European Stone Fruit Yellows: Consequences of the Life Cycle of the Vector and of the Multiplication of the Phytoplasma in the Insect on the Epidemiology of the Disease

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Abstract

***Cacopsylla pruni* is the vector of '*Candidatus* Phytoplasma prunorum', responsible for the disease called European stone fruit yellows. In this work we obtained new data on the overwintering of *C. pruni* and we measured the evolution of the quantity of phytoplasma in the insects after acquisition. The life cycle of *C. pruni* was completed for the first time, demonstrating directly that it is a univoltine species. From the data obtained on the overwintering places, we assume that the overwintering takes place through long distance migrations from *Prunus* to conifers. We demonstrated that when *C. pruni* are grown on an infected *Prunus*, they accumulate the phytoplasma and then multiply it so that after the overwintering period the phytoplasma concentration is at its uppermost value. The young adults of the new generation, although frequently infected, had a low transmission efficiency. Despite multiplying the phytoplasma, the healthy reimmigrant vectors fed on ESFY-infected *Prunus* appeared to die before they could transmit it to other plants. Thus, it seems that the overwintering reimmigrants infected the previous year are the most efficient vectors of the phytoplasma and that the dissemination of the disease should be understood at a regional scale.**

INTRODUCTION

European stone fruit yellows (ESFY) is a disease damaging mainly apricot (*Prunus armeniaca*) and Japanese plum (*P. salicina*) orchards in Europe. It is due to a phytoplasma for which the name '*Candidatus* Phytoplasma prunorum' has been proposed (Seemüller & Schneider, 2004).

This phytoplasma is spread by the psyllid *Cacopsylla pruni* (Carraro *et al*., 1998). *C. pruni* is supposed to be a univoltine species reproducing on *Prunus* sp. and overwintering mainly on conifers (Ossiannilsson, 1992). In France, its distribution, host preference and period of presence on *Prunus* have been studied (Labonne & Lichou, 2004). Two successive morphs were observed on *Prunus*: a dark-winged form corresponding to the reimmigrants coming back for reproduction after overwintering; a light-colored form corresponding to the adults of the new generation. But the knowledge about the overwintering period is very poor and the life cycle of the species has not been completed until now.

During the last six years, the transmission properties of '*Ca*. P. prunorum' by *C. pruni* have been intensively studied, mainly by Carraro *et al.* (1998, 2001, 2002, 2004).

However, two important points remained unclear: the persistence of the phytoplasma in its vector during the overwintering period and the possibility of successful transmission by reimmigrants if they acquire the phytoplasma after their coming back on an infected plant. These two points are epidemiologically important as they determine if transmission from infected plants to healthy plants occurs within a year or between years.

The aim of this work was to obtain the complete life cycle of the insect, to get information about what happens to the phytoplasma in the vector during the overwintering period, to assess the possibility of the reimmigrants to acquire and transmit the phytoplasma during their reproductive period on *Prunus* and to connect the biology of the vector to the transmission processes.

MATERIALS AND METHODS

Overwintering of *C. pruni*

The presence of *C. pruni* on conifers was investigated by searching for the insect in different places at a regional scale. To complete the life cycle of the insect, adults of the new generation were reared on *Prunus marianna* in climatic chambers and then, they were set under sleeve cages on conifer branches at identified natural overwintering sites during the supposed overwintering period (July-February).

Acquisition and Transmission of '*Ca.* P. prunorum' by *C. pruni*

Reimmigrants of *C. pruni* were collected from regional natural populations on *P. spinosa*. The insects were set for acquisition under sleeve cages on infected *P. marianna* plants. These plants were inoculated mainly by grafting with the same isolate of '*Ca*. P. prunorum' one or two years before the experiments. A small number of acquisition experiments were made with other isolates and two other plant species (*P. armeniaca*, *P. salicina)* to avoid any problem which could be linked to the *Prunus* species or to the phytoplasma isolate. After a defined time of acquisition, the insects were set on healthy test plants (young cuttings of *P. marianna*) by groups of 5 to 15 adults. Two sets of transmission experiments were performed (2003 and 2004). A large sample of *C. pruni* from the natural populations was included as control to achieve enough statistical power for detecting an effect of the acquisition on the transmission efficiency.

