

## Floral anatomy, micromorphology and visitor insects in three species of *Aristolochia* L. (Aristolochiaceae)

Sandra Silvina Aliscioni, Andrea Paula Achler & Juan Pablo Torretta

To cite this article: Sandra Silvina Aliscioni, Andrea Paula Achler & Juan Pablo Torretta (2017): Floral anatomy, micromorphology and visitor insects in three species of *Aristolochia* L. (Aristolochiaceae), *New Zealand Journal of Botany*, DOI: [10.1080/0028825X.2017.1380051](https://doi.org/10.1080/0028825X.2017.1380051)

To link to this article: <http://dx.doi.org/10.1080/0028825X.2017.1380051>



Published online: 11 Oct 2017.



Submit your article to this journal [↗](#)



Article views: 2



View related articles [↗](#)



View Crossmark data [↗](#)

RESEARCH ARTICLE



## Floral anatomy, micromorphology and visitor insects in three species of *Aristolochia* L. (Aristolochiaceae)

Sandra Silvina Aliscioni<sup>a,b,c</sup>, Andrea Paula Achler<sup>b</sup> and Juan Pablo Torretta<sup>b,c</sup>

<sup>a</sup>Instituto de Botánica Darwinion (IBODA), Buenos Aires, Argentina; <sup>b</sup>Facultad de Agronomía, Cátedra de Botánica General, Universidad de Buenos Aires, Buenos Aires, Argentina; <sup>c</sup>Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina

### ABSTRACT

The genus *Aristolochia* L. has a specialised pollination system based on flowers that represent traps for insects. The floral anatomy and micromorphology of the perianth were studied to detect structural characters and secretory cells that are essential for the pollination and reproduction in *A. esperanzae*, *A. fimbriata* and *A. triangularis*. Additionally, we identified the insects collected inside the flowers in natural populations of the north of Argentina. Our observations indicate the presence of osmophores in the limbs of *A. esperanzae* and *A. triangularis*, and in the tube of *A. fimbriata*, because in this last species the fimbriae probably play the first optical attraction role. The papillose cells and epicuticular waxes in the limbs and tube entrances, particularly in *A. esperanzae* and *A. fimbriata*, ensure that insects slip into the tube, and the non-secretory conical hairs of the tube, when turgid, guide the insects to the utricle. Idioblasts on the abaxial surfaces of the tubes and utricles were observed in *A. esperanzae* and *A. fimbriata*, and probably represent ethereal oil cells that protect flowers from predation. Secretion of sugar was confirmed on all internal surfaces of the utricle in the three species, and was more abundant at the entrance. The insects were diverse species of Diptera (Sarcophagidae, Muscidae, Ulidiidae, Drosophilidae and Anthomyiidae) and Coleoptera (Staphylinidae), although pollen loads on the body were only detected on specimens of Ulidiidae and Sarcophagidae.

### ARTICLE HISTORY

Received 29 March 2017  
Accepted 11 September 2017

### ASSOCIATE EDITOR

Dr Liliana Katinas

### KEYWORDS

Argentina; *Aristolochia esperanzae*; *A. fimbriata*; *A. triangularis*; ethereal oil cells; nectaries; osmophores; pollination

## Introduction

The Aristolochiaceae family is distributed throughout tropical and temperate regions of the world (Kelly & González 2003) and its largest genus is *Aristolochia* L. (subfamily Aristolochioideae) with c. 400–550 species that are characterised by strongly zygomorphic flowers (Schmidt 1935; Ahumada 1967; González 1999; González & Stevenson 2000; Pabón-Mora et al. 2015). The floral ground plan in *Aristolochia* is relatively constant (Endress 1998); the perianth is constituted of three joined sepals of a petaloid aspect and is morphologically divided into three regions (González & Stevenson 2000; Jaramillo & Kramer 2004; Pabón-Mora et al. 2015). The utricle is a globose structure in the basal zone that contains the gynostemium and acts as a temporary trap; at its base there is a

circular bright area surrounded by a dark line simulating an escape for pollinators (González & Stevenson 2000). The tube is situated in the middle area (internally covered by trichomes) and, in the upper zone, the tube expands to form the limb that is the most morphologically variable part of the perianth. The flowers of *Aristolochia* were described as dichogamous and protogynous (Endress 1998). Herkogamy is also present in this genus due to the particular distribution of the fertile parts, which prevent the anthers or pollen from touching the stigma spontaneously (Trujillo & Sérsic 2006). In general anthesis lasts 2 days; during the first day the flower is in the female phase with receptive stigmas and closed anthers. During the second day, the flower proceeds to the male phase where the stigmatic arms are closed and anthers are dehiscent.

This genus presents a very specialised pollination system because the flowers constitute temporary traps for pollinators. Oelschlägel et al. (2009) summarised this mechanism of pollination in four steps: (1) insect attraction; (2) trapping; (3) retention; and (4) insect release.

Attraction of pollinators in *Aristolochia* depends on different floral fragrances and on limb colour, which varies between green and black with spots and stripes (Vogel 1990). The emitted fragrances are diverse, simulating carrion (*A. grandiflora* Sw.), faeces (*A. macroura* Ortega) or decaying fruits (*A. gigantea* Mart.), or fragrances are inconspicuous (*A. elegans* Mast.) (Endress 1998). Recently, Oelschlägel et al. (2015) demonstrated that freshly killed mirid bugs and flowers of *A. rotunda* L. released the same scent components that chloropid flies use to find their food sources.

Although fragrances emitted by different species of *Aristolochia* are variable, all are produced by secretory structures named by Vogel (1990) as osmophores. There is little information about the anatomical structures of osmophores in *Aristolochia*. Vogel (1990) studied *A. trilobata* L. and *A. aff. cordiflora* Mutis ex Kunth and Costa & Hime (1981) studied the osmophores of *A. gigantea*; however, the location of the osmophores in the perianth is unknown for most species.

More recently, González & Pabón-Mora (2015) and Pabón-Mora et al. (2015) indicated the presence of osmophore patches surrounded by trichomatous nectaries in the inner epidermis of the perianth in flowers of *A. fimbriata*, based on anatomical and micromorphological observations, but without testing of histochemical reactions.

In addition, nectar secretion was also documented in several species of *Aristolochia* (Cammerloher 1923; Petch 1924; Daumann 1959; Costa & Hime 1983; Vogel 1998; Trujillo & Sérsic 2006; Erbar 2014). In most of the species of *Aristolochia* investigated so far, nectar is secreted by trichomatous structures located in the inner walls of the utricle, but in *A. fimbriata* Cham. and Schltdl. the secretion of nectar was also detected outside of the utricle (Daumann 1959). Recently, nectarioles were also reported in three species of *Aristolochia* (Erbar et al. 2017). Diverse studies mentioned that nectar constitutes a reward to ensure the survival of pollinators during their captivity in the utricle (Vogel 1998; Sakai 2002; Trujillo & Sérsic 2006).

The pollinators of *Aristolochia* are species of diverse families of Diptera, including Anthomyiidae, Calliphoridae, Ceratopogonidae, Chloropidae, Cypselidae, Drosophilidae, Heleomyzidae, Milichiidae, Muscidae, Phoridae, Sarcophagidae, Sepsidae, Syrphidae and Ulidiidae (Cammerloher 1923; Petch 1924; Brues 1928; Lindner 1928; Vogel 1978; Brantjes 1980; Costa & Hime 1981, 1983; Wolda & Sabrosky 1986; Razzak et al. 1992; Hall & Brown 1993; Sakai 2002; Burgess et al. 2004; Trujillo & Sérsic 2006; Rulik et al. 2008; Oelschlägel et al. 2015).

Several studies were published on the reproductive biology of different species of *Aristolochia* (Branjtes 1980; Hall & Brown 1993; Endress 1998; Sakai 2002; Bänziger & Disney 2006; Trujillo & Sérsic 2006; Nakonechnaya et al. 2008; Rulik et al. 2008; Stotz & Gianoli 2013; Oelschlägel et al. 2015); however, anatomical studies are more limited (Costa & Hime 1983; Vogel 1990; Erbar 2014; Erbar et al. 2017).

