Viewpoint

How does the nectar of stomata-free nectaries cross the cuticle?

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ABSTRACT

In many glandular structures, departure from the cell is only one step in the process of exudate release to the plant surface. Here the set of events that lead nectar to the external environment is presented and discussed mainly for stomata-free nectaries. After being synthesized, the nectar or some of its component needs to be released to the environment where it performs its functions. Nectar precursors derived from cell metabolism need to cross several barriers, such as the cell membrane and cell wall, in order to become nectar. Then the nectar must cross the cuticle or pass through stomata in order to be offered to plant mutualists. Release through stomata is a simple mechanism, but the ways by which nectar crosses the cuticle is still controversial. Hydrophilic pathways in the cuticle and repetitive cycles of rupture or cuticle detachment are the main routes for nectar release in stomata-free nectaries. In addition to nectar, there are other exogenous secretions that must leave the protoplast and reach the plant surface to perform their function. The ways by which nectar is released discussed herein are likely relevant to understanding the release of other hydrophilic products of the secretory process of plants.

Keywords: animal-plant interactions, cuticular pores, nectar, nectar release, plant secretion

Introduction

For many secretory structures, once the secreted substances cross the cell wall they remain inside the gland or beneath a protective cuticle. Therefore, the question arises: how do substances produced by secretory cells, like nectar, reach the exterior of the plant? In plant secretory systems, if secretory substances are discharged outside of the cell, they are considered to be extracellular secretions (see Fahn 1979 for details). However, there are several ways by which secretory products leave the protoplast of cells and reach their final destination.

Holocrine or merocrine modes of secretion are used to explain how secretory products are released from the protoplast of plant secretory cells, but they do not explain, e.g., how a nectar droplet is offered to an ant over a nectary. What remains to be explained for most secretory systems

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is how secretion passes the barrier imposed by the cell wall and the cuticle. Although a cell-cycle model was recently proposed to explain how secretory products cross the plant cell wall (Paiva 2016), some doubt regarding the way secretory substances reach the surface of the outer cell wall remain. This is particularly true when there is a cuticle that has to be traversed and, in part, results from the complexity of the cuticle, which is often erroneously interpreted.

The term "cuticle" is frequently employed to refer to a cuticle *sensu lato*, which was an undesirable generalization of something quite complex. The cuticle (*sensu lato*) cannot be interpreted as a homogeneous layer as can the cuticle proper (CP), which is basically composed of soluble polymeric lipids (see Jeffree 2006 for details). The cuticle *sensu lato* is a multilayered structure that coats the outer cell walls of the epidermis, being composed of an outermost layer (cuticle proper), the cuticular layer (with embedded cellulose and other cell wall elements), and the innermost pectin layer. The hydrophobic character of the cuticle therefore increases from the innermost to the outermost layers and influences the way in which hydrophilic substances cross this barrier. In order to simplify terminology, the present work will employ "cuticle" to refer to cuticle *sensu lato*, as most authors do.

Therefore, herein some possible ways are proposed by which nectar could move from secretory cells towards the gland surface and perform its function, specifically in stomata-free nectaries (mostly extrafloral).

What about nectar exudation and disposal outside of the plant?

Given the great morphological diversity of nectaries, the possible ways by which material to be secreted can be released from these glandular structures is equally diverse. Among extrafloral and floral nectaries (extranuptials and nuptials, respectively, sensu Delpino 1886) there is a gradation of forms from the simplest, consisting of glandular trichomes, to complex structures encompassing a diversity of plant tissues, including vascular tissues.

Regardless of the type of nectary, there are two cases to consider: nectaries with stomata, and stomata-free nectaries. The release of aqueous solutions, such as nectar, as well as other hydrophilic substances, through stomata is a simple and well-studied mechanism. In general, nectar simply passively flows through the stomata as a result of a concentration gradient and capillarity action. In general, nectaries that exuded nectar through stomata are able to release large volumes of nectar usually in a short time interval, compared to the stomata-free nectaries. In stomata-free nectaries, the production of larger volumes of nectar is relatively slow and nectar accumulation is time dependent (see Gaffal 2012).

