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16 **Maintenance of primary cell cultures of immunocytes from *Cacopsylla* spp. psyllids: a new *in vitro* tool**
17 **for the study of crop pest insects**

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19 **Monti M.¹, Mandrioli M.², Bextine B.³, Hunter W.B.⁴, Alma A.¹, Tedeschi R.¹**

20 ¹Dipartimento di Scienze Agrarie, Forestali e Alimentari (DISAFA), University of Torino, Largo P. Braccini 2, 10095 Grugliasco
21 (TO), Italy, ²Dipartimento di Scienze della Vita, Università degli Studi di Modena e Reggio Emilia, Modena 41125, Italy, 3
22 University of Texas at Tyler, 3900 University Blvd., Tyler, TX 75799, USA, ⁴USDA, ARS, U.S. Horticultural Research Laboratory
23 2001 South Rock Road, Fort Pierce, FL 34945, USA.

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26 **Corresponding Author:**

27 Rosemarie Tedeschi

28 Dipartimento di Scienze Agrarie, Forestali e Alimentari (DISAFA), University of Torino, Largo P. Braccini
29 2, 10095 Grugliasco (TO), Italy.

30 E-mail: rosemarie.tedeschi@unito.it

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32 **Running title:** Immunocyte cell cultures from *Cacopsylla* spp.

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34

35 **Summary**

36 Primary cell cultures of immunocytes have been developed from the three psyllid species *Cacopsylla*
37 *melanoneura*, *Cacopsylla pyri* (vectors of ‘*Candidatus* Phytoplasma mali’ and ‘*Candidatus* Phytoplasma
38 *pyri*’, respectively) and *Cacopsylla crataegi*. The medium most suitable of those evaluated was HH70 psyllid
39 medium. In fact, good survival and proliferation of the *Cacopsylla* immunocytes for over 60 days were
40 observed, with mitosis activities starting at 15 days post culture. Moreover, adhesion and phagocytosis
41 activities were confirmed for all the psyllid cell cultures by functionality tests. Morphological examination of
42 cultured immunocytes revealed the presence of different cell types in all the three psyllid species in
43 accordance to published data about insect immunocytes.

44 The *in vitro* maintenance of psyllid immunocytes represents a powerful tool for a wide range of applications,
45 especially for psyllid cell biology. In particular, in depth studies on the biology of psyllids as vector insects
46 as well as analyses to understand the mechanisms behind the interactions with pathogens and symbionts are
47 now possible. These cultures can be used as an *in vitro* model to study psyllid humoral immune responses,
48 which also will allow in-depth investigations on the abilities of psyllids as vectors of phytoplasmas. All these
49 applications provide new opportunities to develop more focused and specific pest control strategies.

50
51 **Key words:** *Cacopsylla*, *in vitro* culture, immunocytes, adhesion, phagocytosis

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54 **Introduction**

55 Several psyllids are known vectors of plant pathogens (Phytoplasmas and Liberibacters) and as such are
56 regarded as economically important pests (Hodkinson et al. 2009). The availability of psyllid cell cultures
57 will provide a greatly needed resource tool to evaluate and understand the interaction of pathogens at the
58 cellular level and will open new opportunities for expanding management strategies against these devastating
59 pests and diseases.

60 The genus *Cacopsylla* includes the most important vectors of fruit tree phytoplasmas as well as the vector
61 of ‘*Candidatus Liberibacter europaeus*’ (Carraro et al. 1998; 2001; Tedeschi and Alma 2004; Jarausch et al.
62 2007; Raddadi et al. 2011).

63 Building upon the success of Marutani-Hert et al. (2009) with the Asian citrus psyllid, *Diaphorina citri*
64 (Kuwayama) mature embryos, this work focused on developing primary cell cultures from three psyllid
65 species, *Cacopsylla melanoneura* (Förster), *Cacopsylla pyri* (L.), and *Cacopsylla crataegi* (Schrank). The
66 phytoplasmas transmitted by *C. melanoneura* and *C. pyri* cause severe disorders to apple and pear trees, with
67 important losses in terms of quality and quantity of the production. On the contrary, the role of *C. crataegi* in
68 transmitting phytoplasmas has never been proven, despite its possibility of harbouring them (Tedeschi et al.
69 2008). In particular, we focused on immunocytes since previous investigations suggested that the host’s
70 cellular innate immune system mediates the host tolerance to symbionts and pathogens placing circulating
71 immunocytes in a pivotal role (Su et al. 2013).

