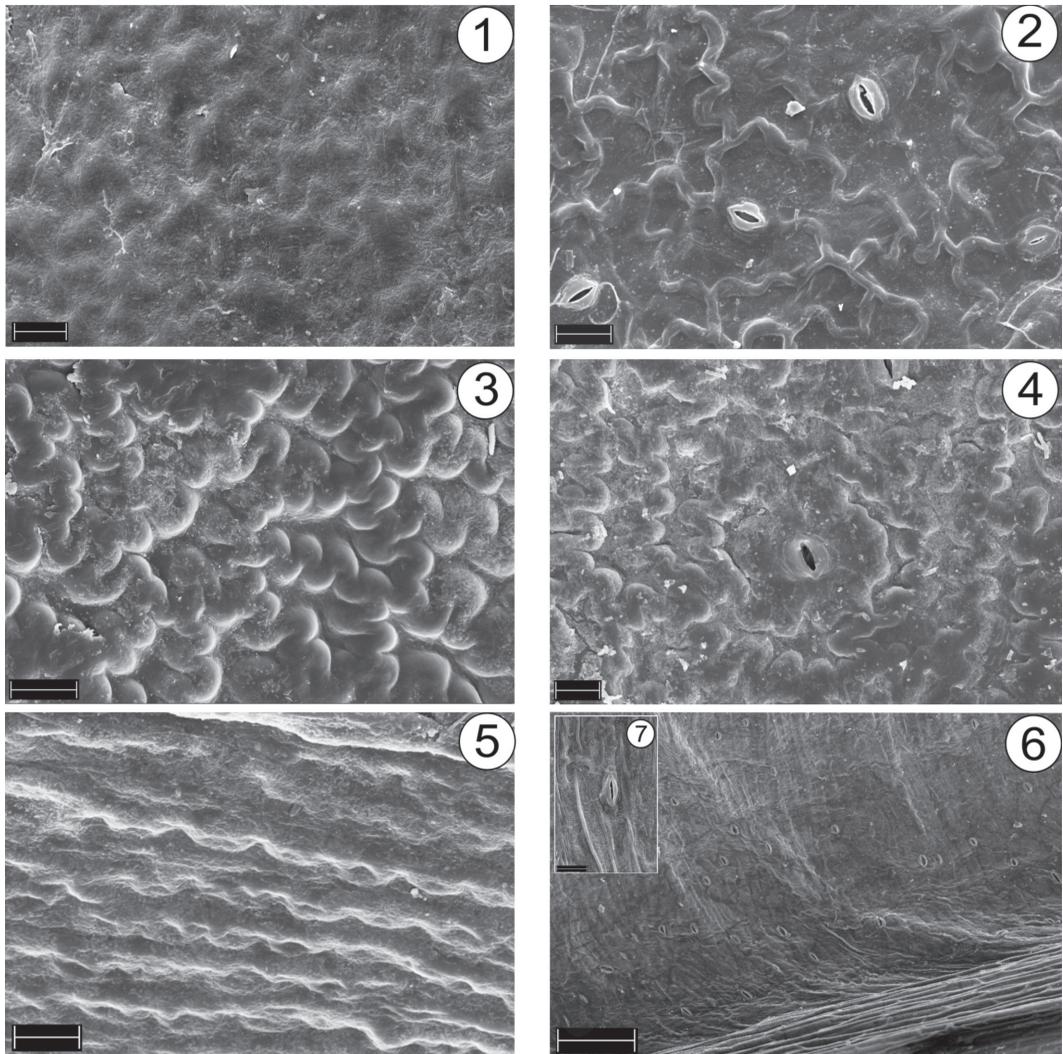


Fig. 1-7. Electron micrographs of leaf surfaces: 1-2. Adaxial and abaxial leaf surfaces of *Smilax siphilitica* Humboldt & Bonpland ex Willdenow (Atlantic Rainforest). 3-4. Adaxial and abaxial leaf surfaces of *S. siphilitica* (Amazon Rainforest). 5-6. Adaxial and abaxial leaf surface of *S. aff. siphilitica* Humboldt & Bonpland ex Willdenow (Amazon Rainforest). 7. Detail of 6, showing membranous platelets and stoma orientation. Scale bars: 1, 2, 4, 5=20µm; 3, 6=200µm; 7=30µm.

