ANATOMY, HISTOCHEMISTRY AND MICROMORPHOLOGY OF LEAVES OF Solanum granuloso-leprosum DUNAL

ANATOMIA, HISTOQUÍMICA E MICROMORFOLOGIA DE FOLHAS DE Solanum granuloso-leprosum DUNAL

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ABSTRACT: In the present work the anatomical, histochemical and micromorphological features of S. granuloso-leprosum leaves were approached in order to evaluate its characteristics associated with its pioneer role. Glandular and non-glandular trichomes were observed on both epidermal surfaces, although in greater number on the abaxial surface. Stellate trichomes presented a thick lignified cell wall. Leaves were amphiestomatic with a single palisade layer and a slightly smaller spongy parenchyma. The epidermal cells of the abaxial surface were shorter than the adaxial ones, both with stomata paracytic. Vascular bundles were bicolateral and idioblasts with conspicuous crystalliferous inclusions were observed in the mesophyll. Lipid drops were evidenced in the spongy parenchyma by Sudan III, Nile Blue, Nadi reagent and Sudan Black histochemical tests. Negative results for alkaloids and phenol compounds were observed. The evaluated anatomical and histochemical data highlights mesophytic characteristics in accordance with S. granuloso-leprosum pioneer plant role.

KEYWORDS: Pioneer plant. “Curvitinga”. Plant anatomy

INTRODUCTION

Solanum granuloso-leprosum Dunal. is a small tree, with simple and entire leaves which produces fleshy fruits with small seeds (PETENATTI et al., 1998, VÁLIO; SCARPA, 2001). This plant species is known in Brazil as “cuvitinga” and “fumeiro”, the botanical synonymy Solanum verbacifolium L. is also acknowledged (International Plant Names Index 2007). S. granuloso-leprosum is considered a pioneer plant (VÁLIO; SCARPA, 2001, FERREIRA et al., 2009), distributed in the northeast of Argentina, Uruguay, Paraguay, and South and Southeast of Brazil, occurring in secondary forests, and colonizing disturbed areas and ecosystems (FERREIRA et al., 2001, CASTELLANI et al., 2008, FERREIRA et al., 2009).

Fowler and Carpanezzi (1997) emphasized that the main interest associated with this species was the recovery of degraded ecosystems. Considered a pioneer species (PETENATTI et al., 1998, FERREIRA et al., 2001, FERREIRA et al., 2009), it was verified that S. granuloso-leprosum fruits attracted a variety of animals, which participated on its seed dispersion (CÁCERES; MOURA, 2003, ALMEIDA et al., 2005). On the other hand, S. granuloso-leprosum was considered one of the potential host of Miridae (Heteroptera), a taxon whose species may cause damages to cultivated plants and also may be predators in potential to be used in biological control (FERREIRA et al., 2001).

This Solanaceae was also described as a medicinal plant (PETENATTI et al., 1998, SHYUR et al., 2005, JOUZIER, 2005, ARAMBARRI et al., 2006). In Argentina, together with other tree medicinal species, S. granuloso-leprosum is known by the same vernacular name “ambay”, and is used as anti-inflammatory and calmative (PETENATTI et al., 1998).

The increase of the worldwide market of biotechnological and pharmaceutical products (SHYUR et al., 2005) evidenced the importance of the knowledge and conservation of the genetic material, besides the need to evaluate, explore and maintain the genetic diversity. In the present work, the anatomical, histochemical and micro morphological characterization of S. granuloso-leprosum leaves were approached.

MATERIAL AND METHODS

Plant Material

Leaves of Solanum granuloso-leprosum Dunal. (Solanaceae) were collected in a secondary forest at the Federal University of Viçosa. The shoots with flowers and fruits were dried, and
exsiccates were deposited at the VIC Herbarium (Federal University of Viçosa), under the numbers 31357 and 31358. The identification was confirmed by Dr. João Renato Stehmann (UFMG), specialist in Solanaceae.

