# Leafhopper Viral Pathogens: Ultrastructure of salivary gland infection of Taastrup-like virus in *Psammotettix alienus* Dahlbom; and a novel Rhabdovirus in *Homalodisca vitripennis* (Germar) Hemiptera: Cicadellidae

**Thorben Lundsgaard[1], Wayne B. Hunter[2], Scott Adkins[2],**

1. Department of Plant Biology, The Royal Veterinary and Agricultural University, Agrovej 10, DK-2630 Taastrup, Denmark. (thorben.lundsgaard@gmail.com *Retired*).

2. USDA, ARS, U.S. Horticultural Research Lab, 2001 South Rock Road, Fort Pierce, FL 34945 USA.

*Author to whom correspondence should be addressed; E-Mail: Wayne.hunter@ars.usda.gov ; Tel.: +1-772-462-5898; Fax: +1-772-462-5960.

*Received:* */ Accepted:* */ Published:*

---

**Abstract**

Viruses that are pathogenic to insect pests can be exploited as biological control agents. Viruses that are pathogenic to beneficial insects and other arthropods, as in honey bees, silk worms, and shrimp, cause millions of dollars of losses to those industries. Current advances in next generation sequencing technologies along with molecular and cellular biology have produced a wealth of information about insect viruses and their potential applications. Leafhoppers cause economic losses as vectors of plant pathogens which significantly reduce the worlds' food crops. Each year more viruses are discovered primarily through the use of next generation sequencing of the leafhopper hosts. The diversity of viruses from leafhoppers demonstrates a wide range of taxonomic members that includes genomes of DNA or RNA from families like: Reoviridae, Iridoviridae, Dicistroviridae, Iflaviridae, and others yet to be classified. Discussed is a recent viral pathogen isolated from the leafhopper *Psammotettix alienus*, name Taastrup Virus. Taastrup virus (TV) is a novel virus with a RNA genome, a Filovirus-like morphology, being tentatively placed within the *Mononegavirales.* Adult *Psammotettix alienus* infected with TV, showed the highest concentration of virions in salivary glands, consisting of a principal gland (type I-VI-cells) and an accessory gland. Examination of thin sections revealed enveloped particles, about 1300 nm long and 62 nm in diameter, located singly or in paracrystalline arrays in canaliculi of type III- and IV-cells. In gland cells with TV particles in canaliculi, granular masses up to 15 µm in diameter were present

in the cytoplasm. These masses are believed to be viroplasms, the sites for viral replication. TV particles were observed at the connection between a canaliculus and the salivary duct system. A TV-like virus with strongly similar morphology was discovered in the ornamental plant, *Liriope*, near Fort Pierce, Florida, USA. When the virus was inoculated to a leafhopper cell culture, HvWH, made from the glassy-winged sharpshooter, *Homalodisca vitripennis* (Germar), the cells rapidly degraded with 100% mortality in 48 hours. These two instances are the only reported cases of this newly discovered viral pathogen of leafhoppers.

**Keywords:** Biological control, Cell culture, *Cicadellidae;* Glassy-winged sharpshooter; *Mononegavirales;* Pathogen*;* Rhabdovirus, Salivary glands; Taastrup virus

---

## 1. Introduction

Insects are commonly infected with multiple viruses and traditional methods of detection and isolation, such as observation of sick or dead insects in the field can miss many pathogens as diseased insects fall to the ground and are quickly scavenged by ants or other predators. The use of insect cell cultures to 'capture' virus by inoculation of crude preparations made from insects collected from the field, can propagate virus if the cell lines are permissive to that specific virus (Hunter et al, 2001) however, these methods only capture a small fraction of the potential pool of viruses that are infecting insects and other arthropods. Mining of the genetic sequences now available, for new insect viruses, has proven to be a rich resource full of undiscovered, insect pathogens (Katsar et al, 2007; Valles et al, 2004, 2008; Liu, et al, 2011). Even so, serendipity still provides many new discoveries for the prepared mind. Recently a Taastrup-like leafhopper-infecting virus was discovered in the ornamental flowering plant, Genus: *Liriope,* Fort Pierce, Florida, USA (Fig. 1). The plant had a mixed infection with a Tospovirus that produced the traditional ring-like symptoms on the leaves (Fig. 2). During these examinations, a Taastrup-like virus was identified. Current research efforts are focused on identifying potentially new leafhopper pathogens which may have a function as biological control agents.

Rhabdoviruses (Family: Rhabdoviridae) in the Order *Mononegavirales*, are enveloped viruses with a nonsegmented negative-strand RNA genome. Currently this Order includes four families— Rhabdoviridae, Bornaviridae**,** Filoviridae, and Paramyxoviridae. Rhabdoviruses have a diverse host range including humans, vertebrate animals, and invertebrates in the aquaculture

62 and agricultural industries. The International Committee on Taxonomy of Viruses, ICTV,
63 recognizes nine genera in the family Rhabdoviridae (Dietzgen et al. 2011). These include
64 viruses considered to be plant viruses transmitted by arthropod vectors of the genera
65 *Nucleorhabdovirus* (10 species) and *Cytorhabdovirus* (9 species); with members in the genera
66 *Ephemerovirus (5 species)*, *Lyssavirus (12 species)*, *Novirhabdovirus (4 species)*, *Perhabdovirus*
67 *(3 species), Sigmavirus (7 species)*, Tibrovirus *(2 species), Vesiculovirus (10 species), and one*
68 *unassigned genus (6 species),* which have been isolated from various vertebrate and invertebrate
69 hosts (www.ictvonline.org/virusTaxonomy.asp). Although more than 150 rhabdoviruses have
70 been identified from a wide range of hosts worldwide, most still remain to be assigned to a genus
71 within a family due to the high amount of genetic diversity among them (Kuwata et al, 2011).

72 Filovirus-like particles of Taastrup virus (TV) have been described from a population originating
73 from France of apparently healthy leafhoppers belonging to the species *Psammotettix alienus*
74 Dahlbom (Hemiptera: Cicadellidae)(Lundsgaard, 1997). *Psammotettix* leafhopper species are
75 extensively listed in the literature as feeding on grasses and occur worldwide. The type specie of
76 the Genus, *Psammotettix maritimus* (Penis), feeds on *Convolvulus* (Haupt, 1929; DeLong, 1973).
77 Examination of plant and leafhopper tissues showed flexuous particles, 55-70 nm in diameter
78 and 600 or 1100 nm long, consist of an inner coiled nucleocapsid about 30 nm in diameter and
79 surrounded by a membrane with pronounced surface projections inserted (Lundsgaard, 1997),
80 thus resembling members of Filoviridae morphologically. The RNA genome has partly been
81 sequenced, which demonstrated both, that TV belongs to the virus Order *Mononegavirales,* but
82 which has not been officially classified into any of the established families of this virus order
83 (Bock *et al*., 2004). However, TV isolated from the leafhopper *P. alienus,* in Denmark,
84 AY423355, was inferred to belong to the family Rhabdoviridae, in the genus *Cytorhabdovirus*
85 based on analyses of the L polymerase gene (Bourhy et al., 2005). The hosts for *P. alienus* are
86 grasses and cereals (Raatikainen and Vasarainen, 1976). The insects salivate during probing,
87 penetration, and plant fluid ingestion (Backus, 1985), thus it is likely that the salivary glands play
88 a key role in the epidemiology of TV. Thus, a more in-depth study was conducted on the
89 ultrastructure of organs from infected leafhoppers with results presented here.

