Interdependence of the ontogeny of two essential foliar structures in a representative of the neotropical genus *Tillandsia* (Bromeliaceae): stomata and absorbing trichomes

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Abstract: Epiphytism in *Tillandsia*, a genus of neotropical monocots of the family Bromeliaceae, is linked to the functioning of absorbing trichomes because the roots of these plants maintain only the anchoring role since the first ontogenetic stages of the individual (seedling). For this reason we assumed that there should exist a temporal and functional coordination between the apparatus through which water loss occurs (stoma) and the apparatus specialized to water assumption (trichome). Otherwise the plant would risk losing water before being able to absorb it. The ontogeny of the two apparata has been investigated in the seedling of *T. aeranthos*. The results confirmed the hypothesis and the ultrastructural aspects characterizing the development stages of the stomata until the full functionality are here discussed. The peculiar strategy of life of Tillands is not a topics of only scientific attractiveness but represents a strong commercial 'appeal' as ornamental plants.

Key words: absorbing trichome, epiphytism, stomata, Tillandsia*, ultrastructure*

Introduction

Terrestrial plants use their roots to absorb water and mineral nutrients from the soil. Among plants adapted to epiphytic life in a neotropical environment, the members of the neotropical family Bromeliaceae, particularly those belonging to genus *Tillandsia*, use roots only to adhere to the host. Instead these plants use their epidermal trichomes for absorbing water and mineral and organic nutrients from the atmosphere (Tukey Jr and Mecklenburg, 1970; Benzing *et al.*, 1976).

The absorbing trichome in *Tillandsia* (Schimper, 1888; Mez, 1904; Francini Corti, 1981; Rauh, 1981) has a nail-like shape and is formed by an anticlinal axis of 1-6 cells, one on top of the other and directly connected to the internal tissues of the leave, and to an external shield (or head). This last structure is mono-layered and formed by dead cells extending their surface over the epidermis. The solutions coming from the external environment go through the shield cells and follow a descending way through the axis cells of the trichome to reach the underlying mesophyll chlorenchyma.

For these reasons it is necessary a strict functional link between the time of stomata development and the ontogeny of the absorbing trichome. In other words stomata must not open until the trichomes are not able to do fully their function, to avoid dehydration of the seedling and hence a slower development and lastly the death of the plant. The best moment to investigate such a relationship is the seedling stage.

On the basis of this assumed link, we report here about the ultrastructural features of the ontogeny of the two essential epidermal structures in seedlings of *Tillandsia aeranthos* (Loiseneur) L.B. Smith. For each stage of ontogeny, a related stage of the trichome ontogeny corresponds. Some attention on the relationship stomata-trichomes has been paid by Benzing (2000), who observed that in species of *Tillandsia* where trichomes are more numerous (xeric species), stomata are in very low number. Moreover Tomlinson (1969) showed that the ratio between stomata and trichomes could give a range of numbers with sufficient discontinuity to separate the three subfamilies of Bromeliaceae (Pitcairnioideae, Bromelioideae, Tillandsioideae) with the lowest ratio observed in xeric tillands.

The first studies on absorption mechanism in trichomes of *Tillandsia* were by Mez (1904). The observations on the ontogeny of *Tillandsia* trichomes are of the same year (Billings, 1904, on *Tillandsia usneoides*). Also because of some methodological difficulty, ultrastructural studies in *Tillandsia* are relatively few and not recent (Dolzmann, 1964, 1965; Benzing *et al.*, 1976; Brighigna, 1974, 1976a, 1976b), and normally dealing with particular ultrastructural features.

In this work we won't discuss in detail ultrastructure and morphogenesis of the trichome, since our paper is more focused on stomata and on the relationships of these last to the trichomes.

The first Bromeliads that have been traded as ornamental plants were those belonging to the terrestrial genera (i. e. *Nidularium, Aechmea, Bromelia,* ecc.). Presently the species of the Tillandsia genus represent the novelty of the market, owing to the singularity of their epiphytic way of life. For this reason these plants are commonly called 'air plants'. The ability to live without roots, not in direct contact with the soil, and even stuck on inert supports is surely at the base of their commercial 'appeal'.