**Light microscopy: Study of leaf surface**

Samples of leaf surface with approximately 0.5 cm² were bleached with chloral hydrate, until the tissues were completely translucent. The samples were washed with distilled water, stained for 30 min with Astra Blue and Safranin (9: 1 v/v) (ROESER, 1962), dehydrated and mounted with synthetic resin (Permount, Fisher). The density of stomata per area was determined in samples from five expanded and healthy leaves, and an average of 33 fields per leaf, and expressed by the number of stomata per mm².

**Histological analysis**

*S. granuloso-leprosum* Dunal. leaves were fixed in FAA₅₀ (formalin, acetic acid, ethanol 50%; 5: 5: 90 v/v), for 24 h. Subsequently, the samples were dehydrated in ethylic series and included in metacrylate (Historesin, Leica). The leaves were sectioned transversally (8 µm thick) in an automatic advance microtome (RM 2155, Leica). The sections were stained with Toluidine Blue pH 4.0 (O’BRIEN; MCCULLY, 1981) for 10 min, and mounted with synthetic resin (Permount, Fisher).

**Histochemical analysis**

The histochemical tests were performed with fresh tissue samples, sectioned with a Microtome LPC (“Rolemberg & Bhering Comércio e Importação Ltda”). The following histochemical tests were carried out: Sudan III (JOHANSEN, 1940) and Sudan Black (LISON, 1960), for lipids; Nile Blue, for neutral and acidic lipids (JENSEN, 1962); NADI Reagent, for essential oils and oleoresins (DAVID; CARDE, 1964); chlorine vanillin, for phenol compounds (MACE;HOWELL 1974); ferric chloride, for phenolic compounds (JOHANSEN, 1940); potassium dichromate, for tannins (GABE, 1968); Wagner reagent and Dittmar reagent, for alkaloids (FURR; MAHLBERG, 1981); XP, for proteins (VIDAL, 1977); floroglucinol, for lignin (JENSEN, 1962); and ruthenium red, for pectic substances (JOHANSEN, 1940). One treatment without any reagent or staining was mounted in water and used as blank test.

All the photographic documentation for light microscopy were performed with a microscope (Olympus AX70) equipped with U-Photo system.

**Micromorphological analysis - Scanning electronic microscopy**

The micromorphological analysis on the basis of the Scanning Electron Microscopy was carried out with completely expanded leaf segments, fixed in Karnovsky mixture (KARNOVSKY, 1965). The samples were submitted to vacuum for 24 h and kept in a 0.05 M potassium phosphate buffer for 30 min. After that, they were dehydrated in ethylic alcohol series. Leaf samples were dehydrated by critical point of CO₂ (BOZZOLLA; RUSSEL, 1992) using the Balzer's Critical Point Dryer (CPD020, Bal-Tec, Balzers, Liechtenstein), and set on stubs previous to the metallization with gold in the Sputter Coater (Balzer FDU010, Bal-Tec, Balzers, Liechtenstein). After the metallization with a fine gold layer (20 nm), the material was photographed using the scanning electron microscope (Model 1430VP, LEO).

**RESULTS**

*Solanum granuloso-leprosum* leaves presented trichomes on the abaxial and adaxial surface, even though they were more frequent on the former and non-glandular trichomes varied from multi seriated and multi cellular to unicellular and glandular trichomes were observed as well (Figures 1 A to K and 2 A to G).