The aim of this research was to study the anatomy and micromorphology of the perianth of three species of *Aristolochia* with different floral morphology (*A. esperanzae* Kuntze, *A. fimbriata* Cham. and Schltdl. and *A. triangularis* Cham.) to detect the structural characters and secretory cells that are essential for pollination and reproduction of these species. We test for the presence and location of osmophores and examine the occurrence of floral nectaries to verify nectar secretion during different floral phases and to estimate the role of nectar during the pollination process. Additionally, we identify the insects collected inside the flowers in natural populations of the north of Argentina and analyse the pollinic loads on the body of floral visitors of these species.

## Materials and methods

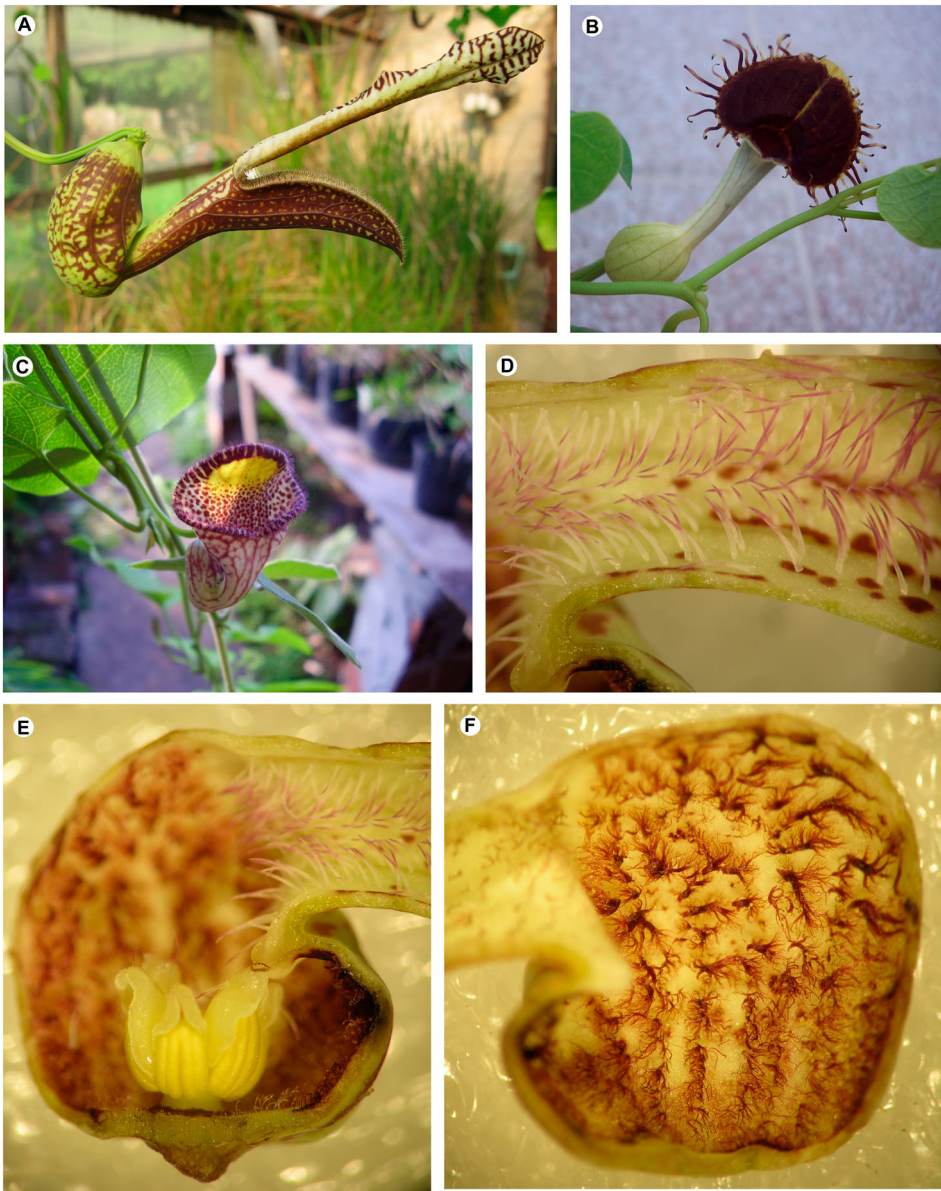
### Studied species

The studied species were selected in order to cover part of the range of floral morphological variability and geographical distribution of the genus in Argentina. *Aristolochia esperanzae* presents a bilobed limb, with unequal lips, the upper lip being thin and lanceolate and the lower ovate and less than  $\frac{3}{4}$  the length of the upper one (Figure 1A). Limbs of *A. fimbriata* and *A. triangularis* are unilobed, *A. fimbriata* presenting a fimbriate margin (Figure 1B) and *A. triangularis* a big yellow spot at the base of the suborbicular limb, near the entrance of the tube (Figure 1C). Natural populations of these species were found, during different field trips, in the provinces of Jujuy and Misiones, in the north of Argentina (Table 1). Plants were cultivated in the Lucien Hauman Botanical Garden of the Faculty of Agronomy, University of Buenos Aires. The reference specimens were deposited in the Xaspar Suarez Herbarium of the Faculty of Agronomy, University of Buenos Aires (BAA).

### Floral anatomy

Fresh flowers of the three species of *Aristolochia* were fixed in FAA (ethyl alcohol 70%: glacial acetic acid: formaldehyde 40%; 90:5:5) for 48 h and stored in 70% ethanol for the anatomical study. Transverse sections of the flowers at different levels of the perianth (limb, tube and utricle) were obtained. The samples were dehydrated in an ethanol series, transferred to xylene, embedded in paraffin (58 °C) and sectioned at a thickness of 6–7 µm on a rotary microtome (Leitz Wetzlar) using conventional methods. Histological samples were stained with Safranin-Fast Green and mounted in Canada balsam (Zarlavsky 2014). Observations were made using a Wild M20 optical light microscope and photomicrographs were taken using Axio Vs40 V 4.8.2.0 (Carl Zeiss).

For scanning electron microscopy (SEM), portions of the perianth were dehydrated and subjected to critical-point drying using liquid CO<sub>2</sub>. The material was then sputter-coated with gold and examined using a Philips XL 30 TMP microscope at an accelerating voltage of 80 kV.



**Figure 1.** A, *Aristolochia esperanzae*; B, *A. fimbriata*; C–F, *A. triangularis*; D, tube with turgid trichome; E, utricle with gynostemium in female phase; F, utricle with trichomes secreting sugar.

### Tests to detect secretory tissues

Different tests and histochemical reactions were performed using fresh flowers to detect the presence of osmophores and nectaries. The first approach to detect osmophores was done with a smell test (Amela García & Hoc 1998; Trujillo & Sérsic 2006). Fragments of limbs, tubes and utricles of the three species were introduced individually into hermetic vials and after 1–2 h each sample was smelled and the intensity of the fragrance was registered (–, without fragrance; +, moderate fragrance; ++, strong fragrance; +++, very strong



**Table 1.** Populations of the studied species with references of geographic positions and herbarium specimens.

| Species                | Locality  | Grid reference             | Specimens                           |
|------------------------|---|----------------------------|-------------------------------------|
| <i>A. esperanzae</i>   | Jujuy, Ledesma  | S 23°47'19"<br>W 64°47'26" | Torretta 42<br>(BAA 27539)          |
| <i>A. fimbriata</i>    | Misiones, L.N. Alem,<br>Provincial Route 4<br>intersection with Arroyo Isabel | S 27°31'04"<br>W 55°27'00" | Aliscioni et al. 890<br>(BAA 27521) |
| <i>A. triangularis</i> | Misiones, Apóstoles,<br>Tres Capones  | S 28°00'24"<br>W 55°36'47" | Aliscioni et al. 895<br>(BAA 27527) |

fragrance). To avoid any potential bias introduced by the authors these smell tests were carried out by people unrelated to this study.

Likewise, lugol staining was used to reveal the presence of starch, based on the assumption that the emission of fragrance requires an energetic cost through starch accumulation and consumption (Gomes da Silva 1990); and neutral red vital staining (1:10,000 in tap water pH 7) was also used as a simple test to localise glandular activity in osmophores.

