Bacterial leaf glands in Styrax camporum (Styracaceae): first report for the family

Silvia Rodrigues Machado, Simone de Pádua Teixeira, and Tatiane Maria Rodrigues

Abstract: In this study, we recorded, for the first time, the occurrence of leaf glands in a member of Styracaceae and their association with bacteria. Samples of Styrax camporum Pohl shoot apices and leaves at different developmental stages were prepared according to the conventional techniques for light and electron microscopy. Glands are emergences constituted by epidermal and parenchyma cells and are differentiated into a secretory body on a short nonsecretory stalk supplied with phloem. Actively secreting glands occur from leaf primordia to mature leaves and produce mucilage that accumulates inside schizogenous intercellular spaces. The epidermal secretory cells have abundant cytoplasm rich in hyperactive dictyosomes, an extensive endoplasmic reticulum, and modified plastids. Bacteria enter the gland via the intact surface and proliferate in the intercellular spaces of the glands. Once inside the intercellular spaces of the glands, bacteria enter the cells owing to the weakening of the anticlinal and inner periclinal cell walls and by phagocytosis. Strands of actin filaments occur near the endocytical vesicles containing degrading bacteria. Accumulations of phenolic compounds and callose could explain the absence of bacteria in the stalk cells. The presence of bacteria inside the leaf glands of S. camporum is a regular and cyclic trait. The significance of the bacteria (not yet identified) and the type of interaction between these two organisms remain unknown.

Keywords: anatomy, bacteria, leaf glands, Styrax camporum, symbiosis, ultrastructure.

Introduction

In this paper, we recorded, for the first time, the occurrence of a glandular structure in a member of Styracaceae and its association with bacteria. Leaf–bacteria symbioses have been described in several tropical genera of three flowering plant families, Myrtales, Rubiaceae, and Dioscoreaceae (Horner and Lersten 1968, 1972; Lersten and Horner 1976; Van Oevelen et al. 2003), but not in Styracaceae.

Styracaceae is a family of woody plants that is best known as the source of benzoin (gum benjamin) and storax resin, which is used as an antisepptic, inhalant, and expectorant, as well as in the manufacture of perfumes and vanilla-cream candies; it is also noted for several beautiful ornamental shrubs and trees. The family occurs in relatively warm parts of the world, such as the Mediterranean, eastern Asia, the Malay Archipelago, and parts of North and South America (Hutchinson 1973; Cronquist 1981). Styrax L., the most important of the 11 genera, contains approximately 130 species, 25 of which occur in Brazil, from the rainforest to the Cerrado (Nakajima and Monteiro 1986; Souza and Lorenzi 2005).

The occurrence of stellate (or lepidote) pubescence is a synapomorphy of the Styracaceae within Ericales (Judd et al. 1999; Fritsch et al. 2001), but glandular structures were not reported. However, a study of leaf indument in Styrax camporum Pohl, a shrubby species that is common in the Brazilian Cerrado (savanna-like vegetation), recorded that young vegetative parts and mature
Figs. 1–9. General features of leaf glands in *S. camporum*. Fig. 1. Photomicrograph, cross section of petiole showing a gland comprised of a short stalk (SL) and a secretory body (SB). Scale bar = 50 μm. Fig. 2. Photomicrograph, cross section of leaf blade showing a prominent gland (arrow) on edge. Scale bar = 500 μm. Fig. 3. Scanning electron micrograph (SEM) of leaf edges showing glands (arrows) immersed in dense stellate indumentum. Scale bar = 300 μm. Fig. 4. SEM showing irregular surface of gland covered by a smooth cuticle. Fig. 5. Photomicrograph showing glands with prominent epidermal cells containing dense bodies. Scale bar = 50 μm. Fig. 6. Photomicrograph showing secretion accumulated inside intercellular channels in apical region of gland (arrow) side by side with broken gland. Scale bar = 50 μm. Fig. 7. Photomicrograph showing distended cuticle (CT) and secretion accumulated (*) in distal region of gland. Scale bar = 50 μm. Fig. 8. Surface view of gland (SEM) showing intact stalk cells and broken body cells. Scale bar = 20 μm. Fig. 9. Transmission electron micrograph showing mucilaginous (MU) content in central space and rupture of cuticle (arrow) in distal region of gland. Scale bar = 50 μm.
leaves have glands producing a sticky exudate (Machado 1991). A detailed analysis over various intervals and for a longer period of time revealed that the glands also occur in the reproductive parts and are always colonized by bacteria. These observations prompted us to investigate these glands and the fate of the bacteria.