In addition to thick and lignified cell walls (Figure 1K, 5E and 5H), stellate trichomes may have own several ramifications (Figure 2A, 2B, 2C, and 2E) which may imprison or provide an appropriate environment to some minute arthropods (Figure 2F). The trichome density is higher on the ab axial surface (Figures 2A, 2B and 2C). A few glandular trichomes were also observed on both surfaces (Figures 2G and 6B), although the density of non-glandular trichomes caused difficulties for the observation of other epidermal cells. The paracytic stomata were at the same level of common epidermal cells (Figure 2H).
Figure 1. Scanning Electron Microscopy photograph of the leaf surface of *Solanum granulosoleprosum* Dunal. A – Unicellular tector trichomes; B – tector trichome, note that there are projections at the trichome base; C – tector trichome, note that there is a larger projection/ramification at the trichome base; D – tector trichome, note that there are two larger projection/ramification at the trichome base; E – tector trichome, note that there are three larger projection/ramification at the trichome base; F – tector trichome, note that there are four larger projection/ramification at the trichome base; G – tector trichome, note that there are five larger projection/ramification at the trichome base; H – tector trichome, note that there are six larger projection/ramification at the trichome base; I – tector trichome, note that there are eight larger projection/ramification at the trichome base; J – another angle from the six ramification tector trichome; and K – multicellular and multisseriated tector trichome, note the thick secondary cell wall. Scale Bars = 20 µm.
The anticlinal walls of epidermal cells were slightly sinuous (Figure 2H), the abaxial cells being shorter than the adaxial ones (Figures 3B and 3C). The epidermis presented only one stratum (Figure 4A) and dimensions for the cells of the superior and inferior surfaces were respectively 12.8±2.4 and 7.1±1.5 µm (Figure 3B) with the corresponding CVs of 18.8 and 20.9%.

A single palisade layer and a slightly smaller spongy parenchyma constituted the mesophyll (Figure 4A). Leaf blade thickness exhibited an average and standard deviation of 131.4±17.2 µm, with a CV of 13.1%, whereas the dimensions for the palisade and spongy parenchyma’s were respectively 63.3±12.0 and 48.2±11.3 µm (Figure 3B). The corresponding CVs were 18.8 and 20.9%.

Except for the sub epidermal layers, the parenchyma in the mid vein was composed by larger cells compared to the mesophyll, some idioblasts with crystal sand and did not present chloroplasts (Figures 4B and 4C). The vascular bundles were bicolateral and the phloem almost surrounds xylem cells completely (Figures 4B and 4C).

Some idioblasts in the palisade and spongy parenchyma, in the midrib parenchyma, and in the phloem contained conspicuous crystalliferous inclusions (Figures 4A, 4B and 4D) which may be depicted in polarized light (Figures 4C and 4E). These inclusions may occupy considerable space in these idioblasts (Figures 4D and 4E)
Figure 3. Histometry and stomata index of *Solanum granuloso-leprosum* Dunal. leaves. A – Percentage of abaxial and adaxial epidermis, and palisade and spongy parenchyma, in relation to the total leaf width. All values are given in percentage (%). Superior Epidermis Ratio (SER); Palisade Parenchyma Ratio (PPR); Spongy Parenchyma Ratio (SPR); and Inferior Epidermis Ratio (IER). B – Abaxial and adaxial epidermis, and palisade and spongy parenchyma heights; All values are given in micrometer (μm). Superior Epidermis (SE); C – Stomata index of adaxial and abaxial epidermis. Palisade Parenchyma (PP); Spongy Parenchyma (SP); and Inferior Epidermis (IE). Averages were taken based on 411 samples each and a mean number of 5 measurements per field. The coefficient of variation (CV) were 21.5, 13.1, 17.3 and 23.0, SER, PPR, SPR and IER, respectively. Average and standard deviation of stomata index were based on average of 182 fields per each face and 36 fields per sampled leaf. The CV for the stomata index were 10.6 and 14.0% for the abaxial and adaxial faces, respectively.
Figure 4. Cross section in *Solanum granulos-o-leprosum* Dunal. leaves. A – Leaf blade section stained with Toluidine Blue, detail of the mesophyll and adaxial and abaxial epidermis. Note the idioblasts with cristaliferous sand in spongy and palisade parenchyma. B – Detail of mid vein under light microscopy; C – detail of the same mid vein under polarized light. Note several idioblasts in the parenchyma and smaller ones associated with phloem. D – Detail of parenchyma cells and some idioblasts with cristaliferous sand under light microscopy; and E – Detail of the same parenchyma cells under polarized light. Scale Bars in Figure A = 100 µm; in Figures B and C = 200 µm; and in Figures D and E = 100 µm.
In the mid vein, a small protuberance in the ad axial surface and a bigger one in the ab axial surface, mostly constituted of collenchyma, parenchyma and vascular tissue (Figures 5A to 5C). Untreated tissues for histochemical tests were documented (Figures 5A, 5D and 5G), whereas the positive reaction for floroglucin (Figures 5B, 5E and 5H) and red ruthenium tests (Figures 5C, 5F and 5I) were observed. Attention is drawn to the xylem elements, lignified and fully differentiated (Figures 5B and 5H), whereas pectic substances were evidenced in parenchyma, collenchyma, and phloem cells (Figures 5E, 5F and 5I). Histochemical test with XP reagent revealed that there were no protein accumulation in a particular cell or location in *S. granuloso-leprosum* leaves (Figure 6A), although a stained background was observed.

