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Transmission characteristics of the European stone fruit yellows phytoplasma and its vector *Cacopsylla pruni*

Luigi Carraro, Nazia Loi and Paolo Ermacora
Dipartimento di Biologia applicata alla Difesa delle Piante, Università di Udine, via Scienze 208, 33100 Udine, Italy (Fax: +390432558501; E-mail: carraro@pldef.uniud.it)

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Abstract

A study was carried out on the transmission parameters of the European stone fruit yellows phytoplasma by the vector *Cacopsylla pruni*. In the greenhouse, using groups of psyllids, the minimum acquisition period was 2–4 days, the minimum latent period 2–3 weeks and the minimum inoculation period 1–2 days. The vectors retained infectivity until their death. Under natural conditions retention of infectivity in *C. pruni* lasts through the winter and the following spring, when the overwintering insects reach the stone fruit trees, they are already infected and infective. The research shows that the vector *C. pruni* transmits the European stone fruit yellows phytoplasma in a persistent manner.

Abbreviations: AAP – acquisition access period; ESFY – European stone fruit yellows; IAP – inoculation access period; LP – latent period; PCR – polymerase chain reaction; RFLP – restriction fragment length polymorphism.

Introduction

The most common vectors of phytoplasmas are leafhoppers (*Hemiptera*: *Cicadellidae*) (Chiykowski, 1981; Sinha, 1984; Tsai, 1979), but the transmission characteristics of only a few of them are known, i.e. aster yellows (Chiykowski and Sinha, 1969), X disease (Purcell, 1979), clover phyllody (Cousin et al., 1968), beet leafhopper transmitted virescence agent (Golino et al., 1987) and maize bushy stunt (Legrand and Power, 1994). It should also be remembered that psyllids are among the vectors of phytoplasmas. Jensen et al. (1964) showed that pear psylla (*Psylla pyricola* Förster, now *Cacopsylla pyricola* Förster) transmitted 'a virus' capable of causing pear decline. More recently transmissions of pear decline-phytoplasma have been obtained in France (Lemoine, 1991) and in Italy (Carraro et al., 1998a) by *Cacopsylla pyri* L. Other *Cacopsylla* spp. have been identified as phytoplasma vectors. Carraro et al. (1998c) ascertained that *Cacopsylla pruni* Scopoli is the vector of ESFY-phytoplasma. Alma et al. (2000) and Frisinghelli et al. (2000) identified respectively *Cacopsylla melanoneura* (Förster, 1848) and *Cacopsylla costalis* (Flor, 1861) as vectors of the apple proliferation-phytoplasma. Pear decline, ESFY and apple proliferation agents are genetically related, all belonging to the phylogenetic group 'apple proliferation' (Seemüller et al., 1998); therefore there is a high correlation between this group of typically epidemic fruit tree diseases and related psyllids. Up to now there has been no precise information regarding the transmission parameters of phytoplasmas by psyllids. A knowledge of the chronological phases of the transmission process, i.e., acquisition, latency, inoculation and retention of infectivity in the vector (Purcell, 1982), is of fundamental importance for epidemiological studies and research aimed at preventing such diseases.

In this paper we report the results of a study aimed at determining the transmission characteristics of ESFY-phytoplasma by using the vector *C. pruni*. To understand the paper better it must be remembered

that the insect has one generation per year and overwinters as adult on shelter plants (conifers). Around the end of March, *C. pruni* moves from shelter plants to stone fruit trees for oviposition. The new generation feed on the primary hosts from May till the beginning of July, when the adults abandon the stone fruit trees (Conci et al., 1992).

Materials and methods

General conditions

All the transmission trials were performed in a conditioned greenhouse, at 22–24 °C under supplementary light. Sources of inoculum were young *Prunus salicina* Lindl. (cv Ozark Premier) micropropagated plants, previously infected by budding. Healthy Myrobalan (*Prunus cerasifera* Ehrh.) micropropagated plants, grown in a separate greenhouse, acted as test plants for psyllid transmissions. After exposure to inoculative *C. pruni* (25 insects per plant), the test plants were sprayed with insecticides and grown in an isolated greenhouse compartment, where they were observed for eight months for symptoms expression and then analyzed by PCR/RFLP.

Acquisition access period (AAP)

The colonies of *C. pruni* used in the transmission trials were derived from overwintering adults and were (10 insects per plant) put onto healthy Myrobalan plants in the greenhouse. One week after oviposition, the adults were killed. Young insects were collected immediately after the eggs had opened and caged for AAPs of 1, 2, 4 and 7 days on ESFY-infected sources of inoculum. After each AAP, ten groups of 25 insects were transferred to test plants, and maintained till their death. Longevity of the insects was checked 15, 30 and 45 days after the beginning of inoculation. Four groups of 25 insects were PCR/RFLP tested for ESFY-phytoplasma before acquisition and other four groups (one for each trial) at the end of each AAP.