The aim of this study was to characterize the nature, structure, and functioning of the bacterial leaf glands in *S. camporum* from a developmental perspective, as well as the mechanism of bacterial entrance in the gland, using light and electron microscopy.

**Materials and methods**

**Study area**

The Cerrado, or the Brazilian savanna, covers nearly 2 million km², approximately 22% of the country’s land surface. The Cerrado biome is extremely variable in terms of physiology, ranging from open grassland to forest with a discontinuous grass layer. A continuum of savanna formations between these two extremes spans the entire range of woody-plant density (Oliveira Filho and Ratter 2002). The Cerrado is characterized by wet (October–April) and dry (May–September) seasons; the deep, acidic, and sandy soils contain low levels of organic matter and phosphorus and are rich in aluminum. Concomitant with the seasonal water deficit, the Cerrado environment experiences a high irradiance load and elevated vapor-pressure deficits (Oliveira and Marquis 2002).

**Plant material**

Terminal and lateral shoot apices and leaves in different developmental stages were collected from five branches of at least 10 individuals of *S. camporum* growing in areas of Cerrado sensu stricto (i.e., dense scrub of shrubs and trees) in the state of São Paulo, Southeast Brazil (22°55'S, 48°30'W), during the 1985–1990, 2003–2005, and 2008–2010 periods.

With a stereoscopic microscope, samples with immature glands, actively secretory glands (turgid and pale yellow), and senescent glands (dark brown and shrunk) were collected for anatomical, histochemical, and ultrastructural studies. Voucher specimens were deposited in the Herbarium at the Department of Botany (BOTU), Botucatu Campus of Universidade Estadual Paulista (UNESP), Brazil.

**Anatomical analysis**

For anatomical studies, the samples were fixed in FAA 50% formaldehyde–acetic acid–alcohol 50% (Johansen 1940) and processed according to the conventional methods for (2-hydroxyethyl)methacrylate resin embedding (HistoResin, Leica, Heidelberg, Germany), sectioning, and mounting of the histological sections. Serial cross and longitudinal sections with a thickness of 5–8 μm were made using a rotatory microtome, stained with 1% toluidine blue in 1% aqueous sodium tetraborate solution (O'Brien et al. 1964), and mounted in synthetic resin. The observations and image documentation were performed using a Leica DMR microscope with a digital camera.

**Histochemical analysis**

Sections of all of the materials were subjected to the following histochemical tests: Periodic Acid–Schiff (PAS) reaction to water-insoluble polysaccharides (Jensen 1962), ruthenium red for pectic substances (Johansen 1940), Sudan IV for total lipids (Johansen 1940), mercuric bromophenol blue for total proteins (Mazia et al. 1953), tannic acid–ferrous chloride for mucilage (Pizzolato and Lillie 1973), 0.1% aniline blue for callose (Kraus and Arduin 1997), and ferric trichloride for phenolic compounds (Johansen 1940). The control samples were tested according to the specifications for each test. For all of the tests, the sections were mounted in glycerin under a coverslip. The observations and image documentation were performed using a Leica DMR microscope coupled with a digital camera.

<table>
<thead>
<tr>
<th>Reagent Substance</th>
<th>Color</th>
<th>Glandular stalk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Periodic Acid–Schiff</td>
<td>Non-cellulosic polysaccharides</td>
<td>Pink</td>
</tr>
<tr>
<td>Acid–Schiff</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sudan IV</td>
<td>Total lipids</td>
<td>Red</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>Proteins</td>
<td>Blue</td>
</tr>
<tr>
<td>Ruthenium red</td>
<td>Mucilage</td>
<td>Pink</td>
</tr>
<tr>
<td>Ferric trichloride</td>
<td>Phenolic compounds</td>
<td>Dark green</td>
</tr>
<tr>
<td></td>
<td>to brown</td>
<td></td>
</tr>
<tr>
<td>Aniline blue</td>
<td>Callose</td>
<td>Blue</td>
</tr>
<tr>
<td>Tannic acid–ferrous chloride</td>
<td>Mucilage</td>
<td>Brown</td>
</tr>
</tbody>
</table>

**Note:** +, positive; −, negative.

**Ultrastructural analysis**

For scanning electron microscopy, the samples were fixed in 2.5% glutaraldehyde in a 0.1 mol·L⁻¹ sodium phosphate buffer, pH 7.3, overnight at 4 °C, dehydrated in a graduated acetone series, critical point-dried using CO₂, mounted on aluminum stubs, gold-coated, and examined using a Philips 515 scanning electron microscope at 20 kV.

