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Salivary gland structure of *Ctenarytaina eucalypti* (MASKELL, 1890) (Hemiptera) and phloem exudate in *Eucalyptus globulus* LABILLARDIÈRE, 1799 (Myrtaceae)

Anamika Sharma

Charles Sturt University, asharma@csu.edu.au

Soundararajan Madhavan

University of Nebraska - Lincoln, msoundararajan1@unl.edu

Anantanarayanan Raman

Charles Sturt University, araman@csu.edu.au

Gary S. Taylor

University of Adelaide, gary.taylor@adelaide.edu.au

Murray J. Fletcher

Orange Agricultural Institute & Graham Centre for Agricultural Innovation, murray.fletcher@dpi.nsw.gov.au

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**Salivary gland structure of *Ctenarytaina eucalypti* (MASKELL, 1890)
(Hemiptera) and phloem exudate in *Eucalyptus globulus*
LABILLARDIÈRE, 1799 (Myrtaceae)**

ANAMIKA SHARMA¹, SOUNDARARAJAN MADHAVAN², ANANTANARAYANAN
RAMAN¹, GARY S. TAYLOR³, MURRAY J. FLETCHER⁴

¹Charles Sturt University & Graham Centre for Agricultural Innovation, PO Box 833,
Orange, NSW 2800, Australia; e-mail: asharma@csu.edu.au, araman@csu.edu.au

²Department of Biochemistry, The University of Nebraska-Lincoln, Lincoln, NE 68588-
0664, U.S.A.; e-mail: msoundararajan1@unl.edu

³Australian Centre for Evolutionary Biology and Biodiversity, School of Earth and
Environmental Sciences, University of Adelaide, SA 5005, Australia;
e-mail: gary.taylor@adelaide.edu.au

⁴Orange Agricultural Institute & Graham Centre for Agricultural Innovation (An alliance
between Charles Sturt University and NSW Department of Primary Industries), Forest
Road, Orange, NSW 2800, Australia; e-mail: murray.fletcher@dpi.nsw.gov.au

ABSTRACT. The structure of the salivary glands of the free-living aphalarid *Ctenarytaina eucalypti*, which infests multiple species of *Eucalyptus* in Australasia and has been introduced into many other regions of the world, is described and illustrated. The principal salivary gland is multi-lobed whereas the accessory gland is tubular. 1-D electrophoresis revealed proteins of approximately 58 and 64 kDa in the salivary gland extracts and proteins of similar molecular weights in the extracted plant exudates, including phloem, from infested leaves and tender shoots of *E. globulus*. Proteins that could fall within this range include, but are not limited to, glucose-methanol-choline-oxidoreductase (53-66 kDa), Zn-binding dehydrogenase (67 kDa) and esterase (65-96 kDa), in addition to cytochrome P-450 (50-55 kDa), trehalase (56 kDa), amylase (50-75 kDa) and lipase (48-52 kDa). Previous studies indicate that glucose-methanol-choline-oxidoreductase, Zn-binding dehydrogenase, cytochrome P-450 and trehalase suppress plant-defence mechanisms, whereas the cell-degrading enzymes such as amylase, lipase and esterase have a possible role in enabling *C. eucalypti* to insert its stylet into leaf and shoot tissues of *E. globulus*.

KEY WORDS: salivary enzymes, phloem proteins, psyllids.