To detect the presence of nectar in flowers of *Aristolochia*, glucose test strips (Hemo-glucotest) were applied in different areas of the perianth interior in fresh flowers (Galletto & Bernardello 2005; Erbar et al. 2017).

### Pollinators and floral visitors

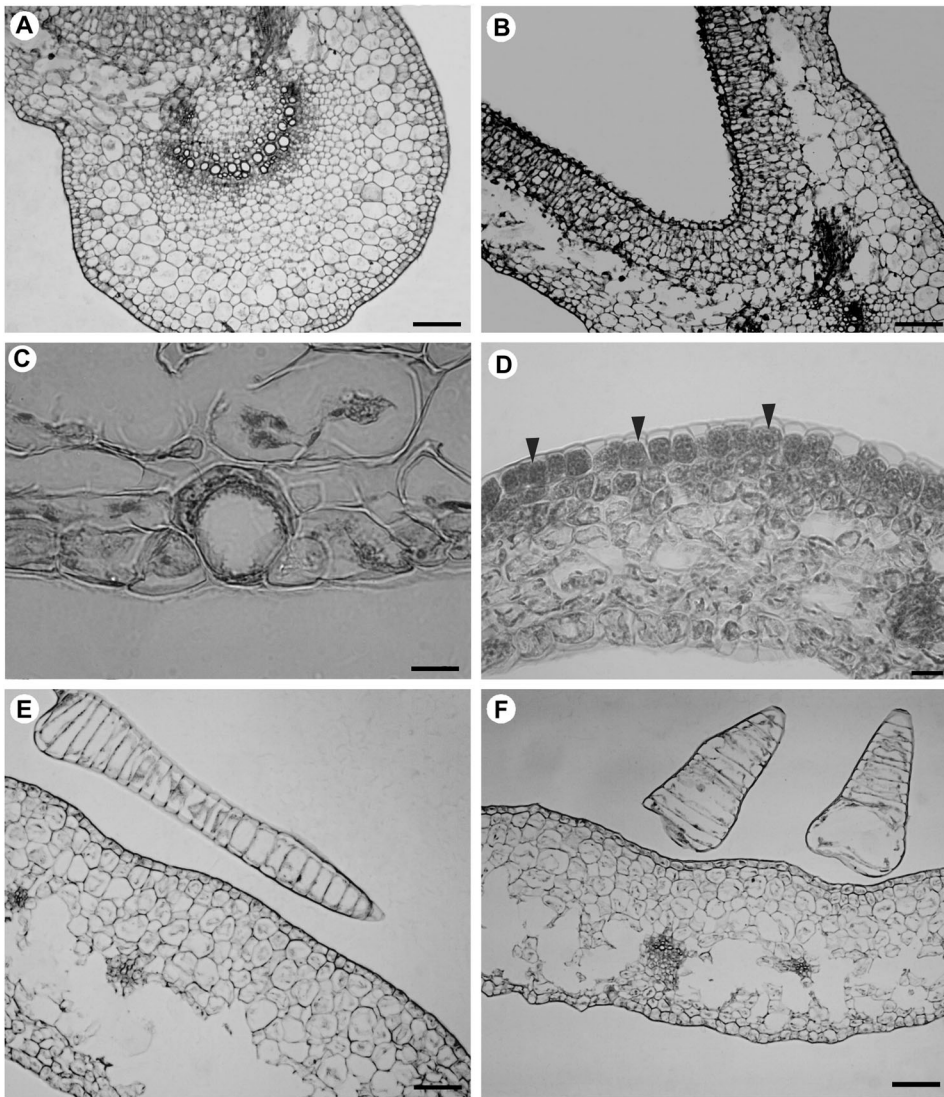
In the field we examined flowers (in both female and male phases) of each studied species looking for pollinators. Insects were captured, sacrificed in situ and preserved to be determined later. Taxonomic determination was carried out to the lowest possible taxonomic level (i.e. species, genus, tribe or family; with the collaboration of specialists, see Acknowledgements). All captured specimens are preserved in the Entomological Collection of the General Botany Unit (FAUBA) at the Faculty of Agronomy, University of Buenos Aires. The pollen load was mechanically extracted, placed in an Eppendorf vial and disaggregated according to conventional techniques (without acetolization), to be determined under the microscope, and compared to reference material (pollen obtained from stamens of the species studied).

## Results

### Floral anatomy and micromorphology

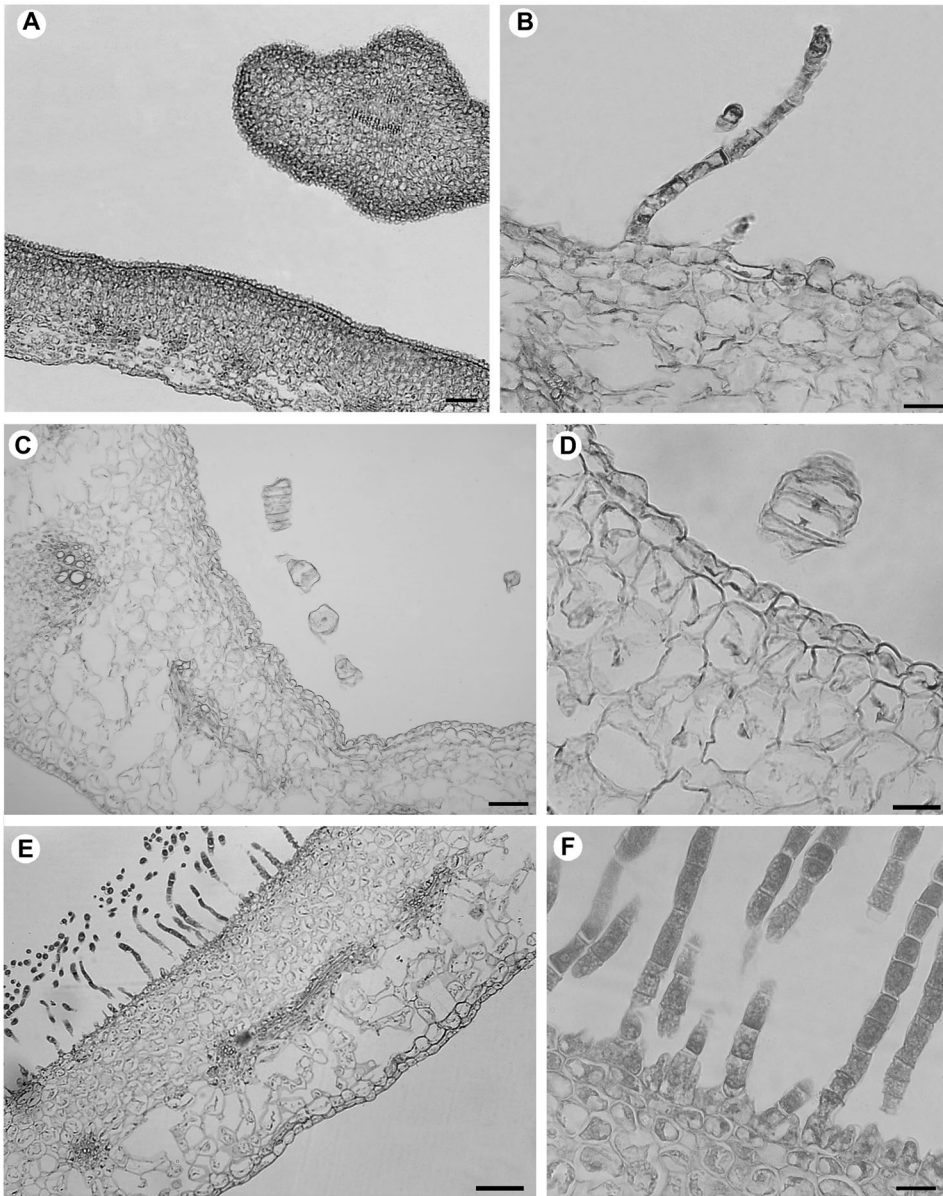
The anatomy and micromorphology of the perianth showed the same general pattern in *A. esperanzae* (Figure 2), *A. fimbriata* (Figure 3) and *A. triangularis* (Figure 4), except for some minor details. The common characteristics are described below, and the species is only mentioned if it differs in some aspect.

