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Mouthparts morphology of the mealybug *Phenacoccus aceris*

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Abstract

The mealybug *Phenacoccus aceris* (Signoret) (Hemiptera Pseudococcidae) is a significant economic pest on grape as a vector of grapevine leafroll-associated viruses (GLRaVs) causing the damaging grapevine leafroll disease. To gain new insights into the feeding behavior and structural anatomy of this mealybug, the structure of the mouthparts was described using scanning and transmission electron microscopy and light microscopy. The piercing-sucking mouthparts of *P. aceris* are composed of a clypeolabral shield and a three-segmented labium with a groove on its anterior face containing the stylet fascicle. Fourteen pairs of different types of sensilla cover the exposed surface of the labium. The stylet fascicle consists of two mandibular stylets, with serrations on their distal extremity, surrounding two maxillary stylets. The maxillary stylets are interlocked and their coapted grooves delimitate the food and the salivary canals. No acrostyle-like structure was observed at the distal extremity of the maxillary stylets; instead, a specific ridge takes place. Stylet pathway was observed from the salivary flange on penetration site and then with the salivary sheath inside plant tissues up to the vascular tissues. This study provides new insights into *P. aceris* mouthparts and opens prospects for the study of the feeding behavior and the retention site of non-circulatively transmitted virions.

Key words: microscopy, SEM, TEM, stylets.

Introduction

The mealybug *Phenacoccus aceris* (Signoret) (Hemiptera Pseudococcidae), also called apple or maple mealybug, has been reported on fruit trees such as maple, apple and cherry trees, and on over 100 host plant species belonging to 28 families and 52 genera, in Western Europe, Canada, United States, Russia, Japan and Korea (García Morales *et al*., 2016). *P. aceris* belongs to the Pseudococcidae family, Coccoidea superfamily, suborder Sternorrhyncha within the Hemiptera (Gullan and Cook, 2007). This species is also known to transmit *Little cherry virus*-2 (Closteroviridae Ampelovirus), causing 'Little cherry disease' (Raine *et al*., 1986; Rott and Jelkmann, 2001). More recently, this highly polyphagous insect has been shown to have a significant economic incidence on grape, by transmitting (i) the *Ampelovirus* species *Grapevine leafroll-associated virus* (GLRaV) -1, -3 and -4-like (Sforza *et al*., 2003; Le Maguet *et al*., 2012; 2013; Martelli, 2014) involved in 'Grapevine leafroll disease', reducing grape yield and quality (Atallah *et al*., 2012), and (ii) the *Vitivirus* species *Grapevine virus A* (GVA) and *Grapevine virus B* (GVB) involved in the 'Rugose wood' complex, a secondary disease of grapevine (Le Maguet *et al*., 2012). *Ampelovirus* and *Vitivirus* members are phloem-restricted. GLRaV-3 was shown to be transmitted according to the semi-persistent and non-circulative mode (Tsai *et al*., 2008; Krüger *et al*., 2015), that involves a virion retention site in mouthparts and/or foregut.

Throughout their evolution, insects have developed an array of different specialized mouthpart types, playing an important role in host choice, in feeding, and in retention and inoculation of pathogens (see Killiny and Almeida, 2009; Blanc *et al*., 2014; Blanke *et al*., 2015; Whitfield *et al*., 2015). The ultrastructure of mouthparts of mealybugs and soft scales (Coccoidea: Pseudococcidae and Coccidae) is only poorly documented, apart from earlier descriptions (e.g. Pesson, 1944) made before the widespread use of electron microscopy. Calatayud and Le Rü (2006) described the organization of the sensilla present on the antennae and the labium of *Phenacoccus manihoti* Matile-Ferrero (Pseudococcidae), and their role to mediate first instar nymphs (crawlers) orientation and the initial steps of plant acceptance. Ahmad *et al*. (2012) studied the mouthparts and stylet penetration of the lac insect *Kerria lacca* (Varshney) (Coccoidea Kerriidae), revealing mouthparts similar to that of other hemipterans and a vertical and intracellular penetration, associated with the production of salivary flanges and sheaths.

The aim of this study was to describe the organization of *P. aceris* mouthparts, including the clypeolabral shield, the labium, the stylet fascicle, the distal extremity of the maxillary stylets, and stylet penetration through plant tissues. Light microscopy, scanning (SEM) and transmission (TEM) electron microscopy were used for observations. This detailed anatomical description will provide new insights to better understand the feeding behavior and the virus transmission process by mealybugs.

Materials and methods

Insect rearing

Cottony egg sacs (ovisacs) were collected from vineyards in spring in Wolxheim and Kienheim (Alsace, France). After hatching in the laboratory, mealybug nymphs were allowed to feed on potato sprouts or on virus-free grape leaves (Pinot noir clone 115), at a temperature of 23 °C and a photoperiod of 16h/8h (day/night).