INTRODUCTION

Ctenarytaina eucalypti (MASKELL, 1890) (Hemiptera: Sternorrhyncha: Psylloidea: Aphalaridae), was first described from New Zealand but is believed to have originated in Australia (FROGGATT 1900). With the introduction of *Eucalyptus globulus* LABILLARDIÈRE, 1799 and other species of *Eucalyptus* L'HERITIER, 1789 for commercial reasons, *C. eucalypti* has spread today as an invasive organism in several regions of the world, such as Italy, Chile, Brazil and Sri Lanka (DE QUEIROZ et al. 2012, CABI 2013). Although *C. eucalypti*'s host range includes several species of *Eucalyptus*, *E. globulus* appears to be its preferred host (SANTANA & BURCKHARDT 2007, SHARMA et al. 2014). *Ctenarytaina eucalypti* feeds on the juvenile leaves of *E. globulus* and all developmental stages occur on the same leaf (SHARMA et al. 2014). Feeding by *C. eucalypti* inflicts physiological damage on *Eucalyptus* (COLLET 2001). A positive correlation occurs in the abundance of the immatures of *C. eucalypti* and concentrations of methionine, valine and threonine in the leaves of *E. globulus* (STEINBAUER 2013). The early instars of *C. eucalypti* feed on mesophyll parenchyma and the later instars and adults feed on leaf phloem sap. During feeding, the immatures and adults of *C. eucalypti* form a proteinaceous sheath around their stylets and the stylet follows an intercellular pathway towards phloem (Figs 1, 2). The feeding action of the early instars inflicts both mechanical and chemical damage and the phloem-feeding late instars and adults induce several signs of degeneration in the leaves of *E. globulus* (SHARMA et al. 2014).

The Hemiptera possess a pair of salivary glands (COBBEN 1978, HORI 2000), each usually comprising a principal gland functioning as a reservoir and an accessory gland supplying the fluid to watery saliva in the form of haemolymph ultrafiltrate (MILES 1999). They secrete both gel-like and watery saliva: the gel-like saliva contains lipoproteins, phospholipids and conjugated carbohydrates, whereas the watery saliva consists of various enzymes (MILES 1999, BACKUS et al. 2005). Immunolocalization techniques indicate that salivary proteins in the principal and accessory glands of Sternorrhyncha (e.g. *Schizaphis graminum* (RONDANI, 1852) [Hemiptera: Aphididae]) vary: 66-69 kDa proteins occur in the watery and gel-like saliva, whereas the watery saliva also contains 154 kDa proteins (CHERQUI & TJALLINGII 2000). In a majority of the Hemiptera the salivary sheath helps to neutralize the plant-defence compounds of the host-cell apoplast. The watery saliva alters the physiology of the host plant by its innate salivary chemistry.

In this paper, we describe the structure of the salivary glands of *Ctenarytaina eucalypti* and the interaction of salivary secretions with the phloem exudate of *Eucalyptus globulus*. Contrary to similar work done on other Hemiptera, the extraction of the salivary glands of *C. eucalypti* was challenging because of their smallness and also because no earlier literature describes the exact location of salivary glands in the Psylloidea. However, cursory

details on stylet movement and feeding action in *Psylla mali* (SCHMIDBERGER, 1836) (Psyllidae) are available in WEBER (1929, 1930), where WEBER explained that stylet action consists of a short protraction of one of the two mandibles followed by the other, each consequently sliding forward along the outside of the maxillae. However, this explanation was subsequently modified by POLLARD (1973). Extraction of phloem exudate was equally daunting especially because the well-known bleeding method (MERCHANT et al. 2010), more suitable for *Eucalyptus* plants growing in natural conditions, could not be used.

MATERIAL AND METHODS

Glasshouse rearing of plant and insect material

Seeds from *Eucalyptus globulus* trees from Charles Sturt University, Orange campus farm (CSU-O), were raised in seedbeds containing commercial seed-raising mix (Debco®, Debco Pty Limited, Tyabb, Victoria, Australia). Individual seedlings were subsequently transplanted to plastic pots (20 cm Ø) with potting mix (Debco®, Debco Pty Limited). Ninety-day old seedlings (~60 cm-long shoots bearing 50-60 leaves) were maintained at 23±2°C and 60±5% RH under natural light in a glasshouse. Field-captured adults of *Ctenarytaina eucalypti* were released onto potted plants maintained in cages (BugDorm-™; BD2400; MegaView Science Co., Ltd., Taichung, Taiwan) to establish populations.