Inoculation access period (IAP)

Psyllids were captured in May from Japanese plum trees in orchards having a high percentage of ESFY-infected trees. Groups of 25 adults were exposed to test plants for IAPs of 1, 2, 4 and 7 days and then removed. For each IAP, ten Myrobalan test plants were used. The survival of the psyllids was checked daily. At the end of each IAP, four groups of 25 insects were PCR/RFLP-tested for ESFY-phytoplasma.

Latent period (LP)

Groups of adults, captured in April from plum tree orchards, were caged inside the greenhouse on sources of inoculum for oviposition. The second instar insects of the new generation were then collected and used for serial transmission trials. Twenty groups of 25 *C. pruni* were moved weekly onto new test plants till their death, for a total of five transfers. Four groups of psyllids were PCR/RFLP-tested before starting the transmission trial in order to ascertain their infectivity.

Retention of infectivity

From previous experience, we knew that longevity of the new generation of *C. pruni* on stone fruit trees in the greenhouse and in the screenhouse can reach a maximum period of two months. In fact, caged insects die in the greenhouse when in nature the new adults are abandoning the primary hosts (July). As it was impossible to complete the life cycle of *C. pruni* under controlled conditions, retention of infectivity in the vectors was studied in insects that had overwintered. During daily surveys in ESFY-infected orchards, in March, we captured the first adults colonizing the stone fruit trees and immediately transferred them, in six groups of 25 insects, onto test plants in the greenhouse. Meantime, four other groups of the same psyllids were used for PCR/RFLP analyses. The six groups of *C. pruni* were transferred weekly onto new test plants. After 28 days (four transfers) the surviving psyllids, split into two groups of 20 individuals, were confined to two other test plants.

Testing for the presence of phytoplasmas in test plants and in psyllids

As the Myrobalan test plants do not show clear symptoms of ESFY (Carraro et al., 1998b), positivity of transmission using *C. pruni* was based on the results of PCR/RFLP analyses. We examined all the inoculated test plants, the sources of inoculum, a representative number of negative and positive controls, as well as groups of *C. pruni* collected during each transmission

trial. DNA was isolated from approximately 1 g of leaf petioles and midrib tissues of each test plant and from individual groups of psyllids. A modification of the phytoplasma enrichment procedure developed by Kirkpatrick (Malisano et al., 1996) was used for the DNA extraction from plants and Doyle and Doyle method (1990) for extraction of insect DNA. The presence of ESFY-phytoplasma was determined by PCR using the ribosomal primers f01/r01 (Lorenz et al., 1995). Five microlitre 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 the presence of 0.5 μg/ml ethidium bromide. Ten microlitre of the PCR products were digested with *Bsa*AI, 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

Acquisition access period

None of the test plants inoculated with *C. pruni* that had fed on the ESFY-sources of inoculum for 1 or 2 days became infected. In contrast, three out of 10 of the test plants inoculated by *C. pruni* that had fed for 4 or 7 days were infected (Table 1). The mortality of the insects was equally distributed among the groups of *C. pruni*: it was 10%, 20% and 55%, respectively, 15, 30 and 45 days after the beginning of the inoculations. The groups of insects tested by PCR/RFLP before acquisition or 1 day after, were ESFY-free; however, the insects tested after AAPs of 2, 4 or 7 days were ESFY-infected.

Inoculation access period

C. pruni transmitted ESFY-phytoplasma after an inoculation period of 2 days (one test plant infected out of the 10 inoculated) but not after 1 day of IAP. The percentage of infected test plants increased when inoculated for 4 days (30%) and 7 days (50%) (Table 1). No mortality of the test insects was observed at the end of each IAP. All the four groups of insects analyzed by PCR/RFLP at the conclusion of the experiment were ESFY-infected.

Latent period

Among the twenty groups of insects used in this experiment, six transmitted at least once ESFY-phytoplasma to the test plants. The data of these multiple transmissions are shown in Table 2. One group began to transmit during the second week of exposure, one during the third, two during the fourth and, finally, two groups during the last week. Three of the four groups of *C. pruni* PCR/RFLP tested at the beginning of the experiment were ESFY-infected.

Retention of infectivity

The results concerning retention of infectivity in *C. pruni* are shown in Table 3. Three out of the

Table 1. Reaction of Myrobalan test plants inoculated by groups of 25 *C. pruni* after different acquisition access periods (AAP)(1) and inoculation access periods (IAP)(2). Positivity of transmission to test plants was based on PCR analyses for ESFY-phytoplasma. The data of PCR analyses on test psyllids are also reported (3)

AAP (days)	Infected/ inoculated plants		Infected/ tested groups of insects N°	IAP (days)	Infected/ inoculated plants		Infected/ tested groups of insects N°
	N°	%			N°	%	
1	0/10	0	0/1	1	0/10	0	4/4
2	0/10	0	1/1	2	1/10	10	4/4
4	3/10	30	1/1	4	3/10	30	4/4
7	3/10	30	1/1	7	5/10	50	4/4

(1): For AAP, healthy first instar *C. pruni* were confined on sources of inoculum for 1, 2, 4 or 7 days and than exposed to test plants.
(2): For IAP, infective *C. pruni* adults were exposed to test plants for 1, 2, 4 or 7 days.
(3): Groups of test psyllids were PCR/RFLP analyzed for ESFY-phytoplasma at the end of each AAP and IAP.