Several developing countries of the Caribbean area and of central America (Nicaragua, Guatemala, Honduras, ecc) gain benefit from the export trade of Tillandsias. The previous investigations on the biological aspects and mechanisms of Tillandsias are hence useful to increase their attractiveness on the marked and to provide useful informations to breeders, traders and collectors.

Materials and methods

Seeds of *Tillandsia aeranthos* germinated in a Petri's dish. The first leaves of the seedling were dissected, than prefixed overnight in 1.25 % glutaraldehyde at cold temperature (4° C) in 0.1 M phosphate buffer (pH 6.8). The samples were fixed in OsO_4 1% in the same buffer for 1 hr. After dehydration in an ethanol series the samples were embedded in Spurr's epoxy resin (Spurr, 1969). Transverse sections approximately 80 nm thick were cut with a diamond knife, stained with uranyl acetate (Gibbons and Grimstone, 1960) and lead citrate (Reynolds, 1963), then examined with a Philips EM300 transmission electron microscope at 80 KV. Some sections were stained with Toluidine Blue, observed and photographed with a Light Transmission Microscope.

Results

In Figure A one *Tillandsia aeranthos* seedling with the first three leaves is shown. The embryo root was degenerating while a small adventitious root could be observed. In figures B and C respectively a stoma and a trichome, well developed and in the same stage and epidermis zone, are represented.

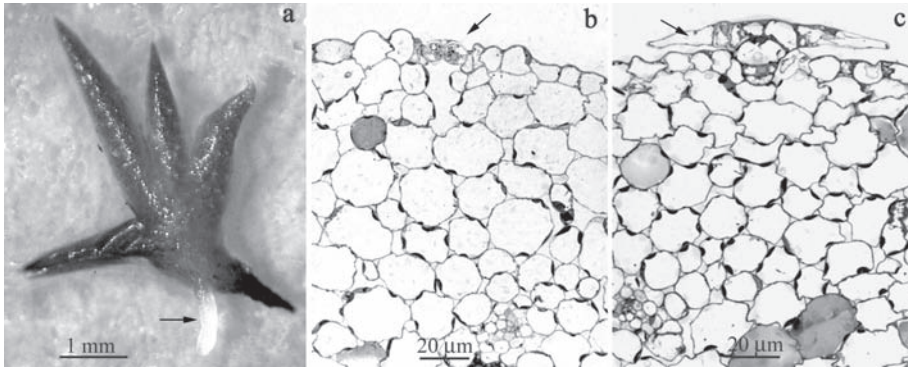We subdivided the ontogeny of the stoma into four phases.

Figure a - Tillandsia aeranthos *seedling with the first three leaves. An adventitious root (arrow) can be observed near the degenerating embryo root*
Figure b - Functional stoma (arrow) with stomatal chamber in Tillandsia *seedling*
Figure c - Developed trichome (arrow) in Tillandsia *seedling*

In the first phase the two guard cells of the stoma resulted from an anticlinal division of an epidermal mother cell. The stomatal type appeared to be perigenous since the cell division was symmetrical (Fig. 1). At first, in the prestomatic stage (Fig. 1), the two guard cells resulted still fused through a thin wall that appeared thickened only at the apex towards the outside, where the external closing 'beaks' will be formed. The cytoplasm was not particularly dense and contained a big nucleus in central position, many small and medium dimension vacuoles, small roundish proplastids with a very electrondense matrix and often budding and long mitochondria, with well developed cristae. The long elements of Rough Endoplasmatic Reticulum (RER) were particularly significant since in both cells they ran parallelly to the dividing wall (Fig. 1 and 2).

In this phase the trichome ontogeny appeared to be at a very early stage: a periclinal division caused a cell to move towards the outside of the epidermis surface (Fig. 9). Afterwards other periclinal divisions caused the formation of the trichome vertical axis (stalk), connecting the mesophyll to the external structures of the trichome (the shield and the wing) (Fig. 10, 11 and 12).