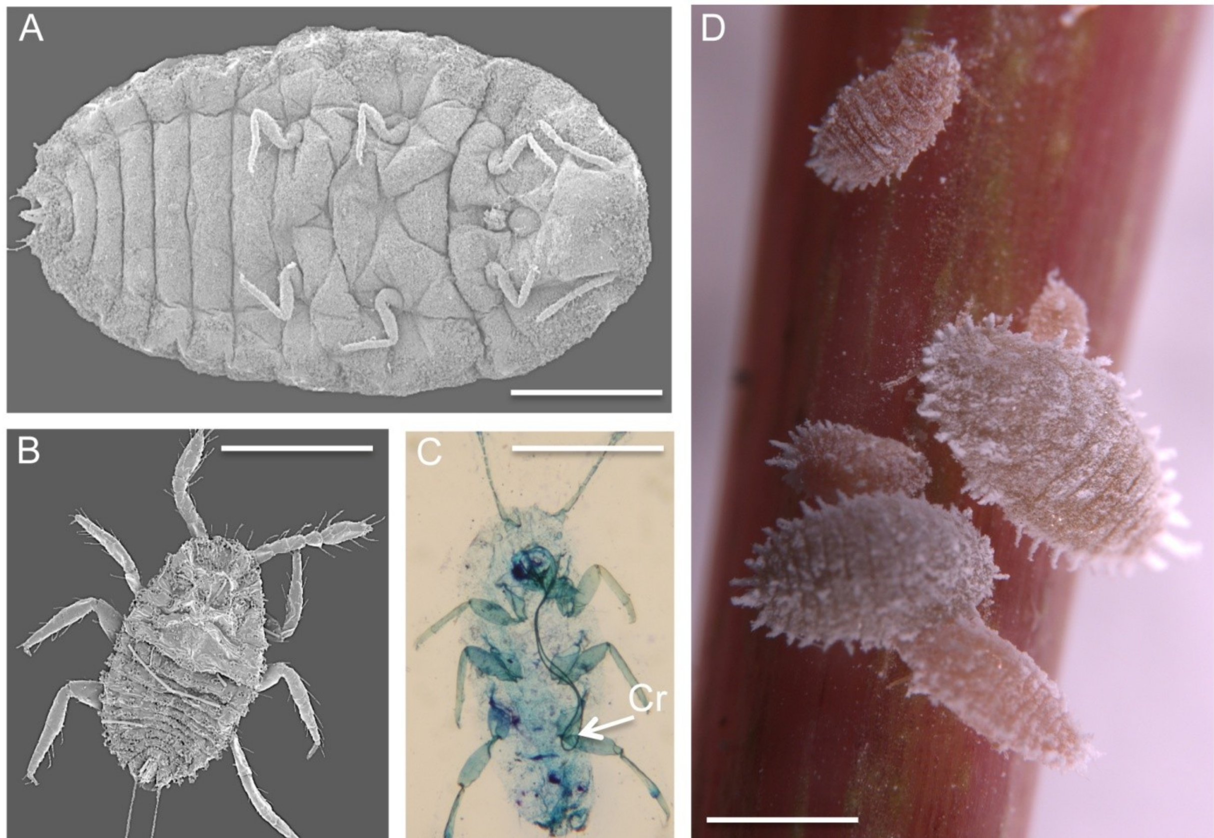

Figure 1. Micrographs of *P. aceris* on grape. **(A)** Scanning electron micrograph of a ventral view of a third instar nymph; **(B)** SEM of a dorsal view of a first instar nymph; **(C)** Ventral view in light microscopy of a second instar showing the crumena (Cr); **(D)** Light micrograph of a mealybug settlement on a petiole. Bars: A = 350 µm; B = 175 µm; C = 300 µm; D = 900 µm.

Scanning electron microscopy (SEM)

For SEM, the wax of first and third instar nymphs was removed gently with a paintbrush and a solution composed of 25% ethanol and 1% Triton X100 (Cid and Fereres, 2010). Insects were then cleaned in an ultrasonic cleaner for 1 min in 70% ethanol, followed by dehydration in a gradual series of 70, 80, 90 and 100% ethanol. They were finally dried for 2 h at room temperature. Samples were mounted on aluminium stubs, coated with gold-palladium and observed with a Hitachi S800 scanning electron microscope operating at 5 kV.

Stylets of third instar nymphs stored in 70% ethanol were isolated from the rest of the insect body under a dissecting microscope using thin tweezers (Dumont Tweezer N° 5). The four stylets were then separated using insect pins (0.1 mm; Agar Scientifics) and put on siliconized coverslip (22 mm squared; D. Dutscher). The stylets were heated at 50 °C for 2 h for adhesion, washed three times in water and dried for 15 min at 37 °C. Samples were coated with gold-palladium and observed with a Hitachi S4000 scanning electron microscope operating at 10 kV.

Transmission electron microscopy (TEM)

Third instar nymphs were fixed in 5% glutaraldehyde and 2% paraformaldehyde, in 0.1 M phosphate buffer pH 7.4 for 20 h at 4 °C, as modified from Reinbold *et al*. (2001). After three 5-min rinses in phosphate buffer, the samples were post-fixed in 1% osmium tetroxide for 1 h in the dark at 4 °C. Samples were then gradually dehydrated in a series of acetone solutions (30, 50, 70, 95 and 100%), embedded in epoxy araldite resin and polymerized for 60 h at 65 °C. They were oriented in order to perform longitudinal and cross sections. Ultrathin sections (80 nm) were collected on copper grids and counterstained with uranyl acetate (3%) and lead citrate (4 mg/ml). Observations were made using a Philips EM208 TEM operating at 80 kV. Micrographs were taken on Kodak films and scanned.