Microscopy

To determine the feeding sites and characterize changes consequent to feeding, leaf segments were processed for wax microtomy. Leaf segments were fixed in FAA (formalin, ethanol [95%], glacial acetic acid, distilled water – 10:50:5:35/100 ml), followed by processing through an alcohol series (30, 50, 70, 80, 90, 100%, each 12 h), histolene and paraffin-wax embedding done at 65°C. The wax-embedded tissue was sectioned at 8 µm, deparaffinized in histolene, contrasted with either 1% toluidine blue (in 1% aqueous borax solution) or 1% aqueous acid fuchsin, and mounted in DPX. Photomicrographs were made using a photomicroscope (BX-51, Olympus Optical Co., Ltd, Tokyo, Japan).

For scanning-electron microscopy, the adults and nymphs of *Ctenarytaina eucalypti* were fixed in 2.5% glutaraldehyde in 0.1 M PO₄ buffer for 4 h and dehydrated in a graded-alcohol series (25-100%). The specimens were cleaned in a sonicator (Branson 2510, Danbury, Connecticut, USA) for 5 min, and critical-point dried in a CPD (# 030, Bal-Tec AG, Schalksmühle, Germany). The material was then coated with gold in a sputter coater (SC7620, Quorum Technologies Limited, Kent, UK) and viewed in an SEM (S-4500, Hitachi Scientific Instruments, Tokyo, Japan) at 5 kV.

Extraction of *C. eucalypti* salivary glands

Salivary glands ($n=105$) of *Ctenarytaina eucalypti* adults were dissected in PO_4 buffered saline (PBS, pH 7.0). *C. eucalypti* adults were placed dorsal side down on the slide so that the mouthparts were facing upward. The head segment of each adult was carefully detached using a needle, and another needle was held inserted between the first pair of legs. Following confirmation that the excised salivary glands were devoid of any obvious muscle and/or fat tissues, which were plentiful, they were transferred to the resolubilization buffer (8 M urea, 65 mM dithiothreitol [DTT] and 16 mM 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulphonate [CHAPS]) and preserved at -80°C . The chemicals used were from Sigma Aldrich (Missouri, USA) and Fisher Scientific (Massachusetts, USA). Photomicrographs of salivary glands were taken using an Olympus photomicroscope (BX-51, Olympus Optical Co., Ltd, Tokyo, Japan).

Extraction of the phloem exudate

Because of the established feeding by mature-nymphal instars and adults of *Ctenarytaina eucalypti* on the shoot and leaf phloem of *Eucalyptus globulus*, we used the EDTA-chelator method, which is considered one of the valid methods for phloem-exudate extraction (PALMER et al. 2014). We followed TURGEON & MEDVILLE (2004) and DAFOE et al. (2009) to achieve the best possible outcomes. These methods were chosen because TURGEON & MEDVILLE (2004) verified this extraction using excised shoots and petioles for phloem extraction and DAFOE et al. (2009) used parts of plants raised *ex situ*.