In a second phase the formation of the stomatal aperture began by separation of the apexes of the sister cells (Fig. 3). The periclinal walls showed traces of localized thickenings. The cuticle beaks were evident even if still fused together. Significant changings were evident in the cytoplasm of the guard cells: the vacuome was restricted to a big central element to which smaller vacuo-
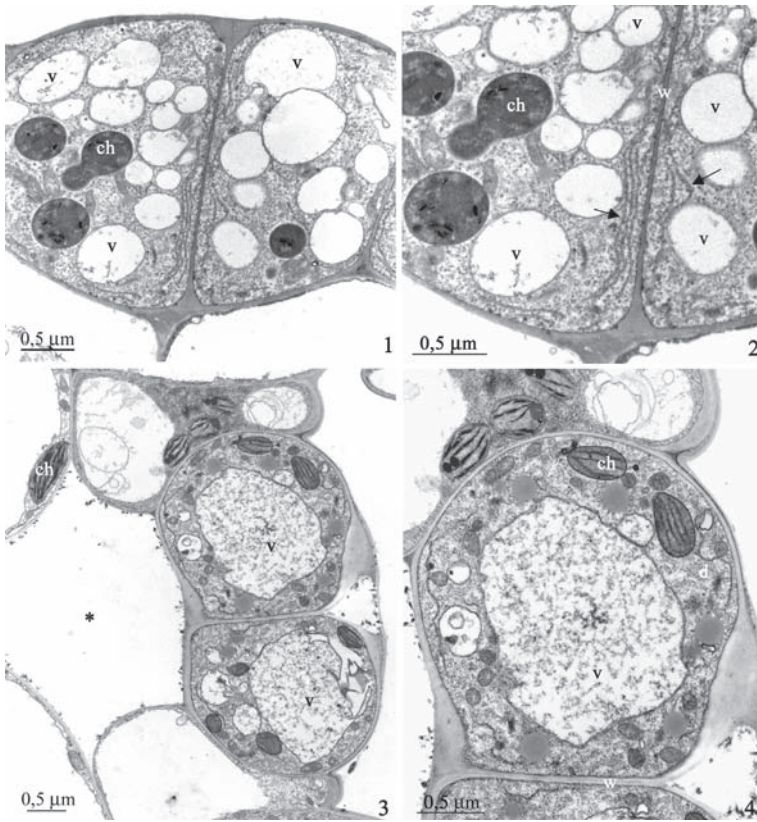
Figure Legends: Key to labelling: ch = chloroplast; d = dictyosome; m = mitochondrion; s = starch;   v = vacuole; w = wall; * = stomatal chamber.

Figure 1 - Prestomatic stage. The two guard cells, resulted from a symmetrical division of an epidermal mother cell, are still connected by a thin wall. Small and medium dimension vacuoles are evident

Figure 2 - Prestomatic stage. Detail of fig. 1. Long RER Elements (arrows) run parallely to the thin wall on both sides of the dividing wall. Roundish chloroplasts have a strong osmiophylic matrix

Figure 3 - Beginning of the formation of the stomatal aperture. The beaks of the periclinal outher wall are evident. In the chlorenchyma under the guard cells the substomatal chamber (asterisk) is in formation

Figure 4 - Detail of fig. 3. Plastids of the guard cells and of a subsidary cell show well formed tylakoids. Lipid droplets are present in the cytoplasm of the guard cell. The large vacuole contains finely dispersed electrondense matherial. Dictyosomes are budding

les tended to converge (Fig. 3, 4). Inside the central element heterogeneous electrondense material was dispersed. Tylakoids appeared in the plastids while in the cytoplasm a limited presence of lipid droplets was remarkable. Dictyosomes active in budding vesicles were observed in the peripheral cytoplasm. In the chlorenchyma under the guard cells the substomatal chamber was in formation (Fig. 3 and 4). In the subsidiary cells the presence of plastids with well formed tylakoids is significant (Fig. 4).

