

## THE 'LIFE CYCLE' OF PEAR DECLINE PHYTOPLASMA IN THE VECTOR *CACOPSYLLA PYRI*

L. Carraro, N. Loi and P. Ermacora

Dipartimento di Biologia Applicata alla Difesa delle Piante,  
Università degli Studi di Udine, Viale delle Scienze 208, I-33100 Udine, Italy

### SUMMARY

The 'life cycle' of pear decline phytoplasma in the vector *Cacopsylla pyri* has been studied. For two consecutive years, groups of insects caught monthly from March to October were used for transmission trials. The psylla was equally infective throughout the experimental period. In the second year, insects were fed on dormant plants from November to March and regularly tested. Retention of infectivity of the pear decline phytoplasma in the vector lasted through the winter. The overwintering adults of *C. pyri* did not transmit the phytoplasmas to plants in dormancy, but they did, without any previous recharge feeding, after bud break in the spring. Control of the overwintering adults of *C. pyri* appears to be of fundamental importance for preventing the spread of pear decline.

*Key words:* epidemiology, phytoplasmas, retention of infectivity, PCR-RFLP, detection.

### INTRODUCTION

Pear decline (PD) is one of the most dangerous diseases of pear trees. The causal agent is a phytoplasma belonging to the apple proliferation group (Seemüller *et al.*, 1998). In the late 1950s, PD affected more than a million pear trees along the Pacific Coast of North America (Woodbridge *et al.*, 1957). The disease has long been known as 'moria' in northern Italy (Refatti, 1948) and has been reported in other European and extra-European fruit areas (Blattny and Vana, 1974; Davies *et al.*, 1992; Lorenz *et al.*, 1995; Jarausch and Dosba, 1995; Malinowski *et al.*, 1996; Battle *et al.*, 1999). Recently, PD has also been detected in southern Italy, in areas previously considered free from the disease (Marcone *et al.*, 1996).

Although the aetiology of PD has been established,

some problems concerning the epidemiology remain. Jensen *et al.* (1964) showed that pear psylla (*Psylla pyricola* Förster, now *Cacopsylla pyricola* Förster) transmits 'a virus' capable of causing PD disease. More recently transmission of the PD agent by *C. pyricola* in England (Davies *et al.*, 1992) and by *C. pyri* L. in France (Lemoine, 1991) and Italy (Carraro *et al.*, 1998a), has been reported. Some aspects of the epidemiology were also studied in England using the vector *C. pyricola* (Davies *et al.*, 1998). These authors detected PD phytoplasma in vectors collected in PD-affected orchards, over an entire year.

In Italy, *C. pyri* is the predominant psyllid in pear orchards, but the relationship between this vector and the PD phytoplasma has yet to be clarified. The insect produces 3-5 generations per year on pear trees and overwinters as an adult on the same host (Conci *et al.*, 1992).

In this paper we report the results of a two-year investigation, intended to resolve some epidemiological features. In particular, presence of PD phytoplasma in *C. pyri* during the vegetative season has been compared to their actual ability to infect test plants and retention of the phytoplasma in overwintering adult vectors.

### MATERIALS AND METHODS

**Natural infection and infectivity of *C. pyri*.** From March to October 1997 and 1998 adults of *C. pyri* were captured monthly from orchards with a high percentage (70-90%) of PD-affected pear trees (Carraro *et al.*, 1998a) and each sample split into two groups. One group was used for polymerase chain reaction/restriction fragment length polymorphism (PCR-RFLP) analyses and the other to inoculate young pear seedlings. Each month at least 200 psyllids were transferred to four young seedlings of *Pyrus communis* L. protected by a plastic-screen cage. After the inoculation period the test plants were treated with an insecticide and kept in the greenhouse for observation. During the two years of trials a total of 56 pear seedlings were inoculated. PCR-RFLP analyses, adopting the procedure described

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Corresponding author: L. Carraro  
Fax: +39.0432.558501  
E-mail: luigi.carraro@pldef.uniud.it

below, were carried out each time on four groups of 25 insects.

#### Retention of infectivity in overwintering *C. pyri*.

Early in November 1998 about 2000 overwintering *C. pyri* were collected in PD-affected orchards and transferred, in a greenhouse, to four healthy pear seedlings in winter dormancy. These test plants on which the psyllas were reared were replaced each month up to March 1999 making a total of 20 pear seedlings. Every fortnight, two groups of 25 psyllas were analyzed by PCR-RFLP for the PD phytoplasma (20 groups of 25 individuals each). In April 1999 the surviving *C. pyri* (approximately 300 insects) were used to inoculate four pear seedlings in the vegetative stage. After inoculation, all the test plants were treated with insecticide and maintained in the greenhouse. Six months later the plants were analyzed by PCR-RFLP for the phytoplasma.