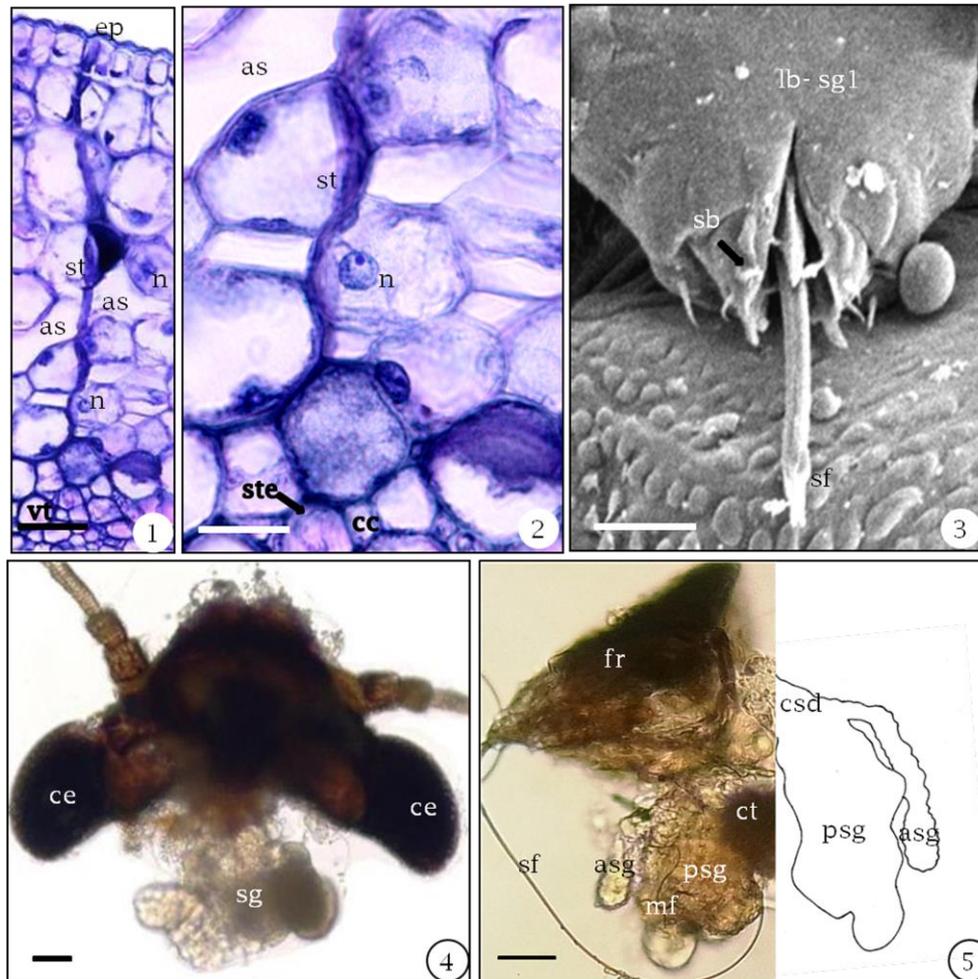
The uppermost 10-cm long stem portions ($n= 20$) were excised from 90-day-old *E. globulus* and the leaves were detached. Following TURGEON & MEDVILLE (2004), the cut segment was re-cut within the next 30 seconds, removing a further segment of 0.5 cm from the stem, while retaining the cut end of the 10 cm long stem sunk into 20 ml of the Tris-EDTA buffer solution (300 μL of 50 mM Tris [pH 8.0] +2 mM EDTA) in Petri dishes (10 cm \varnothing x 1.5 cm deep). Excision of a 0.5-cm long segment while standing the cut end in Tris-EDTA buffer, with a high probability, prevents any possible blocking of sieve-plate pores by phloem, thus enabling the flow of phloem exudate (TETYUK et al. 2013). Following DAFOE et al. (2009), the 9.5 cm long segments were quickly transferred to Falcon tubes (50 mL) containing 300 μL of the Tris-EDTA buffer solution. This process was completed in the next 180 sec for each sample. To collect phloem exudate from leaves, <1.0 mm segments from the free end of the petioles ($n= 100$) were sloughed off from petioles (0.5 cm long) following the TURGEON & MEDVILLE (2004) protocol. The petioles (c. 0.4 cm length each) were immediately transferred individually to Falcon tubes (50 mL) containing Tris-EDTA buffer solution.