Light microscopy

Insect clearing

In order to clear the exoskeleton and observe inner organs, second instar nymphs were placed in 10% KOH and heated at 95 °C for 15 min. They were subsequently rinsed in distilled water, stained with Azure B, and finally rinsed in distilled water and in 95% ethanol. Stained insects were mounted on microscopy slide, covered with coverslip, and observed under light microscopy using a Zeiss Axio Imager M2 microscope. Pictures were taken with Axiocam 105 color digital camera.

Cross-sections

Semi-thin sections (500 nm) were incubated for 5 min in a drop of Azure B, and washed with water. Sections were then mounted on microscope slides covered with

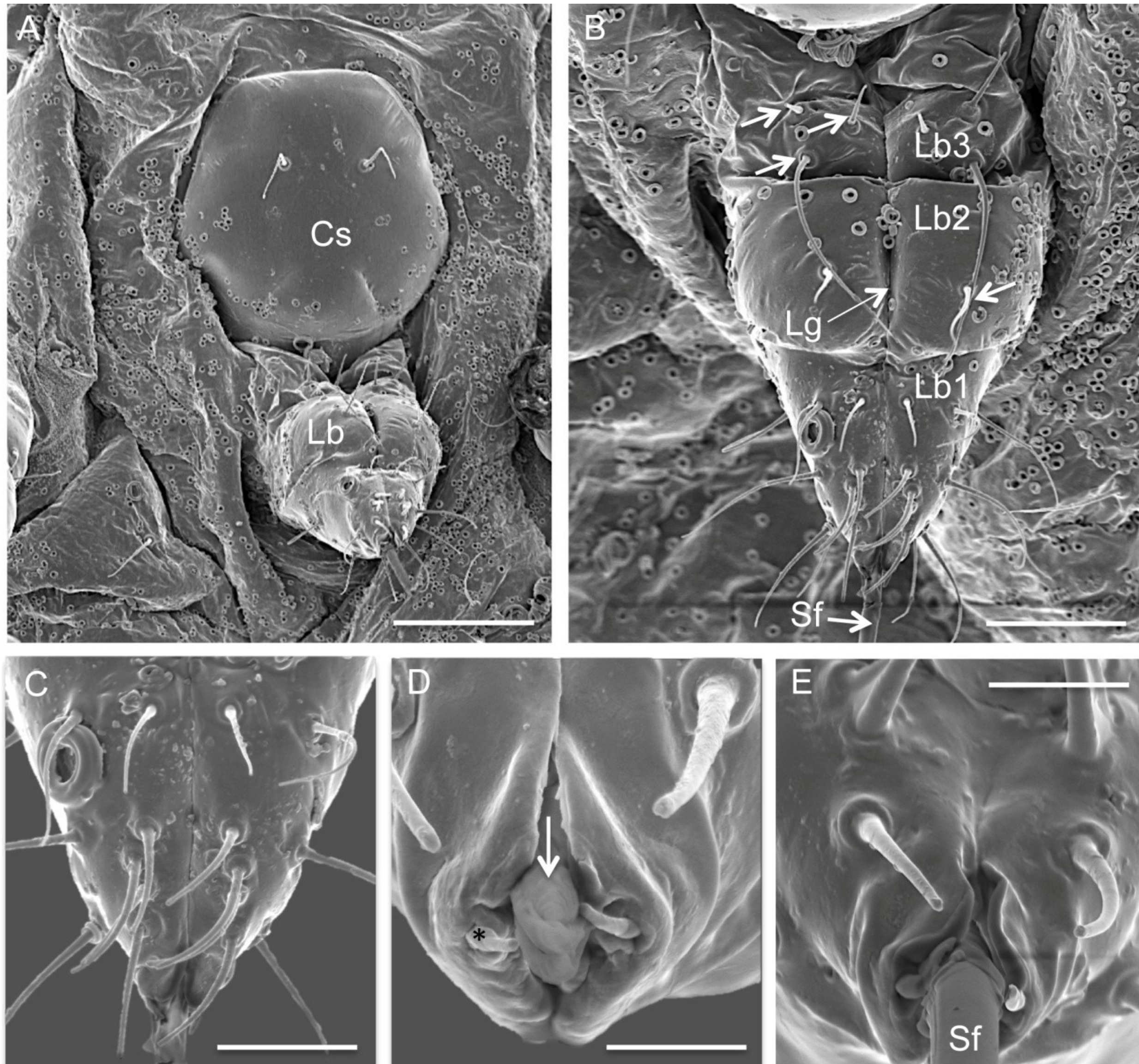

Figure 2. SEM on *P. aceris* mouthparts. **(A)** Front view of the clypeolabral shield (Cs) and the labium (Lb); **(B)** Front view of the three segmented labium (Lb1, Lb2, Lb3) showing the labial groove (Lg), sensilla of the second and third segments (white arrows) and the stylet fascicle (Sf); **(C)** Enlarged view of the first segment of the labium (Lb1) showing sensilla; **(D)** Opened labial tip showing the mandibular stylets (white arrow) and a pair of sensilla (*). **(E)** Opened labial tip showing the stylet fascicle (Sf). Bars: A = 50 µm; B = 20 µm; C = 12 µm; D = 3 µm; E = 3 µm.

coverslip, and observed under a Zeiss Axio Imager M2 microscope. Pictures were taken with Axiocam 105 color digital camera.