#### Phytoplasma detection in test plants and in psyllids.

All test plants used for inoculation trials, a representative number of healthy and PD-affected pear tree controls, and groups of 25 *C. pyri* were analysed by PCR/RFLP. Both apple trees and *C. costalis* Förster infected by apple proliferation (AP) as well as apricot plants and *C. pruni* Scopoli infected by European stone fruit yellows (ESFY) were used for comparison (Carraro *et al.*, 1998b). DNA was isolated from approximately 1 g of leaf petiole and midrib tissues from each of the test plants and from individual groups of psyllas. A modification of the phytoplasma enrichment procedure developed by Kirkpatrick (Malisano *et al.*, 1996) was used for DNA extraction from plants and the method of Doyle and Doyle (1990) for extraction of insect DNA. The presence of AP-group phytoplasmas was determined by PCR using the ribosomal primers f01/r01 (Lorenz *et al.*, 1995). Five µl of the PCR products were analyzed by electrophoresis in 1.5% agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8) in presence of 0.5 µg ml<sup>-1</sup> ethidium bromide. Ten µl of the PCR products were digested separately with *SspI* and *BsaAI*, according to the manufacturer's instructions (BioLabs). Restriction fragments were resolved in a 5% polyacrylamide gel. After electrophoresis, the DNA was stained with silver nitrate.

## RESULTS

**Natural infection and infectivity of *C. pyri*.** The results obtained in the transmission trials conducted in

1997 and 1998 using naturally infected groups of psylla and the % infection of the insects collected are shown in Table 1. From March to October, in both years, we consistently obtained some positive transmissions of the PD agent using *C. pyri*. Only the results of the transmission trial carried out in September 1997 were negative, although the groups of psylla were found to be PD-infected by PCR. This result was not confirmed in September 1998. Of the 56 pear seedlings inoculated over the two years, 17 (30%) were positive by PCR-RFLP analysis. Five of the PD-infected test plants also showed typical disease *i.e.* leaf reddening and enlarged brown veins; the others remained without symptoms under greenhouse conditions. In both years, from March to October, at least one of the four groups of 25 psyllas captured in the field each month were PD-positive by PCR-RFLP, *i.e.* 35 out of 64 (55%). More specifically, these were 13 out of 32 positive reactions (41%) among the groups of insects collected from March to June and 22 out of 32 (69%) among those captured from July to October.

**Table 1.** Results of PCR analyses to detect the presence of pear decline phytoplasma in groups of *C. pyri*, captured monthly in 1997 and 1998 during the vegetative season in pear decline-infected orchards, and the transmission trials of pear decline to pear seedlings using groups of the same psyllas.

Month	1997		1998	
	<i>C. pyri</i> <sup>a</sup>	Test plants <sup>b</sup>	<i>C. pyri</i>	Test plants
March	+ ---	n.d.	+ ---	+ ---
April	+ + --	+ ---	+ ---	n.d.
May	+ + --	+ ---	+ + --	+ ---
June	+ + --	+ ---	+ + --	+ ---
July	+ + +-	+ + --	+ + +-	+ ---
August	+ + +-	+ ---	+ + +-	+ ---
September	+ + +-	----	+ + +-	+ + --
October	+ + --	+ + --	+ + --	+ + --

<sup>a</sup> Each month four groups of 25 *C. pyri* were captured and immediately analyzed by PCR.

<sup>b</sup> Four pear test plants were inoculated by *C. pyri* and then analyzed by PCR.

+: positive PCR reaction.

-: negative PCR reaction.

n.d.: not done.

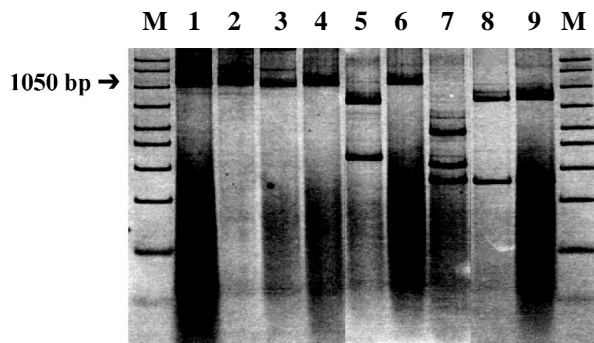
#### Retention of infectivity in overwintering *C. pyri*.

