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

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

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

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

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

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

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

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

Insects and primary cell cultures

Adults of *C. pyri* (collected on pear trees), *C. melanoneura* (collected on apple trees) and *C. crataegi* (collected on hawthorn plants) in Northwestern Italy, were used to establish psyllid cell cultures. Insects were washed in 0.115% sodium hypochlorite, 75% ethanol and MilliQ sterile water for 10, 30 and 20 seconds (sec) respectively. After drying on a filter paper for a couple of seconds, they were put in a single well of a sterile 24-well cell culture plate (Costar®, Corning, NY, USA) containing 1 ml of Hert-Hunter 70 (HH70) medium (Marutani-Hert et al. 2009). The abdomen was cut apart and gently shaken with a pair of sterile forceps for 5-10 seconds in order to release the immunocytes, paying attention to avoid tissue ruptures. Then, together with the other solid parts of the insect, the abdomen was removed in order to avoid
migration into the medium of different cell types from the remaining tissues. To avoid dehydration of the cell
culture, 1 ml of sterile MilliQ water was used to fill adjacent wells. Two female adults were used for each
species for each well. Plates were incubated at 24-26°C and 0.2ml of medium was added every 48h if
necessary, whereas observation of cell cultures and the evaluation of the cell growth was carried out daily
using an inverted Leica DMI3000 light microscope.

Media and supplements

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

Cell morphological characterization

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

Functional assays by adhesion test and phagocytosis assay

An aliquot of 200 µl from each immunocyte cell culture was collected and placed on a glass slide in an
aseptic Lab-Tek Chamber Slide system (Nunc, Naperville, IL, USA). Immunocytes were allowed to attach
for 30 min in presence of HH70 medium. Thereafter, the slide was removed from the chamber slide system,
stained with a 200 ng/ml propidium iodide solution and observed with a Zeiss Axioplan epifluorescence microscope. Photographs were taken using a CCD camera as previously reported.

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

Results and Discussion

Maintenance of psyllid immunocytes in culture medium gave different results in the three different media evaluated (Fig. 1). The Sf-900™III medium did not support psyllid cells which shrivelled and died in the first two days post culture. Better results were obtained with the Ex-Cell ® 405 medium, since cells remained viable for more than one month even though with a low growth rate. On the contrary, extremely positive results were obtained with HH70 medium, which kept cells alive for more than sixty days. Cell counts showed a slightly declining cell number in the first 15 days with mitosis observed in cells cultured from the three psyllid species starting at 15 days post culture (Figs. 2, o-q) with a maximum amount of 3x10^4 cell/ml at day 45. In all three Cacopsylla species different cell types were observed (Fig. 2), in accordance to published data (Lavine and Strand 2002; Pandey and Tiwari 2012). Most of the observed cells in the three studied species were small in size with the nucleus occupying the central part of the cellular body and they resembled typical insect plasmatocytes (Figs. 2b, c, f, g, l, m). A second type observed in all three studied species (Figs. 2a, c, e, h, i, n) consisted of cells larger than the previous with abundant cytoplasm containing cytoplasmic inclusions varying in shape from round to irregular or elongated. The third cell type (observed in C. crataegi only) (Figs. 2d, g) consisted of small cells with a thin cytoplasm layer without granules.

The presence of three different cell types and their shape and presence/absence of cytoplasmic inclusions is in agreement with previous studies indicating that prohemocytes, plasmatocytes and granulocytes represent the typical immunocyte types observed in insects (e.g. Manfredini et al. 2008). Adhesion tests showed that more than 80% of the psyllid immunocytes were able to adhere to a glass slide after 30 min
incubation (Figs 3a-c), losing their spherical shape. Moreover, more than 75% of the cultured cells were able
to phagocytize fluorescent microspheres (Figs. 3d-g) indicating that they are functional despite their *in vitro*
maintenance. These results are in agreement with published data suggesting that most part of immunocyte is
able to adheres to the glass and to phagocytize fluorescent microspheres (e.g. Manfredini et al. 2008).

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

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

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

**References**


31: 263-278.


**Figure legends**

**Figure 1.** Comparison of the growth trends of *C. pyri* in the SfR900™III (dashed line), Ex-Cell ® 405 (dotted line) and HH70 (continuous line) exemplifying the immunocytes growth observed in psyllid immunocytes cultures. For each medium, datapoints represent a mean of three replicates.

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

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