For transmission electron microscopy, the samples were fixed in 2.5% glutaraldehyde in a 0.1 mol·L⁻¹ sodium phosphate buffer, pH 7.3, for 6–8 h at 4 °C, post-fixed with 1% osmium tetroxide solutions in the same buffer for 2 h at room temperature, dehydrated in a graduated ethanol series, and embedded in epoxy resin (Araldite). The ultrathin sections were contrasted with 5% uranyl acetate in 50% ethanol and lead citrate (Reynolds 1963) and examined using a Philips CM 100 transmission electron microscope at 80 kV.

**Results**

**General characteristics of the glands**

Leaf glands are emergences differentiated into a glandular body on a nonsecretory short stalk. The body shape varies according to the developmental stage of the gland. The body of mature glands presents one layer of secretory epidermal cells arranged around a parenchymatous central axis and covered by a continuous cuticle (Fig. 1).

Actively secreting glands are present from leaf primordia to mature leaves. As leaf maturation progresses, the glands commonly dry up and fall off but persist on the petiole (Fig. 1) and on the margin of the mature leaf blade (Fig. 2). These glands can be solitary or grouped and are partially hidden by a dense indumentum formed by stellate trichomes (Fig. 3).

The glands reach maturity and become secretory before the leaf reaches 5 mm in length. Mature glands have an uneven surface (Fig. 4) due to the irregular size and shape of the epidermal cells (Fig. 5). Mature glands are turgid, pale yellow, and secrete hyaline and fluid exudates but display a gradual shrinking and color changes to dark brown toward senescence. As maturation progresses, the epidermal cells of the glands become loosely arranged and contain abundant dark bodies (Fig. 5). The secretion, composed of mucilage rich in proteins and polysaccharides, is stored inside intercellular spaces in the distal portion of the gland (Fig. 6). Following the accumulation of the secretion, the intercellular spaces enlarge, leading to the detachment of the cuticle (Figs. 6 and 7) and posterior rupture of the outer cell wall and cuticle of the epidermal cells (Figs. 6, 8, and 9). The secretion covers the leaf primordia and young leaves in the shoot tip.

The results of the histochemical tests are presented in Table 1.
Figs. 10–18. Transmission electron micrographs of epidermal secretory cells in S. camporum leaf glands. Fig. 10. Immature glands are comprised of thin-walled cells with prominent nucleus (NU) and nucleolus, abundant cytoplasm, and poorly developed vacuoles (VA). Arrows indicate plasmodesmata in anticlinal walls. Scale bar = 2.5 μm. Fig. 11. Mature glands showing cluster of vesicles (VE) in the apical region of epidermal cells, nucleus with irregular outline, and vacuole (VA) containing electron-dense material or filled with oil (OL). Scale bar = 2 μm. Fig. 12. Detail of outer epidermal cell wall (CW) showing cuticle (CT) with electron-dense ramifications in cuticle layer. Scale bar = 0.3 μm. Fig. 13. Plastid with prolamellar body and oil drops (OL). Scale bar = 0.3 μm. Fig. 14. Endocytical vesicles (VE) containing electron-dense bodies. Scale bar = 0.3 μm. Fig. 15. Cytoplasm rich in free ribosomes, rough endoplasmic reticulum (RER), dictyosomes (DI), mitochondria (MI), and small vacuoles (VA) with electron-dense bodies. Scale bar = 0.3 μm. Fig. 16. Proliferate smooth endoplasmic reticulum (SER) surrounding vacuole filled with oil (OL), NU, nucleus. Scale bar = 0.2 μm. Fig. 17. Sinuous plasma membrane and numerous vesicles in peripheral cytoplasm. Arrow indicates vesicle juxtaposed to plasma membrane. Scale bar = 0.3 μm. Fig. 18. Strands of actin-filaments (AC) near vacuoles (VA) containing electron-dense bodies. Some dictyosomes (DI) are highly active. Scale bar = 0.3 μm.