The midrib exhibited one sub epidermal chlorenchyma and several layers of angular collenchyma, approximately 12 and 5 cell layers in the ad axial and ab axial faces, respectively (Figures 5F and 5G). The grayish cells corresponded to the idioblasts with crystal sand (Figures 2D, 4, 5A, 5C and 5I).

Occasional glandular trichomes were observed (Figure 6B). A weak reaction with potassium dichromate was observed suggesting the presence of phenolic substances in these trichomes. Contrarily to non-glandular trichomes, the base and head of glandular trichomes cell walls were thin and non-lignified (Figure 6A). Except for floroglucinol (Figure 6C), no other test were positive for the non glandular trichomes.

**Figure 5.** Histochemical tests in *Solanum granuloso-leprosum* Dunal. leaves (transversal sections). A – Control treatment, detail of the mid vein; B – tissue treated with floroglucinol, detail of the mid vein; C – mid vein treated with ruthenium red; D – leaf blade control treatment; E – leaf blade treated with floroglucinol; F – mid vein treated with ruthenium red, detail of collenchyma tissue in the adaxial (upper figure) and in abaxial face (lower figure), note a layer of chlorophyll parenchyma above and beneath the collenchyma, respectively; G – non treated sub epidermal collenchyma layers in the abaxial face of the leaf mid vein; H – detail of xylem (mid vein) treated with floroglucinol; and I – detail of phloem in mid vein treated with ruthenium red. Scale Bars in Figures A, B and C = 500 µm; in Figures D, E, G, H and I = 100 µm; in Figure F= 100 µm.
Figure 6. Histochemical tests in *Solanum granulosoleprosum* Dunal. leaves (transversal sections). A – leaf blade treated with XP reagent, note that the mesophyll, although stained, did not present a conspicuous cell or organelle marked in particular; B – detail of a glandular tricome; C – leaf blade treated with Sudan III, note that the spongy parenchyma exhibit lipid bodies stained with this reagent; D – detail of a trichome tector multiseriated, note the lignified secondary cell wall of the trichomes; E – leaf blade treated with Nile Blue reagent, note the lipid bodies stained; F – leaf blade treated with Nadi reagent, note the lipid bodies stained; G – leaf blade treated with Sudan Black, note the lipid bodies stained. Scale Bars in Figures A to G = 50 µm; in Figures E, F and G details = 100 µm.

Sudan III (Figure 6D), Nile Blue (Figure 6E), Nadi reagent (Figure 6F) and Sudan Black (Figure 6G) tests indicated the presence of lipid bodies in the spongy parenchyma, which suggests the presence of a mixture of essential and or neutral oils and acidic lipids. Additionally, *S. granulosoleprosum* cuticle was more evident when using Sudan III, Nile Blue and Nadi, and corresponded to one sixth to one tenth of the epidermis height, and was thicker in the ad axial surface. Negative results for the chlorine vanillin, ferric chloride, Wagner and Dittmar reagents suggest that phenol compounds and alkaloids were not stored in *S. granulosoleprosum* leaves.
DISCUSSION

The different trichomes observed on the epidermal surfaces of *S. granuloso-leprosum* leaves are in accordance with the Solanaceae literature (SIDDIAS, 1980; D’ARCY et al., 2001; MAITI et al., 2002; ELIAS et al., 2003; ALIERO et al., 2006; ARAMBARI et al., 2006; ALVES et al., 2007). Following Metcalfe and Chalk’s (1979) classification of stellate trichomes, stellate multiangulate sessile and stalked over the surface were found in *S. granuloso-leprosum*. Considering the number of ramifications and the differences on trichome density, it is hypothesized that the trichome and this morphogenesis process started at different times, what would account for the differences observed.