Fibers, columnar sclereids, and astrosclereids, the latter of which is not observed in *S. aff. siphilitica*, are the types of lignified cells that were found (Fig. 24). In *S. siphilitica* from the Atlantic Rainforest, the parenchyma cells have a conspicuous thickening of the cell wall (Fig. 15) not observed in other samples (Fig. 18, 21).

The mid-vein region has between three-eight collateral vascular bundles (Fig. 16, 19, 22), and sclerenchyma sheaths shared

by mid-vein vascular bundles were observed only in *S. siphilitica* from the Atlantic Rainforest (Fig. 16).

Phenolic, protein, and starch grain content (the latter two are only observed in the mid-vein) are present only in leaf blade cells of *S. siphilitica* from both sources.

Thorns (Fig. 25-27) occur in nodes and internodes of *S. siphilitica* but only in the internodes of *S. aff. siphilitica*.

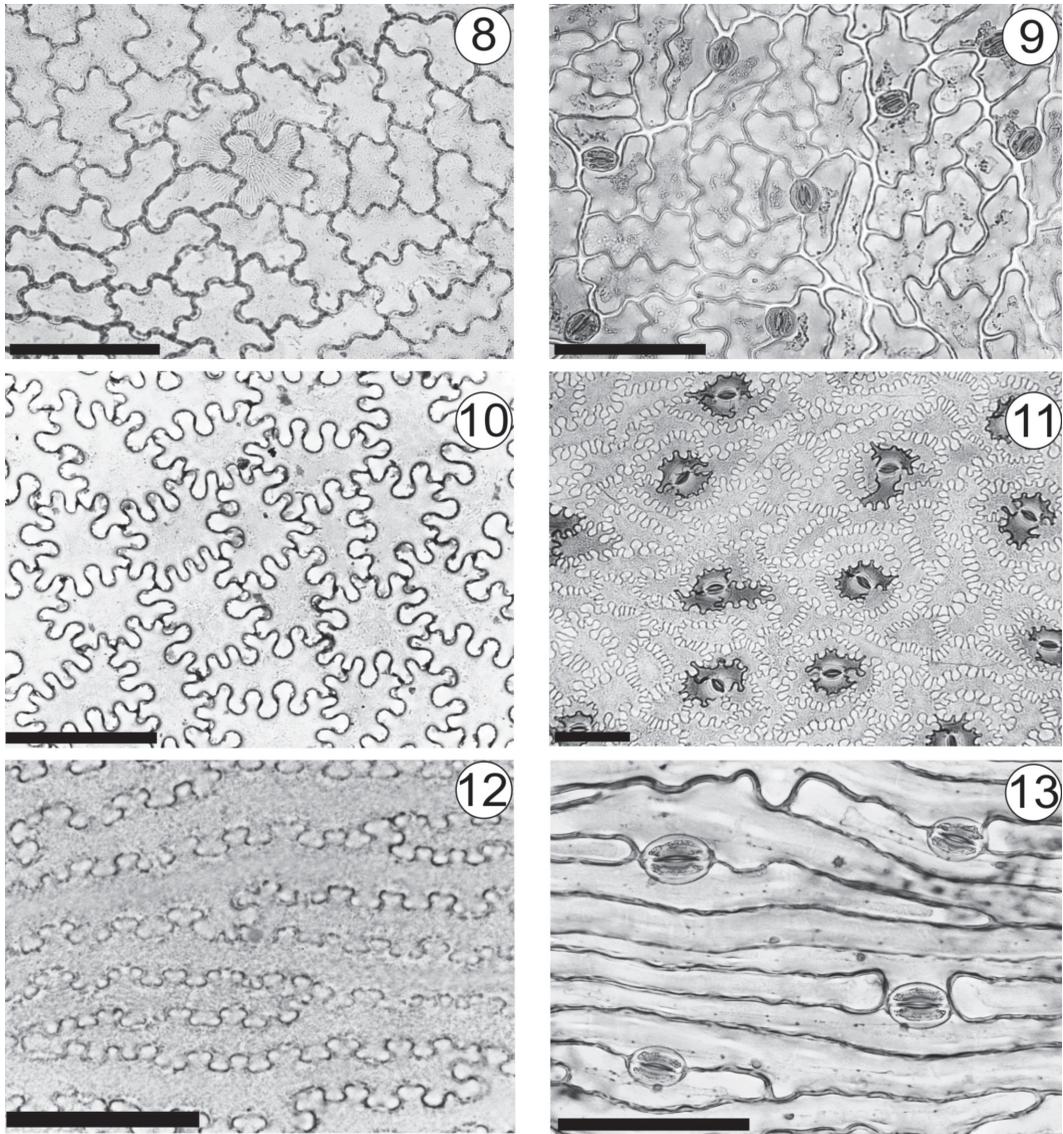


Fig. 8-13. Frontal view of the epidermis on adaxial (8, 10, 12) and abaxial (9, 11, 13) leaf surfaces of *Smilax syphilitica* Humboldt & Bonpland ex Willdenow from the Atlantic Rainforest (8-9), the Amazon Rainforest (10-11), and *S. aff. syphilitica* Humboldt & Bonpland ex Willdenow (12-13) from the Amazon Rainforest. Scale bars=100µm.

In the three internode regions that were analyzed, the epidermis is uniseriate with stomata and covered by a thick cuticle. In *S. syphilitica*, the epidermis has cells with phenolic and protein content. We observed that the hypodermis, which underlies the epidermis (Fig. 28-29), has different degrees of lignification and many protein and phenolic idioblasts. This

tissue was not observed in plants that attach themselves to tree trunks; this characteristic is typical of vines, but only in those that remain small shrubs. In the third internode of *Smilax syphilitica* and *S. aff. syphilitica*, there are between three-five chlorenchyma layers and a continuous sclerenchymatic ring enveloping the vascular cylinder (Fig. 28, 30).