Stomata-free nectaries

In stomata-free nectaries, after a hydrophilic substance (nectar) crosses the cell wall, how does it cross the usually hydrophobic cuticle? For some nectaries and other plant glands, the cuticle constitutes the last barrier to be crossed by secretions. According to Jeffree (2006), cuticular pores capable of functioning as pathways for the passage of fluid are practically confined to secretory cells in higher plants, and despite the possible presence of these pores, the release of secretion may still require the rupture of the cuticle. However, Tresmondi *et al*. (2017) presented evidences that secretion crosses the cuticle by micro-channels, without breaking the cuticle in some colleters.

Extranuptial nectaries, which are mostly stomata-free nectaries, commonly possess a barrier surrounding the secretory tissue, which impedes the apoplastic transport of secretion products. This barrier prevents the reflux of material into the inner tissues, and thus forces the unidirectional displacement of nectar towards the external environment (see Paiva 2009; Paiva 2011). This barrier is commonly a juxtaposed cell layer that exhibits changes to the cell wall that prevent the transport of solutes into the free space of the cell wall. Thus, the solutes are forced through the gland at a critical location, and drive the flow of secretion. According to Lüttge (1971), the cell wall incrustations in these barriers involve suberization, cutinization, and possibly lignification of the gland cell wall, which alters its chemical composition and structural features. This view is supported by the development of such an apoplastic barrier in a variety of plant structures (Fahn 1979 and examples therein), and the presence of similar barriers in animal gland systems.

The model proposed by Paiva (2016) to explain how some secretory products cross the cuticular barrier, is largely based on the restriction to secretion reflux inside glands. This reflux restriction is facilitated by a Casparian strip, an endoderm or an endoderm-like layer. So, the accumulation of secretion products inside glands appears to produce pressure that permits the flux of secretion across the cuticle (Fig. 1), as pointed out by Paiva (2016).

The role of an endoderm-like layer that prevents the reflux of secretion has been described for a wide variety of extranuptial nectaries devoid of stomata, where it is frequently considered a boundary or endodermic layer (Grout & Williams 1980; Paiva 2011) or as filter tissue (Chakravarty 1948). In carnivorous plants, digestive glands develop an endodermal layer in order to restrict apoplastic flow (Fineran & Gilbertson 1980; Owen & Lennon 1999).