72

73 *Insects and primary cell cultures*

74 Adults of *C. pyri* (collected on pear trees), *C. melanoneura* (collected on apple trees) and *C. crataegi*
75 (collected on hawthorn plants) in Northwestern Italy, were used to establish psyllid cell cultures. Insects
76 were washed in 0.115% sodium hypochlorite, 75% ethanol and MilliQ sterile water for 10, 30 and 20
77 seconds (sec) respectively. After drying on a filter paper for a couple of seconds, they were put in a single
78 well of a sterile 24-well cell culture plate (Costar®, Corning, NY, USA) containing 1 ml of Hert-Hunter 70
79 (HH70) medium (Marutani-Hert et al. 2009). The abdomen was cut apart and gently shaken with a pair of
80 sterile forceps for 5-10 seconds in order to release the immunocytes, paying attention to avoid tissue
81 ruptures. Then, together with the other solid parts of the insect, the abdomen was removed in order to avoid

82 migration into the medium of different cell types from the remaining tissues. To avoid dehydration of the cell
83 culture, 1 ml of sterile MilliQ water was used to fill adjacent wells. Two female adults were used for each
84 species for each well. Plates were incubated at 24-26°C and 0.2ml of medium was added every 48h if
85 necessary, whereas observation of cell cultures and the evaluation of the cell growth was carried out daily
86 using an inverted Leica DMI3000 light microscope.

87

88 *Media and supplements*

89 In order to assess the best growth conditions for *C. pyri*, *C. melanoneura* and *C. crataegi* immunocyte
90 cultures, three media were evaluated: Ex-Cell ® 405 (Sigma) and Sf-900™III SFM (Invitrogen, Carlsbad,
91 CA) with addition of 10 ml/L L-Glutamine200mM solution (Invitrogen) and the psyllid medium HH70
92 (Marutani-Hert et al. 2009). Each medium received antibiotics Gentamicin (at a final concentration of 50
93 µg/ml, Sigma-Aldrich, MO, USA) and Penicillin/Streptomycin (Sigma-Aldrich, MO, USA) at a final
94 concentration of 50U/ml and 50 µg/ml, respectively. The antimycotic agent Nystatin (Sigma-Aldrich, MO,
95 USA) was also added to each medium at a final concentration of 100 U/ml. Cell counts were performed with
96 a Bürker chamber (Brand GmbH, Wertheim, Germany).

97

98 *Cell morphological characterization*

99 An aliquot of cells (in a volume ranging from 40 to 120 µl on the basis of the cell culture density) was
100 cytocentrifuged onto slides with a Shandon Instrument Cytospin II running at 400 rpm for 2 min, then
101 stained with a 200 ng/ml propidium iodide solution and observed with a Zeiss Axioplan epifluorescence
102 microscope. Images were taken using a CCD camera (Spot, Digital Instrument, Madison, USA) and the Spot
103 software supplied with the camera and processed using Adobe Photoshop (Adobe Systems, Mountain View,
104 CA).

105

106 *Functional assays by adhesion test and phagocytosis assay*

107 An aliquot of 200 µl from each immunocyte cell culture was collected and placed on a glass slide in an
108 aseptical Lab-Tek Chamber Slide system (Nunc, Naperville, IL, USA). Immunocytes were allowed to attach
109 for 30 min in presence of HH70 medium. Thereafter, the slide was removed from the chamber slide system,

110 stained with a 200 ng/ml propidium iodide solution and observed with a Zeiss Axioplan epifluorescence
111 microscope. Photographs were taken using a CCD camera as previously reported.

112 For each cell culture a phagocytosis assay was performed. Briefly, a 200 µl aliquot was sampled and
113 added to 100 µl HH70 medium in a 0.2 ml tube previously covered and the material was then incubated with
114 0.1 µl of a fluorescent beads suspension for 30 min in soft oscillation, according to Manfredini et al. (2008).
115 After incubation, cells were cytocentrifuged onto glass slides, stained with a 200 ng/ml propidium iodide
116 solution and observed with a Zeiss Axioplan epifluorescence microscope.