Stylet penetration

Mealybug third instar nymphs were caged for 5-7 days on fresh grapevine petioles. A small wet cotton piece was wrapped around the end of cut petioles and tightly fixed inside Parafilm™ pieces to keep water supply. They were immobilized through a piece of paper soaked in chloroform for 5 min under a fume hood. Then mealybugs were removed from petioles by pulling them out with a fine forceps. Then petioles were placed between two pieces of dry elderberry cane and transversally sectioned by hand, using a razor blade to the thinnest possible sections. These sections were immediately transferred to a drop of phosphate buffer saline (PBS, pH 7.4). Sections were transferred in a small cup with 4% paraformaldehyde in PBS for 1 h under a fume hood. They were then washed three times for 5 min with PBST (PBS with 0.1% Triton X100). Sections were additionally incubated for 5 min in a drop of fuchsin acid and washed with water. Sections were then mounted on microscope slides covered with coverslip, and observed under Zeiss Axio Imager M2 microscope. Pictures were taken with Axiocam 105 color digital camera.

Results

P. aceris (figure 1) shows mouthparts very similar to those of other piercing-sucking phytophagous insects. They are composed of a clypeolabral shield (Cs), a labium (Lb) and a stylet fascicle (Sf) (figure 2A, 2B, 2E).

When mealybugs are at rest, the stylet fascicle remains as an internal loop inside a membrane called crumena (Cr) within the insect's body (figures 1C, 4C, 4D).

Clypeolabral shield (Cs)

The clypeolabral shield is formed by the fusion of the clypeus and the labrum, resulting in a convex and smooth surface of 80 µm long and 79 µm wide in *P. aceris* third instar nymph. A single pair of trichoid sensilla is centrally located on the surface with a symmetrical distribution (figure 2A).

Labium (Lb)

The labium is composed of three segments (Lb1, Lb2, Lb3), each having on its anterior surface a continuous groove, the labial groove (Lg), enclosing the stylet fascicle (figure 2B). The first (Lb1) and third (Lb3) segments have the same width and seem to form a unique segment, surrounded by the second segment (Lb2), wider than the others. The Lb1 segment is conical and its distal extremity is the thinnest part of the labium. The labium of *P. aceris* third instar nymph has a mean length of 81 ± 6 µm (n = 5, range 74.3-88.3 µm) and a mean width of 48 ± 0.9 µm (n = 5, range 46.7-48.9 µm) (figure 2B).

All parts of the labium are covered with different types of sensilla (figure 2B) mainly symmetrically distributed along the labial groove. Three pairs of trichoid sensilla cover the Lb3 segment while the Lb2 segment shows only one pair. The Lb1 part (figure 2C) is the segment most covered by trichoid sensilla. Five pairs are distributed on either side of the labial groove, four pairs are on the lateral surface of the segment, one pair is on the posterior surface of the labium and one pair is located on either side of the labium distal extremity (figure 2D, 2E).

Stylet fascicle (Sf)

The needle-like stylet fascicle (Sf) is composed of two distinct pairs of mandibular and maxillary stylets. The stylet fascicle has a mean diameter of 3 ± 0.71 µm (n = 10, range 2.43-4.29 µm) and a length of about 720 µm for the third instar nymph.

The mandibular stylets (Md), located on either side of the maxillary stylets, are convex externally and concave internally. They form a groove holding the maxillary stylets (figure 3A). The surface of mandibular stylets is