Given their hydrophilic character, the fraction of the nectar produced by the protoplast diffuses through the outer periclinal cell wall (see Paiva 2016 for this) and accumulates, transiently, in the subcuticular space or, depending on the chemical nature of the cuticle or even the presence of cuticular canals, passes through it. When accumulated in the
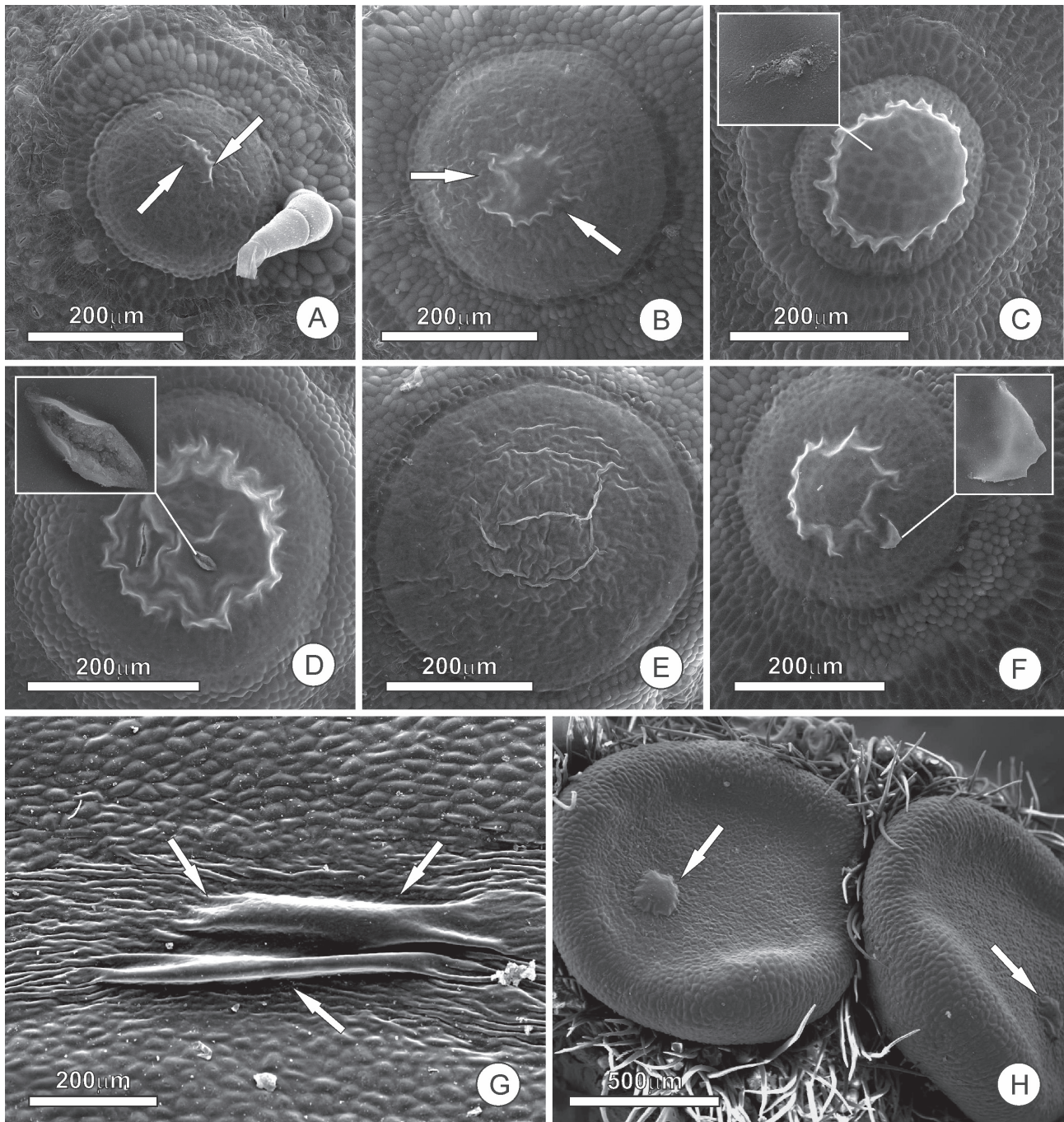
Figure 1. Accumulation of nectar and formation of the subcuticular space in extrafloral nectaries (NEFs); samples were prepared following standard SEM methods, fixed in 2.5 % glutaraldehyde in 0.1 M phosphate buffer, dehydrated in an ascending ethanol series and dried to the critical point in liquid CO_2, metalized with gold and observed using a Quanta 200 scanning electron microscope. **A-F** - NEFs of *Luffa cilindrica* (Cucurbitaceae) showing different stages of nectar release. In A-B, the arrows indicate the beginning of nectar accumulation and the subsequent formation of the subcuticular space. In C, the subcuticular space is full of nectar, nearly reaching the limits of the gland; the inset shows indications of cuticular ruptures. Cuticular ruptures and reduction in the subcuticular space due to the departure of nectar can be seen in D. In E, the cuticle, still distended, resumes its original position shortly after the extravasation of nectar. After the cuticle is recomposed and a new nectar accumulation cycle begins, a small cuticular fragment can be seen, in F, indicating the restoration of the cuticle from the previous cycle. In G and H, the secretory face of NEFs in *Ouratea castaneifolia* (Ochnaceae) and *Anadenanthera* sp. (Fabaceae) respectively, showing the accumulation of nectar below the cuticle (arrows).

subcuticular space, the volume of the nectar is defined by the extent of the cuticle that covers the gland and, naturally, the expandability of the cuticle. Nectar accumulation beneath the cuticle was reported by Pacini *et al.* (2003), Gama *et al.* (2016) and Nepi (2007), the latter of which explained some modes of nectar release. This process of nectar release was described by Wunnachit *et al.* (1992) who stated: "cuticle became distended by secretion of nectar (...) no cuticular pores were observed and the nectar must tear the cuticle". Reaching its expansion threshold, the cuticle ruptures and the nectar is released to the external environment (Fig. 1).

Nectar accumulation in subcuticular space, and cuticle rupture for the release of nectar, were described by Roshchina & Roshchina (1993) for nectaries with thick cuticles and the absence of pores. It should be noted that the fact that the subcuticular space does not extend beyond the limits of the gland (Rocha *et al.* 2009; Possobom *et al.* 2015; Gama *et al.* 2016) allows us to infer that pectinases, which are responsible for the weakening of the pectin layer and the consequent displacement of the cuticle, act on secretory cells and not neighboring cells. If this were not the case, the subcuticular space would tend to expand beyond the limits of the gland due to cuticular resistance. Although there is no experimental evidence, it seems quite likely that in most cases of merocrine secretion this cuticular rupture is cyclical, with the cuticle being restored after each nectar release event, thus beginning a new cycle. If this were not the case, and it occurred in any other way, the extrafloral nectary, whose secretory activity extends for long periods of time, would have persistent breaks in the cuticle, thereby allowing pathogen attacks or water loss from the gland tissue. However, this is not what is observed.