90

## 2. Experimental Section

The following experiments were performed in order to determine the distribution of virus in leafhoppers taken from a TV-infected population. Two fractions were made from ten randomly chosen insects. One fraction consisted of heads (including salivary glands) and the other fraction of thorax, abdomen, wings and legs. Each of the fractions was extracted in 1 ml distilled water. After one cycle of differential centrifugation (5 min at 1800 *x* g and 20 min at 13,000 *x* g), the sediments were dissolved in 20 µl (head fraction) or 100 µl (body fraction) of negative staining solution (0.5% ammonium molybdate, pH 7). Preparations were made on Formvar® mounted nickel grids (400-mesh) and the number of TV particles in ten grid squares was counted in a transmission electron microscope (JEOL JEM-100SX). The mean number of particles for the head fraction was 9.1 [standard deviation (SD) = 4] and zero particles for the body fraction. In the next experiment, the heads from ten insects were severed and two fractions prepared, namely: a salivary gland fraction (SG) and a fraction representing the remaining part of the head (H). After differential centrifugation, both sediments were dissolved in 20 µL staining solution. The mean number of particles per grid square was 34.3 (s = 5) for SG and 0.3 (s = 0.7) for H, showing the salivary glands to be the most important site for accumulation of TV particles.

Ten adults taken in random from the TV infected population were dissected in Ringer's solution (0.7% NaCl, 0.035% KCl, 0.0026% $CaCl_2$). Each pair of salivary glands was divided in a left and a right part with the purpose of checking one part for presence of particles by negative staining electron microscopy (extraction in 10 µl 0.5% ammonium molybdate) and the other corresponding part by ultrastructural analysis. The salivary glands were fixed, dehydrated, and embedded essentially according to Berryman and Rodewald (1990). In brief, the specimens were transferred to fixative (3% formaldehyde, 0.3% glutaraldehyde, 100 mM phosphate buffer, pH 7.0) and maintained in fixative for at least 2 h. After washing (3.5% sucrose, 0.5 mM $CaC1_2$, 100 mM phosphate buffer, pH 7.4), the aldehyde groups were quenched with 50 mM $NH_4Cl$ dissolved in washing solution) for 1 h. The specimens were then washed (3.5% sucrose, 100 mM maleate buffer, pH 6.5) and post-fixed with 2% uranyl acetate in the same maleate buffer for 2 h. After dehydration in acetone (50%, 70%, 90%), the specimens were infiltrated in LR Gold (The London Resin Co., England) containing 0.5% benzoin methyl ether (3 changes) and polymerized under near ultraviolet light (Philips TW6). Fixation and dehydration (including 50% acetone) was done

121 at 5-10 °C and dehydration (from 70% acetone), embedding, and polymerization performed at -
122 20°C. Thin sections (60-70 nm) were collected on 200-mesh nickel grids, stained with saturated
123 uranyl acetate for 5 min and lead citrate (1 mM, pH 12) for 1 min, and examined with a JEOL
124 100SX transmission electron microscope operated at 60 kV. As controls, specimens were prepared
125 in the same way from a healthy population of *P. alienus.*

126 Field collected *Liriope* which had symptoms of a tospovirus-like infection (Figs. 1, 2) were
127 prepared for examination by TEM as mentioned above. Upon discovery of a Taastrup-like virus
128 infection a crude sap inoculum was prepared. Solutions were prepared from *Liriope* plants with
129 and without symptoms and the resulting inoculum dispensed onto leafhopper cell cultures
130 produced from the glassy-winged sharpshooter, *Homalodisca vitripennis*, HvWH, Hunter, USDA.
131 The crude plant sap preparation used 2 g of leaf tissues homogenized in Histidine, monohydrate
132 buffer, pH 6.5, which was the same buffer used for insect cell cultures (Hunter and Polston 2001;
133 Hunter et al, 2001; Marutani et al, 2009). The solution was then centrifuged in 15 mL tubes at 600
134 xg for 4 min to pellet the plant debris. The supernatant was collected and filtered through a 0.45
135 µm filtration unit, and then filtered again through a 0.22 µm filtration unit. The filtered sap
136 inoculum was then added to adherent leafhopper cell cultures at 1 mL per 25 $cm^2$ flask (Corning®,
137 Inc), and let stand for 10 minutes. After this time 4 mL of fresh insect medium was added (Table
138 1). Leafhopper cell cultures were observed daily for cell pathogenicity.

139 Leafhopper cell line maintenance: HvWH, were cultivated in H2G Leafhopper medium, modified
140 from WH2 honey bee media (Hunter 2010) (Table 1). Culture methods in (Kamita et al, 2005;
141 Biesbock et al, 2014). Prior to adding the fetal bovine serum, the medium was passed through
142 sterilizing filtration units (500 mL, 0.22 µm filter, Corning), then fetal bovine serum was added to
143 make a 10% concentration. Aliquots of 5 mL of medium were placed on the counter for three days
144 at room temperature to test for bacterial contamination. Fungin™ a new soluble formulation of
145 Pimaricin (InvioGen, Cat. No. ant-fn-2, 200mg) was added to the culture medium to inhibit mold
146 growth. Cultures were maintained in Corning 25 $cm^2$ tissue culture flasks treated with
147 CellBIND™ to promote cell attachment (Corning®, Lowell, MA) (Hunter 2010). Culture flasks
148 were kept in an incubator at 24°C and examined using an inverted microscope (Olympus IX70) at
149 10x, 20x, 60X magnifications. Complete medium change was done every 10 days without
150 disturbing the culture surface and cultures were passed when approximately 80% confluent. Cell

151 passage used 0.25% Trypsin EDTA solution (Invitrogen™, Carlsbad, CA) to dissociate cells by
152 exposure for 2 to 5 min to achieve complete dissociation. Adherent cells had medium gently
153 pipetted across the culture surface to detach cells. Once cells were dissociated, they were collected
154 into 15 mL centrifuge tubes and centrifuged for three min at a force of 400xg, in a clinical
155 centrifuge, (IEC, Centra CL2, Thermo Electron Corp., Milford, MA 01757). The supernatant was
156 drawn off and the cell pellet gently resuspended in 4 mL of fresh medium. Cells were seeded at 2
157 mL per 25 $cm^2$ flask, being split at a 1:2 ratio. Fresh medium was added to make a final volume of
158 5 mL per flask. Newly passed cultures were left untouched for 48 hours to let cells settle and attach
159 to the flask substrate.

160

161 **3. Results and Discussion**

162 Sogowa (1965) has described the morphology of leafhopper salivary glands in detail, but *P. alienus*
163 was not included and has not been described elsewhere. The morphology of these glands was
164 therefore studied and described here. The naming of the glandular cells made by Sogowa (1965)
165 is followed. The salivary glands of P. *alienus* are lying in the head and prothorax and consist of
166 two pairs of glands, each made up of a complex principal gland (Fig. 3, I-VI) and an U-formed
167 accessory gland (Fig. 3, AG). The lettering III, IV, and V all represents single cells, but the areas
168 designated I, II, and VI in Fig. 3, represents one to more cells each (the exact number not
169 determined). The accessory salivary gland (AG) consists of many cells. According to the
170 ultrastructure of the canaliculi in type III-cells (see later), these cells are subdivided in four type
171 IIIa-cells and two type IIIb-cells. The orientation of the principal salivary gland within the insect
172 showed one IIIb-cell pointing downward and the other IIIb-cell against the front. The I- and II-
173 cells (anterior lobe) form the proximal part and the IV- through VI-cells (posterior lobe) the distal
174 part of the gland. The overall length of a typical principal salivary gland (from the tip of the lower
175 IIIb-cell to the tip of the upper IIIa-cell) is about 550 µm.