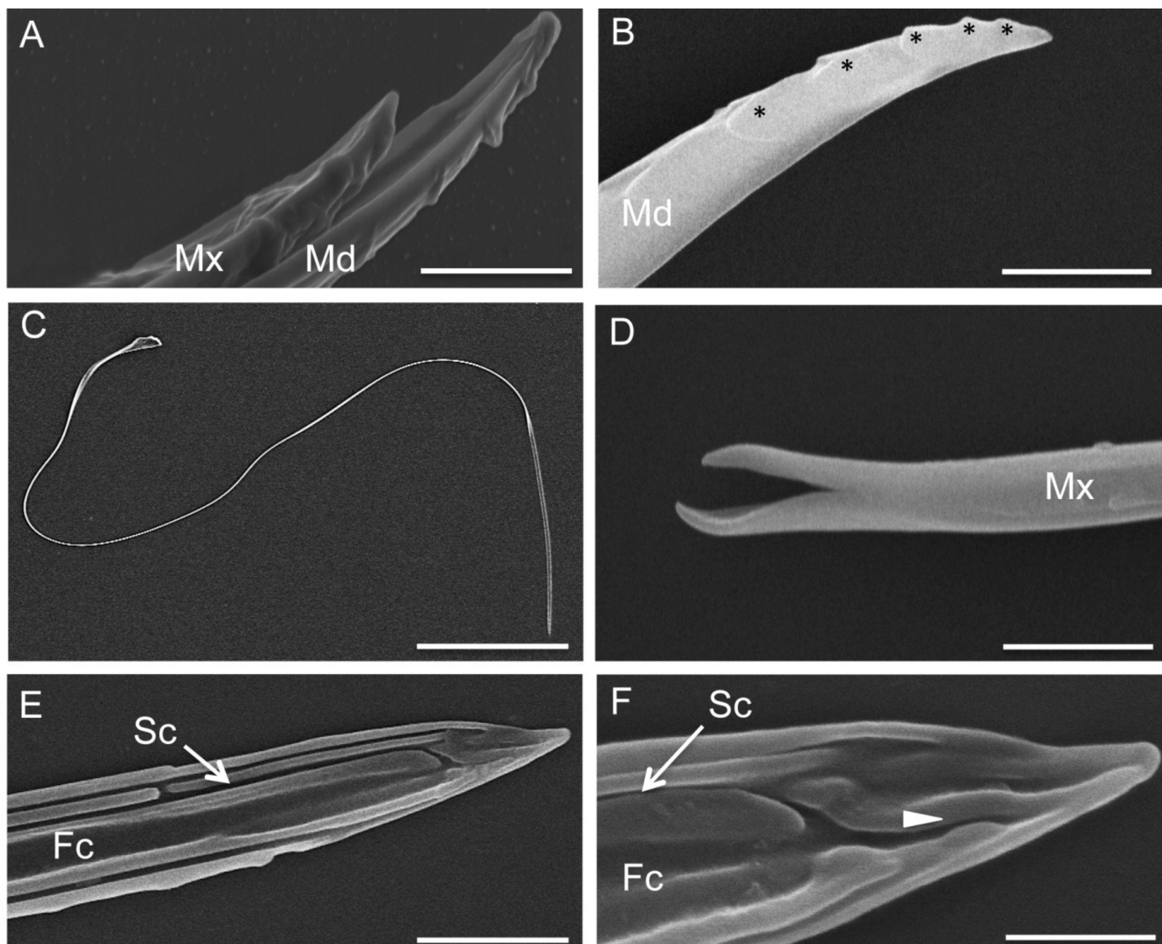

Figure 3. SEM of *P. aceris* stylets. **(A)** Tip of a mandibular stylet (Md) beside a maxillary stylet (Mx); **(B)** Front view of a mandibular stylet tip showing serrated edges (*); **(C)** Full maxillary stylet; **(D)** Tip of coapted maxillary stylet (Mx); **(E)** Tip of a maxillary stylet showing inner surface with food canal (Fc) and salivary canal (Sc); **(F)** Enlarged view of the tip of a maxillary stylet showing inner surface, food and salivary canals and the ridge at the distal extremity (white arrowhead). Bars: A = 4 µm; B = 3 µm; C = 150 µm; D = 4 µm; E = 3 µm; F = 1 µm.

