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Possible phytoplasma transovarial transmission in the psyllids *Cacopsylla melanoneura* and *Cacopsylla pruni*

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The possible transovarial transmission of two phytoplasmas, '*Candidatus* Phytoplasma mali' and '*Candidatus* Phytoplasma prunorum', through their respective psyllid vectors *Cacopsylla melanoneura* and *Cacopsylla pruni*, was investigated. Different life stages of the progeny of infected female psyllids were analysed by PCR detection of phytoplasma DNA. While '*Ca.* Phytoplasma mali' could not be detected in any of the *C. melanoneura* life stages tested, '*Ca.* Phytoplasma prunorum' could be detected in eggs, nymphs and newly emerged adults of *C. pruni*. Infectivity tests using both nymphs and newly emerged adults of *C. pruni* showed that '*Ca.* Phytoplasma prunorum' inherited from infected females can be transmitted to healthy plum plants. Although further validations are required, these findings open up new perspectives on the study of the epidemiology of diseases associated with European stone fruit yellows.

Keywords: apple, apple proliferation, European stone fruit yellows, phytoplasma vectors, psyllids, stone fruits

Introduction

In Europe, psyllids belonging to the genus *Cacopsylla* are among the major insect pests of fruit trees because of their role as phytoplasma vectors. Phytoplasmas belonging to the apple proliferation (AP) cluster (Seemüller *et al*., 1998) represent a serious economic problem for pome and stone fruit trees, and are specifically transmitted by different species of psyllid: the pear decline (PD) agent by *Cacopsylla pyricola* in the UK (Davies *et al*., 1992) and by *Cacopsylla pyri* in France (Lemoine, 1991) and Italy (Carraro *et al*., 1998a); the European stone fruit yellows (ESFY) agent by *Cacopsylla pruni* (Carraro *et al*., 1998b, 2001); and the AP agent by *Cacopsylla picta* (syn. *Cacopsylla costalis*) in north-eastern Italy and Germany (Frisinghelli *et al*., 2000; Jarausch *et al*., 2003) and by *Cacopsylla melanoneura* in north-western Italy (Tedeschi *et al*., 2002; Tedeschi & Alma, 2004). The epidemiology of these diseases relies on the phytoplasma–vector relationship and, in particular, on the persistent-propagative mechanism of transmission, because of which psyllids retain infectivity up to their death (Davies *et al*., 1998; Carraro *et al*., 2001). Those psyllids that are able to transmit both as nymphs and as adults (Carraro *et al*., 1998b; Tedeschi & Alma, 2004) complete one or more generations per year on wild and cultivated rosaceous plants (Pomaceae and Drupaceae), and usually overwinter as adults. Recent studies indicate that fruit tree phytoplasmas are able to overwinter in the body of their insect host (Davies *et al*., 1998; Carraro *et al*., 2001; Tedeschi *et al*., 2003), but nothing is known of the possibility of their transovarial transmission.

For many years it was considered that phytoplasmas were not transmitted vertically to the progeny of infected insects, although an aster yellows (AY) phytoplasma has been reported in eggs, nymphs and adults of the experimental vector *Scaphoideus titanus* reared on healthy plants (Alma *et al*., 1997). Only recently, the possibility of transovarial transmission was confirmed with the leafhopper *Hishimonoides sellatiformis* as a vector of the mulberry dwarf phytoplasma (Kawakita *et al*., 2000), and with *Matsumuratettix hiroglyphicus* as a vector of the sugarcane white leaf phytoplasma (Hanboonsong *et al*., 2002). The vertical transmission of phytoplasmas through the eggs of psyllids has never been investigated. The aim of the present work was to investigate the occurrence of transovarial transmission in two psyllid species, *C. melanoneura* and *C. pruni*, as representative examples of vectors of pome and stone fruit phytoplasmas, respectively. Recently, following phylogenetic analyses and according to the convention proposed by Murray & Schleifer (1994) for prokaryotes that can be only incompletely described, the novel designations of '*Candidatus* Phytoplasma mali' and '*Candidatus* Phytoplasma prunorum' were proposed (Seemüller & Schneider, 2004) to describe the AP and