The cyclic model for cuticle restoration was suggested by Findlay & Mercer (1971), in trichome nectaries of *Abutilon* (Malvaceae)*,* wherein the nectar accumulates in a subcuticular space; the hydrostatic pressure within the hairs causes the opening of fine pores in the cuticle and the concomitant release of a nectar droplet with a sudden decrease in the hydrostatic pressure, whereupon the pores close and the periodic process starts again. Referring to Findlay & Mercer (1971), Jeffree (2006) interpreted these pores to be a kind of valve: "these pores seemed to have valve-like action, periodically releasing nectar accumulated between the cuticle and the cell wall". This view is very interesting and we must consider that cuticles, due to their lipidic nature, are sufficiently plastic in order to restore their integrity after the release of any secretory products or after any other kind of mechanical damage. Some nectaries possess lipid droplets inside secretory cells even when lipid was not detected in the nectar (Paiva & Machado 2006; Gama *et al.* 2016). Thus, the role of lipids inside nectary cells during the cuticle restoration process needs to be investigated.

Some floral nectaries that lack stomata exhibit the formation of a kind of subcuticular space where nectar accumulates transiently before release (see Weryszko-Chmielewska & Chwil 2016). Interestingly these subcuticular spaces are very small, being just vesicles on the cell surface (for details see Stpiczynska *et al.* 2003), and their formation is completely independent of the endodermal layer as discussed above and suggested for *Luffa* extrafloral nectaries (as shown in Fig. 1). These small cuticular swellings are thus distinct from large subcuticular spaces in both development and shape, but yet are similar in function and indicate the presence of an impermeable cuticle on the cell surface.

However, according to Paiva (2016), there are nectaries without stomata and from which nectar passes without the formation of a subcuticular space. In these cases, the presence of pores or hydrophilic pathways must be considered.

While there have been reports of the occurrence of cuticular canals in nectaries, many of them are questionable or not supported by convincing evidence. Nonetheless, the existence of such cuticular canals must be considered, although they seem to be restricted to the nectaries of just a few plant species. Indeed, Findlay & Mercer (1971) described such cuticular pores in the nectaries of *Abutilon*. Cuticular pores or canals are terms employed to describe hydrophilic pathways through the plant cuticle; however, these terms are vague because they do not always refer to a duct with a free lumen. In addition, most of them just cross the cuticular layer, and do not reach the cuticle proper.

In most cases, canals are hydrophilic pathways composed of cell wall elements (usually cellulose and pectins), which extend into the cuticular layer to the proximity of the cuticle proper, but without reaching the outer surface of the cuticle. So, in fact these canals are note true ducts, but hydrophilic pathways into the cuticular layer, since ducts imply in a structure with an empty lumen. These hydrophilic pathways into the cuticular layer have been described for some nectaries, such as those of *Platanthera chlorantha* (Orchidaceae) (Stpiczynska 2003), *Galanthus nivalis* (Amaryllidaceae) (Weryszko-Chmielewska & Chwil 2016), nectary spurs of four representatives of Ranunculaceae (Antón & Kaminska 2015) and several other examples in the literature, mostly in stomata-free floral nectaries. Similar cuticular canals, which are polysaccharide material from cell wall protruded inside the cuticular layers, seems to act increasing the porosity of the cuticle and, consequently, constituting pathways for the secretion release in colleters (see Tresmondi *et al.* 2017).