117

118 **Results and Discussion**

119 Maintenance of psyllid immunocytes in culture medium gave different results in the three different media
120 evaluated (Fig. 1). The Sf-900TMIII medium did not support psyllid cells which shrivelled and died in the
121 first two days post culture. Better results were obtained with the Ex-Cell ® 405 medium, since cells
122 remained viable for more than one month even though with a low growth rate. On the contrary, extremely
123 positive results were obtained with HH70 medium, which kept cells alive for more than sixty days. Cell
124 counts showed a slightly declining cell number in the first 15 days with mitosis observed in cells cultured
125 from the three psyllid species starting at 15 days post culture (Figs. 2, o-q) with a maximum amount of
126 3×10^4 cell/ml at day 45. In all three *Cacopsylla* species different cell types were observed (Fig. 2), in
127 accordance to published data (Lavine and Strand 2002; Pandey and Tiwari 2012). Most of the observed cells
128 in the three studied species were small in size with the nucleus occupying the central part of the cellular body
129 and they resembled typical insect plasmatocytes (Figs. 2b, c, f, g, l, m). A second type observed in all three
130 studied species (Figs. 2a, c, e, h, i, n) consisted of cells larger than the previous with abundant cytoplasm
131 containing cytoplasmic inclusions varying in shape from round to irregular or elongated. The third cell type
132 (observed in *C. crataegi* only) (Figs. 2d, g) consisted of small cells with a thin cytoplasm layer without
133 granules.

134 The presence of three different cell types and their shape and presence/absence of cytoplasmic inclusions
135 is in agreement with previous studies indicating that prohemocytes, plasmatocytes and granulocytes
136 represent the typical immunocyte types observed in insects (e.g. Manfredini et al. 2008). Adhesion tests
137 showed that more than 80% of the psyllid immunocytes were able to adhere to a glass slide after 30 min

138 incubation (Figs 3a-c), losing their spherical shape. Moreover, more than 75% of the cultured cells were able
139 to phagocytize fluorescent microspheres (Figs. 3d-g) indicating that they are functional despite their *in vitro*
140 maintenance. These results are in agreement with published data suggesting that most part of immunocyte is
141 able to adhere to the glass and to phagocytize fluorescent microspheres (e.g. Manfredini et al. 2008).

142 The application of immunocyte cell cultures in insect science has been widely increased in the last
143 decades. In particular most of the interest has been focused on mechanisms underlying the cellular and
144 humoral immunity and the interaction with microorganisms (Fallon and Sun 2001; Smaghe 2009).

145 The *in vitro* maintenance of *Cacopsylla* spp. immunocytes increases the opportunity for studying insect
146 humoral immune responses, pathogen/host interactions, and host hemolymph microbe fauna. Evaluation of
147 all three species of *Cacopsylla*, demonstrated that they can carry phytoplasmas and two of them are
148 acknowledged vectors (Carraro et al. 1998; Tedeschi and Alma 2004; Tedeschi et al. 2008). A better
149 understanding of the interplay between symbionts and immunocytes may explain in part, why some species
150 are vectors and others are not or solve contradictions concerning the vector ability of some species. That is
151 the case of *C. melanoneura*, the main vector of ‘*Ca. Phytoplasma mali*’ in Italy (Tedeschi and Alma 2004),
152 while in Germany it has no relevance as a vector (Mayer et al. 2009).

153 Moreover, the availability of primary and continuous cell cultures from psyllids will open new
154 opportunities for gene expression studies (Marutanti et al. 2009; Hunter et al. 2009) providing a new tool for
155 screening and discovering novel management chemistries against psyllid phytoplasma vectors. In particular,
156 these cultures aid in current efforts to discover and develop viral pesticides against psyllids.

157

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193 **Figure legends**

194

195 **Figure 1.** Comparison of the growth trends of *C. pyri* in the Sf-900™III (dashed line), Ex-Cell ® 405 (dotted
196 line) and HH70 (continuous line) exemplifying the immunocytes growth observed in psyllid immunocytes
197 cultures. For each medium, datapoints represent a mean of three replicates.

198

199 **Figure 2.** *In vitro* maintained immunocytes of *C. pyri* (a-c), *C. melanoneura* (d-h) and *C. crataegi* (i-n)
200 unstained at light microscopy (a-b, d-f, i-l) and after propidium iodide staining (c, g-h, m-n) can be
201 distinguished in three types: *i.* small cells with the nucleus occupying the central part of the cellular body (b,
202 c, f, g, l, m) (indicated by asterisks); *ii.* large cells with abundant cytoplasm containing cytoplasmic
203 inclusions varying in shape from round to irregular or elongated (a, c, e, h, i, n) (indicated by arrows); *iii.*
204 small cells with a thin cytoplasm layer around a highly defined nucleus (d, g) (indicated by arrow heads).
205 Mitotic cells were frequently observed in *C. pyri* cultures (o-q) after propidium iodide staining. Bars
206 correspond to 10 µm.

207

208 **Figure 3.** Cultured immunocytes of *C. pyri* (a-b, d, e) and *C. melanoneura* (c, f-g) are able to adhere to glass
209 slides losing their spherical shape during adhesion test (a-c) and to phagocytize fluorescent microspheres (d-
210 g) indicating that they are active cells despite their *in vitro* growth.

211

212