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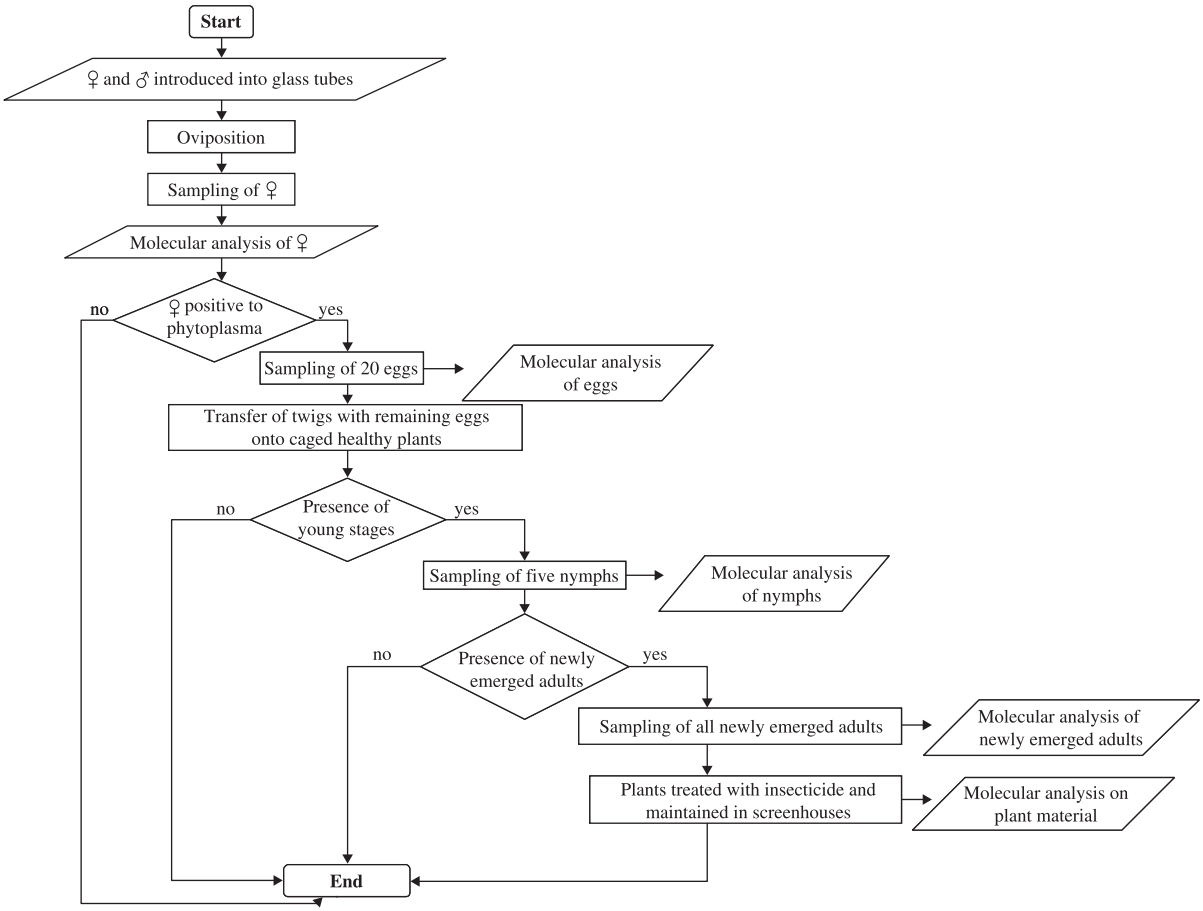

Figure 1 Flow chart of trials to verify phytoplasma transovarial transmission in *Cacopsylla melanoneura* and *Cacopsylla pruni*.

ESFY phytoplasmas, respectively; these names are used in the present work.

Materials and methods

Insect rearing

Overwintered *C. melanoneura* and *C. pruni* adults were collected, by the beat-tray method, in apple orchards located in north-western Italy in the first half of March, and in plum orchards in north-eastern Italy in the second half of March, respectively, where the two phytoplasmas are endemic (Carraro *et al*., 2002; Tedeschi *et al*., 2003).

Pairs of adult *C. melanoneura* or *C. pruni* were isolated in glass tubes (25 × 120 mm) containing a small apple or plum twig, respectively, bearing two or three buds. These plant substrates, which provided nutrition and oviposition sites for the psyllids, originated from healthy plants grown from seeds obtained from apple cv. Golden Delicious and plum cv. Black Diamond. Tubes containing pairs of psyllids were maintained in the laboratory at 25°C (±2°C) and 60% relative humidity.