The transversal sections of the limb are from flat to conduplicate or V-shaped. The limb presented a dorso-ventral structure and a developed central midrib with a prominent and acute abaxial keel (Figures 2A–B, 4A). The adaxial surface presented epidermal cells with dense cytoplasm and big nuclei, from flat to slightly papillose (Figures 2D, 3A). There were trichomes with approximately 10–28 cylindrical cells and rounded or acute apices on the epidermis of the adaxial surface of the lower lip of *A. esperanzae*, towards the



**Figure 2.** *Aristolochia esperanzae*, cross sections of the perianth. **A–B**, Upper lip of the limb; **C**, ethereal oil cell in abaxial surface of the tube; **D**, lower lip of the limb, black arrowheads indicate epidermal cells; **E–F**, tube. Scale bars A–B, F = 500  $\mu\text{m}$ ; C = 200  $\mu\text{m}$ ; D = 125  $\mu\text{m}$ ; E = 600  $\mu\text{m}$ .

margin of the limb of *A. triangularis* (Figure 4B) and in the central area of the limb of *A. fimbriata* (Figure 3B). This last species also differs in presenting fimbriate projections from the margin, that in transection were circular to elliptical, covered with papillose epidermal cells and a central periphloematic vascular bundle (Figure 3A). The subepidermal parenchyma was arranged in several layers of isodiametric to shortly columnar cells with dense cytoplasm, compactly positioned (Figure 4C). Below this parenchyma was the lacunose tissue composed of vacuolated cells with small nuclei; this tissue was lax, with intercellular space ranging from low to highly developed (Figure 4E). Several well developed collateral vascular bundles with xylem and phloem were observed approximately

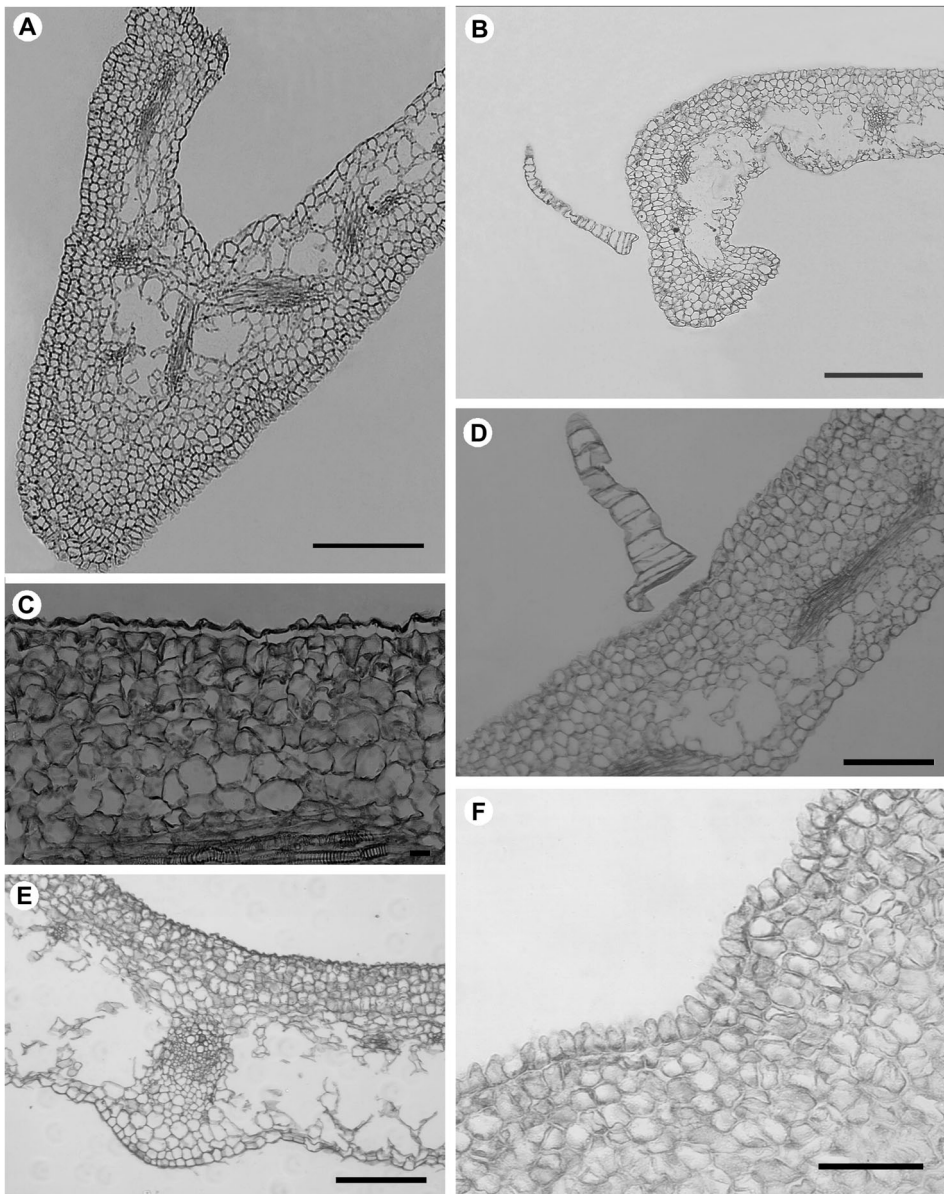


**Figure 3.** *Aristolochia fimbriata*, cross sections of the perianth. **A**, Margin of the limb with fimbriae; **B**, apex of the limb with trichome; **C–D**, apex of the tube; **E–F**, utricle. Scale bars A, C, E = 500  $\mu\text{m}$ ; B, F = 125  $\mu\text{m}$ ; D = 200  $\mu\text{m}$ .

equidistant from the epidermis. The epidermal cells on the abaxial surface were flat, contained a less dense cytoplasm and were generally bigger than the cells of the adaxial surface.

The tube of the perianth was circular or subcircular in transection, with a dorsi-ventral structure, and a developed midrib area forming a prominent abaxial keel, similar to the limb. The adaxial surface presented flat epidermal cells with a thin cuticle in





**Figure 4.** *Aristolochia triangularis*, cross sections of the perianth. **A**, Central keel in the apex of the limb; **B**, margin in the base of the limb with trichome; **C**, median zone of the limb; **D**, apex of the tube; **E**, median zone of the limb; **F**, utricle. Scale bars A–B = 500  $\mu\text{m}$ ; C = 100  $\mu\text{m}$ ; D–E = 250  $\mu\text{m}$ ; F = 100  $\mu\text{m}$ .

*A. esperanzae* (Figure 2E–F), or globose to papillose cells in *A. fimbriata* and *A. triangularis* (Figures 3C–D, 4D). This epidermis presented a greater amount of trichomes in the middle and basal zones of the tube, either conically shaped (*A. esperanzae* and *A. triangularis*) or somewhat globose (*A. fimbriata*); the basal cell of the trichome was slender in all cases (Figures 2E–F, 3C–D). During the female phase these trichomes were turgid and erect (Figure 1D). The subepidermal parenchyma was

made up of 2–5 layers of isodiametric cells in a compact disposition, generally with less dense cytoplasm than the subepidermal parenchyma of the limb; below, lacunose parenchyma with large intercellular spaces was observed and several collateral vascular bundles with differentiated xylem and phloem. The abaxial surfaces showed flat to somewhat globose epidermal cells; big and spherical isolated idioblasts were observed in the abaxial surfaces of *A. esperanzae* and *A. fimbriata* (Figure 2C).

The utricle was nearly circular in the transverse section of the middle zone, with a similar dorsi-ventral aspect in the limb and tube; the adaxial surface presented papillose to flat epidermis cells and numerous trichomes constituted of 15–25 cylindrical cells that were darkly stained, with big nuclei. Below, there were several layers of subepidermal parenchyma composed of closely arranged isodiametric and vacuolated cells, containing abundant amyoplasts. The lacunose parenchyma was well developed, vascularised by collateral bundles with differentiated xylem and phloem, and contained a lower proportion of amyoplasts. The abaxial surface presented flat, translucent epidermal cells; only spherical idioblasts were observed in *A. fimbriata* (Figures 3E–F, 4F).