Infected nymphs and adults of the new generation were produced in climatic chambers from eggs laid by the reimmigrants on the previously described source plants. They were set for transmission on healthy *P. marianna* as previously described.

The psyllids which were still alive were collected 20 days after inoculation. Test plants were then sprayed with an insecticide and incubated in an insect-proof greenhouse.

Detection of '*Ca.* P. prunorum'

Total DNA was extracted from the phloem of *Prunus* using CTAB as described by Maixner et al. (1995). Total DNA of each insect was extracted by the same procedure modified following Marzachi et al. (1998). Detection of '*Ca*. P. prunorum' in plants and insects was performed by PCR using the specific primer pair ESFYf/r (Table 1). The inoculated plants were checked for phytoplasma infection after at least 6 months following the inoculation.

Quantification of '*Ca.* P. prunorum' in *C. pruni*

A real-time PCR method using TaqMan® chemistry was designed, calibrated and used to measure the evolution of the phytoplasma titer inside the insects after

phytoplasma acquisition. A 18S rDNA fragment (GenBank accession numbers DQ778629 to DQ778635) of *C. pruni* was used as an internal standard. Serial dilutions of cloned rDNA fragments from the phytoplasma and of the standard gene were used to obtain the calibration curves. Primers and probe are listed in Table 1.

RESULTS AND DISCUSSION

Overwintering of *C. pruni*

During the years 2002-2005 a survey was carried out on the conifers around Montpellier. We were unable to detect *C. pruni* on the *Pinus halepensis* surrounding the blackthorn hedges and bushes in the plain. *C. pruni* was found on *P. halepensis* in small numbers but regularly on the first line of hills north of Montpellier. It was found with a greater abundance on the plateau (altitude: 700 m) and the mountainous area (altitude: 1100-1400 m) on *Abies alba*, *Abies* sp, *Picea abies, and Pinus sylvestris*.

Three sites were chosen to try an artificial overwintering of *C. pruni*: 2 natural sites on *Abies* sp. and *P. abies* and in the plain on *P. halepensis*. The 3370 adults of the new generation obtained in climatic chambers were enclosed on conifer shoots under sleeve cages. Surviving *C. pruni* were recovered at all the 3 sites at the end of their natural overwintering period. The proportion of surviving adults inside the cages was irregular (0% to 54%) but generally small (mean: 4%). For each site, a sample of surviving *C. pruni* was recovered and set on *P. marianna* plants. The eggs laid on each plant developed normally in nymphs and new adults.

Thus, for the first time, the biological cycle of *C. pruni* has been completed, demonstrating directly that the individuals found on conifers are the same than those reproducing on *Prunus* and that there is only 1 generation per year. Moreover, the distance of several tenths of km between the plain and the plateau and mountains seems to imply large migration movements of this insect.

Detection of '*Ca*. P. prunorum' in Overwintering *C. pruni*

Samples of *C. pruni* collected on conifers in winter were checked for the presence of the phytoplasma. Eight out of 256 were detected infected. This result demonstrates that the phytoplasma persists in its vector during the overwintering period.

From 5 adults reared on an infected plant, set to overwinter on conifers and recovered after the overwintering period, 4 were detected still infected and 3 out this 4 were able to transmit the phytoplasma to a healthy plant. The transmission efficiency of the infected reimmmigrants after overwintering seems thus very high.