In the pericarpial nectaries of *Spathodea campanulata* (Bignoniaceae), the cuticle (*sensu lato*) has very evident ducts with completely free lumina (Fig. 2); the cuticle is interspersed by an extensive network of ducts that open to the surface, thus allowing the release of nectar. In fact, in this species subcuticular spaces are not formed and the production of nectar persists for a long period of time, from post-pollination until fruit ripening. In the absence of stomata, just the presence of an undamaged cuticle possessing ducts allows the protection of the secretory tissue when nectar is continuously released.
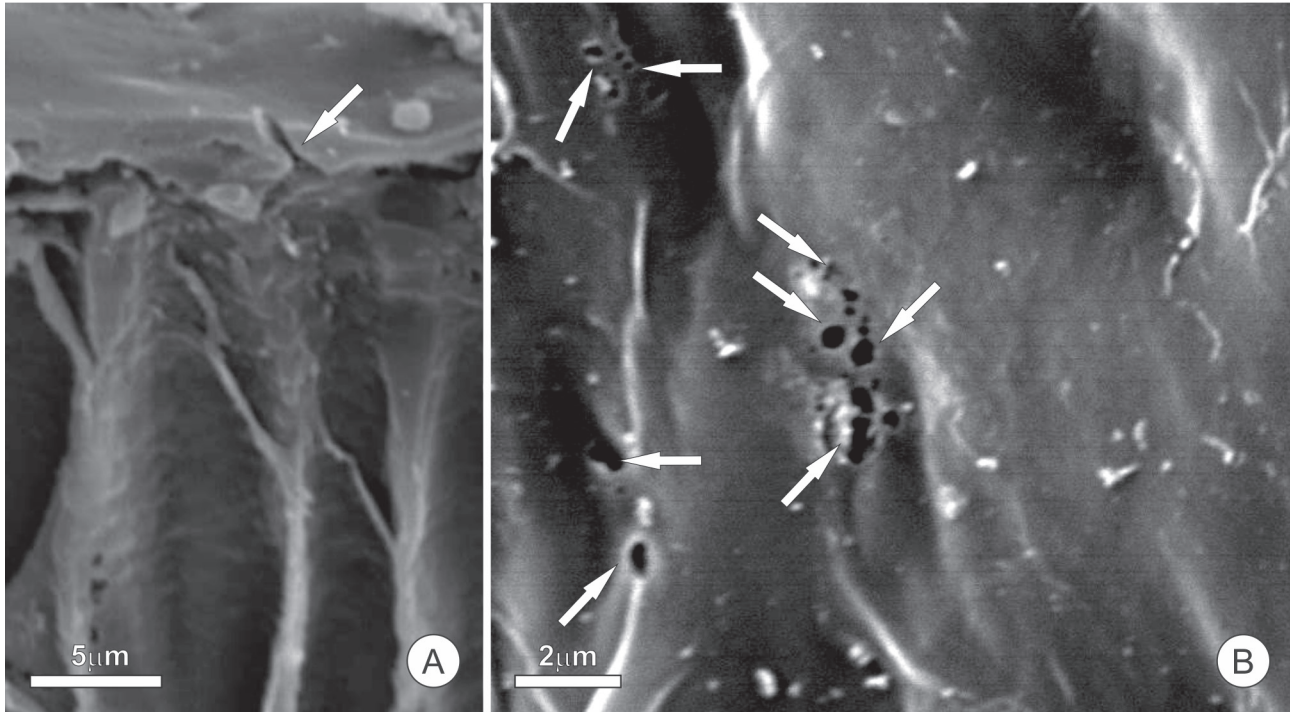
Figure 2. Cuticular canals in pericarpal nectaries of *Spathodea campanulata* (Bignoniaceae), seen using a scanning electron microscope; samples were prepared as described in figure 1. In A, a transverse section showing detail of the secretory epithelium; the arrow indicates a channel through the cuticle. In B, frontal view of the secretory face showing the cuticle with the opening of the cuticular canals (arrows).

For both nectaries with and without stomata, the accumulation of sugars inside the gland establishes the "water potential gradient" required for exuding nectar. According to Lüttge & Schnepf (1976), an alternative driving force may be the modification of sugars by gland-cell metabolism, or by the presence of invertases on the gland surface or in the released nectar itself. Cell wall invertases are important for phloem sugar unloading (Roitsch 1999) and are required for nectar production in *Arabidopsis* (Ruhlmann *et al*. 2010).

Final remarks

Nectar release occurs differently in nectaries with stomata compared to stomata-free nectaries. Simply crossing the plasma membrane and cell wall is insufficient for the provision of nectar to consumers as the hydrophobic barrier of the cuticle must be surpassed. Stomata, cuticular pores, cuticle rupture or detachment (in repetitive cycles or not) are the main routes for nectar release, and are easily recognized by structural features, thus avoiding unnecessary speculation.

Acknowledgements

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