SEM observations (Figures 5–6) revealed that the three species presented papillose to dome-shaped cells on the adaxial surface of the limb, covered by epicuticular waxes, cubic in *A. esperanzae* and in platelets in *A. fimbriata* (Figures 5A–B, 6C); epicuticular waxes were not observed on the limb of *A. triangularis*. Some stomata were observed dispersed on the limbs of the three species (Figure 5E) and, towards the entrance of the tubes, the epidermal cells continued being papillose but conical trichomes and closed stomata were also observed (Figures 5D, 6A, 6D). The adaxial surface of the tubes presented numerous conical trichomes, somewhat striate cuticles, and epicuticular waxes were scarce or absent.

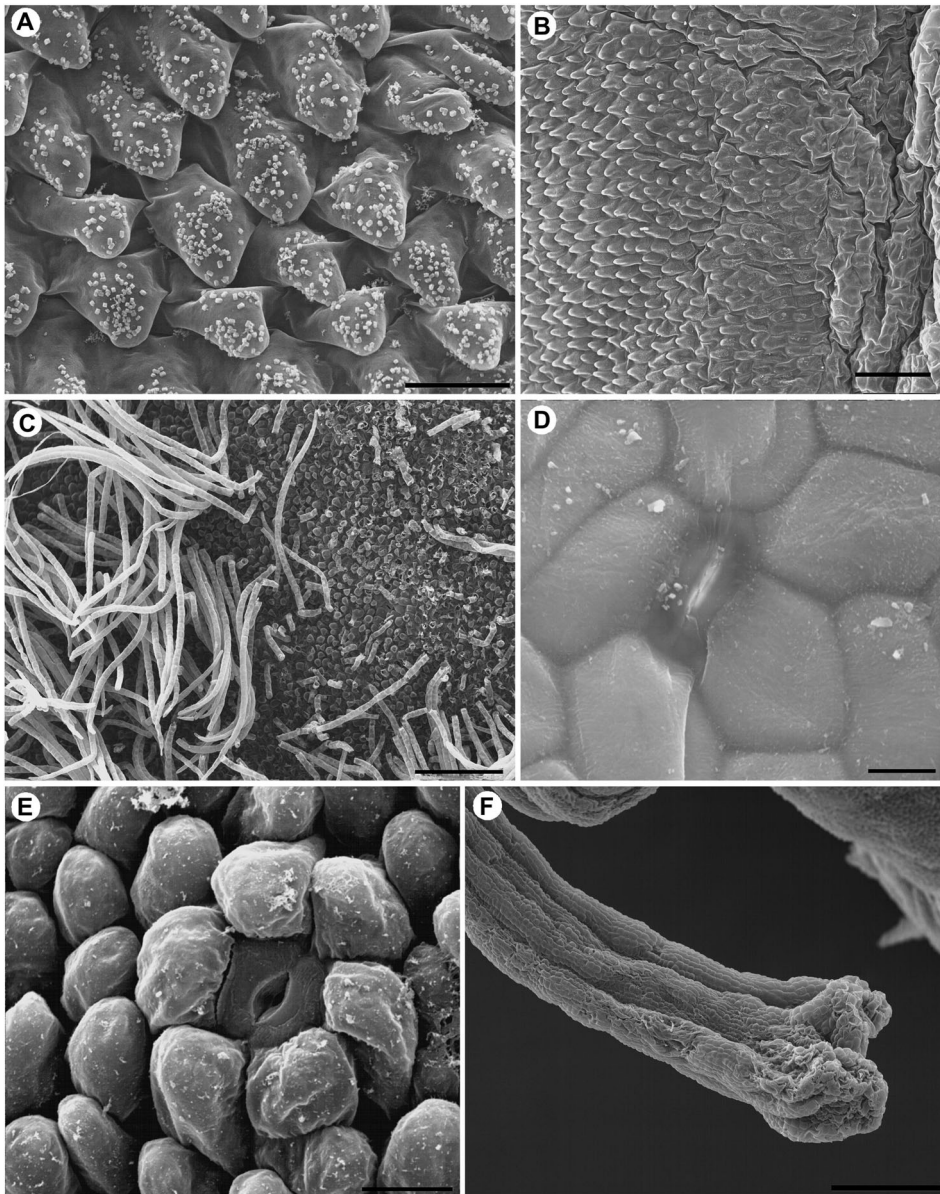
The utricle zone presented globose epidermal cells and open stomata were observed (Figure 6E). Abundant multicellular trichomes covered the internal surface of the utricle in the three species (Figures 5C, 6B, 6F), distributed more or less irregularly, with two groups of trichomes more densely packed on the adaxial side, near the entrance of the utricle, being more notorious in *A. esperanzae* and *A. triangularis*.

Contrary to the limbs and tube entrances, epicuticular waxes were not observed in the utricles of the three species. The fimbriae of the limb margin present only in *A. fimbriata* were covered by globose cells, with neither secretory aspects nor epicuticular waxes (Figure 5F).

### **Presence of secretory structures**

Using the smell test with hermetic vials, the most fragrant fragments were found in limbs of *A. esperanzae* and *A. triangularis* (in this last species, principally the portion of the limb that presents the yellow spot); while in *A. fimbriata* the tube presented more odour.

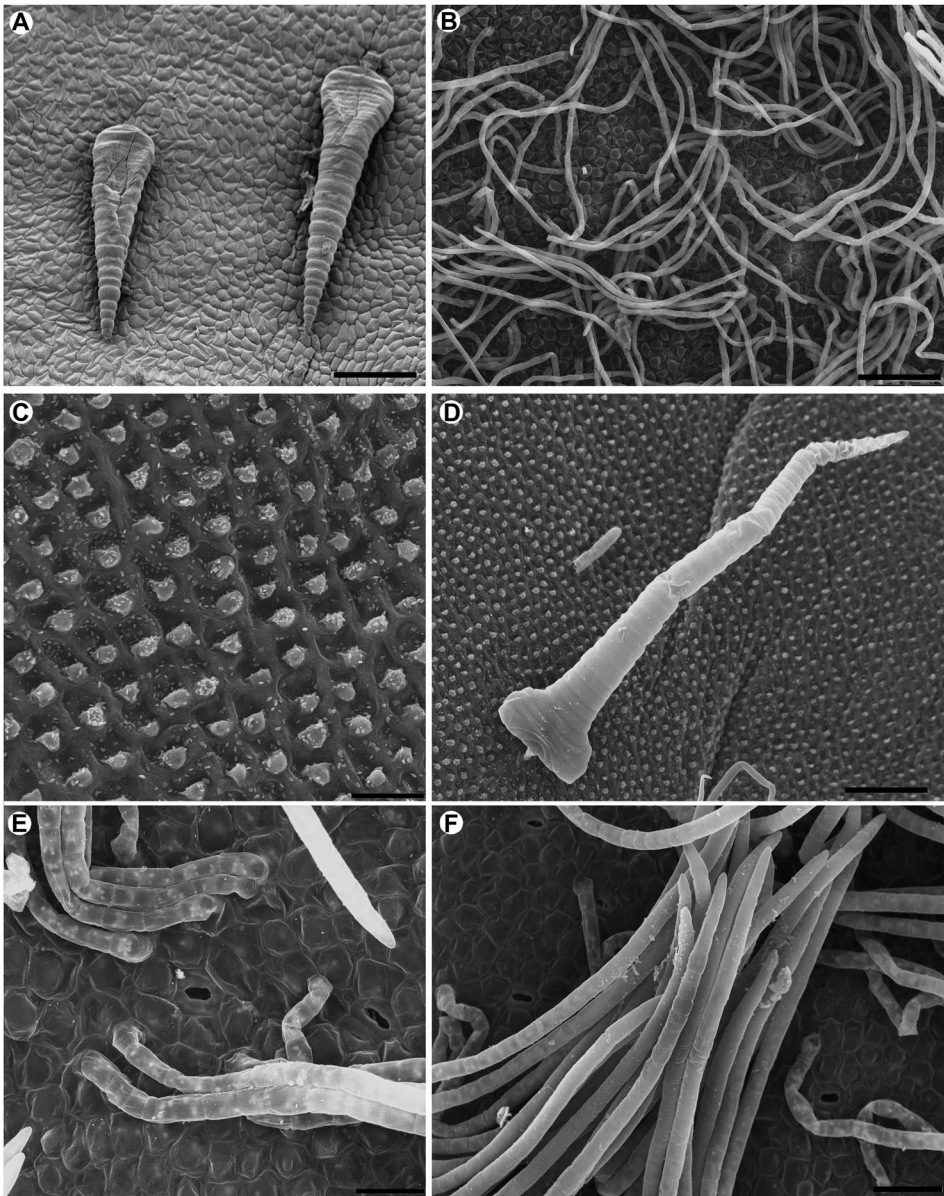
Reactions to lugol and neutral red were positive in the limbs of *A. esperanzae* and *A. triangularis*; in *A. fimbriata* both reactions were positive in the tube and also in the utricle but only to lugol (Table 2). Regarding the glucose test band applied on the internal surfaces of the perianth, positive reactions were observed in the utricles of the three species, indicating sugar secretion from the adaxial surfaces. Our observations show the presence of two trichomatous areas on the adaxial surface, near the entrance of the utricle, very well defined in *A. esperanzae* and *A. triangularis*, and more diffuse in *A. fimbriata*; these represent nectaries. The trichomes of the utricle of *A. esperanzae* and *A. fimbriata* were whitish, whereas in *A. triangularis* trichomes were reddish