Forty-four pairs of *C. melanoneura* and 52 pairs of *C. pruni* were established. Only females that laid at least 40–50 eggs were maintained for further steps in the trial. After eggs were laid, females were removed for individual DNA extraction and molecular analysis. If the DNA analysis was negative for phytoplasma, rearing of offspring ceased (Fig. 1). Conversely, if the female was positive for phytoplasma, rearing continued and 20 eggs were collected per female for DNA extraction and molecular analysis.

The twigs, with the remainder of the eggs, were taken from the glass tubes and individually tied to branches of young, potted, healthy plants grown from seed. These plants were isolated in plexiglas cages (200 × 200 × 300 mm) in which two of the walls and the top were covered with a fine nylon gauze. As soon as all the new nymphs had emerged and moved on the new plant, the original twig was removed. After 40–45 days, a sample of five nymphs (IV–V instar) was collected for DNA extraction and molecular analysis to test for phytoplasmas. In all the trials, all newly emerged adults were collected and analysed in batches of five (Fig. 1).

Proportions of infected samples for *C. pruni* ('*Ca.* Phytoplasma prunorum') and *C. melanoneura* ('*Ca.* Phytoplasma mali') were compared using Fisher's exact test ($P > 0{\cdot}05$).

At the end of the experiments, test plants were treated with insecticides (0·08% permethrin, 0·15% bromopropilate) (Reckitt & Colman, Italy) and maintained inside a leafhopper- and psyllid-proof screenhouse (20/10 net).

At the end of the summer, when the titre of phytoplasmas is normally higher in woody plants (Schaper & Seemüller, 1982), samples of leaves were collected from each test plant and analysed for the presence of phytoplasmas.

DNA extraction and molecular analysis

Total DNA was extracted from all stages of *C. melanoneura* and *C. pruni*. The parental females were tested individually; eggs laid by each infected female were tested in batches of 20; while the nymphs and the newly emerged adults were tested in batches of five. Total insect DNA was extracted following a procedure developed for leafhoppers (Marzachì *et al*., 1998) and already applied to psyllids (Tedeschi *et al*., 2002).

Plant DNA was isolated from phloem tissue according to the phytoplasma-enrichment procedure described by Ahrens & Seemüller (1992). Approximately 0·5 g fresh plant material was used.

Phytoplasma detection

'*Candidatus* Phytoplasma mali' and '*Ca*. Phytoplasma prunorum' were detected in plant and insect DNA samples by nested PCR assays. The universal phytoplasma primer pair P1/P7 (Schneider *et al*., 1995) was used, then the amplicons were diluted 1/40 and 2-μL aliquots were primed with the AP-specific primers fO1/rO1 (Lorenz *et al*., 1995). Reaction and cycling conditions were as in the original papers cited for both primer pairs. Amplification products were separated in 1% agarose gels and stained with ethidium bromide. To identify '*Ca*. Phytoplasma mali' 7 μL fO1/rO1 amplicons (48–230 ng nucleic acid μL^{-1}) from *C. melanoneura* specimens or exposed plants were digested with 3 U *Ssp*I (Sigma-Aldrich, USA) for 04·30 h at 37°C (Lorenz *et al*., 1995). To identify '*Ca*. Phytoplasma prunorum', 10 μL fO1/rO1 amplicons (48–230 ng nucleic acid μL^{-1}) from *C. pruni* specimens or exposed plants were digested with 2 U *Bsa*AI (New England Biolabs, USA) for 3 h at 37°C (Carraro *et al*., 1998b).

Results

Cacopsylla melanoneura

Thirty-nine *C. melanoneura* females laid 40–50 eggs, and these females were subjected individually to DNA extraction. Of these, 19 were positive for '*Ca*. Phytoplasma mali' and gave amplicons of the expected size (1050 bp) when primed with the fO1/rO1 pair specific for the AP group phytoplasma. Moreover, the presence of '*Ca*. Phytoplasma mali' was confirmed by RFLP analysis using the endonuclease *Ssp*I (Fig. 2).