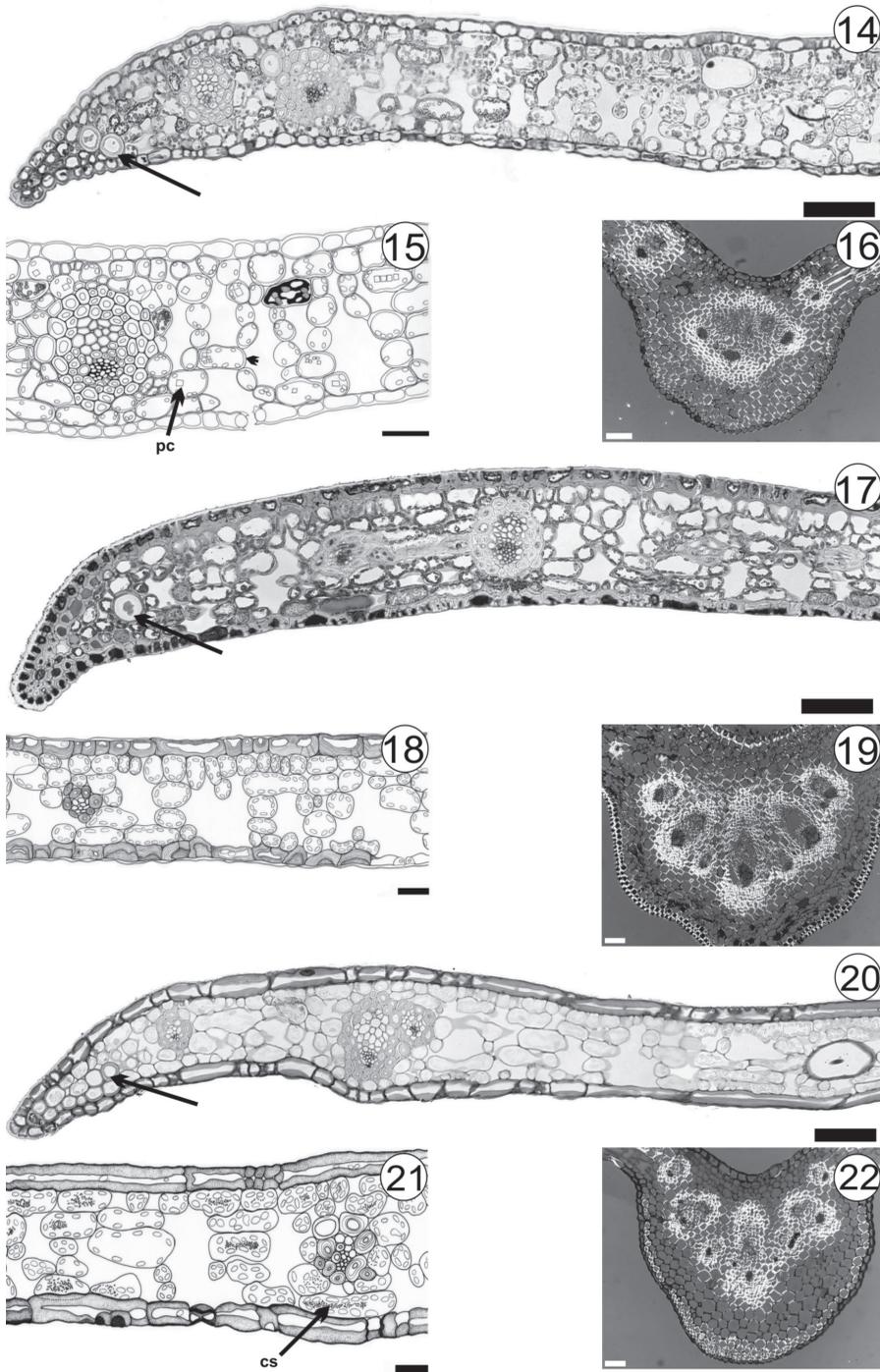


Fig. 14-22. Cross sections of leaf blades: *Smilax syphilitica* Humboldt & Bonpland ex Willdenow from the Atlantic Rainforest (14-16), the Amazon Rainforest (17-19), and *S. aff. syphilitica* Humboldt & Bonpland ex Willdenow from the Amazon Rainforest (20-22). Arrows in 14, 17, and 20 indicate idioblasts containing raphides close to the margin. 16, 19, and 22 show the mid-vein under polarized light. cs=crystal sand; pc=prismatic crystal. Scale bars=100μm.