176 Negative staining electron microscopy revealed TV particles in glands from two of 10 examined
177 insects. By ultrastructural analysis, unusual structures, as described below, were detected in glands
178 from the same two individuals. Rod-shaped particles were observed either singly or in
179 paracrystalline arrays in canaliculi of type III- and IV-cells (Fig. 4 -5). The type III-cells are easily

180 distinguished from other salivary gland cells by not having secretory vesicles in the cytoplasm.
181 However, two types of III-cells could clearly be distinguished in *P. alienus* according to the
182 structure of canaliculi. In the one type, here designated IIIa, the content of the canaliculi is granular
183 (Fig. 3 and 4A), whereas the other, designated IIIb, has canaliculi filled with many small
184 membrane-bound spherules containing electron-opaque granules (Fig. 3 and 4B). The IV-cells
185 could easily be identified from all other salivary gland cells by a visible line pattern in their
186 secretory vesicles (Fig. 4C).

187 The particles in the canaliculi were often seen with a membrane-like bleb at the one end (Fig. 4D).
188 These blebs are probably remnants of viral envelopes remaining after a presumed budding process.
189 For particles for which both ends could be seen, the mean length - not including the bleb - was
190 calculated to be 1294 nm (s = 22). In cross section, an electron-opaque inner hollow core (about
191 31 nm in diameter) is seen surrounded by an outer faint electron opaque layer, separated by a
192 translucent layer (Fig. 4D, 5A). The diameter of particles was calculated to be 62 nm from particles
193 laying in paracrystalline arrays. The morphology and dimensions for the particles observed here
194 in canaliculi are in agreement with the negative stained particles presented previously
195 (Lundsgaard, 1997). This taken together with the correlation found between presence of particles
196 in extracts and presence of particles in canaliculi among the 10 examined leafhoppers. The
197 particles observed in canaliculi are identical to those described in the former paper. So, the inner,
198 hollow core is probably the helical nucleocapsid of TV and the outermost faint layer the G protein
199 spikes. In cross sections through particles of Rhabdovirus and Filovirus (Murphy and Harrison,
200 1979; Geisbert and Jahrling, 1995), an electron opaque layer, thought to be the virus membrane,
201 is present between the layer of spikes and the inner nucleocapsid core. The analogous position of
202 this electron opaque layer is translucent in the present TV particles. This discrepancy can be
203 explained by use of osmium tetroxide for post-fixation (known to preserve and stain membranes)
204 in the studies on rhabdo- and filoviruses, whereas osmium tetroxide was omitted in the present
205 investigation. Osmium tetroxide was omitted in order to preserve the antigenic activity for future
206 research on localization of viral antigens in these specimens. Aggregates of particles, 75-80 nm in
207 diameter, were described from head cells of *P. alienus* (Lundsgaard, 1997). These particles were
208 seen located in the cytoplasm (not in canaliculi as presented here) and their central, electron opaque
209 core was not seen hollow (as are the present TV particles), suggesting that the particles described
210 previously were not TV particles.

211 In the cytoplasm of type III- and IV-cells, possessing canaliculi with TV particles, fine granular
212 masses up to 15 µm in diameter were observed (Fig. 4A). These masses do not contain ribosomes
213 or other cellular organelles and their presence was positive correlated with presence of TV particles
214 in nearby canaliculi. Granular masses (viroplasms), believed to be viral replication sites, have been
215 observed in cells infected with Rhabdovirus (Murphy and Harrison, 1979; Conti and Plumb, 1977)
216 and Filovirus (Geisbert and Jahrling, 1995). The granular masses present in the cytoplasm of
217 salivary glands with TV particles are thus suggested to be viroplasms, the sites for TV replication.
218 Because a nuclear localization signal has been identified in the G-protein gene of TV (Bock *et al*.,
219 2004), nuclei close to viroplasms were carefully examined for abnormalities as compared with
220 uninfected controls. Neither budding through nuclear membranes nor abnormality of nucleoplasm
221 were observed during the present investigation.

222 In several cases, two glandular cells in contact with each other could contained both, viroplasms
223 in the cytoplasm and TV particles in canaliculi, while the other neighboring cell was uninfected
224 (Fig. 4B). These uninfected cells were of a type, which were infected elsewhere. TV particles were
225 never seen between such cells or in the space between the plasma membrane and the basal lamina,
226 so, the delivery of mature TV particles appeared only to take place by budding of nucleocapsids -
227 synthesized in viroplasms - into canaliculi destined for saliva excretion. TV-particles or viroplasms
228 were neither observed in type I-, II-, V-, and VI-cells of principal salivary glands, nor in accessory
229 salivary glands from infected leafhoppers. TV-particles or viroplasms were not seen in salivary
230 glands from leafhoppers originating from a TV-free population of *P. alienus.*

231 This investigation shows that salivary glands are the primary site of TV synthesis in adult *P.*
232 *alienus.* One possible mode of TV acquisition relies on virions excreted with saliva into the plant
233 tissue, which are passively delivered into the plant during feeding. Then TV is being ingested by
234 uninfected nymphs and/or adults which become infected. Former investigations (Lundsgaard,
235 1997) have shown that host plants used for rearing TV infected leafhoppers, do not become
236 infected, and so, the plants probably function as passive host acquisition for leafhopper vectors
237 which then are able to transmit TV post replication latent period.

238 A Rhabdovirus, with strong similarity to the Taastrup virus was discovered in an ornamental plant,
239 *Lirope spp.*, grown in Florida, USA (Hunter and Adkins 2009; Hunter et al, 2009). A crude sap
240 extract was prepared and screened on leafhopper cell cultures, *Homalodisca vitripennis* (Germar)

241 glassy-winged sharpshooter (Hemiptera: Cicadellidae), for pathogenicity (Fig. 5C,D). The plant,
242 *Liriope*, belongs to the lily family and is one of the most popular ground covers in Florida (Fig.
243 1). *Liriope* is a perennial, herbaceous, mat-forming plant that grows to about 30 cm tall.
244 Leafhopper cell cultures inoculated with the virus-laden sap from infected *Liriope* plants resulted
245 in massive cell death, 100% mortality in 48 hrs. Control cells inoculated with non-symptomatic
246 plant sap inoculum showed no significant changes in cell morphology, adherence, or survival.
247 Examination using TEM showed viroplasms-like structures in inoculated leafhopper cells treated
248 with virus-infected *Liriope* sap, at 30 hrs post treatment (Fig. 5D). Attempts to sap inoculate non-
249 infected *Liriope*, from the same field plots failed to produce symptoms. However, in the original
250 *Liriope* plant with symptoms, the TV-like virions were observed at significant numbers in plant
251 tissues to suggest replication (Fig. 2).

252

253 **4. Conclusions**

254 As genomic techniques continue to improve more viruses are rapidly being discovered (Babayan
255 et al, 2018; Hunter et al, 2006; Hunnicutt et al, 2008; Liu, et al, 2011; Nouri et al, 2018; Rosario
256 et al, 2018). The taxonomic group which includes Taastrup viruses and other related virus
257 (Taastrup virus---Viruses; ssRNA viruses; ssRNA negative-strand viruses; Mononegavirales;
258 Rhabdoviridae; Cytorhabdovirus; unclassified Cytorhabdovirus) continues to have new members
259 discovered across a diverse range of insect Orders, from Hemiptera to Lepidoptera (Ma, et al,
260 2014). Insect infecting viruses are still examined for use as biological control agents (Lacey et
261 al, 2015; Szewczyk et al, 2006), but applications of viruses have moved beyond just being
262 pathogens. The advances in molecular biotechnology, low cost sequencing, bioinformatics
263 software, and virus discovery pipelines to analyze large genetic datasets (Babayan et al, 2018;
264 Bigot et al, 2018) have made virus discovery easier (Greninger 2018). Along with the rapid
265 increase of virus discovery, has been the expansion of novel molecular biology applications for
266 viruses, used in whole, or in part (promoters/peptides) across agriculture, human medicine, and
267 material sciences (Hunter et al, 2019; Kolliopoulou et al, 2017; Wen and Steinmetz 2016). Virus
268 can be used to delivery 'genetic' payloads, to produce RNA suppression of specific genes such
269 as toxins in plants, or critical genes in an insect pest (Andrade and Hunter 2016, 2017;
270 Kolliopoulou et al, 2017; Zotti and Smagghe 2015; Zotti et al, 2018). Since sap inoculation into

uninfected plants was not successful, it may be that the virus requires inoculation by an infected leafhopper. If this is the mode of transmission, the rate of transmission would be low, and may explain the severity of infection due to random exposure in nature. The analyses of the infection pathway in leafhopper salivary glands (*Psammotettix alienus* Dahlbom), along with the capacity to induce 100% mortality in leafhopper cell cultures [*Homalodisca vitripennis* (Germar)] suggests Taastrup-like virus may have an early evolutionary association with leafhoppers. The rapid mortality observed in leafhopper cells when inoculated may also be why infected leafhoppers are rare, and difficult to collect in field sampling efforts.