The Falcon tubes containing cut stem segments and petioles were closed immediately using the Falcon screw caps, enabling exudation to take place in the Tris-EDTA buffer solution for 120 min at room temperature ($22^{\circ}\text{C}\pm 1^{\circ}\text{C}$). The exudate-containing Tris-EDTA buffer solution was dialysed overnight (Spectra/Por, 23 mm, MWCO: 6000-8000, Spectrum Laboratories, Rancho Dominguez, CA, USA) against 200 ml of deionized water followed by one water change. Samples were lyophilized and stored at -80°C until later analysis. Proteins from the lyophilized samples were resolubilized in water and precipitated in two volumes of cold acetone at -20°C (two volumes of cold acetone were added to the protein samples) for 60 min. Samples were then vortexed and held at -20°C for 60 min. The precipitate, obtained as a pellet after centrifugation (15 min, 16000 g [3000 rpm]) at 4°C , was washed twice with 80% cold acetone, dried and resuspended in the resolubilization buffer (8M urea, 65 mM dithiothreitol [DTT] and 16 mM 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulphonate [CHAPS]) at room temperature for 30 min prior to protein quantification applying the Bradford method (BRADFORD 1976, NOBLE & BAILEY 2009).

1-D gel electrophoresis

For SDS polyacrylamide gel electrophoresis (SDS-PAGE), salivary gland proteins and phloem-exudate proteins were treated in Laemmli sample buffer (BioRad, Hercules, CA) and loaded on to 4-15% polyacrylamide gels under a 100 V current in a Mini PROTEAN II system (BioRad, Hercules, CA). A 10-250 kDa molecular mass ladder (BioRad) was run on each gel. Proteins were stained in Coomassie Blue for 20 min and destained in methanol: acetic acid (3:1) for 60 min. The gels were scanned using the ProXPRESS Proteomic Imaging System (Perkin-Elmer, Waltham, MA). The strength of running the SDS-PAGE is that it would have characterized any contaminant proteins from non-phloem sources as well.

Approximate sizes of protein bands in samples were calculated by constructing a standard curve using the distance to which each band in the standard molecular mass ladder had run from the bottom of the well. Zero point was the distance from the bottom of the well to the end of the gel. Linear regression was prepared from the plot constructed between molecular weights and the distance of each band. Molecular weights of protein bands in samples were calculated using the regression equation ($y=mx+b$).



Figs 1-5. Stilet track and salivary glands in adults of *Ctenarytaina eucalypti*. 1. Leaf of *Eucalyptus globulus* (cross section) and stilet track terminating in phloem. as – air space; ep – epidermis; n – nucleus; st – stilet track; vt – vascular trace (bar=50 μ m). 2. Enlarged view of phloem. as – air space; cc – companion cell; n – nucleus; st – stilet; ste – sieve-tube element (bar=50 μ m). 3. Mouthparts. lb-sg1 – labium-segment 1; sb – labialsensilla; sf – stilet fascicle (bar=10 μ m). 4. Position of the salivary glands (dorsal view). ce – compound eye; sg – salivary gland (bar=500 μ m). 5. Principal and accessory salivary gland detail shown in a reconstructed sketch. Abbreviations: asg – accessory salivary gland; csd – common salivary duct; ct – connective tissue; mf – muscle fibre; psg – principal salivary gland; fr – frons; sf – stilet fascicle (bar=500 μ m).

RESULTS

Adult *Ctenarytaina eucalypti* feed on phloem of *Eucalyptus globulus* (Figs 1, 2). The mouthparts of *C. eucalypti* include a postclypeus and a labium forming the rostrum. At the distal end of the rostrum there are four equidistantly placed sensilla on either side. Distal mouthparts also show the first segment of the labium and stylet fascicle (Fig. 3). The salivary glands are multi-lobed and translucent and occur proximally to the rostrum (Fig. 4). The principal and accessory lobes of the salivary gland are not clearly differentiated, but we interpret the larger lobe as the principal gland and the smaller lobe as the accessory one (Fig. 5).

About 150 $\mu\text{g/ml}$ protein was extracted from the 105 salivary glands. About 1500 $\mu\text{g/ml}$ ($n=100$ uninfested leaves), 1400 $\mu\text{g/ml}$ ($n=100$ infested leaves), 1200 $\mu\text{g/ml}$ ($n=20$ uninfested shoots) and 1300 $\mu\text{g/ml}$ ($n=20$ infested shoots) protein material was collected from the exudate. Phloem collection from the uppermost 9.5 cm segments of shoots was less productive, which could have been due to the plants being raised in pots, making them water deficient and thus influencing exudate flow from the cut end (A. MERCHANT, personal communication, email, 8 May 2013). Collection of exudates using a pressure bomb did not yield useful quantities of proteins either.