Transmission of '*Ca*. P. prunorum' by *C. pruni*

Transmission experiments carried out in 2003 and 2004 with reimmigrants indicated that about 0.5% of the sampled populations of *C. pruni* were infectious (95% confidence intervals: [0.19% - 1.13%] in 2003; [0.04% - 1.14%] in 2004). Whatever the duration of the acquisition period on infected plants and the duration of the transmission period, we were unable to find out any significant increase of the transmission efficiency, despite the large number of insects tested (Table 2).

The adults of the new generation reared on infected plants were able to infect a few test plants but they showed a transmission efficiency of only 0.6% (Table 3). As the emerging adults of *C. pruni* exhibit a strong emigration behavior (quick takeoff from their *Prunus* hosts), it can be thought that the feeding behavior of the new adults on *Prunus*

plants can prevent the transmission of the phytoplasma. A similar proportion of the old nymphs reared on infected plants was able to transmit the phytoplasma (Table 3).

Quantification of '*Ca*. P. prunorum' in *C. pruni*

The growth of the number of phytoplasma in *C. pruni* after a defined acquisition period was measured either for reimmigrants arriving on an infected plant or for the new generation reared on an infected plant and overwintering afterward on conifers (Fig. 1).

When *C. pruni* was reared on infected plants, the quantity of phytoplasma increased with time from a mean of 5.4×10^4 phytoplasma in the nymphs collected 19 to 21 days after hatching to 2.0×10^7 phytoplasma in the adults at the end of the overwintering period. As the highest phytoplasma titer was found in *C. pruni* overwintering on conifers, it demonstrates that the phytoplasma is conserved or multiplied in the insects outside their reproduction host.

After acquisition on an infected plant (even after only 1 day), the reimmigrants contained a measurable quantity of phytoplasma, clearly differentiating them from the control insects. The mean quantity of phytoplasma measured after 1, 2, 10 or 21 days of acquisition remained around 10^4 and the quantities measured in different individuals were quite similar. After the reimmigrants vectors were transferred from infected plants to healthy plants, the quantity of phytoplasma differed greatly depending on the individuals. Some insects lost almost completely the phytoplasma while in the others the quantity of phytoplasma reached values between 10^6 and 10^7. This 100-fold increase relatively to the previous values can be explained only by a multiplication of the phytoplasma inside the insects after an initial delay. Through these results, this delay can be evaluated around 30 days after acquisition.

From the tested individuals, 4 were able to transmit the phytoplasma to a healthy plant. In these individuals, the amount of detected phytoplasma was at its uppermost value (between 10^7 and 10^8) in the 4 cases.

CONCLUSION

For the first time, the entire life cycle of *C. pruni* was experimentally completed, the persistence of the phytoplasma in the vector during overwintering was demonstrated and the growth of the phytoplasma concentration was measured all along the life cycle of the vector.

The synthesis of the results on the life cycle of *C. pruni* and of its transmission efficiency at different stages of its life suggests the following scenario for the dissemination of '*Ca*. P. prunorum': the young psyllids would get infected when living on an infected plant, either cultivated or wild; although infected, they would be poor vectors at this stage; after migrating to overwinter on conifers, the insects would either lose, conserve or multiply the phytoplasma; most reimmigrants still infected at the end of the winter would be infectious and able to inoculate susceptible plants when they return to reproduce on *Prunus*. This scenario implies that the reimmigrants would be the most efficient vectors of the phytoplasma. The wild *Prunus* may play a central role because they produce numerous vectors and they are reservoirs of the phytoplasma (Carraro *et al.*, 2002). Questions remain about the distances and trajectory of the migratory flights, but the scenario implies a regional scale for the spread of the phytoplasma, as the migrations of *C. pruni* seem to occur at distances of several tens of kilometers, at least in southeastern France.