**Figure 5.** Micromorphology of the adaxial surface of the perianth. **A–D**, *Aristolochia esperanzae*; **A–B**, papillose epidermal cells of the limb, note epicuticular waxes in **A**; **C**, multicellular trichomes and papillose epidermal cells in the utricle; **D**, closed stomata in the tube; **E–F**, *A. fimbriata*; **E**, open stomata in the limb; **F**, fimbriae on the limb margin. Scale bars **A** = 20  $\mu\text{m}$ ; **B** = 100  $\mu\text{m}$ ; **C**, **F** = 200  $\mu\text{m}$ ; **D**, **E** = 10  $\mu\text{m}$ .

contrasting with the remaining epidermal cells of the utricle. Flowers of the three species in the female phase presented erect and turgid trichomes in the utricle, and obvious secretions could be appreciated (**Figure 1E**). Later on, when flowers entered the male phase, the internal cavity of the utricle had a sticky aspect, and the trichomes lost turgidity and were fixed to each other by the presence of nectar (**Figure 1F**).





**Figure 6.** Micromorphology of the adaxial surface of the perianth. **A**, *Aristolochia fimbriata* trichomes with conical shape and basal foot cell in the entrance of the tube; **B**, *A. fimbriata* multicellular trichomes and papillose epidermal cells of the utricle; **C**, *A. triangularis* papillose epidermal cells of the limb, note epicuticular waxes; **D**, *A. triangularis* trichome with conical shape and basal foot cell in the entrance of the tube; **E**, *A. triangularis* opened stomata in the utricle; **F**, *A. triangularis* multicellular trichomes in the utricle. Scale bars A–B, D = 100  $\mu\text{m}$ ; C, E–F = 50  $\mu\text{m}$ .

### ***Floral visitors and potential pollinators***

The insects observed in flowers of the three studied species of *Aristolochia* were mainly diverse species of Diptera (Anthomyiidae, Drosophilidae, Muscidae, Sarcophagidae,



**Table 2.** Results of the tests to identify secretory tissues.

| Species         | <i>A. esperanzae</i> |      |         | <i>A. fimbriata</i> |      |         | <i>A. triangularis</i> |      |         |
|-----------------|----------------------|------|---------|---------------------|------|---------|------------------------|------|---------|
|                 | Limb                 | Tube | Utricle | Limb                | Tube | Utricle | Limb                   | Tube | Utricle |
| Perianth region |                      |      |         |                     |      |         |                        |      |         |
| Smell intensity | ++                   | +    | –       | ++                  | +++  | +       | ++ (x)                 | +    | –       |
| Lugol           | ++                   | +    | –       | +                   | ++   | ++      | ++                     | –    | +       |
| Neutral red     | ++                   | +    | –       | +                   | ++   | –       | +                      | (x)  | –       |
| Glucose test    | –                    | –    | ++      | –                   | –    | ++      | –                      | –    | ++      |

–, negative reaction; +, moderate reaction; ++, intense reaction; +++, very intense reaction; (x), more intense reaction in the spot area.

Ulidiidae) and a few individuals of Coleoptera (Staphylinidae). In flowers of *A. esperanzae*, in female phase, six species/morphospecies were found, of which *Acrosticta* sp. (Ulidiidae) and *Tricharaea occidua* (Sarcophagidae) seem to be potential pollinators because of the presence of pollen on the body. In flowers of *A. fimbriata* only two species of Drosophilidae (principally *Zaprionus indanum*) and one beetle of the genus *Platandria* (family Staphilinidae) were found. In the analysed flowers of *A. triangularis*, in female phase, only five individuals of one indeterminate species of Anthomyiidae were found.

Regarding pollen loads on the body, only some of the specimens of Ulidiidae and one of the species *Tricharaea occidua* (Sarcophagidae) were observed with abundant pollen of *A. esperanzae* on the dorsal surface of the thorax (Figure 7). All flowers contained living visitors (no dead insects were found within the utricles). Table 3 summarises the information of the floral visitors observed in each species of *Aristolochia*.

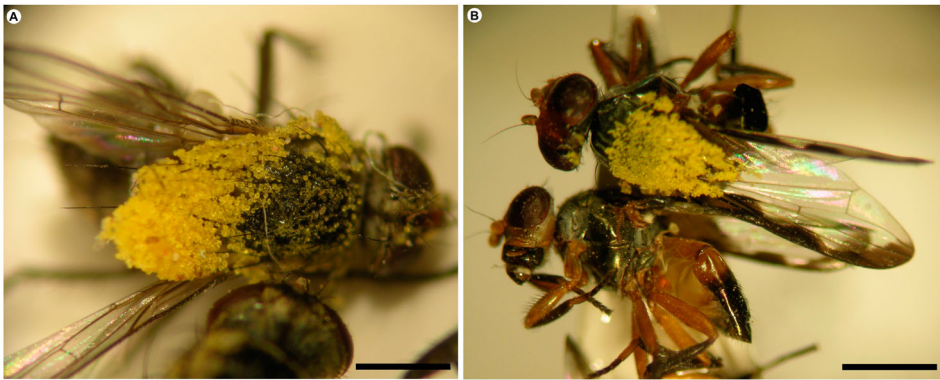
## Discussion

The studied species have the same general anatomical structure in the perianth and similar histological organisations from the limb up to the utricle, with the mesophyll arranged in a dorsi-ventral structure and the more compact parenchyma adaxially located. The epidermis of the adaxial surface is associated with different types of trichomes, in all cases uniseriate, multicellular and unbranched. These trichomes present dissimilar aspects and functions depending on the zone of the perianth or state of the flower.

We observed big idioblasts in the epidermis of the abaxial surfaces of the tubes and utricles of *A. esperanzae* and *A. fimbriata*; and although no histochemical test was performed, their anatomical characteristics coincide with the so-called ethereal oil cells. These cells can contain terpenoids, and it has been suggested that they form a chemical defense mechanism against predators and pathogens.

Our observations with the SEM revealed that papillose cells and epicuticular waxes are presents on limbs and tube entrances (except in *A. triangularis*). Wax crystals make leaves unsurmountable or at least difficult to walk on for some insect species (Eigenbrode & Jetter 2002), but in flowers of *Aristolochia* epicuticular waxes, along with the papillose epidermal cells, probably play a much more structural role, explaining the surfaces' anti-adhesive and slippery effects (Poppinga et al. 2010). Thus, the limbs would act as a landing zone for insects which would slide towards the tube due to the presence of epicuticular waxes and/or epidermal papillary cells. After this, the pollinators pass through the tubes, due to the orientation of the conical hairs, to fall into the utricles.