Ultrastructure of the glands

Immature glands are formed by a mass of thin-walled cells covered by a thin cuticle; these cells are characterized by conspicuous spherical nuclei and nucleoli, an abundant granular cytoplasm, and a poorly developed vacuole system (Fig. 10). Numerous mitochondria, scattered small lipid bodies, and plastids with starch grains are noticeable in the cytoplasm.

The epidermal cells of mature glands (Fig. 11) exhibit cuticle with two distinctive strata: the cuticle proper and the cuticular layer with electron-dense ramifications, which extends from the polysaccharide portion of the outer cell wall constituting a pectin reticulate network (Fig. 12). Numerous mitochondria, a large number of dictyosomes with many cisternae and associated vesicles, an extensive rough endoplasmic reticulum, and modified plastids are the main ultrastructural features of the cytoplasm of epidermal cells (Figs. 11 and 13-15). The plastids are ovoid, exhibit a dense granular stroma and poorly developed inner membranes, and start to produce oil droplets (Fig. 13). In the same epidermal cell, some of the vacuoles are electron-lucent and contain fibrillar material and dense inclusions (Figs. 14 and 15), whereas others are filled with oils (Fig. 16). Cytoplasmic regions containing only smooth endoplasmic reticulum surround the vacuoles filled with lipids (Fig. 16). The plasma membrane is sinuous, and small vesicles are observed in the peripheral cytoplasm very close to the plasma membrane (Fig. 17). Clusters of vesicles and small vacuoles are visible in the peripheral cytoplasm in the distal pole of the epidermal cell (Fig. 11). Strands of actin-like filaments are observed near the small vacuoles containing electron-dense inclusions (Fig. 18).

Throughout gland development, epidermal secretory cells with a dark cytoplasm and low definition of organelles occur side by side with epidermal secretory cells exhibiting a proliferation of hyperactive dictyosomes, an abundance of endoplasmic reticulum, amoeboid plastids filled with abundant oil drops (Figs. 19 and 20), and cells displaying signs of senescence (Fig. 21). Such cells are characterized by a reduced nucleus, clearer areas in the cytoplasm, the proliferation of vesicular elements, the rupture of vacuolar membranes, degenerating mitochondria, and dense bodies surrounded by membranes (Fig. 21). During gland maturation, intercellular spaces are continuously formed by the dissolution of the middle lamella along the anticlinal and periclinal cell walls of both the epidermal and parenchyma cells (Fig. 22). Glands at the post-secretory stage are characterized by the disorganization of cell walls, protoplast retraction, the reduction of the cytoplasm to a very thin film, and the degradation of the internal system of membranes, in addition to the rupture of the outer epidermal cell walls and cuticle (Fig. 23).

The stalk is composed of thin-walled cells with a reduced cytoplasm and a large central vacuole containing flocculant material and (or) dense inclusions identified as polysaccharides and phenolic compounds, respectively. In the middle region of the stalk, sieve elements with a narrow lumen and thick walls are connected to large companion cells (Fig. 24).

Gland–bacteria association

The bacteria (not identified here) associated with the glands are variously rod or ovoid in shape. The bacterium typically contains a conspicuous chromatic region, a surrounding capsule, and a number of light-staining inclusions (Fig. 25, insert).

Bacteria were observed on the intact surface (Fig. 26) and inside the mature glands (Fig. 25). Bacteria were restricted to the body of the gland and were immersed in the mucilage that fills the schizogenous intercellular spaces, which are wider in the central region of the gland (Fig. 25). A layer of thick-walled parenchyma cells encompassed the bacteria in the wider intercellular space localized in the central zone of the mature glands. The sheath cells were oval and juxtaposed and displayed a reduced cytoplasm and large vacuole with electron-dense phenolic compounds. This sheath is discontinuous on the distal side (Fig. 25), favoring the connection of this central intercellular space to the tubular spaces in the distal region of the gland.