**Conflict of Interest**

"The authors declare no conflict of interest".

**Acknowledgements**

The authors are indebted to Prof. David B. Collinge for helpful advice during the preparation of the manuscript. Critical reviews by Dr. Xiomara Sinisterra-Hunter, AgTec, Plant Biotechnology, LLC and Dr. Catherine Katsar, PPQ, APHIS; Diann Achor, for electron microscopy work, University of Florida, IFAS, Lake Alfred, FL. Maria T. Gonzalez, Biological Science Technician, USDA, ARS, Fort Pierce, FL, for technical support.

290 **References and Notes**


291 1. Ammar, el-D., Tsai, C.W., Whitfield, A.E., Redinbaugh, M.G., Hogenhout, S.A. Cellular
292 and molecular aspects of rhabdovirus interactions with insect and plant hosts. *Annu. Rev.*
293 *Entomol*. **2009,** 54, 447–468.
294 2. Andrade, E.C., Hunter, W.B. RNA interference – Natural gene-based technology for
295 highly specific pest control (HiSPeC). *RNA Interference* (ed. by IY Abdurakhmonov), pp.
296 391–409. InTech, Rijeka, Croatia. **2016.** Doi:10.5772/61612.
297 3. Andrade, E.A., Hunter, W.B. RNAi feeding bioassay: development of a non-transgenic
298 approach to control Asian citrus psyllid and other hemipterans. *Entomol. Exper. et*
299 *Applic.* **2017,** 162, 389-396. Doi:10.1111/eea.12544.
300 4. Babayan, S.A., Orton, R.J., Streicker, D.G. Predicting reservoir hosts and arthropod
301 vectors from evolutionary signatures in RNA virus genomes. *Science* **2018**, 362, 577-
302 580.
303 5. Backus, E.A. Anatomical and sensory mechanisms of leafhopper and planthopper feeding
304 behavior. In: Nault, L.R. and Rodriguez, J.G. (Eds.), *The Leafhoppers and Planthoppers*.
305 John Wiley & Sons, New York,**1985**; pp. 163-194.
306 6. Bigot, D., Atyame, C.M., Will, M., Justy, F., Herniou, E.A., Gayral, P. Discovery of
307 *Culex pipiens* associated Tunisia virus: a new ssRNA(1) virus representing a new insect
308 associated virus family. *Virus Evolution* **2018,** 4(1), vex040. Doi:10.1093/ve/vex040
309 7. Berryman, M.A., Rodewald, R.D. An enhanced method for post-embedding
310 immunocytochemical staining which preserves cell membranes. *J. Histochem. Cytochem*.
311 **1990**, 38, 159-170.
312 8. Biesbock, A.M., Powell, C.M., Hunter, W.B., Bextine, B.R. Propagation of Homalodisca
313 Coagulata Virus-01 *via Homalodisca vitripennis* Cell Culture. JOVE **2014**, 91, e51953–
314 e51953. Doi:10.3791/51953
315 9. Bock, J.O., Lundsgaard, T., Pedersen, P.A., Christensen, L.S. Identification and partial
316 characterization of Taastrup virus: a newly identified member species of the
317 Mononegavirales. *Virology* **2004**, 319, 49-59.
318 10. Bourhy, H., Cowley, J.A., Larrous, F., Holmes, E.C., Walker, P.J. Phylogenetic
319 relationships among rhabdoviruses inferred using the L polymerase gene. *Jour. Gen.*
320 *Virology* **2005**, 86, 2849-2858.
321 11. Conti, M., Plumb, R.T. Barley yellow striate mosaic virus in the salivary glands of its
322 planthopper vector *Laodelphax striatellus* Fallen. *Jour. Gen. Virology* **1977**, 34, 107-114.
323 12. Geisbert, T.W., Jahrling, P.B. Differentiation of filoviruses by electron microscopy. *Virus*
324 *Res*. **1995**, 39, 129-150.
325 13. Haupt, H. Neueinteilung der Homoptera-Cicadina nach phylogenetisch zu wertenden
326 Merkmalen. *Zool. Jahr. Abt. Fur Syste. Okolesine Georgr. Tierre.* **1929**, 58, 173-286.
327 *Psammotettix.* [Type by original designation, *Athysanus maritinus* Perris, 1857.]
328 14. DeLong, D.M. A New Species of *Psammotettix* (Homoptera: Cicadellidae) from Mexico.
329 *The Ohio Journal of Science* **1973**, 73(n4), 237-239. http://hdl.handle.net/1811/21991

15. Dietzgen, R.G., Calisher, C.H., Kurath, G., Kuzmin, I.V., Rodriguez, L.L., Stone, D.M., Tesh, R.B., Tordo, N., Walker, P.J., Wetzel, T. Rhabdoviridae. In Andrew M. Q. King, Michael J. Adams, Eric B. Carstens and Elliot J. Lefkowitz (Ed.), *Virus taxonomy: Ninth report of the International Committee on Taxonomy of Viruses,* Oxford, United Kingdom: Elsevier**, 2011**; pp. 654-681.
16. Greninger, A.L. A decade of RNA virus metagenomics is (not) enough. *Virus Research* **2018,** 244, 218–229. https://doi.org/10.1016/j.virusres.2017.10.014.
17. Hogenhout, S.A., Redinbaugh M.G., Ammar el-D. Plant and animal rhabdovirus host range: a bug's view. *Trends Microbiol*. **2003**, 11, 264–271.
18. Hunter, W.B. Medium for development of bee cell cultures (*Apis mellifera*: Hymenoptera: Apidae). *In Vitro Cell. Develop. Biol.-Animal* **2010**, 46, 83-86. Doi:10.1007/s11626-009-9246-x.
19. Hunter, W.B., Adkins, S. Leafhopper-infecting Rhabdovirus: New Taastrup-like virus. *Proc. Ann. Meeting Florida Entomol. Soc*. **2009**, Dsp21. July 26-29, Fort Myers, FL. http://flaentsoc.org/09feshunter_taastrup.pdf
20. Hunter, W.B., Katsar, C.S., Chaparro, J.X. Molecular analysis of capsid protein of Homalodisca coagulata Virus-1, a new leafhopper-infecting virus from the glassy-winged sharpshooter, *Homalodisca coagulata*. *Journal of Insect Science* **2006,** 6:28, available online: insectscience.org/6.28
21. Hunter, W.B., Polston, J.E. Development of a continuous whitefly cell line [Homoptera: Aleyrodidae: *Bemisia tabaci* (Gennadius)] for the study of Begomovirus. *Jour. Inverte. Pathol.* **2001**, 77, 33-36. Doi:10.1006/jipa.2000.4993.
22. Hunter, W.B., Patte, C.P., Sinisterra, X.H., Achor, D.S., Funk, C.J., Polston, J.E. Discovering new insect viruses: Whitefly iridovirus (Homoptera: Aleyrodidae: *Bemisia tabaci*). *Jour. Inverte. Pathol.* **2001**, 78, 220-225. https://doi.org/10.1006/jipa.2001.5060.
23. Hunter, W.B., Clarke, S.V., Paris, T., Gonzalez, M.T., Lopez, S.P.,---Pelz-Stelinski. Advances in RNA suppression of the Asian Citrus Psyllid Vector and Bacteria (Huanglongbing Pathosystem). Chapter 18, In *Asian Citrus Psyllid: Biology, Vector Ecology, Management*. (Phil Stansly and Jawwad Qureshi, eds). CABI Press. **2019.**
24. Hunnicutt, L.E., Mozoruk, J.J., Hunter, W.B., Crosslin, J.M., Cave, R.D., Powell, C.A. Prevalence and natural host range of *Homalodisca coagulata virus*-1 (HoCV-1). *Archives of Virology* **2008,** 153, 61-67. Doi:10.1007/s00705-007-1066-2.
25. ICTVdB Virus Description - 00.000.2.00.036.Taastrup virus. *ICTVdB - The Universal Virus Database*, developed for the International Committee on Taxonomy of Viruses (ICTV) by Dr Cornelia Büchen-Osmond, is written in DELTA. The virus descriptions in ICTVdB are coded by ICTV members and experts, or by the ICTVdB Management using data provided by the experts, the literature or the latest ICTV Report. 2012. http://ictvdb.bio-mirror.cn/ICTVdB/00.000.2.00.036.htm.
26. Katsar, C.S.; Hunter, W.B.; Sinisterra, X.H. Phytoreovirus-like sequences from glassy-winged sharpshooter salivary glands. *Florida Entomologist* **2007**, 90(1), 196-203. 7