We confirm the presence of osmophores in all studied species based on anatomical observations, olfactory and histochemical tests. We observed these secretory structures



**Figure 7.** Floral visitor with pollen loads. **A**, *Trichareae occidua* (Sarcophagidae); **B**, cf. *Acrosticta* sp. (Ulidiidae). Scale bars = 1 mm.

to consist of an epidermis covered by a thin cuticle and two to five subepidermal parenchymatous layers. Vogel (1990) observed that osmophores were innervated by bundle endings of just phloem, but we observed vascular bundles with well developed xylematic tissue. Moreover, we observed open stomata surrounded by papillose cells only at the tube entrance of *A. fimbriata*, but these structures were either absent or scarcely dispersed in the osmophores of *A. esperanzae* and *A. triangularis*.

The limbs of *A. esperanzae* and *A. triangularis* emitted stronger fragrances and, coincidentally, this zone of the perianth showed a dark stained epidermis and subepidermal parenchyma. The reactions with lugol and neutral red were positive in these zones for both species. This anatomical–functional concordance was not observed in *A. fimbriata* due to the more compact parenchyma, with more stainable cells observed in the limb but a stronger fragrance detected from the tube. The fimbriae would be visual and tactile attractions to pollinators. Similar observations were made in *A. pilosa* Kunth whose fimbriae are necessary to attract flies, but once the insects arrive at the flowers they are apparently not interested in the limb because they immediately try to enter the tube (Wolda & Sabrosky 1986).

Pabón-Mora et al. (2015) reported that, in *A. fimbriata*, the inner epidermis of the limb and utricle are formed by osmophores, as well as by the tips of the fimbriae. Even though we detected high emission smells in the tube, some odour was also perceived in the limb and utricle, which partially coincides with the observation of Pabón-Mora et al. (2015). However, there was no smell from the fimbriae in the present study.

The glucose test band applied on the internal surfaces of the perianth probe the presence of nectar in the utricle of the three studied species. Daumann (1959) stated that the trichomes of the utricle are erect due to turgid pressure exerted by sugar content during the female phase; once the nectar is secreted, trichomes lose pressure and they become sticky, as we observed during the male phase. Erbar (2014) studied flowers of *A. gigantea* and described nectaries at the upper end of the utricle consisting of multicellular hairs and also commented that the papillose epidermis between the hairs probably participates in the secretion of sugar. In a more exhaustive work, Erbar et al. (2017) indicated that the nectar-secreting areas are always situated near the junction between utricle

**Table 3.** Analysis of the floral visitors.

|                        | No. studied plants | No. flowers and phase analysed | Diptera                     |  |   |               |              | Coleoptera<br>Staphylinidae |
|------------------------|--------------------|--------------------------------|-----------------------------|--|---|---------------|--------------|-----------------------------|
|                        |                    |                                | Sarcophagidae               | Muscidae   | Ulidiidae   | Drosophilidae | Anthomyiidae |                             |
| <i>A. esperanzae</i>   | 2                  | 5 female                       | 3 <i>Tricharaea occidua</i> | 1 <i>Gymnodia</i> sp.<br>1 <i>Limnophora</i> sp. | 26 cf. <i>Acrosticta</i> sp.<br>1 indeterminate sp. | 0             | 0            | 0                           |
| <i>A. fimbriata</i>    | 6                  | 4 male<br>14 female            | 0                           | 0  | 0   | 0             | 0            | 0                           |
| <i>A. triangularis</i> | 1                  | 6 male<br>4 female             | 0                           | 0  | 0   | 0             | 0            | 0                           |
|                        |                    | 8 male                         | 0                           | 0  | 0   | 0             | 0            | 0                           |

and tube. These areas can have two, four or numerous nectary-hair patches on the adaxial side. Also, nectarioles were described in *A. grandiflora*, *A. arborea*, *A. salvadorensis* and *A. tricaudata* (Erbar et al. 2017) and *A. fimbriata* (González & Pabón-Mora 2015). Taking into account the types of nectaries established by Erbar et al. (2017) in *Aristolochia*, the species studied here match the '*A. gigantea* type' due to the observed two trichomatous areas on the adaxial surface of the utricle, near to the entrance. These patches of hairs are very well defined in *A. esperanzae* and *A. triangularis*, but are somewhat more diffuse in *A. fimbriata*. However, we also observed lines of trichomes more irregularly dispersed on all surfaces with secretory aspects, separated by papillose cells and open stomata called nectarostomata (Smets & Cresens 1988). These observations indicate that all surfaces of the utricle could contribute to the secretion of sugar. Vogel (1998) assumed that the presence of nectaries on the utricles aims to guarantee the survival of the pollinators inside the utricle; moreover, Sakai (2002) proved that nectar is an important pollinator reward in *Aristolochia*. The presence of mostly living and few dead insects within the flowers of the three species of *Aristolochia* analysed is congruent with these ideas.

Regarding floral visitors, our observations match those of other *Aristolochia* spp. (Proctor et al. 1996; Endress 1998) because flowers were visited by small flies of different saprophagous families. Most of the insects were found in the flowers during the female phase, in coincidence with the stage when the trichomes of the tube are erect and curved towards the utricle. We observed conical pluricellular trichomes along the tubes, composed by translucent and vacuolated cells, without secretory aspects, in the three studied species. Oelschlägel and collaborators (2009) described the function of these trichomes associated with maintaining pollinators. Furthermore, we observed conical, pluricellular trichomes scarcely dispersed on limbs and at the tube entrances in the three species. Pabón-Mora et al. (2015) mention that the conical hairs in the tube of *A. fimbriata* are secretories during preanthesis.

Numerous fly families were cited as pollinators of *Aristolochia* spp. (Berjano et al. 2009; Hipólito et al. 2012). In our observations, we could identify several species but not all were efficient pollinators. It is important to note that our results about floral visitors and pollinators of the three studied species of *Aristolochia* correspond only to one population of each species in Argentina, but as these species have wide geographical distributions the floral visitor richness should be bigger. The flowers of *Aristolochia* show a pronounced protogyny and attract flies by odour only during the female phase (Oelschlägel et al. 2009). The presence of insects with pollen loads inside the flowers in this phase proves that these insects must have visited at least one different flower during its male stage beforehand (Rulik et al. 2008). A study in *A. bracteolata* Lam. revealed anther rupture to be explosive and the whole utricle is dusted with pollen (Razzak et al. 1992). Accordingly, the finding of flies with pollen loads observed in flowers of *A. esperanzae* during the female phase proves that these insects previously visited other flowers in their male phase and reveals that they are potential pollinators of this species of *Aristolochia*. The presence of beetles of the Staphylinidae can be explained because some species of this family are attracted to sites where saprophagous flies oviposit, and even some genera are typically predators of eggs and larvae of Diptera (M. Chani-Posse, Instituto Argentino de Investigaciones de las Zonas Áridas; Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina, pers. comm. 2016). Other studies have reported different species of



Staphylinidae and other beetle families in flowers of *Aristolochia* spp. (Sakai 2002; Burgess et al. 2004; Trujillo & Sérsic 2006; Nakonechnaya et al. 2008; Rulik et al. 2008).

## Acknowledgements

We thank L. Patitucci (Sarcophagidae, Muscidae, Anthomyiidae), M. Chani-Posse (Staphylinidae), R. L. Mello (Drosophilidae) and E. Kameneva (Ulidiidae) for collaboration in the determination of insects, and A. Torretta, M. Zietsman and R. Saurral for helping with the English revision of the manuscript.

## Disclosure statement

No potential conflict of interest was reported by the authors.

## Funding

The fieldwork was supported by CONICET [grant number PIP 11220110100312], ANPCyT [grant number PICT 2013-1867] and UBA [grant number UBACyT 20020130200203BA] Argentina. SSA and JPT are affiliated with CONICET.