In addition, bacteria were also embedded in the middle lamellae (Fig. 27), in the cell wall matrix of the epidermal cells (Fig. 28), and in wall protuberances located on the inner surface of the epidermal cells (Fig. 29), indicating a loosening of the middle lamellae. These protuberances form tube-like projections that extend toward the protoplast of the epidermal cell (Fig. 30). Pockets containing bacteria and heterogeneous secretion, seen as myelin figures, were observed between the plasma membrane and cell wall (Fig. 31). In the intracellular compartment, degenerating bacteria (seen as dark bodies with irregular shape and size) were observed in the cytoplasm of the epidermal cells completely surrounded by membrane originating from the host plasma membrane (Fig. 32). These vesicles gradually expand, creating electron-lucent vacuoles containing electron-dense bodies (Figs. 11, 14, and 22). These phagosomes are internalized in the cytoplasm and coalesce, forming larger vacuoles. Degenerate bacteria were also observed to be entrapped in the callose deposit located between the cell wall and plasma membrane of the stalk cells (Figs. 33 and 34). No intact bacteria were observed in the stalk cells.

Discussion

In this study, we describe, for the first time, the occurrence of a glandular structure and its association with bacteria in a member of the family Styracaceae. The leaf glands in S. camporum share features of colleters and bacterial leaf nodules, as discussed below. From the ontogenetical, structural, and functional perspectives, the leaf glands in S. camporum can be classified as colleters ( Lersten 1974, 1975; Klein et al. 2004). Similar to colleters, the leaf glands of S. camporum are multicellular secretory structures characterized by the production of protein and carbohydrate-based mucilage and lipids and are active in secretion beginning during the initial developmental stages of leaves (Fahn 1979, 2000; Evert 2006; Paiva and Machado 2006a, 2006b; Paiva 2009, 2012; Coelho et al. 2013). However, in contrast with most of the reports of the functioning time of colleters (Thomas and Dave 1989; Thomas et al. 1989; Thomas...
the leaf glands in *S. camporum* are persistent and active in secretion, even in the mature leaves. The presence of sieve elements with large companion cells in the gland stalks of *S. camporum* provides strong evidence of the transport of substances from the glands toward the leaf tissues. Oils are produced throughout gland development, whereas mucilage is produced only in the mature glands; oil is both released via the outer cell wall and accumulates in the vacuoles of the epidermal cells, whereas mucilage is stored in the intercellular channels formed by schizogenesis. The formation of intercellular...
Figs. 19–24. Transmission electron micrographs of *S. camporum* leaf glands. Fig. 19. Secretory epidermal cells with different electron-density. Observe loose and reticulate appearance of middle lamellae (ML) in the anticlinal cell walls. Scale bar = 2 μm. Fig. 20. Epidermal cell with irregular nucleus (NU) and proliferation of dictyosomes (DI) and modified plastids (PL). Scale bar = 0.7 μm. Fig. 21. Epidermal cells with senescence. Note irregular nucleus (NU) and electron-dense bodies (*) surrounded by membrane. Scale bar = 1 μm. Fig. 22. Intercellular spaces (IS) among epidermal cells. VA, vacuole; OL, oil. Scale bar = 2 μm. Fig. 23. Senescent gland showing cells with sinuous walls, reduced cytoplasm, and large vacuoles (VA). Arrowhead indicates broken outer cell wall. Scale bar = 6 μm. Fig. 24. Gland stalk showing epidermal and parenchyma cells with reduced cytoplasm and large vacuoles with accumulations of phenolic compounds. Note phloem constituted by sieve tube members (ST), large companion cells (CC), and parenchyma cells (PC). Scale bar = 3 μm.