370 https://Doi.org/10.1653/0015-4040(2007)90[196:PSIFSG]2.0.CO;2
371 27. Kamita, SG., Do, ZN., Samra, AI., Hagler, JR., Hammock, BD. Characterization of cell
372 lines developed from the glassy-winged sharpshooter, *Homalodisca coagulata*
373 (Hemiptera: Cicadellidae). *In Vitro Cell Dev. Biol,-Anim*. **2005,** 41(5-6), 149-53.
374 Doi:10.1290/0501002.1
375 28. Kolliopoulou, A., Taning, C.N.T., Smagghe, G., Swevers, L. Viral delivery of dsRNA for
376 control of insect agricultural pests and vectors of human disease: Prospects and
377 challenges. *Front. Physiol.* **2017,** 8, 399. Doi:10.3389/fphys.2017.00399.
378 29. Kuwata, R., Isawa, H., Hoshino, K., Tsuda, Y., Yanase, T., Sasaki, T., Kobayashi, M.,
379 Sawabe, K. RNA splicing in a new rabdovirus from *Culex* mosquitoes. *J. Virol*. **2011**, 85,
380 6185-6196.
381 30. Lacey, L.A., Grzywacz, D., Shapiro-Ilan, D.I., Frutos, R., Brownbridge, M., Goettel,
382 M.S. Insect pathogens as biological control agents: back to the future. *J. Inverte. Pathol.*
383 **2015**, 132, 1-41.
384 31. Liu S., Vijayendran, D., Bonning, B.C. Next generation sequencing technologies for
385 insect virus discovery. *Viruses* **2011**, *3*, 1-x manuscripts; Doi:10.3390/v30x000x.
386 32. Lundsgaard, T. Filovirus-like particles detected in the leafhopper *Psammotettix alienus*.
387 *Virus Research* **1997**, 48, 35-40.
388 33. Ma, H., Galvin, T.A., Glasner, D.R., Shaheduzzaman, S., Khan, A.S. Identification of a
389 novel Rhabdovirus in Spodoptera frugiperda cell lines. *J. Virol.* **2014,** 88(12), 6576-6585.
390 Doi:10.1128/JVI.00780-14.
391 34. Marutani-Hert, M., Hunter, W.B., Hall, D.G. Establishment of Asian Citrus Psyllid
392 (*Diaphorina citri*) primary cultures. *In Vitro Cell. Develop. Biol.-Animal*. **2009**, 45, 317-
393 320.
394 35. Murphy, F.A., Harrison, A.K., Electron microscopy of the rhabdoviruses of animals. In:
395 Bishop, D.H.L. (Ed.), *Rhabdoviruses*, Vol. I, CRC Press, Boca Raton, Florida, **1979**; pp.
396 65-106.
397 36. Nouri, S., Matsumura, E.E., Kuo, Y-W., Falk, B.W. Insect-specific viruses: from
398 discovery to potential translational applications. *Curr. Opin.Virol.* **2018**, 33, 33–41.
399 https://doi.org/10.1016/j.coviro.2018.07.006
400 37. Raatikainen, M., Vasarainen, A. Composition, zonation and origin of the leafhopper
401 fauna of oatfields in Finland. *Ann. Zool. Fennici* **1976**, 13, 1-24.
402 38. Rosario, K., Fierer, N., Breitbart, M. Near-complete genome sequence of a novel single-
403 stranded RNA virus discovered in indoor air. *Genome Announc*. **2018**, 6(12):e00198-18.
404 Doi:10.1128/genomeA.00198-18
405 39. Szewczyk, B., Hoyos-Carvajal, L., Paluszek, M., Skrzecz, I., Lobo de Souza, M.
406 Baculoviruses – re-emerging biopesticides. *Biotechnol. Adv.* **2006,** 24, 143-160.
407 40. Sogawa, K. Studies on the salivary glands of rice plant leafhoppers. I. Morphology and
408 histology. *Jap. J. Appl. Ent. Zool.* **1965**, 9, 275-290.

41. Valles, S.M.; Strong, C.A., Dang, P.M., Hunter, W.B., Pereira, R.M., Oi, D.H., Shapiro, A.M., Williams, D.F. A picorna-like virus from the red imported fire ant, *Solenopsis invicta*: initial discovery, genome sequence, and characterization. *Virology* **2004**, 328, 151-157.
42. Valles, S.M., Strong, C.A., Hunter, W.B., Dang, P.M., Pereira, R.M.; Oi, D.H.; Williams, D.F. Expressed sequence tags from the red imported fire ant, *Solenopsis invicta*: Annotation and utilization for discovery of viruses. *Jour. Inverte. Pathol.* **2008**, 99, 74-81.
43. Wen, A.M., Steinmetz, N.F. Design of virus-based nanomaterials for medicine, biotechnology, and energy. *Chem. Soc. Rev.* **2016,** 45(15), 4074–4126. Doi:10.1039/c5cs00287g
44. Zotti, M.J., Smagghe, G. RNAi technology for insect management and protection of beneficial insects from diseases: lessons, challenges and risk assessments. *Neotrop. Entomol.* **2015**, 44, 197–213. Doi.10.1007/s13744-015-0291-8
45. Zotti, M., Santos, E.A., Cagliari, D., Christiaens, O., Taning, C.N.T., Smagghe, G. RNA interference technology in crop protection against arthropod pests, pathogens and nematodes. *Pest Manag. Sci.* **2018.** Doi.10.1002/ps.4813.

© 2013 by the authors; licensee Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution license (http://creativecommons.org/licenses/by/3.0/).

431 **Tables and Figures**

432 **Table 1.** Hunter's Leafhopper Cell Culture Medium, H2G, components. **[1A, B]** *Homalodisca*
433 *vitripennis* (Germar) (Hemiptera: Cicadellidae) cells in culture started from embryonic tissue
434 from eggs. Adult glassy-winged sharpshooter leafhopper.