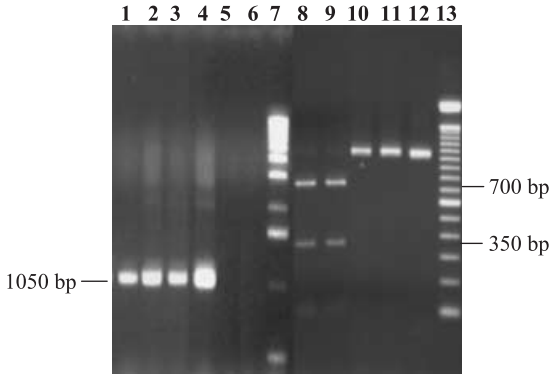

Figure 2 PCR amplification with the specific primer pair fO1/rO1 in nested PCR after a direct PCR with the primer pair P1/P7 from field-collected *Cacopsylla melanoneura* females (lanes 1–4) and *C. melanoneura* eggs (5,6); 1-kb DNA ladder (Gibco BRL) (7). RFLP analysis with *Ssp*I restriction enzyme of fO1/rO1 amplicons from field-collected *C. melanoneura* females (8,9); pear decline control (10); European stone fruit yellows control (11); undigested apple proliferation control (12); 100-bp DNA ladder (Gibco BRL) (13).

Table 1 Proportion of samples positive for '*Ca*. Phytoplasma mali' and '*Ca*. Phytoplasma prunorum' among all instars of *Cacopsylla melanoneura* and *Cacopsylla pruni*, respectively

Insect instar	*C. melanoneura*	*C. pruni*
Overwintered adults	19/39	18/31
Eggs	0/19 a	3/18 a
Nymphs	0/6 a	3/16 a
Newly emerged adults	0/6 a	3/25 a

Within rows, proportions followed by a common letter do not differ significantly (Fisher's exact test, $P \leq 0.05$).

Samples of 20 eggs were collected from each of the latter 19 females and analysed by PCR, but none of them was positive for AP. In six of the 19 rearings, sufficient young stages were obtained to allow testing of nymphs and newly emerged adults, but again none gave positive results after nested PCR (Tables 1 and 2).

No amplification products were obtained from eggs, nymphs and newly emerged adults of *C. melanoneura* used as negative controls.

Cacopsylla pruni

Thirty-one *C. pruni* females laid 40–50 eggs, and these females were subjected individually to DNA extraction. Of these, 18 were positive for ESFY. Samples of 20 eggs were collected from each of the 18 females and tested for phytoplasma by PCR. Three of these egg samples were positive for ESFY after nested PCR with the primers P1/P7 and fO1/rO1, and RFLP analysis confirmed the presence of '*Ca*. Phytoplasma prunorum' (Fig. 3). No amplification products were obtained from eggs, nymphs and newly emerged adults of *C. pruni* used as negative controls.

In most of the rearings, a sufficient number of nymphs and newly emerged adults were obtained for further testing.

Table 2 Vertical succession of positive PCR results in the brood of all overwintered females of *Cacopsylla melanoneura* and *Cacopsylla pruni*, and in test plants

Female ID no.	Eggs (*n* +ve/1)	Nymphs (*n* +ve/*n* tested)	Newly emerged adults (*n* +ve/*n* tested)	First-year plants (*n* +ve/*n* tested)	2nd year plants (*n* +ve/*n* tested)
C. melanoneura					
1	0	0/1	0/1	–	–
2	0	0/1	0/1	–	–
3	0	0/1	0/1	–	–
4	0	0/1	0/1	–	–
5	0	0/1	0/1	–	–
6	0	0/1	0/1	–	–
7	0	–	–	–	–
8	0	–	–	–	–
9	0	–	–	–	–
10	0	–	–	–	–
11	0	–	–	–	–
12	0	–	–	–	–
13	0	–	–	–	–
14	0	–	–	–	–
15	0	–	–	–	–
16	0	–	–	–	–
17	0	–	–	–	–
18	0	–	–	–	–
19	0	–	–	–	–
C. pruni					
1	1	–	–	0/1	–
2	1	1/2	1/1	0/1	1/1
3	1	1/1	1/2	0/1	0/1
4	0	1/1	1/2	0/1	0/1
5	0	0/1	0/1	0/1	–
6	0	0/1	0/1	0/1	–
7	0	0/1	0/1	0/1	–
8	0	0/2	0/2	0/1	–
9	0	0/1	0/2	0/1	–
10	0	0/1	0/2	0/1	–
11	0	0/5	0/6	0/1	–
12	0	–	0/1	0/1	–
13	0	–	0/2	0/1	–
14	0	–	0/1	0/1	–
15	0	–	0/1	0/1	–
16	0	–	–	0/1	–
17	0	–	–	0/1	–
18	0	–	–	0/1	–

Overwintered adult females were tested individually; eggs in batches of 20 from the same female; nymphs and newly emerged adults in batches of five each.