Electrophoresis offered a useful and representative comparison between phloem exudates of uninfested and infested plant sources and salivary proteins of *Ctenarytaina eucalypti*. The protein bands A, B of 50-75 kDa and C, D of 10-15 kDa were manifested in the saliva of *C. eucalypti* and also in the phloem exudate of infested leaves (Fig. 6). Similar molecular weight proteins were also evident, but faintly, in the phloem exudate of plants of infested shoots (Fig. 6), but the sample from the uninfested leaves was smudged, resulting in poor resolution of the contents. The approximate masses of protein bands A and B were 64 kDa and 58 kDa respectively. Similarly, the masses of protein bands C and D were 16 kDa and 10 kDa respectively.

DISCUSSION

This is the first report presenting the detailed structure of the salivary glands of a species of Psylloidea, although the structure of the salivary glands of a few other hemipteroids is known (e.g. CHAPMAN 1985, DEL BENE et al. 1999, TAYLOR & MILES 1994). In general, the principal gland in the Hemiptera is often subdivided into two or more lobes. The principal and accessory glands are serviced by their respective ducts, and the ducts from each lobe fuse to form a lateral salivary duct. Considerable variation in the

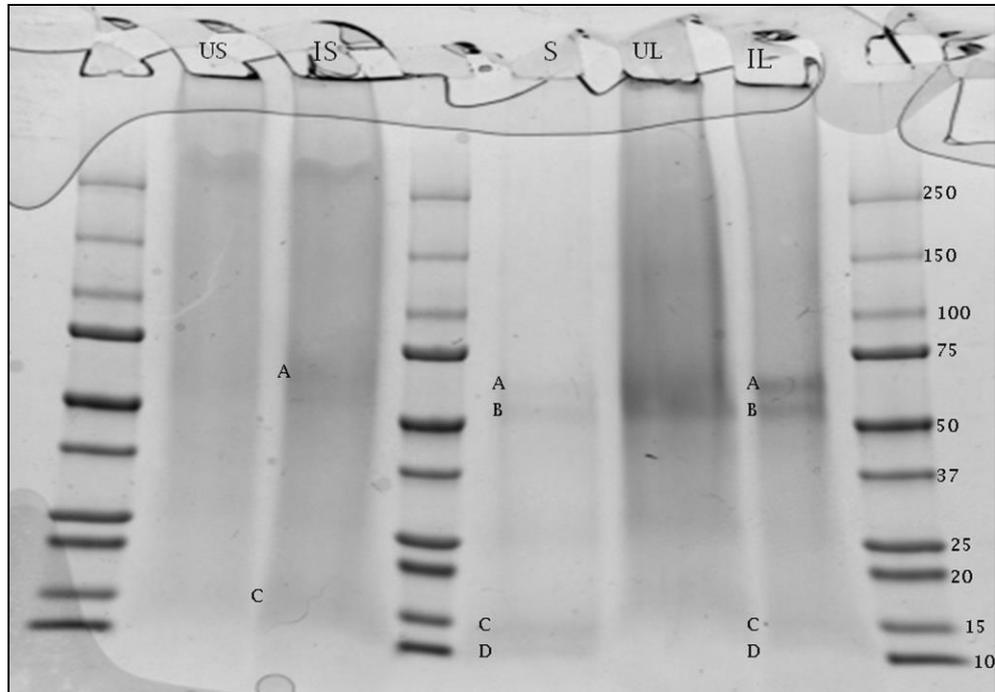


Fig. 6. SDS-PAGE analysis of salivary proteins of adult *Ctenarytaina eucalypti* (0.05 g total protein/lane) from an uninfested shoot (US); Infested shoot (IS); S – salivary protein; UL – uninfested leaves; IL – infested leaves of *Eucalyptus globulus*. Unlabelled lanes (3x) contain a 10 kDa molecular mass ladder (BioRad).