Figs. 25–34. Transmission electron micrographs of *S. camporum* leaf glands. Fig. 25. General view showing epidermal cells (EP) with varying sizes, intercellular spaces (IS), and a wide central space filled with bacteria (BT) surrounded by a sheath (SH) of distinctive cells. Note interruption of sheath on distal side of gland. Dividing bacteria shown in insert. Scale bar = 4 μm. Fig. 26. Oil drops (OL), cell debris, and bacteria (BT) embedded in mucilage (MU) on surface of epidermal cell. CT, cuticle. Scale bar = 0.3 μm. Fig. 27. Reticulate middle lamellae with embedded bacteria (BT). The arrow indicates plasma membrane surrounding degenerating bacteria. Scale bar = 0.3 μm. Fig. 28. Bacteria (BT) embedded in cell wall matrix. Scale bar = 0.3 μm. Fig. 29. Bacteria (BT) embedded in protuberances of wall. Scale bar = 0.3 μm. Fig. 30. Tube-like cell wall projection containing bacteria (BT). Scale bar = 0.5 μm. Fig. 31. Pocket containing mucilage (MU) and immersed bacteria (BT) between cell wall (CW) and the plasma membrane (arrow). Scale bar = 0.3 μm. Fig. 32. Phagosomes (*) with bacteria inside protoplast of the epidermal cell. CW, cell wall; MI, mitochondria. Scale bar = 0.2 μm. Fig. 33. Stalk cells showing vacuole (VA) filled with phenolic compounds and accumulations of callose (CA) between plasma membrane and cell wall. BT, bacteria in central space of the body gland adjacent to stalk cells. Scale bar = 2 μm. Fig. 34. Detail of callose accumulation showing entrapped bacteria. Scale bar = 0.7 μm.
channels during colleter development is common in different species (Thomas and Dave 1989; Paiva and Machado 2006a, 2006b; Coelho et al. 2013). Additionally, enzymatic processes involving the dissolution of the middle lamellae and the degradation of cell wall-bound polysaccharides have been reported during bacterial infection in several plant tissues (Huang 1986; Quadt-Hallmann et al. 1997). In S. camporum, the schizogenous process provides a microenvironment for the residence and proliferation of bacteria and a route for their spreading in the middle lamellae region. The residence of bacteria inside the leaf glands of S. camporum appears advantageous to the symbiont (which has not yet been identified), considering the high temperatures and light intensities and low humidity of the Brazilian Cerrado. As per Van Oevelen et al. (2003), extreme environmental conditions cause mucilage dehydration, rendering the bacteria unable to actively grow and prohibiting further inoculation.

Our results demonstrate that the pathways of secretion release in the leaf glands of S. camporum involve three distinct mechanisms: granulocrine, eccrine, and holocrine processes sensu Fahn (1979). Oil drops are able to cross the plasma membrane and the outer cell wall of epidermal cells, characterizing the eccrine process of liberation. The mucilage exits the protoplast of epidermal cells by exocytosis and first accumulates in the periplasmic space, characterizing the granulocrine process of secretion. Later, the mucilage passes through the cells wall and is discharged into the intercellular spaces. The accumulation of mucilage in these spaces generates a pressure that causes the rupture of both the cell wall and cuticle in the distal portion of the gland body, disintegrating the secretory cells, typical of the holocrine process. In fact, cell debris is mixed with mucilage and oils on the gland surface.

The ultrastructural features observed in the epidermal secretory cells of S. camporum mature leaf glands are typical of cells that synthesize protein and carbohydrate-based mucilage in that a subcellular compartment consisting of hyperactive dictyosomes, a rough endoplasmic reticulum, vesicles, and small storage vacuoles are involved (Meyberg 1988; Evert 2006). The presence of a smooth endoplasmic reticulum and modified plastids characterized by the presence of oil droplets and a poorly developed inner membrane system is associated with lipid synthesis and has been described for many glands secreting lipophilic substances, including monoterpenes (Evert 2006 and references therein). Some characteristics that were observed in the gland cells of S. camporum, such as the proliferation of vesicular cytoplasmic elements, mitochondrial alterations, the accumulation of dark globules, and the loose appearance of the cell wall, are cytological alterations that are frequently associated with gland senescence (Miguel et al. 2010 and references therein).

Concerning the initiation, development, and structure of the leaf structures involved in bacterial symbiosis, significant differences occur between S. camporum and the other three plant families that present the leaf–bacteria association. In the two nodulated dicotyledonous families, leaf nodules are initiated in the shoot tip during the early stages of leaf development by the entry of bacteria-laden mucilage through the stomata (Rubiaceae or hydathodes (Myrsinaceae), and the bacteria are in close contact with the internal tissues of the host plant (Miller et al. 1983). In the Dioscoreaceae, bacteria are harbored in ducts formed by the invagination of the adaxial epidermis of the leaf acumen lined with secretory trichomes, and the bacteria are being held out of the host plant tissue (Miller and Reporter 1987). In S. camporum, the glands are a constitutive trait, originating in the early stages of leaf development, and all of the glands are colonized with bacteria during the mature stage.