Batches each of five nymphs and five newly emerged adults were tested by PCR. The number of batches analysed for each trial was related to the total number of specimens available, and when the number of young stages was very low, they were not tested in order to permit adult emergence. In one of the rearings, the female did not lay very well, so it was possible to test only the eggs.

In 14 rearings, newly emerged adults were observed and several samples were analysed. Altogether, 25 samples of newly emerged adults were tested and three were positive for ESFY after nested PCR with the specific primer and digestion with *Bsa*AI (Table 1).

In two of the rearings, there was a sequence of eggs, nymphs and newly emerged adults positive for the ESFY phytoplasma. In one case the eggs were not positive, but the nymphs and newly emerged adults were phytoplasma-positive (Table 2).

Test plants

As none of the samples of eggs, nymphs and newly emerged adults of *C. melanoneura* were positive for '*Ca.* Phytoplasma mali', none of the test plants was subjected to molecular analysis. Because of the positive results obtained with *C. pruni*, at the end of the summer following the experiments all test plants were tested for phytoplasma to assess the transmission of '*Ca.* Phytoplasma prunorum' after vertical acquisition. None of the test plants was positive, but in the

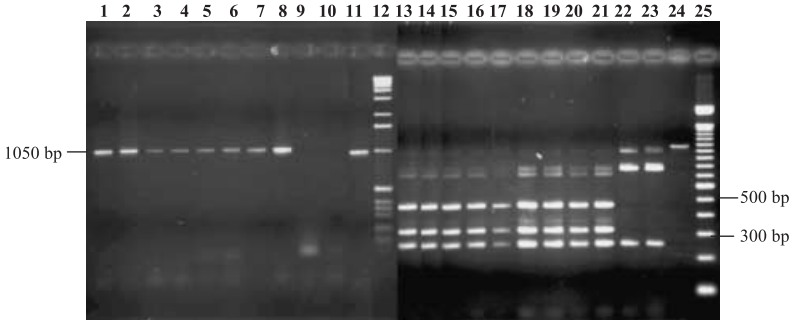

Figure 3 PCR amplification with the specific primer pair fO1/rO1 of total DNA from field-collected *Cacopsylla pruni* females (lanes 1–2); *C. pruni* eggs (3–4); *C. pruni* nymphs (5–6); *C. pruni* newly emerged adults (7–8); *C. pruni* eggs obtained from healthy females (9–10); experimentally infected plum test plant (11); 1-kb DNA ladder (Gibco BRL) (12). RFLP analysis with *Bsa*AI restriction enzyme of fO1/rO1 amplicons from field-collected *C. pruni* females (13–14); *C. pruni* eggs (15–16); *C. pruni* nymphs (17–18); *C. pruni* newly emerged adults (19–20); experimentally infected plum test plant (21); pear decline control (22); apple proliferation control (23); undigested European stone fruit yellows control (24); 100-bp DNA ladder (Gibco BRL) (25).

following year the tests were repeated on the plants on which infected specimens of *C. pruni* had been isolated, and one gave positive results after nested PCR (Table 2). RFLP with *Bsa*AI confirmed the presence of '*Ca.* Phytoplasma prunorum' (Fig. 3, lanes 11 and 21).

Discussion

To date, transovarial transmission has been reported for mulberry dwarf phytoplasma (*Hishimonoides sellatiformis*, Kawakita *et al.*, 2000) and sugarcane white leaf phytoplasma (*Matsumuratettix hiroglyphicus*, Hanboonsong *et al.*, 2002). The AY phytoplasma has also been reported in eggs, nymphs and adults of the experimental vector *Scaphoideus titanus* (Alma *et al.*, 1997). In all three cases, the insects belong to the order Hemiptera and to the family Cicadellidae. In addition to many species of the families Cicadellidae, Cixiidae and Delphacidae efficient phytoplasma vectors are also found in some species belonging to the family Psyllidae.