## References

- Ahumada Z. 1967. Revisión de las Aristolochiaceae argentinas. *Opera lilloana*. 16:1–145.
- Amela García MT, Hoc PS. 1998. Biología floral de *Passiflora foetida* (Passifloraceae). *Revista de Biología Tropical*. 46:191–202.
- Bänziger H, Disney RHL. 2006. Scuttle flies (Diptera: Phoridae) imprisoned by *Aristolochia baenzigeri* (Aristolochiaceae) in Thailand. *Mitteilungen der Schweizerischen Entomologischen Gesellschaft*. 79:29–61.
- Berjano R, Ortiz PL, Arista M, Talavera S. 2009. Pollinators, flowering phenology and floral longevity in two Mediterranean *Aristolochia* species, with a review of flower visitor records for the genus. *Plant Biology*. 11:6–16.
- Brantjes NBM. 1980. Flower morphology of *Aristolochia* species and the consequences for pollination. *Acta Botanica Neerlandica*. 29:212–213.
- Brues CT. 1928. Some Cuban Phoridae which visit the flowers of *Aristolochia elegans*. *Psyche*. 35:160–161.
- Burgess KS, Singfield J, Melendez V, Kevan PJ. 2004. Pollination biology of *Aristolochia grandiflora* (Aristolochiaceae) in Veracruz, Mexico. *Annals of the Missouri Botanical Garden*. 91:346–356.
- Cammerloher H. 1923. Zur biologie der blute von *Aristolochia grandiflora* Swartz. *Plant Systematics and Evolution*. 72:180–198.
- Costa EL, Hime NC. 1981. Biología floral de *Aristolochia gigantea* Mart. & Zucc. (Aristolochiaceae). *Rodriguésia*. 33:23–44.
- Costa EL, Hime NC. 1983. Observacoes sobre a biología floral de *Aristolochia macroura* Gomez (Aristolochiaceae). *Atas da Sociedade Botânica do Brasil. Sección Rio de Janeiro*. 1:63–66.
- Daumann E. 1959. Zur Kenntnis der Blütennektarien von *Aristolochia*. *Preslia*. 31:359–372.
- Eigenbrode SD, Jetter R. 2002. Attachment to plant surface waxes by an insect predator. *Integrative and Comparative Biology*. 42:1091–1099.
- Endress PK. 1998. Diversity and evolutionary biology of tropical flowers. Cambridge: Cambridge University Press.
- Erbar C. 2014. Nectar secretion and nectaries in basal angiosperms, magnoliids and non-core eudicots and a comparison with core eudicots. *Plant Diversity and Evolution*. 131:63–143.
- Erbar C, Heiler A, Leins P. 2017. Nectaries in fly-deceptive pitcher-trap blossoms of *Aristolochia*. *Flora*. 232:128–141.

- Galetto L, Bernadello G. 2005. Nectar. In: Dafni A, Kevan PK, Husband BC, editors. Practical pollination biology. enviroquest. Cambridge, Canada: Enviroquest Ltd; p. 261–328.
- Gomes da Silva A. 1990. Osmóforos: retrospectiva dos últimos 20 anos de pesquisa. *Rodriguesia*. 42:7–20.
- González F. 1999. Inflorescence morphology and the systematics of Aristolochiaceae. *Systematics and Geography of Plants*. 68:159–172.
- González F, Pabón-Mora N. 2015. Trickery flowers: the extraordinary chemical mimicry of *Aristolochia* to accomplish deception to its pollinators. *New Phytologist*. 206:10–13.
- González F, Stevenson DW. 2000. Gynostemium development in *Aristolochia*. *Botanische Jahrbücher für Systematik, Pflanzengeschichte und Pflanzengeographie*. 122:249–291.
- Hall DW, Brown BV. 1993. Pollination of *Aristolochia littoralis* (Aristolochiales: Aristolochiaceae) by males of *Megaselia* spp. (Diptera: Phoridae). *Annals of the Entomological Society of America*. 86:609–613.
- Hipólito J, Viana BF, Selbach-Schnadelbach A, Galetto L, Kevan PG. 2012. Pollination biology and genetic variability of a giant perfumed flower *Aristolochia gigantea* Mart. and Zucc., (Aristolochiaceae) visited mainly by small Diptera. *Botany*. 90:815–829.
- Jaramillo MA, Kramer EM. 2004. APETALA3 and PISTILLATA homologs exhibit novel expression patterns in the unique perianth of *Aristolochia* (Aristolochiaceae). *Evolution and Development*. 6:449–458.
- Kelly LM, González F. 2003. Phylogenetic relationships in Aristolochiaceae. *Systematic Botany*. 28:236–249.
- Lindner E. 1928. *Aristolochia lindneri* Berger und ihre Bestäubung durch Fliegen. *Biologisches Zentralblatt*. 48:93–101.
- Nakonechnaya OV, Sidorenko VS, Koren' OG, Nesterova SV, Zhuravlev YN. 2008. Specific features of pollination in the Manchurian birthwort, *Aristolochia manshuriensis*. *Biology Bulletin*. 35:459–465.
- Oelschlägel B, Gorb S, Wanke S, Neinhuis C. 2009. Structure and biomechanics of trapping flower trichomes and their role in the pollination biology of *Aristolochia* plants (Aristolochiaceae). *New Phytologist*. 184:988–1002.
- Oelschlägel B, Nuss M, von Tschirnhaus M, Pätzold C, Neinhuis C, Dötterl S, Wanke S. 2015. The betrayed thief—the extraordinary strategy of *Aristolochia rotunda* to deceive its pollinators. *New Phytologist*. 206:342–351.
- Pabón-Mora N, Suárez-Baron H, Ambrose BA, González F. 2015. Flower development and perianth identity candidate genes in the basal angiosperm *Aristolochia fimbriata* (Piperales: Aristolochiaceae). *Frontiers in Plant Science*. 6:1095. doi:10.3389/fpls.2015.01095.
- Petch T. 1924. Notes on *Aristolochia*. *Annals of the Royal Botanic Gardens, Peradeniya*. 8:1–108.
- Poppinga S, Koch K, Bohn HF, Barthlott W. 2010. Comparative and functional morphology of hierarchically structured anti-adhesive surfaces in carnivorous plants and kettle trap flowers. *Functional Plant Biology*. 37:952–961.
- Proctor M, Yeo P, Lack A. 1996. The natural history of pollination. Orgeon: Timber Press.
- Razzak MA, Ali T, Ali SI. 1992. The pollination biology of *Aristolochia bracteolata* Lam. (Aristolochiaceae). *Pakistan Journal of Botany*. 24:79–87.
- Rulik B, Wanke S, Nuss M, Neinhuis C. 2008. Pollination of *Aristolochia pallida* Willd. (Aristolochiaceae) in the Mediterranean. *Flora*. 203:175–184.
- Sakai S. 2002. *Aristolochia* spp. (Aristolochiaceae) pollinated by flies breeding on decomposing flowers in Panama. *American Journal of Botany*. 89:527–534.
- Schmidt OC. 1935. Aristolochiaceae. In: Engler A, Prantl K, editors. *Die Naturlichen Pflanzenfamilien* 2 ed., Vol. 16. Leipzig: Engelmann; p. 204–242.
- Smets EF, Cresens EM. 1988. Types of floral nectaries and the concepts character and character-state, a reconsideration. *Acta Botanica Neerlandica*. 37:121–128.
- Stotz GC, Gianoli E. 2013. Pollination biology and floral longevity of *Aristolochia chilensis* in an arid ecosystem. *Plant Ecology & Diversity*. 6:181–186.
- Trujillo CG, Sérsic AN. 2006. Floral biology of *Aristolochia argentina* (Aristolochiaceae). *Flora*. 201:374–382.

- Vogel S. 1978. Pilzmückenblumen als Pilzmimeten. Erster Teil. Flora. 167:329–366.
- Vogel S. 1990. The role of scent glands in pollination: on the structure and function of osmophores. New Delhi: Engelmann.
- Vogel S. 1998. Remarkable nectaries, structure, ecology, organophyletic perspectives II: nectarioles. Flora. 193:1–29.
- Wolda H, Sabrosky CW. 1986. Insect visitors of two forms of *Aristolochia pilosa* in Las Cumbres, Panama. Biotropica. 18:295–299.
- Zarlavsky GE. 2014. Histología vegetal. Técnicas simples y complejas. Buenos Aires: Sociedad Argentina de Botánica.