In this phase the trichome ontogeny was going on by the formation of the wing through a double anticlinal division of the distal cell of the axis (the dome cell). At the beginning the four cells constituting the centre of the trichome head are formed. Starting from these last, concentric arrays of living cells constituting the wing of the trichome were formed through a series of anticlinal divisions (Fig. 13).

The wall thickening localized in the guard cells were increasing and contemporaneously the formation of the stomatal aperture was going on: the detachment of the guard cells was proceeding in a centripetal direction. The aperture was closed only by a subtle remnant of cuticle film (Fig. 5). The chloroplasts of the guard cells showed in this stage big starch storages (Fig. 6).

In the last stage of stoma development the differentiation of the guard cells appeared completed (Fig. 7). The aperture was open. The huge thickenings of the periclinal walls due to the contribution of more layers were evident. In the cytoplasm (which is peripheral owing to the presence of the big central vacuole) the chloroplasts contained big starch grains. The dictyosomes maintained their budding activity (Fig. 8) while microtubules were evident close to the plasmamembrane. Microorganisms entering the open stoma were often observed (Fig. 7a). A large intercellular space interrupted the compactness of the clorenchyma underneath the stoma (Fig. 7).

Only in this phase the trichomes adjacent to stomata appeared fully developed with a wing enlarged to cover the largest surface and made only of dead cells (Fig. 14). The two foliar organs, stomata and trichomes, appeared to be at the same degree of anatomical and morphological maturity at the end of the developmental process.

Discussion

The ultrastructural features characterizing the four stages of stomatal development are of simple interpretation and coherent with the known general
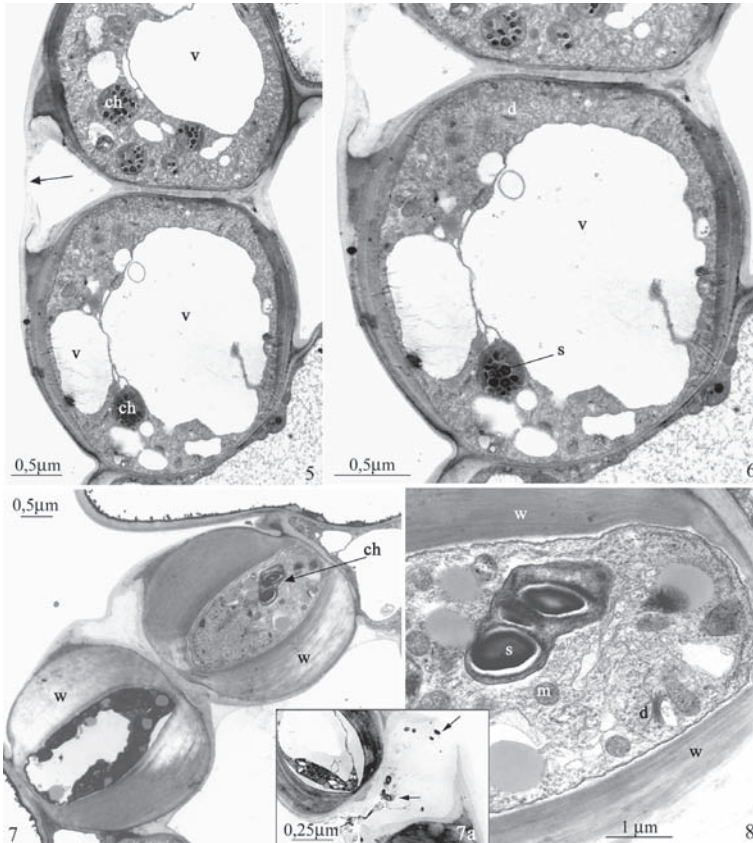
Figure Legends: Key to labelling: ch = chloroplast; d = dictyosome; m = mitochondrion; s = starch;　v = vacuole; w = wall; * = stomatal chamber.