The present work is the first demonstration that a psyllid, *C. pruni*, can transmit phytoplasmas transovarially. Eggs, nymphs and newly emerged adults of this psyllid reared on phytoplasma-free plants were phytoplasma-positive. Although only 3/18 samples of eggs, 3/16 samples of nymphs and 3/25 samples of newly emerged adults were positive for '*Ca.* Phytoplasma prunorum', a good correspondence was observed between the positive samples (Table 2). In one of the rearings, '*Ca.* Phytoplasma prunorum' was not found in the eggs, but it was detected in nymphs and newly emerged adults, probably because the titre of phytoplasmas in the eggs was too low to be detectable, or the 20-egg sample was not representative of the actual distribution of phytoplasmas in eggs. The amplification signal observed in the newly emerged adults, stronger than that in eggs and nymphs, could be the result of multiplication of phytoplasma in the insect body.

The presence of '*Ca.* Phytoplasma prunorum' in the eggs of *C. pruni* strongly supports the hypothesis of transovarial transmission. It is unlikely that the eggs can acquire phytoplasma from the phloem through the pedicel. The phloem is a system under pressure, in which solution moves from an area of high solute production to an area of high consumption (Salisbury & Ross, 1994), and it seems unlikely that the egg can actively enter this system. White (1968) asserted that eggs take up water through the pedicel inserted into the mesophyll. The egg replenishes the water lost by evaporation, acting as an integral part of the xylem through the continuity between the pedicel and the xylematic veins of the mesophyll.

Cacopsylla melanoneura and *C. pruni* have similar life cycles. Both complete one generation per year; they overwinter as adults on alternative host plants, mainly conifers; and they start to colonize orchards at the end of winter. Overwintered adults lay eggs on fruit trees and nymphs and new adults emerge progressively. New adults migrate to their alternative hosts in late spring or early summer and remain there until the end of winter (Domenichini, 1967; Ossiannilsson, 1992; Poggi Pollini *et al.*, 2002; Tedeschi *et al.*, 2002; Schaub & Monneron, 2003).

The completion of the life cycle and occurrence of different life stages (eggs, nymphs and newly emerged adults) were similar in the two species in the field and in laboratory experiments. Despite the affinities between the two species, considering the preliminary results obtained in the present work, it appears that their relationships with the phytoplasmas they transmit differ: while *C. pruni* was able to transmit the ESFY phytoplasma transovarially, the same was not observed for *C. melanoneura* and AP. However, in the light of the statistical analysis, which did not reveal any significant differences between the two species, transovarial passage cannot be ruled out in the second system.

Kawakita *et al.* (2000) suggested a connection between the genome size of the phytoplasma and the capacity to be transmitted transovarially. These authors noted that both the agent of aster yellows and the agent of mulberry dwarf belong to the ribosomal group 16SrI (AY), a group in which there is considerable variability in genome size. On the basis of the latter consideration, they suggested that

AY-group phytoplasmas vary in their phenotypic characteristics, including survival in genital organs and successive transovarial transmission of phytoplasmas. Marcone *et al*. (1999) estimated the sizes of full-length chromosomes of 71 strains of phytoplasmas belonging to 12 major phylogenetic groups. On the basis of these studies, the genome size of phytoplasmas belonging to the AP group appeared to range between 630 and 690 kb, with a low size variation and small genome.

At present the different behaviour of *C. melanoneura* and *C. pruni* in relation to the transovarial transmission of '*Ca*. Phytoplasma mali' and '*Ca*. Phytoplasma prunorum', respectively, cannot be easily interpreted, considering also the affinities between the two vectors and the two phytoplasmas. It would be interesting to carry out further experiments with the system *C. melanoneura*–'*Ca*. Phytoplasma mali' using a larger sample size, and then to study the behaviour, in this context, of the other vector of '*Ca*. Phytoplasma mali', *C. picta*, which occurs in north-eastern Italy and Germany (Frisinghelli *et al*., 2000; Jarausch *et al*., 2003).

The present work confirms the ability of phytoplasmas to be transmitted transovarially in their insect vectors, extending this – until now restricted to insects belonging to the family Cicadellidae – to a psyllid species. Moreover, it was possible to assess the capacity of psyllids that acquired the phytoplasma transovarially to transmit it by feeding to a healthy plum seedling.

The fact that the insect is not only the vector, but also a reservoir of the phytoplasma has implications for disease management. In addition, the possibility of transovarial passage adds to the difficulty of disease control. The implication is that insect control should extend to the wild vegetation where it spends the summer and most of the winter.

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