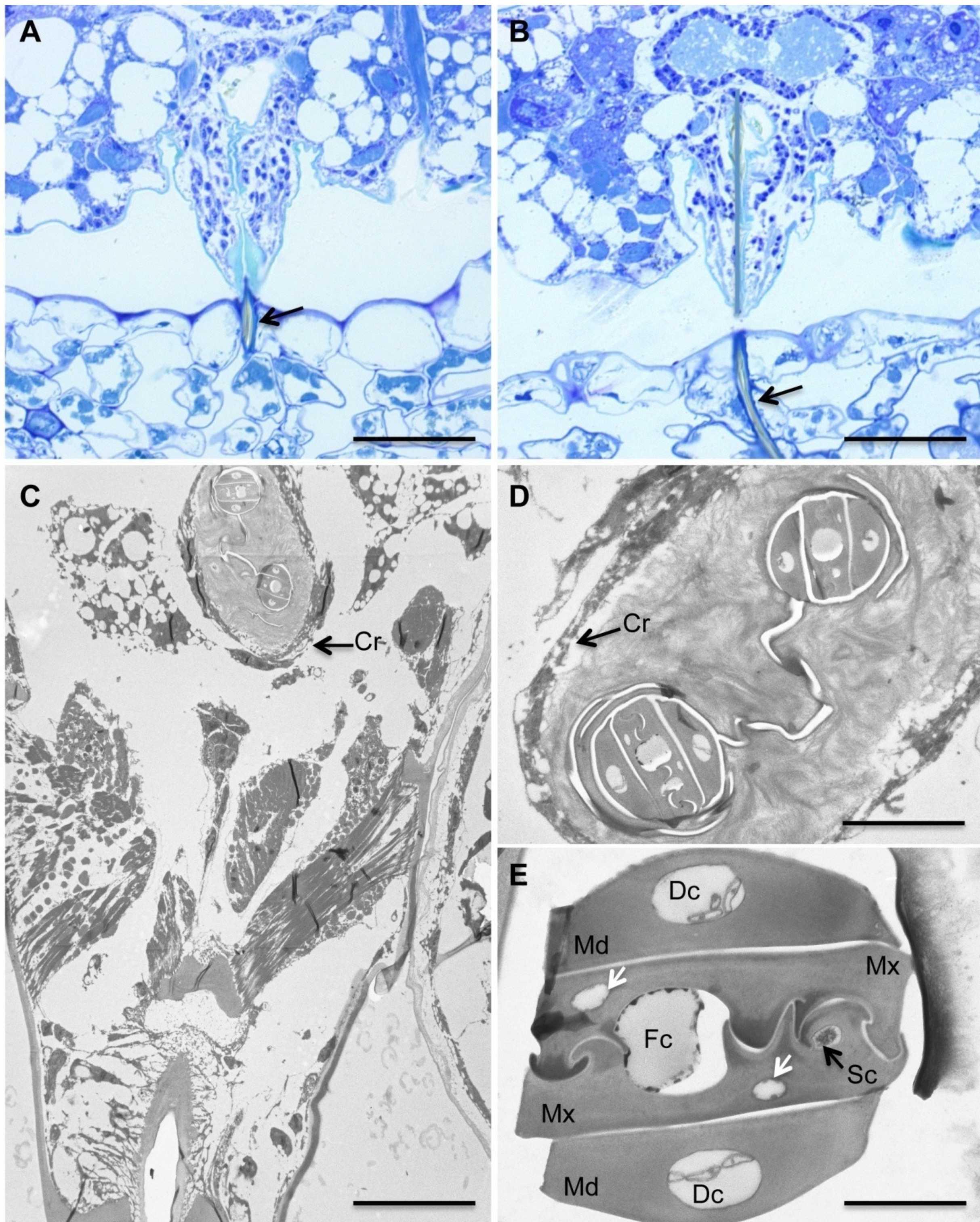

Figure 4. Micrographs of *P. aceris* labium and stylet cross section. **(A)** and **(B)** Light micrographs of a labium showing the stylet fascicle (black arrow) penetration in grape leaf; **(C)** TEM micrograph of a labium showing the crumena (Cr) embedding the stylet fascicle; **(D)** TEM micrograph showing a cross section in the crumena (Cr); **(E)** TEM micrograph of a cross section in the stylet fascicle showing mandibular stylets (Md), maxillary stylets (Mx), food canal (Fc), salivary canal (Sc), dendritic canals (Dc) and dendritic-like canals (white arrows). Bars: A = 40 µm; B = 40 µm; C = 10 µm; D = 3 µm; E = 1 µm.

smooth except at the external distal extremity, which is covered with serrated ridges (figure 3B), probably involved in the laceration of plant tissues for a better penetration of the stylet fascicle into the feeding sites. Each mandibular stylet bears a dendritic canal present all along the stylet length, on the thickest part of the structure harboring three dendrites (figure 4E).

The maxillary stylets (figure 3C, 3D, 3E, 3F) are

slightly shorter and have a more complex architecture. While the outer surface of the maxillary stylets is smooth and without any peculiar feature (figure 3D), their inner surface displays a succession of longitudinal ridges and grooves with an asymmetric distribution. As shown on figure 3E, three ridges take place on one side and two on the opposite side, allowing full-length coaptation of the maxillae in a zip-like system, that delimits the food canal and the salivary canal (figure 4E, 4F). The food canal is centrally located and has a diameter of 0.85 µm. The salivary canal is smaller with a diameter of 0.25 µm. This canal is hosted in one side of the two maxillae, and closed by one ridge of the opposite maxilla (figure 4E). At the tip of maxillary stylets, a protruding 1.8 µm long ridge takes place (figure 4E, 4F). This structure starts on a side of the distal extremity and curves to join the opposite side where the food and salivary canals end. The presence of a canal inside each maxillary stylet was also observed (figure 4E, white arrows). However, this canal is smaller than the dendritic canal of the mandibular stylets and does not seem to be associated with any dendrite-like structure.

Feeding sites and stylet penetration

On grape leaves, *P. aceris* first and second instar nymphs are located almost exclusively on veins and margins of the abaxial side of the leaf, while third instar nymphs and adults are also found on stems and petioles (figure 1D). When insects are feeding on grape leaves, stems or branches, the mouthparts are perpendicular to the plant surface (figure 4A, 4B). During first steps of epidermis penetration, mealybugs secrete a gelling saliva, the 'salivary flange', deposited at the feeding sites on the plant surface and which hardens very rapidly (figure 5B, 5C). This salivary sheath is characterized by its tubular structure surrounding the stylet fascicle (figure 5A) and may facilitate the stylet penetration through the plant tissues towards the vascular bundles. Plant penetration was found to occur through the epidermal cells (figure 5A, 5B), while stylet penetration pathway through cortical tissues was found to be mainly extracellular (electropenetrography data not shown). Close to vascular tissues, the salivary sheath presents branches (figure 5A, 5C) indicating mealybug attempts to reach the phloem or xylem vessels.