The role of the mucilage in the nourishment of bacteria and in their transportation, enabling the infection of the leaves and seeds of the host plants, has been discussed previously (Lersten and Horner 1967; Whitmoyer and Horner 1970; Miller et al. 1983; Miller and Reporter 1987; Van Oevelen et al. 2003). In the three plant families that maintain bacterial leaf symbioses, the bacterial colony resides in the stem apical region, which is permanently filled with the mucilage secreted by specialized trichomes (dendroid colleters in Rubiaceae, Miller et al. 1983; stellate trichomes in Myrsinaceae, Miller et al. 1984; multicellular trichomes in Dioscoreaceae, Miller and Reporter 1987). In S. camporum, the bacteria-laden mucilage, which is released by gland rupture, surrounds the developing leaves and infects each newly formed gland. The protein and carbohydrate-based mucilage produced by S. camporum glands appears to have a key function in this symbiosis, as bacteria are absent in immature glands not yet producing mucilage.

In S. camporum, bacteria enter the mature gland through the intact surface of the epidermal secretory cells. The ability of bacteria to penetrate both the cuticle and cell wall in the absence of wounds was experimentally shown in cotton seedlings by Quadt-Hallmann et al. (1997). The spread of bacteria inside the plant tissue via intercellular spaces originating via a schizogenous process, as observed in S. camporum glands, is a well-documented phenomenon during leaf nodule development in Rubiaceae species (Miller et al. 1983; Miller 1990). This schizogenous process, involving the secretion of pectolytic enzymes of plant or bacterial origin, loosens the middle lamellae between the cells and provides a pathway for bacterial dispersion.

In S. camporum epidermal cells, the invading bacterium is separated from the cytoplasm by the host-plasma membrane, forming an intracellular compartment. Thus, the bacterium may be intracellular but is always extracytoplasmic (Parniske 2000). The engulfment of the bacteria by the host-derived membrane occurs in a manner that resembles phagocytosis in animal cells (Parniske 2000; Yutin et al. 2009) and has been well-documented in the root-nodule symbiosis (Peleg-Grossman et al. 2009). The strands of actin filaments near the endocytod vesicles in S. camporum are consistent with the phagocytosis process, as this process is an actin-dependent process that occurs in all eukaryotic cells (Yutin et al. 2009). Concerning the intracellular accommodation of bacteria by the leaf glands of S. camporum, some processes, such as cell wall reorganization, membrane synthesis, and cytoskeleton involvement, are likely to be similar between different systems and constitute the basic cellular traits of endosymbioses (Parniske 2000).

The thick-walled parenchymal sheath observed around the bacterial population in the leaf glands of S. camporum is very similar to that reported for the bacterial leaf nodules of Photinia bacteriophila Valeton (Whitmoyer and Horner 1970) and may serve as a passive physical barrier that controls the expansion of the gland and limits the spread of bacteria through the foliar organs. In fact, bacteria are restricted to the gland body. The absence of bacteria in the stalk cells of the glands in S. camporum may also be associated with the presence of phenolic compounds in these cells, as these substances are related to protection against microbial attack (Harborne 1993). In addition, callose accumulations in the stalk cells can provide an additional protective barrier that prevents the invasion of the host cells by bacteria (Evert 2006).

Based on a large number of material samples and long-term observation, this study demonstrates that the presence of bacteria in the leaf glands of S. camporum is a regular and cyclic trait. In its broadest sense, symbiosis refers to organisms living together, whether the interaction is mutualistic, commensal, or parasitic (Parniske 2000). The significance of bacteria inside S. camporum leaf glands and the type of interaction between these two organisms remain unknown. The association between the leaf glands and bacteria in S. camporum is a valuable system for symbiosis research and can open new avenues for research into plant–microorganism interactions.
Acknowledgements

We thank FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo – Proc. 2008/55434–7) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico – Proc. 470643/2006-4) for their financial support; the staff of the Centro de Microscopia Eletrônica (CME), Institute of Biosciences, UNESP - Botucatu for helping in the sample preparation; and Daniela Carvalho dos Santos, CME, for her helpful suggestions. S.R. Machado received financial support from the CNPq Council.

References


Evert, R.F. 2006. Esau’s plant anatomy; meristems, cells, and tissues of the plant body—their structure, function, and development. 3rd ed. John Wiley & Sons, New Jersey.