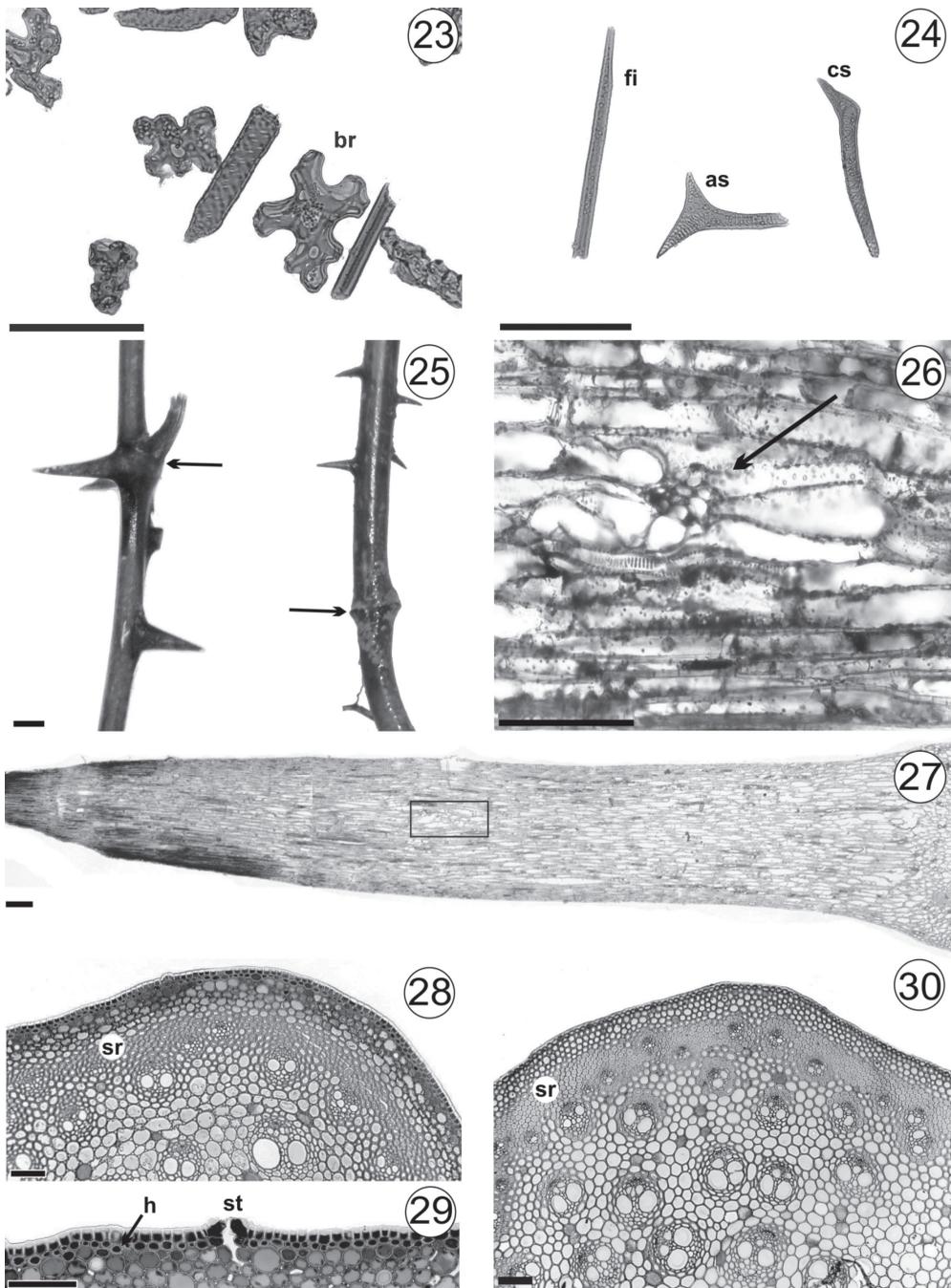


Fig. 23-30. *Smilax syphilitica* Humboldt & Bonpland ex Willdenow and *S. aff. syphilitica* Humboldt & Bonpland ex Willdenow. Dissociated mesophyll from *S. syphilitica* (23, 24). Overview of thorn distribution in *S. syphilitica* on the left and *S. aff. syphilitica* on the right (25). Nodal regions are indicated by arrows. Longitudinal section of the thorn (26-27). In 26, observe vascularization detail (arrow) indicated by the rectangle in 27. Cross section of the third internode in *S. syphilitica* (28-29) and *S. aff. syphilitica* (30). Note the presence of hypodermis in *S. syphilitica*. as=astroclereids; br=braciform cells; cs=columnar sclereids; fi=fibers; h=hypodermis; sr=sclerenchymatic ring; st=stomata. Scale bars: 23, 24, 26, 28-30=100µm; 25=1cm; 27=200µm.

In the cortex of *S. syphilitica*, phenolic and protein idioblasts are common, and spherical and polyhedral starch grains are observed exclusively in the central parenchyma.

Idioblasts containing raphides are restricted to the cortex in *S. syphilitica* and *S. aff. syphilitica*. Table 1 summarizes the distinguishing features between them.

TABLE 1