Results of the PCR-RFLP analyses, carried out from November 1998 to March 1999, on the 20 groups of 25 overwintering *C. pyri* adults (sampled among the 2000

reared in the greenhouse) were consistently PD-positive. At least one of the two groups of insects analyzed fortnightly, for a total of 17/20 (85%), were PD-infected. In contrast, the 20 test seedlings exposed to infected vectors during winter dormancy and replaced every month, were all negative by PCR analysis and showed no symptoms. Two of the four plants exposed in April – during the vegetative period – to the surviving overwintering psyllas became infected; about four months after inoculation they showed typical PD symptoms and reacted positively on PCR analysis.

#### Phytoplasma detection in test plants and in psyllids.

Using the primer pair f01/r01, PD-phytoplasma DNA was amplified from all the positive controls – that is PD-infected pear trees, AP-infected *C. costalis* and apple trees, ESFY-infected *C. pruni* and apricot trees – as well as from part of the test plants and groups of psyllas used in the trials. After digestion with *SspI* and *BsaAI* respectively, the PCR products obtained from all the specific samples always showed the same restriction profiles, by which the three different phytoplasmas could be distinguished (Fig. 1).



**Fig. 1.** Native products (lanes 1-3) of 16S rDNA amplified with primer pair f01/r01 and their RFLP profiles after digestion with *SspI* (lanes 4-6) and *BsaAI* (lanes 7-9). The template DNA was from: ESFY-infected *C. pruni* (lanes 1, 4 and 7); AP-infected *C. costalis* (lanes 2, 5 and 8); PD-infected *C. pyri* (lanes 3, 6 and 9). M: marker (BIO-RAD). The same profiles were obtained with the infected test plants and positive controls (not shown).

#### DISCUSSION

Our results confirm that *C. pyri* is a particularly active vector of the PD agent. Its presence was detected in 35 out of 64 (55%) groups of 25 psyllas collected in orchards. In terms of single insects, the minimum percentage of psyllids carrying phytoplasmas was 2.2% during the whole experimental period and, in particu-

lar, 1.60% from March to June and 2.75% from July to October. Likewise, 17 out of 56 (30%) of the inoculated test plants during the vegetative phase (using a total of about 3000 insects) became infected. These results explain the rapid spread of pear decline in Italy, where *C. pyri* is the predominant species in pear orchards. A pear tree can be visited every year by thousands of individuals of the vector and consequently the possibility of PD transmission is very high.

Table 1 shows that the transmission efficiency of the groups of psylla gathered during July-October was higher (69%) than that for March-June (41%). During the latter period, the first two generations of psylla were present, and the lower transmission efficiency could be attributed to a weaker capacity of the first psylla generations. Interestingly, the lowest percentage of insects carrying PD phytoplasma occurred when the overwintering generation had died; this is in accordance with the results obtained in England using *C. pyricola* (Davies *et al.*, 1998). It is likely that during spring the aerial part of PD-affected pear trees is not completely colonized or recolonized by the phytoplasmas (Seemüller, 1988), and consequently the plants are not efficient sources of inoculum.

Our trials also showed that *C. pyri* retained infectivity during winter but could not transmit PD to dormant test plants. However, the following spring the same overwintering adults could transmit the phytoplasma to test plants, without recharge feeding. The lack of transmission may be because pear trees are not infectable at this time and/or because the vector is unable to inoculate the host during dormancy. All plants colonized by infected psyllas in winter during the retention experiment were negative by PCR-RFLP. It is clear that such plants can be considered as 'hosts for the vector but completely resistant to the PD agent'; therefore they could be ideal for investigations on retention of infectivity in a potential vector.

We conclude that, in addition to the roots of PD-affected pear trees (Schaper and Seemüller, 1982) the PD agent can overwinter in *C. pyri*; this confirms the results obtained in England with *C. pyricola* (Davies *et al.*, 1998). *C. pyri* is thus of fundamental importance in the natural 'life cycle' of the phytoplasma. This is particularly true if pear trees are grafted with quince rootstocks in which winter survival of the PD agent is difficult or even impossible (Seemüller *et al.*, 1986).

To prevent the spread of PD, suitable strategies to control the overwintering generations of the vector (which are infected and infective) are thus of fundamental importance. A practical suggestion is to break the phytoplasma cycle by choosing quince as rootstock and also controlling the overwintering psylla.

## ACKNOWLEDGEMENTS

We thank Prof. C. Rapisarda and Prof. P. Zandigiacomo for identifying the insects; Prof. R. Osler and Prof. E. Refatti for helpful discussion and critical reading of the manuscript.

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Received 6 October 2000

Accepted 1 February 2001