435 **Figure 1.** Healthy non-infected *Liriope* is a plant belonging to the lily family and is one of the
436 most popular ground covers in Florida. It is also called 'Lilyturf' or 'monkey grass' and is a
437 native plant of Eastern Asia.

438 **Figure 2.** Flowering plant, genus, *Liriope,* with ring-like symptoms typical of a tospovirus
439 infection-(A). Electron microscopy examination of plant tissues however, showed a mix
440 infection with one virus having distinct morphology similar to Taastrup virus (B- Taastrup-like
441 virus Florida strain); Plant tissues showed typical virus infection associated with the plant-
442 infecting Rhabdoviruses (C).

443 **Figure 3.** Drawing of salivary glands from *Psammotettix alienus* seen from the insect axis (left)
444 or from outside (right). The salivary glands consist of a principal gland (cell type I to VI) and an
445 accessory gland (AG), both connected to the principal duct (PD). According to ultrastructure of
446 canaliculi, the type III-cells are differentiated in four type IIIa-cells (pointing upwards and
447 backwards) and two type IIIb-cells (pointing forwards and downwards).

448 **Figure 4.** Section through a type IIIa-cell from *P. alienus* infected with Taastrup virus. Aggregates
449 of virus particles (white arrowheads) are located in canaliculi **(c)** close to granular masses **(gm)** in
450 the cytoplasm. Bar = 2 µm. **[4A];** Section through a type IIIb- (right) and a type IV-cell (left) from
451 *P. alienus* infected with Taastrup virus. Aggregates of virus particles (black arrowhead) are located
452 in a canaliculus of the type IIIb-cell (inset). Note the vesicles with electron opaque granules in the
453 canaliculus, characteristic of type IIIb-cells. The neighboring cell (type IV) has cytoplasm **(cp)**
454 with rough endoplasmic reticulum together with electron opaque secretory vesicles (sv). No virus
455 particles were observed in any of the canaliculi (c) of this cell. Bar = 2 µm. Inset bar = 500 nm.
456 **[4B];** Section through a type IV-cell from another infected leafhopper other than the one examined
457 in Fig. 4. Virus particles (white arrowhead) are located in canaliculi **(C)** of this cell. Note the dark
458 lines in the secretory vesicles **(sv)**, which are characteristic for the type IV-cells. **Cp** =cytoplasm.
459 Bar = 1 µm**. [4C];** Longitudinal section through aggregates of virus particles located in a

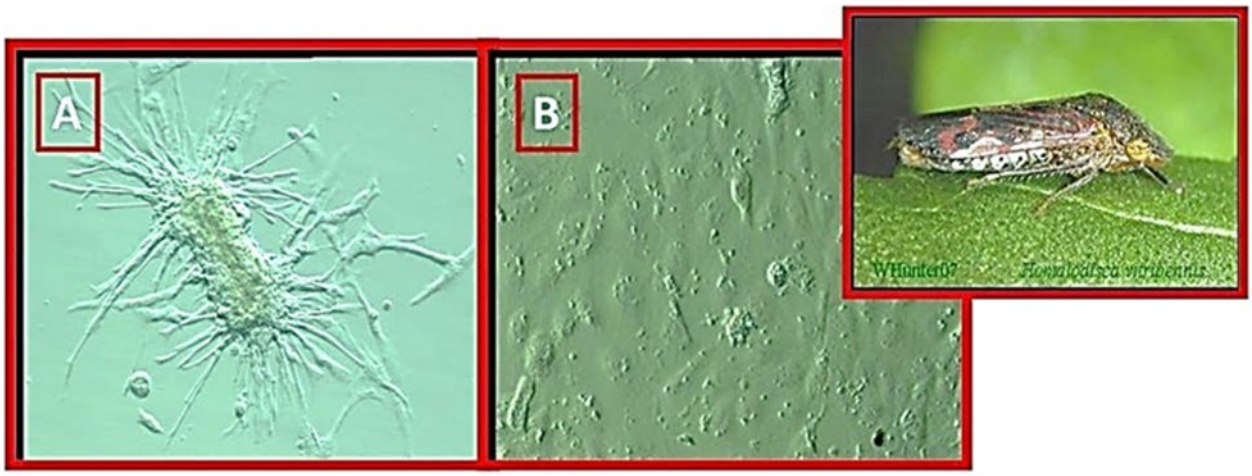
460 canaliculus of a type IIIa-cell from *P. alienus* infected with Taastrup virus. Small blebs (white
461 arrowheads) are seen at one end of several particles. Bar = 1 µm. **[4D].**

462

463 **Figure 5.** Transverse section through virus particles located in a canaliculus of a type IIIb-cell
464 from *P. alienus* infected with Taastrup virus. An electron opaque tube with a hollow centre is
465 seen surrounded by a translucent layer and outermost a faint electron opaque layer. TEM
466 micrograph, Bar = 100 nm. **[5A]**; Close-up where a canaliculus opens into the salivary duct
467 system. Oblique sections through some virus particles are seen (white arrowhead). Bar = 500
468 nm. **[5B]**; Light microscope image of Taastrup-like virus infected leafhopper cells (*H.*
469 *vitripennis*) (**5C**) and transmission electron microscopy image (**5D**) of cell culture from glassy-
470 winged sharpshooter, *Homalodisca vitripennis* (Germar), HvWH infected with a Taastrup-like
471 virus (**5C**, 30x magnification) (Hunter and Adkins 2009). TEM of leafhopper cell with
472 viroplasms-like structures in infected cells (**5D**, image provided by Diann Achor, University of
473 Florida, Electron Microscopy Core, Lake Alfred, FL).