Distinctive features between *Smilax syphilitica* Humboldt & Bonpland ex Willdenow and *S. aff. syphilitica* Humboldt & Bonpland ex Willdenow

Characteristics	<i>S. syphilitica</i>	<i>S. aff. syphilitica</i>
Epicuticular wax ornamentation – adaxial surface	Granules and platelets; Network of rounded ridges	Rounded ridges in parallel
Epicuticular wax ornamentation – abaxial surface	Network of rounded ridges	Rounded ridges in parallel, interspersed by membranous platelets
Epidermal cells – surface view	Random arrangement	Parallel arrangement
Epidermal cells – cross-section	Slightly thick to thickened in anticlinal and inner periclinal walls	Thick periclinal and anticlinal walls
Stomata	Paracytics; random orientation	Paracytics and anisocytic; poles oriented towards the main vein
Lignified cells	Fibers, columnar sclereids, and astroclereids	Fibers and columnar sclereids
Calcium oxalate crystals	Prismatic crystals	Sand crystals
Thorns	Present in stem nodes and internodes	Present in stem internodes

DISCUSSION

First, it is noteworthy that very few individuals of the species examined could be located in the field. The reasons behind this distribution are uncertain, although as stated by Lacy (2000), a small population of scattered individuals is more vulnerable to extinction.