Discussion

Plant acceptance and feeding behavior are complex processes where physical and/or chemical cues play key roles. Therefore, the sensilla on *P. aceris* labium are likely to be involved in the early phases of host-plant choice (Backus, 1988). The pre-feeding behavior of the cassava mealybug, *P. manihoti*, has been studied and described by Catalayud and Le Rü in 2006. Repeated drums and rubs of the plant surface with the tip of the antennae, the labium and the forelegs characterize the initial recognition steps. Vibrations of the antennae follow this, while the labium distal tip remains in contact with the plant surface, probably to evaluate the feeding sites. The authors hypothesized that *P. manihoti* would perceive odors above the leaf surface, with the repeated contacts of its olfactory and gustatory organs mainly located on the antennae and the labium. The labia of these two related *Phenacoccus* species are morphologically very similar, even though the cassava mealybug has fifteen pairs of sensilla on the labium, while *P. aceris* displays only fourteen pairs, at least in the specimens observed.

During the first steps of penetration into host-plant tissues, *P. aceris*, like other sternorrhynchans, secretes a gelling saliva which forms a salivary flange around the stylet bundle on the leaf surface. The salivary sheath was found to form a continuum with the external portion of the flange as demonstrated for *K. lacca lacca* (Ahmad *et al*., 2012). The salivary sheath can branch into leaf plant tissue as it was already shown for *P. manihoti* and *Ferrisia virgata* Cockerell (Pseudococcidae) (Calatayud and Le Rü, 2006; Morgan *et al*., 2013). Various functions for these salivary secretions have been proposed, such as lubrification, physical support to anchor the stylet fascicle and avoidance of leakage, directional control, or prevention of air contact with injured tissues (Leopold *et al*., 2003; Miles, 1999; Pollard, 1973).

Serrations or 'mandibular teeth' observed at the distal outer extremity of the mandibular stylets are believed to cut and lacerate leaf tissues (Rosell *et al*., 1995), and thus help the stylet fascicle to penetrate plant tissues. The variation in the number of serrations among Hemipteran species (Forbes, 1977; Rosell *et al*., 1995; Zhao *et al*., 2010; Garzo *et al*., 2012; Liang *et al*., 2013; Wang *et al*., 2015) could reflect an adaptation of feeding behavior to various plant species or targeted tissues.

The presence of a dendritic canal within the mandibular stylets indicates that the innervation of the fascicle probably provides a mechanoreceptive function, as shown for other Hemipterans (e.g. Tjallingii, 1978; Leopold *et al*., 2003). Various numbers of dendritic canals have already been reported in mandibular stylets of *K. lacca* (Ahmad *et al*., 2012). In *P. aceris*, one canal containing three dendrites was observed in each stylet. A dendritic canal with 2 to 5 dendrites is usually found also in the maxillary stylets of Hemipteran Auchenorrhyncha (Backus and McLean, 1982; Leopold *et al*., 2003; Wang *et al*., 2015), while our observations in the Sternorrhyncha *P. aceris* reveal a similar canal in each maxillary stylet without dendrite-like structure. Unless the absence of dendrite-like structure is merely an artifact, it can be suggested that the canal has a physical function, in providing the stylet fascicle with more flexibility, in relation to the protruding from and withdrawal into the crumena. More generally, the length of the stylets in scale insects could be an adaptation to feed on woody plants, as it has also been described in a tree-adapted aphid species (Brożek *et al*., 2015).

While we showed that the general morphology of the mouthparts of the maple mealybug is highly similar to that of other hemipterans' maxillae (Forbes, 1969; Pollard, 1973; Tjallingii and Hogen Esch, 1993; Garzo *et al*., 2012; Liang *et al*., 2013), our ultrastructural study reveals a new distinctive feature at the distal extremity of the maxillary stylets. A unique ridge with a curved