Figure 5 - The detachment of the guard cells is proceeding in a centripetal direction. The stomatal aperture is closed only by a subtle remnant of cuticle (arrow) and by a portion of wall

Figure 6 - Detail of fig. 5. The chloroplasts of the guard cells show big starch grains. Vacuoles are in fusion

Figure 7 - Last stage of stoma development. The guard cells are now fully differentiated and the aperture is open
Fig. 7a. - Microorganisms (arrows) entering through the aperture of the stoma

Figure 8 - Detail of fig. 7. The chloroplasts contain big starch grains while the dictyosomes maintain their budding activity
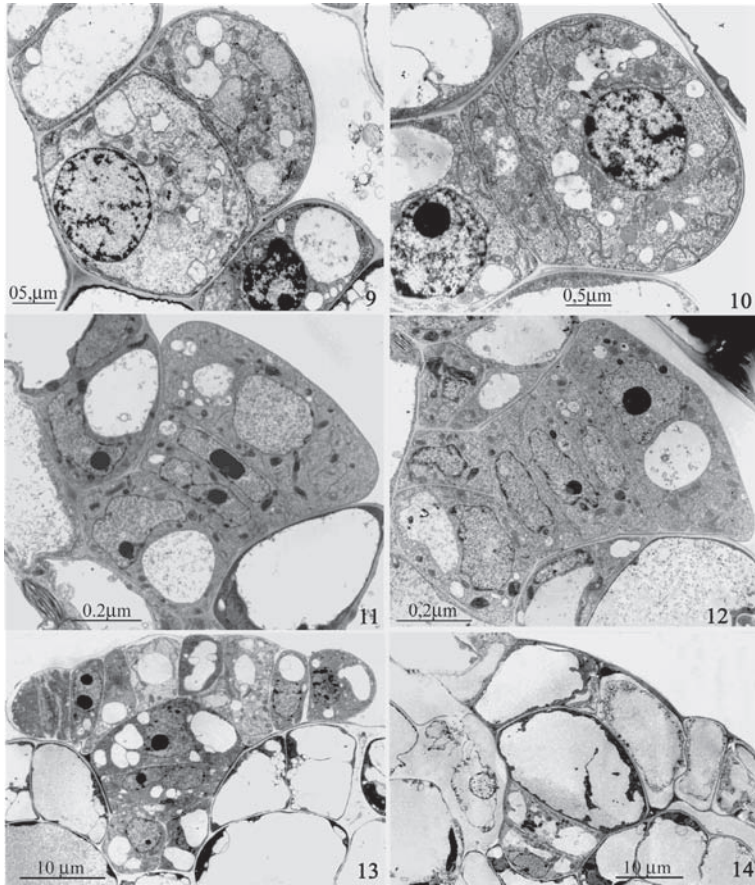
Figure 9 – 12 - Trichome ontogeny. 9) Very early 2-cells stage: a periclinal division caused a cell to move towards the outside of the epidermis surface. 10) Another periclinal division leads to the 3-cells stage of development. 11) 4-cells stage of trichome ontogeny. The trichome vertical axis (stalk) is in formation. 12) 5-cells stage of trichome ontogeny. The vertical axis is now formed. The most external cell is increasing in dimension

Figure 13 - Anticlinal divisions of the distal cell of the axis leads to the formation of the trichome wing

Figure 14 - Fully developed trichome. The wing is enlarged to cover the largest surface and is made only of dead cells

patterns of stomata ontogeny (Buvat, 1989). The first cellular division of the stomatal formation is symmetrical and hence the determinants of the cellular destiny (sensu Serna *et al.*, 2002) wouldn't be of the intrinsic type (linked to the cell line) but by interactions with the neighbouring cells.

In the first stage thin walls, fragmented vacuome and dividing plastids indicated the early life stage of the two sister cells. The (polarized) position of the long RER profiles in both cells was evidence of production of enzymes dedicated to the open the future aperture by degradation of the middle lamella.

The cells of the underlying parenchyma didn't show any preferential wall thickening, as already observed by Billings (1904). Hence there is insufficient evidence for the statement (Benzing, 1980) that the closing of stomata in *Tillandsia* wouldn't happen because of changes in cell shape of the guard cells, but interactions with cells of the underlying parenchyma. On the contrary adult stomata of seedlings *T. aeranthos* showed ultrastructural features (plastids containing starch grains, open stoma aperture) indicating their full functionality.