**Table 1. Hunter's Leafhopper Medium, H2G, components.**

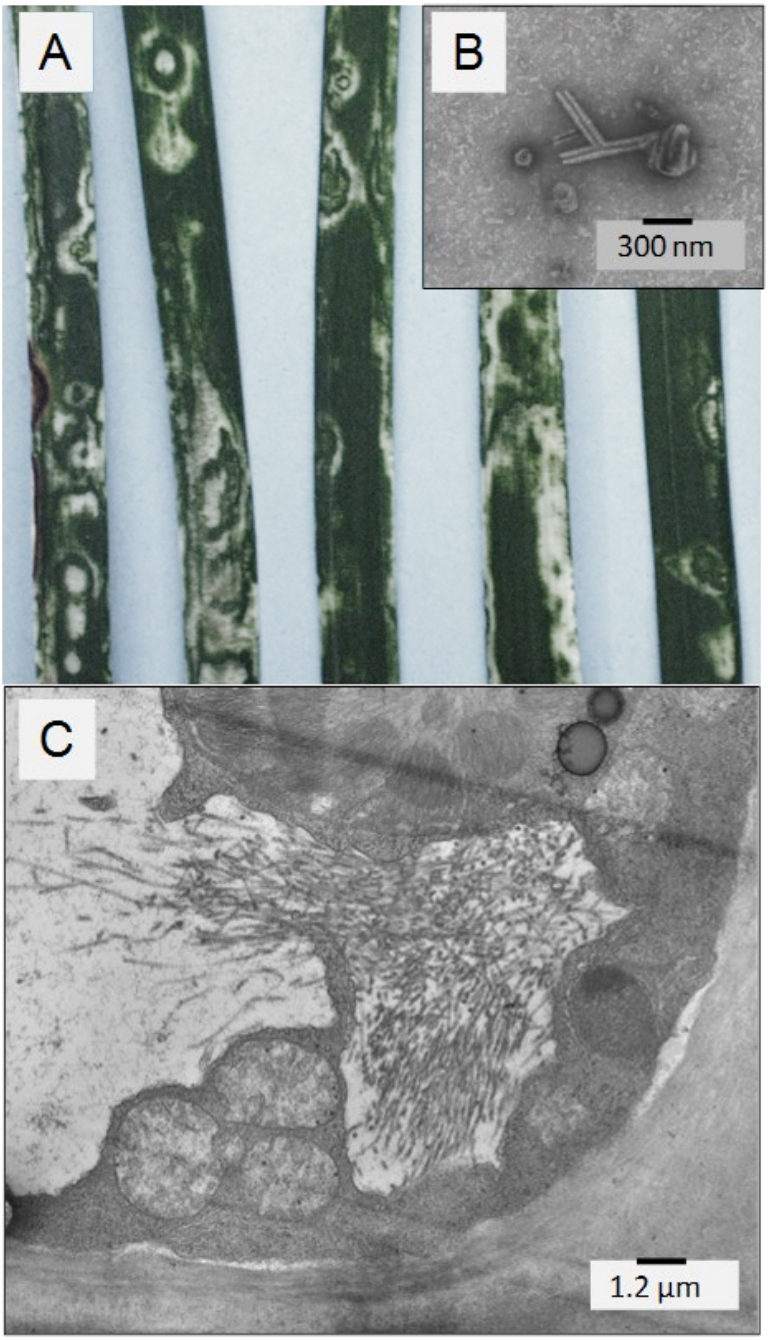
| | |
|---|---|
| Grace's Insect medium (supplemented, 1X) | 210 mL |
| 0.06M L-histidine monohydrate buffer solution pH=6.5 | 290 mL |
| Medium 199 (10X) | 10 mL |
| Medium 1066 (1X) | 17 mL |
| Hank's Balanced Salts (1X) | 33 mL |
| L-Glutamine (100X) | 1.5 mL |
| MEM, amino acid mix (50X) | 1.5 mL |
| Pen-Strep (w/ Glutamine) | 2.5 mL/500 mL |
| Nystatin | 1.0 mL/500 mL |
| Gentamycin | 1.5 mL/500 mL |
| Dextrose | 1.8 g |
| Fetal Bovine Serum | 10% of final volume |




474

**Figure 1.** Healthy non-infected *Liriope* is a plant belonging to the lily family and is one of the most popular ground covers in Florida. It is also called 'Lilyturf' or 'Monkey grass' and is a native plant of Eastern Asia.

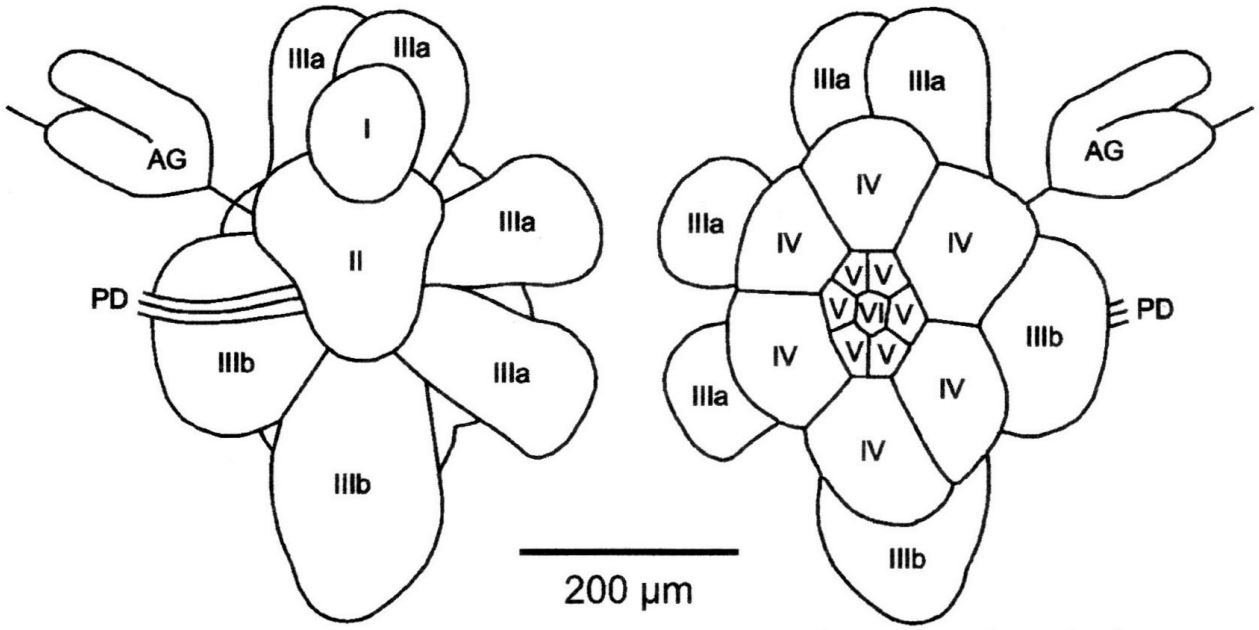
482 **Figure 2.** Flowering plant, genus, *Liriope,* with ring-like symptoms typical of a tospovirus
483 infection-(A). Electron microscopy examination of plant tissues however, showed a mix infection
484 with one virus having distinct morphology similar to Taastrup virus (B- Taastrup-like virus Florida
485 strain); Plant tissues showed typical virus infection associated with the plant-infecting
486 Rhabdoviruses (C). [Adkins, S., USDA, ARS; Achor, D., Univ. Florida].