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Tables

Table 1. Primers and probes used for '*Ca*. P. prunorum' detection

Name	Sequence (5'→ 3')
'*Ca*. P. prunorum' 16S rDNA	
ESFYf (forward)	CCATCATTTAGTTGGGCACT
ESFYr (reverse)	ATAGGCCCAAGCCATTATTG
Quantitative PCR:	
'*Ca*. P. prunorum' 16S rDNA	
ECAQf (forward)	AAACGACTGCTAAGACTGGATATGAA
ECAQr (reverse)	TTACCAACTAACTAATGTGCCGCA
ECAQp (probe)	**VIC**-CCCGCAAGGGTATGCTGAGAGATGGG
C. pruni 18S rDNA	
CPf (forward)	CAAGTACGTCCCCGTTGATCA
CPr (reverse)	GCTGGCTGACATCGTTTATGG
CPp (probe)	**FAM**-TTAGAGGTTCGAAGGCGATCAGATACCGC

Table 2. Transmission of *'Ca*. P. prunorum' by *C. pruni* reimmigrants collected on *P. spinosa* and previously set on an infected plant for acquisition.

	2003 experiment *	2004 experiment *
control (natural pop.)	6 / 1155	2 / 630
1 day acquisition	4 / 400	
7-10 days acquisition	6 / 1235	
20 days acquisition	1 / 200	4 / 480

* : x / y = infectious *C. pruni* / total *C. pruni* tested *;* transmission period : 20 days

Table 3. Transmission of *'Ca.* P. prunorum' by new generation of *C. pruni* after breeding on healthy or infected *Prunus*.

Rearing host plant	Stage at deposition	Result *
healthy (control)	adult	0 / 620
infected	nymph	3 / 300
infected	adult	2 / 600

* : x / y = infectious *C. pruni* / total *C. pruni* tested

Figure

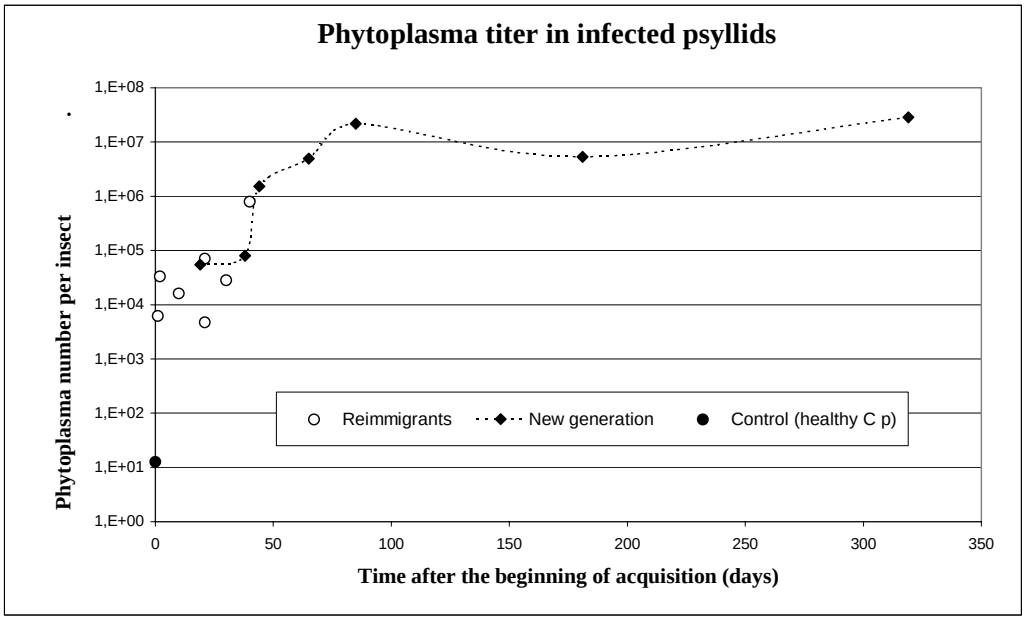

Fig. 1. Kinetics of '*Ca.* P. prunorum' in its vector *Cacopsylla pruni* after the beginning of acquisition. Each point represents the mean number of phytoplasmas measured per insect from 5 to 20 insects (the insects where the phytoplasma had disappeared were discarded).