The first growth of a seedling of *Tillandsia* from a seed is concomitant with the fast degeneration of the embryo root (Harms, 1930) and with the growth of an adventitious root adhering to the host surface. This last adventitious root will then dry fastly. For this reason water absorption is executed by the absorbing trichomes on the leaves and not by root apparata. The seedling is the fundamental stage of the individual in which the epiphytic strategy becomes stable (Tomlinson, 1969; Adams and Martin, 1986).

The results confirm the hypothesis that during the first stages of the seedling development in *Tillandsia* the opening of stomata and hence their role in supporting the gas exchange between plant and environment, cannot precede the achievement of full absorbing functionality by the trichomes. The necessity of a functional relationship between a foliar absorption carried out by glandular trichomes (structurally simpler than those of Tillands) and the stomatal transpiration function was already hypothesized by Pridgeon (1981) about the representatives of subfamily Pleurothallidinae, neotropical epiphytic orchids whose leaves own a thick layer of water-storing hypodermis. This layer indicates a possible absorbing capability of the leaf epidermis, even if of low amount. Anyway in the seedling of *Tillandsia* the full development of the trichome head affects the normal functionality of stomata by superposing of the wings on the stomatal aperture, as observed by Adams e Martin

in *Tillandsia deppeana* (1986). The risk of such an interference is lower in the adult leave, where the expansion of the leave surface reduces the density of trichomes (Roguenant *et al.*, 2001), so that stomata apertures are freer. It should be observed that in the adult plant the presence of the water-storing hypodermis reduces noticeably the dehydration risk.

In the second stage the cell development went on by the achievement of the big central vacuole by fusion of smaller vacuoles. In this stage the product of the photosynthetic activity is mainly devolved to the formation of the walls thickenings by the activity of vesicles budded from the dictyosomes. This phenomenon explains the scarce accumulation of starch in the plastids.

The hypothesis that the two anatomical structures here investigated in *Tillandsia* seedling (stomata and trichomes) are functionally and temporally related was confirmed by ultrastructural data. The signs of the achievement of full physiological functionality by the stoma were the features exhibited by the walls and the vacuome and the presence of remarkable starch storage in the chloroplasts of the guard cells and of the subsidiary cells. Also the presence of lipid droplets is common in functional stomata. The capability to open the aperture actually depends on possibility to increase the ions concentration (prevailingly glucose coming from plastids) in the guard cells that, through the following water influx, permit them to swell. This swelling, thanks to the lesser thickening of the cell walls on the equatorial plain, permits the opening of the stomatal aperture. The full structural functionality is reached through the separation of the cuticle 'beaks' on the external side of the guard cells.

It appeared of particolar interest that the cuticle film is the last remnant preceding the opening of the stomatal aperture. It might indicate that, while the demolition of the middle lamella between the guard cell is a biochemical event, the rupture of the cuticle film is due to simple mechanical causes.

In cytoplasm of stomatal cells the maintenance of an evident secretory activity of dictyosomes and the presence of peripheral microtubules might indicate the prosecution of the process of thickening of the periclinal walls also at the adult stage.

This last stage is contemporaneous to the formation of the trichome wing and to the cytoplasmatic death of the cells that form it. The completion of this anatomical periphery indicates that the trichome is now fully organized and ready to absorb water. This physiological mechanism is so specialized to capture the water steam from the atmosphere (De Santo *et al.*, 1976).

The functional stoma not only is a necessary structure for gas-exchange, but it is also a way of entrance of microorganisms with which the plant established an ecological relationship important for his life. It appears to be particularly important the nitrogen-fixing phyllospherical microbial component (Favilli *et al.*, 2001; Brighigna *et al.*, 1992).

In conclusion the ultrastructural data confirm the initial hypothesis that the full functionality of the stomata is obtained only contemporaneously to the full functionality of the absorbing trichomes.

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