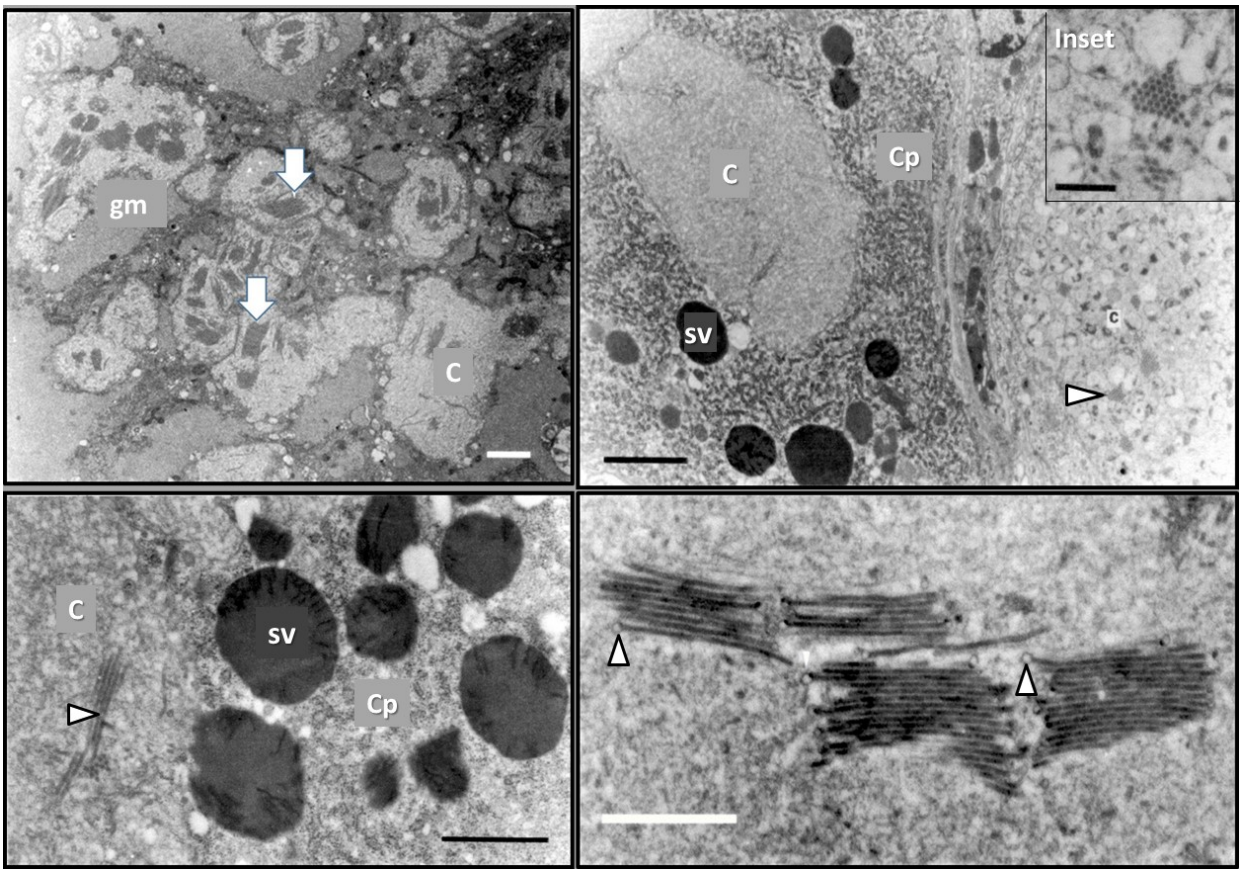

487

488

**Figure 3.** Drawing of salivary glands from *Psammotettix alienus* seen from the insect axis (left) or from outside (right). The salivary glands consist of a principal gland (cell type I to VI) and an accessory gland (AG), both connected to the principal duct (PD). According to ultrastructure of canaliculi, the type III-cells are differentiated in four type IIIa-cells (pointing upwards and backwards) and two type IIIb-cells (pointing forwards and downwards).

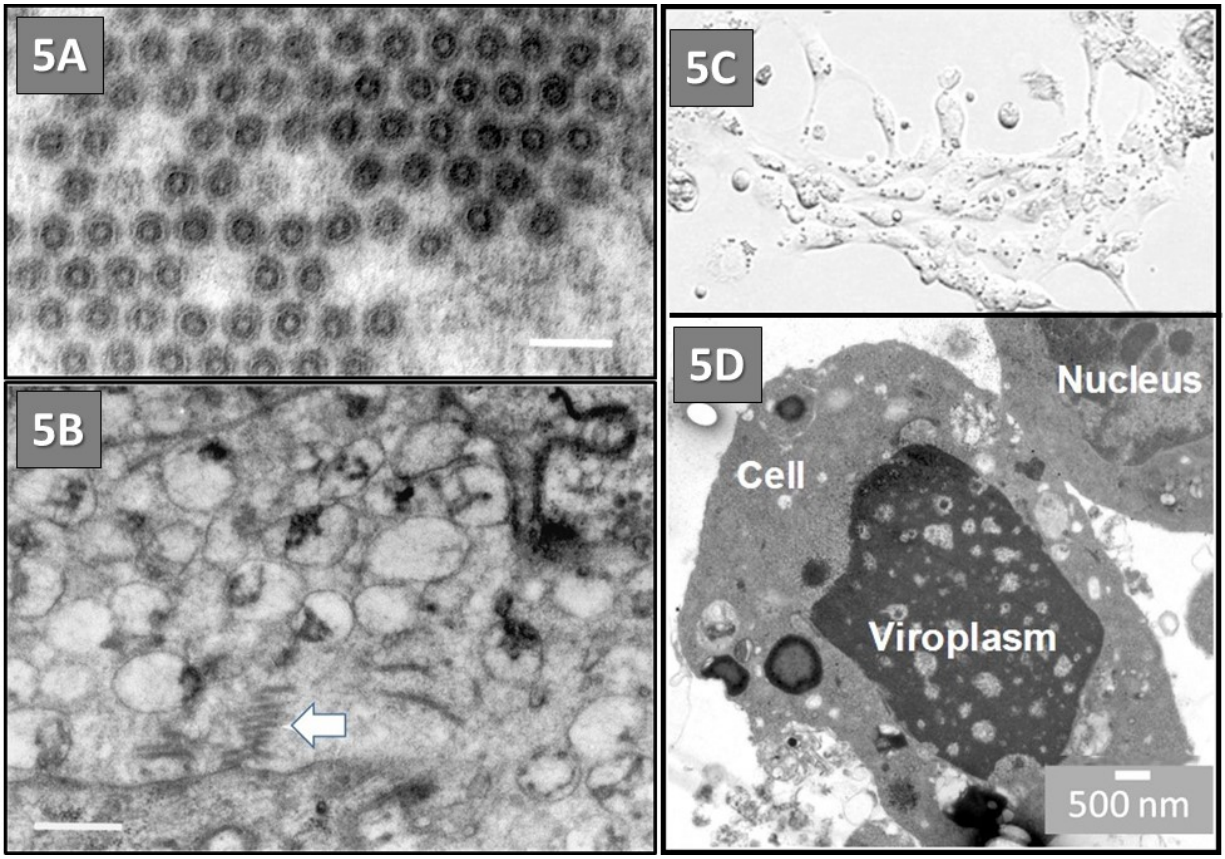


498 **Figure 4.** Section through a type IIIa-cell from *P. alienus* infected with Taastrup virus. Aggregates
499 of virus particles (white arrowheads) are located in canaliculi **(c)** close to granular masses **(gm)** in
500 the cytoplasm. Bar = 2 µm. **[4A];** Section through a type IIIb- (right) and a type IV-cell (left) from
501 *P. alienus* infected with Taastrup virus. Aggregates of virus particles (black arrowhead) are located
502 in a canaliculus of the type IIIb-cell (inset). Note the vesicles with electron opaque granules in the
503 canaliculus, characteristic of type IIIb-cells. The neighboring cell (type IV) has cytoplasm **(cp)**
504 with rough endoplasmic reticulum together with electron opaque secretory vesicles (sv). No virus
505 particles were observed in any of the canaliculi (c) of this cell. Bar = 2 µm. Inset bar = 500 nm.
506 **[4B];** Section through a type IV-cell from another infected leafhopper other than the one examined
507 in Fig. 4. Virus particles (white arrowhead) are located in canaliculi **(C)** of this cell. Note the dark
508 lines in the secretory vesicles **(sv)**, which are characteristic for the type IV-cells. **Cp** =cytoplasm.
509 Bar = 1 µm**. [4C];** Longitudinal section through aggregates of virus particles located in a
510 canaliculus of a type IIIa-cell from *P. alienus* infected with Taastrup virus. Small blebs (white
511 arrowheads) are seen at one end of several particles. Bar = 1 µm. **[4D].**




512

513 **Figure 5.** Transverse section through virus particles located in a canaliculus of a type IIIb-cell
514 from *P. alienus* infected with Taastrup virus. An electron opaque tube with a hollow centre is seen
515 surrounded by a translucent layer and outermost a faint electron opaque layer. TEM micrograph,
516 Bar = 100 nm. **[5A]**; Close-up where a canaliculus opens into the salivary duct system. Oblique
517 sections through some virus particles are seen (white arrowhead). Bar = 500 nm. **[5B]**; Light
518 microscope image of Taastrup-like virus infected leafhopper cells (*H. vitripennis*) (**5C**) and
519 transmission electron microscopy image (**5D**) of cell culture from glassy-winged sharpshooter,
520 *Homalodisca vitripennis* (Germar), HvWH infected with a Taastrup-like virus (**5C**, 30x
521 magnification) (Hunter and Adkins 2009). TEM of leafhopper cell with viroplasms-like structures
522 in infected cells (**5D**, image provided by Diann Achor, University of Florida, Electron Microscopy
523 Core, Lake Alfred, FL).




524