Alves et al. (2007) observed the occurrence of glandular and non-glandular trichomes in *Solanum cernuum*, although unicellular non-glandular trichome was not reported. Previous work on *Solanum* anatomy established that the trichomes are very diverse in shape and size (EDMONDS, 1982).

A few glandular trichomes were also observed in the ad axial (Figure 2G) and on the abaxial epidermis, although the density of non-glandular trichomes made difficult the observation of glandular trichomes and stomata. This is especially useful when approaching its use as a medicinal plant, considered the different trichomes observed in *S. granuloso-leprosum*, and *Solanum* trichome diversity (EDMONDS, 1982; MAITI et al., 2002; ALIERO et al., 2005; ALVES et al., 2007).

Although considered a pioneer plant, *S. granuloso-leprosum* exhibited, in general, mesophytic characteristics. It displayed large leaves with one palisade layer, and a thinner leaf and epidermis, and a higher number of stomata per mm² than *Capparis spinosa*, a plant adapted to prolonged summer drought (RHIZOPOULOU; PSARAS, 2003). Despite the great density of non-glandular trichomes on *S. granuloso-leprosum* leaves, it probably would not be as adapted to a drought condition as it is to high light intensities. Nevertheless, Chaves Filho and Staccianrini-Seraphin (2001) observed that the osmotic adjustment was one of the mechanisms of drought resistance detected in a related species *S. lycocarpum*. Additionally, in xeric conditions, the gaseous exchange may be favored by means of a greater stomatic area (MEDRI; LLERAS, 1980).

*Solanum granuloso-leprosum* epidermis had only one stratum with slightly sinuous cell walls, what may be associated with an adaptation to reduce the excessive waist of water (MEDRI; LLERAS, 1980). Irregular epidermal cells with different sizes are observed, what also reflected on the coefficient of variation (CV) of the epidermis height in the histometry data (Figures 3B and 3C).

The paracytic stomata were at the same level of common epidermal cells, and, even though the number of stomata per area was different, they were observed on both leaf epidermal surface. Contrarily, Pentenatti et al. (1998) observed only anisographic stomata in *S. granuloso-leprosum*.

Maiti et al. (2002) observed two types of stomata, anisographic present in *D. inoxia*, *D. stramonium*, *N. glauca*, *P. viscosa*, *S. americanum*, and *S. rostratum*, and anomocytic in *L. esculentum*, *S. nitida*, *S. eleagnifolium*, *S. erianthus*, *S. nigrescens*. Micromorphology of *S. pseudocapsicum* revealed amphystomatic leaves presenting anisographic stomata (ALIERO et al., 2005). *S. lycocarpum*, displayed the same variation in the types of stomata observed (D’ARCY et al., 2001; ELIAS et al., 2003) observed for *S. granuloso-leprosum*, similar number of stomata per area for the ab axial epidermis, although a higher number of stomata for the ad axial surface (ELIAS et al., 2003).

As the abaxial epidermis displayed a higher number of stomata per area, the trichomes may be just protecting the leaf from losing too much water, what is considered to be a xerophytic feature (FAHN; CUTLER, 1992). Moreover, the trichomes may be also indirectly associated with the transpiration decrease, reducing the solar radiation that reaches the leaves (FAHN, 1986).

*S. granuloso-leprosum* mesophyll was constituted of a single palisade layer, with a spongy parenchyma cell layers slightly smaller than the palisade, and the abaxial epidermis shorter than the adaxial (Figure 4A). Some idioblasts with cristaliferous sand were present in the parenchyma and in the phloem and may occupy considerable space in these cells (Figure 4).