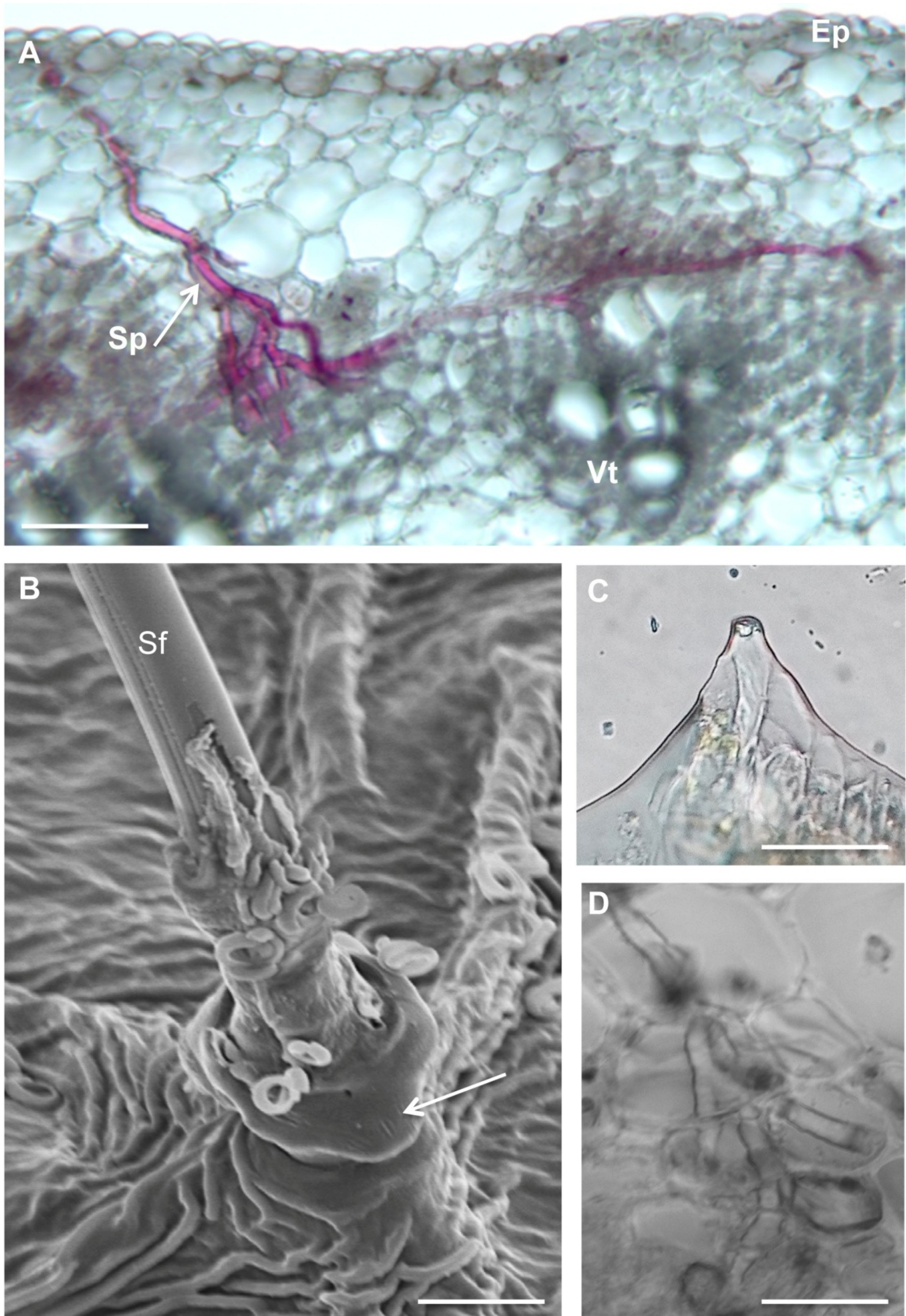

Figure 5. Micrographs of *P. aceris* stylet penetration pathway in grape. **(A)** Light micrograph of a grapevine petiole cross section showing the stylet pathway (Sp) from epidermis (Ep) to vascular tissues (Vt); **(B)** SEM of a penetration site on a grape leaf showing the stylet fascicle (Sf) and the salivary flange (white arrow); **(C)** Light micrograph of a penetration site showing the salivary flange; **(D)** Light micrograph of a branched stylet pathway extremity. Bars: A = 100 µm; B = 3 µm; C = 10 µm; D = 15 µm.

shape, different from the straight one previously described in two psyllid species *Cacopsylla chinensis* (Yang et Li) and *Diaphorina citri* Kuwayama (Garzo *et al*., 2012; Liang *et al*., 2013), was observed at the extreme tip of the maxillary stylets of *P. aceris*. The specific shape of the ridge might confer flexibility to the distal extremity of the maxillae during plant tissue penetration. Furthermore, it seems that one extremity of the ridge ends inside the salivary canal at its extreme end (figure 3F). We could therefore speculate that this ridge acts as a valve opening and closing during appropriate feeding phases (e.g secretion of salivary sheath) as the stylet tip bends to penetrate plant tissues, and facilitates or optimizes a mixing of saliva and plant sap for a better assimilation of nutrients. This could compensate the very short common canal present in *P. aceris* as compared to the wider common canal described in aphids (Forbes, 1969). Additionally, a structure resembling the acrostyle located in the common duct of aphid species (Uzest *et al*., 2010) and shown to be involved in non-circulative and semi-persistent virus transmission (Uzest *et al*., 2007; Garzo *et al*., 2012) has not been observed in *P. aceris* maxillary stylets in our study.

P. aceris is able to transmit viruses such as GLRaVs on the semi-persistent and non-circulative transmission mode (Tsai *et al*., 2008; Le Maguet *et al*., 2012; Herrbach *et al*., 2016). However, the precise virus retention site in mealybug vectors, presumably in mouthparts and/or foregut, remains unknown. To conclude, this study sheds new light on *P. aceris* mouthparts and will open the way to a better understanding of the feeding behavior and the virus transmission by *P. aceris*.

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