Leaf morphology did not vary between *S. syphilitica* and *S. aff. syphilitica* or between sampling areas. Although there are differences in the length and width of the leaf blades, the shape and venation patterns are the same. Guaglianone & Gattuso (1991) observed differences in leaf shape in *S. campestris* Grisebach and recognized that this character is subject to phenotypic variation among species according to its distribution.

According to Jenks & Ashworth (1999), variations in the deposition patterns of

epicuticular wax may be attributable to different stressors at various stages of plant development. However, Moore *et al.* (2008) only used these patterns to taxonomically differentiate the species for the first time (according to the authors). The ornamental patterns of epicuticular wax should not be used as the sole taxonomic tool for distinguishing species of *Smilax*; in *S. syphilitica* and *S. aff. syphilitica*, there are variations between individuals of *S. syphilitica* from the Atlantic and Amazon Rainforests. However, the patterns observed in *S. syphilitica* and *S. aff. syphilitica* differ from those described in *S. spicata* Vell., *S. rufescens* Griseb., *S. fluminensis* Steudel (Marquete & Pontes 1994), and *S. polyantha* (Martins & Apezatto-da-Glória 2006).

Laakso *et al.* (2000) found an increase in secondary wall thickness of *Pinus* L. leaf epidermis that had been subjected to high UVB

radiation. Wall thickness is dependent on variations in gene regulation (Caño-Delgado *et al.* 2000, Kim *et al.* 2002), thickness, and composition, which involve differentiation and communication between neighboring cells (Roberts 2001). This becomes clearer when examining the DNA of *Arabidopsis thaliana* (L.) Heynh, in which approximately 1 000 genes are involved in cell wall formation (Roberts 2001). Therefore, this mechanism appears to be more related to genomic characteristics of the *S. siphilitica* and *S. aff. siphilitica* specimens we analyzed rather than to environmental influences, suggesting that cell wall formation may consequently be of taxonomic value.

Analysis of epidermal cell walls in surface views has been a widely used method of characterizing species of the genus *Smilax* L. (Guaglianone & Gattuso 1991, Marquete & Pontes 1994, Guimarães *et al.* 2010), and we also considered that these cell walls have taxonomic value; both *S. siphilitica* and *S. aff. siphilitica* have sinuous walls but only in *S. aff. siphilitica* they are elongated in the cross-section of the blade and arranged in parallel. According to Haberlandt (1928) the cell wall sinuosity of the epidermis increases contact among adjacent cells, and may help to maintain leaf structure under mechanical stress. Variation in the degree of sinuosity in epidermal cell walls observed in *S. siphilitica* is related to stress during leaf differentiation (Avery 1933), the cuticle hardening process (Watson 1942), and variations in environmental conditions (Watson 1942, Omosun *et al.* 2008).

Classification of stomata and mesophyll has also been used for distinguishing species of *Smilax* (Guaglianone & Gattuso 1991, Marquete & Pontes 1994, Andreato 1997, Martins & Appezzato-da-Glória 2006, Palhares *et al.* 2009, Guimarães *et al.* 2010). Thus, leaves of *S. siphilitica* and *S. aff. siphilitica* can also be differentiated by this criterion. Among other criteria, Moore *et al.* (2010) used the orientation of the stomatal poles to differentiate six species (*S. verruculosa*, *S. megacarpa*, *S. bracteata*, *S. pottingeri*, *S. micro-china*, and *S. corbularia*). This analysis of stomatal poles also

enhanced our ability to distinguish between *S. siphilitica* and *S. aff. siphilitica*.