structure of salivary glands occurs in the Hemiptera (WALKER 2009). For example, in *Oncopeltus fasciatus* (DALLAS, 1852) (Hemiptera: Heteroptera: Lygaeidae), the salivary system consists of a pair of three-lobed principal glands divided into discrete lobes and a pair of tubular accessory glands with associated ducts (BRONSKILL 1958). In contrast, the principal salivary gland of *Circulifer tenellus* (BAKER, 1896) (Hemiptera: Auchenorrhyncha: Cicadellidae) is divided into an anterior and posterior lobe, each serviced by independent ducts. The accessory gland is not subdivided and its duct joins to form the lateral duct in a few of the Cicadellidae (WAYADANDE et al. 1997). The salivary system of *Myzus persicae* (SULZER, 1776) (Aphididae) consists of two lobes of the principal gland and accessory glands in pairs (PONSEN 1972). We consider the paired larger lobes in *Ctenarytaina eucalypti* to be the principal glands and the smaller lobes to be accessory glands. The large lobe of the salivary gland of *C. eucalypti* appears similar in structure to that known in *Myzus persicae*.

SDS-PAGE analysis of samples revealed bands ranging between 50 and 75 kDa in the salivary secretions and in the phloem exudate (which could be 'plant exudate' due to potential exudates from non-phloem tissues) extracted from infested leaves and shoots of *E. globulus*. The protein labelled 'band A' is approximately 64 kDa in molecular weight and may represent glucose-methanol-choline-oxidoreductase (53-66 kDa); Zn-binding dehydrogenase (67 kDa) and esterase (65-96 kDa). The protein labelled band B (58 kDa) could be cytochrome P-450 (50-55 kDa), trehalase (56 kDa), amylase (50-75 kDa) and lipase (48-52 kDa) (SHARMA et al. 2013).

The salivary proteins glucose-methanol-choline-oxidoreductase, Zn-binding dehydrogenase, cytochrome P-450 and trehalase enable suppression of plant-defence mechanisms by detoxifying host-plant allelochemicals that *Ctenarytaina eucalypti* could be encountering while feeding on *Eucalyptus globulus* leaf and shoot cells. Hardened bulbous droplets of the gel-like saliva have been shown in cells encircling the stylet track of *C. eucalypti* (SHARMA et al. 2014). The gel-like saliva is implicated as an avoidance tactic to circumvent allelochemicals of the host plant during feeding (MILES 1999). For instance, oxidoreductases in the gel-like saliva polymerizes the phenolics sequestered in the plant-cell apoplast as an induced defence mechanism and generates quinones (SHARMA et al. 2013). Production of quinones in the leaves of *E. globulus* leaves when infested by *C. eucalypti* was reported by TRONCOSO et al. (2012). Cell-degrading enzymes, such as amylase, lipase and esterase, may enable *C. eucalypti* to pierce tissues of *E. globulus* (OTEN et al. 2014, SHARMA et al. 2013). Amylase and protease have been demonstrated in the saliva of *Trioxa jambolanae* CRAWFORD, 1917 (Hemiptera: Psylloidea: Triozidae), which lives on *Syzigium cumini* LINNAEUS, 1753 (Myrtaceae) (RAJADURAI et al. 1990). However, these salivary proteins can only be unequivocally identified by probing these bands with antibodies raised against the possible candidate proteins using an immunoblotting technique.

CONCLUSION

The salivary glands of *Ctenarytaina eucalypti* are multi-lobed and occur proximally to the rostrum. The salivary proteins (from 50 to 75 kDa) interact with the phloem sap of *Eucalyptus globulus*, which could contain cell degrading and/or detoxifying enzymes. Nevertheless, more sophisticated techniques are needed to generate definite protein profiles of the saliva of *C. eucalypti* and the phloem sap of *E. globulus*.

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