Prominent midrib and the presence of crystal sand in this region were reported for other Solanaceae, like *Tubocapsicum anomalum* (D’ARCY et al., 2001). Pentenatti et al. (1998) reported the same bifacial structure, although also mentioned the presence of lipids and mucilage in addition to the crystal sand. Occasional druses were also reported at spongy and palisade parenchyma (D’ARCY et al., 2001). Different forms of crystals were also observed for ten *Solanum* species, clustered in the form of sands in *L. esculentum*, *N. glauca*, *P. viscosa*, *S. americanum*, *S. eleagnifolium*, *S. erianthus*, *S. rostratum* and *S. triquetrum*; druses...

The mid vein of S. granuloso-leprosum leaves was evident in transverse sections, with a small protuberance in the ad axial surface and a bigger one in the ab axial surface (Figures 5A to 5C), mostly constituted of vascular tissue, parenchyma and collenchyma. Pectic substances were evidenced in fundamental tissues (Figures 5C, 5F and 5I) whereas stellate trichomes and xylem cells presented lignified cell walls (Figures 5B, 5H and 6C). Similarly to Arambarri et al. (2006), no sclerenchymatic tissue was observed in S. granuloso-leprosum leaves (Figures 5B, 5E and 5H).

Histochemical test with XP reagent did not detect protein accumulation in S. granuloso-leprosum leaves (Figure 6A). Despite the weak reaction with potassium dichromate in glandular trichomes, no other leaf part was stained. Contrarily, high tannin contents were reported for D. stramonium, N. glauca, Solandra nitida, S. americanum and S. triquetrum (MAITI et al., 2002).

The positive results with Sudan III (Figure 6D), Nile Blue (Figure 6E), Nadi reagent (Figure 6F) and Sudan Black (Figure 6G) tests indicates a good characteristic to discriminate S. granuloso-leprosum, since only for Solanum nitida the presence of lipids had been reported (MAITI et al., 2002).

Although Solanaceae is known for containing alkaloids (MAITI et al., 2002), negative results for the histochemical tests suggests that phenol compounds and alkalis were not stored in S. granuloso-leprosum leaves. Other substances, such as solanin, saponin and tannins were reported in Petenatti et al. (1998) review, nevertheless, it was not found in S. granuloso-leprosum leaves. It was hypothesized that the seasonal or environmental influence on the production might have accounted for the absence of such substances in leaf tissues.

Maiti et al. (2002) observed that the majority of the species studied presented variations on the alkaloid and protein content.

The present histochemical and anatomical approach allowed us to verify that S. granuloso-leprosum presented mesophytic and xerophytic characteristics, features that are in accordance with its phytossociological role as a pioneer plant. A well differentiated dorsiventral mesophyll, amphiestomatic leaves, the presence of a great density of non-glandular trichomes on the epidermis, and idioblasts with crystal sand were observed. Curiously, substances such as alkaloids were not observed in leaves, although lipid drops were present in the chlorophyll parenchyma and may represent important features in an ecological sense, or for medicinal usage. The histochemical and micro morphological features also suggest that the non-glandular trichomes grant leaves with some protection role.

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RESUMO: No presente trabalho foi abordada a anatomia, histoquímica e micromorfologia de folhas de S. granuloso-leprosum no intuito de se levantar características associadas a sua função de espécie pioneira. Tricomas glandulares e não glandulares foram observados em ambas as faces da epiderme, apesar de maior número na superfície abaxial. Tricomas estrelados apresentaram uma parede espessa e lignificada. As folhas são anfíestomáticas com uma única camada de paliçada e um parênquima lacunoso com células menores que as do paliçádio. As células da face abaxial da epiderme são menores, e ambas as faces apresentam estômatos paracíclicos. Os feixes vasculares são bicilaterais e idioblastos com inclusões cristalinas conspicuas. Gotas de lipídeo foram evidenciadas no parênquima lacunoso com os testes histoquímicos de Sudan III, azul do Nilo, reagente de Nadi e Sudan Black. Foram observados resultados negativos para alcaloides e fenóis. Os dados anatômicos e histoquímicos avaliados permitiram verificar que S. granuloso-leprosum apresentam características mesófilas, os quais estão em conformidade com o seu papel fitossociológicos como pioneira.


REFERENCES

Anatomy, histochemistry...