The presence of idioblasts with raphides on the leaf blade margins of *S. siphilitica* and *S. aff. siphilitica* is common for the genus (Yates & Duncan 1970, Guaglianone & Gattuso 1991, Marquete & Pontes 1994, Martins & Appezzato-da-Glória 2006). The prismatic and sand crystals observed throughout the mesophyll of *S. siphilitica* and *S. aff. siphilitica*, respectively, are tiny structures that together with the styloids, raphides, and druse crystals make up the five main forms of calcium oxalate crystals (Metcalf & Chalk 1950). Prychid & Rudall (1999) noted in a review that the absence, presence, and shape of calcium oxalate crystals are “useful taxonomic features” in monocotyledon classification. Crystal morphology can vary between different plant organs and tissues, whereas within the same species, this feature remains constant (Franceschi & Nakata 2005). Differences in crystal type were observed between *S. siphilitica* and *S. aff. siphilitica*.

The analysis of mesophyll type, lignified cell type, vascular bundle type, and sclerenchyma sheaths shared along the mid-vein are commonly used for identifying species of the genus (Yates & Duncan 1970, Guaglianone & Gattuso 1991, Marquete & Pontes 1994, Gattuso 1995, Martins & Appezzato-da-Glória 2006, Guimarães *et al.* 2010) and can also be used to differentiate *S. siphilitica* and *S. aff. siphilitica*.

The presence of hypodermis in the stem of *S. siphilitica* from the Atlantic Rainforest can be explained by the habitats in which individuals were collected; in other words, hypodermis was absent in epiphytes but present in shrubs. This is because in individuals from the Amazon Rainforest (all shrubs), the hypodermis is similar to *Lycopodiella cernua* (L.) Pic. Serm (Lycopodiaceae), a vine that keeps this tissue until it reaches a height of approximately one meter and needs mechanical support from adjacent plants to develop an epiphytic habit (Rowe *et al.* 2004). The presence of hypodermis is considered useful for differentiating between

species of Indian palm (Arecaceae) (Mathew & Bhat 2008). Similarly, paleobotany studies use this tissue for taxonomic distinction between extinct vine species (Li & Taylor 1998, Dunn *et al.* 2003). Here, the presence or absence of hypodermis as it relates to species, environment of origin, habit, and stem portion is a major caveat regarding use of this character to differentiate between groups.

Based on anatomical differences between *S. siphilitica* and *S. aff. siphilitica* we suggest further morphological studies in all *Smilax* species occurring in the Amazon region in order to verify if *S. aff. siphilitica* is in fact a new taxon.

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RESUMEN

Smilax L. en Brasil está representado por 32 táxones y es un género difícil en lo que respecta a su taxonomía porque las plantas son dioicas y presentan mucha variación fenotípica. El análisis y uso de los caracteres anatómicos de la hoja es reconocido frecuentemente como un exitoso método taxonómico para distinguir entre los táxones individuales cuando las flores están ausentes o hay diminutas diferencias. El objetivo de este estudio fue caracterizar los rasgos anatómicos de los órganos aéreos en *Smilax siphilitica* recolectados en el Bosque Atlántico en Santa Teresa-ES y de *Smilax aff. siphilitica* del Amazonas, Manaus, Brasil. Un total de tres muestras fueron recolectadas por sitio. Las muestras fueron fijadas en FAA 50, sumergidas en historesina, seccionadas en un micrótomó rotatorio, teñidas y montadas en resina sintética. Se hicieron pruebas histoquímicas y la ornamentación de la cutícula fue analizada con microscopía electrónica de barrido estándar. *S. siphilitica* y *S. aff. siphilitica* difirieron en la ornamentación de la cutícula, la disposición de células epidérmicas y el grosor de la pared, tipo y orientación de estomas, tipo de cristal de oxalato de calcio y la posición de las espinas del tallo. Estas características han probado ser de valor

diagnóstico y pueden apoyar la separación de dos especies, pero se necesitan futuros estudios para confirmar que *S. aff. siphilitica* es un nuevo taxon.

Palabras clave: tallos aéreos, cristales, cutículas, hojas, Smilacaceae.

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