Table 2. Latent period of ESFY-phytoplasma in *C. pruni*: six groups of insects born on infected sources of inoculum were moved weekly onto Myrobalan test plants till their death, for a total of five transfers. Four groups of 25 psyllids were PCR/RFLP analyzed before starting the transmission trial (1)

Group of *C. pruni*	Reaction of test plants inoculated during five transfers (2)				
	1°	2°	3°	4°	5°
1	−	+	+	−	+
2	−	−	+	−	+
3	−	−	−	+	+
4	−	−	−	+	+
5	−	−	−	−	+
6	−	−	−	−	+

(1): Three groups of test psyllids resulted ESFY-infected. (2): + = positive reaction to PCR analyses for ESFY-phytoplasma; − = negative reaction to PCR analyses for ESFY-phytoplasma.

Table 3. Retention of ESFY-phytoplasma in *C. pruni*: overwintering adults were captured in an infected area, divided into six groups of 25 individuals each, and immediately put onto test plants. Every week the test insects were moved to a new plant for a total of four transfers. Four groups of 25 psyllids were PCR/RFLP analyzed before starting the transmission trial (1)

Group of *C. pruni*	Reaction of test plants inoculated during four transfers			
	1°	2°	3°	4°
1	+	+	+	+
2	+	+	+	+
3	+	−	−	−
4	−	+	−	−
5	−	+	−	−
6	−	−	−	−

(1): All the four groups of test psyllids resulted ESFY-infected. (2): + = positive reaction to PCR analyses for ESFY-phytoplasma; − = negative reaction to PCR analyses for ESFY-phytoplasma.

six groups of *C. pruni* transmitted the ESFY-agent during the first week of inoculative feeding. It should be noted that two of these groups of psyllids transmitted the ESFY-phytoplasma to all the exposed plants; one group of insects never transmitted the agent; two groups transmitted it only during the second week of exposure to test plants. Also the two test plants inoculated using the insects that survived the four weeks of transmission trials were infected. The ESFY-agent was also detected in the four groups of insects tested at the beginning of the experiment.

Phytoplasma detection in plants and psyllids

Using the primer pair f01/r01, ESFY-phytoplasma DNA was amplified from all the positive controls and sources of inoculum as well as from part of the test plants and groups of psyllids used in the trials. After digestion with *Bsa*AI, the PCR products obtained from all the samples always showed the same ESFY-restriction profile.

Discussion

The work carried out was difficult mainly, because *C. pruni* is not easy to handle. It is impossible to get the insects to complete their life cycle in the greenhouse and woody test plants must be used. This is why we decided to use groups and not single insects, as previously with other vectors and phytoplasmas (Carraro et al., 1996). Besides, we chose *P. cerasifera* as the test plant because it is a good host both for the phytoplasma and its vector, though it is tolerant to ESFY. In spite of these difficulties the data obtained show the transmission characteristics of ESFY-phytoplasma by *C. pruni*.

The minimum period of acquisition ranges from 2 to 4 days; the efficiency of transmission does not increase by lengthening the AAP. Healthy *C. pruni*, after 2 days of AAP, were ESFY-infected but they did not succeed in transmitting the agent to the test plants. Non-transmission by vectors carrying phytoplasmas, already observed in *C. pyri* and pear decline (Carraro et al., 2001) could be due to several factors e.g. susceptibility of the test plant, long latency period in the vector or intermittent transmission.

We proved the existence of a measurable latent period and consequently the transmission is of a persistent type. As can be deduced from the first group of *C. pruni* in Table 2, the minimum latent period lasts between 2 and 3 weeks. In fact, the insects that could acquire the phytoplasma immediately after egg opening, were used for inoculation trials one week later and they transmitted ESFY-phytoplasma during the second weekly transfer onto test plant. Other groups of insects transmitted for the first time during the third, fourth or fifth transfer; this indicates that the latent period can be variable in length. The fact that all the six groups of psyllids transmitted ESFY during the last transfer indicates that retention of infectivity does not decrease up to that period and consequently it is certainly longer.

By using naturally infected *C. pruni* collected from ESFY-infected orchards, the length of the minimum inoculation period was between 1 and 2 days; increasing IAP also enhanced the efficiency of transmission.

To complete the study on retention of ESFY-phytoplasma in *C. pruni*, in addition to the information obtained from 'latency' experiments conducted in the greenhouse, we used the first overwintering insects recolonizing the stone fruit trees. Carraro et al. (1998c) proved that in July, when the adults of the new generation of *C. pruni* abandon the stone fruit trees infected by ESFY to move to the shelter plants, they are highly infective. In this research we demonstrated that in the spring, when the overwintering insects reach the primary hosts, they are already infected and infective. In fact, three out of six of the groups of *C. pruni* used in the specific experiment were able to transmit the phytoplasma to the first test plant inoculated in the serial transmission trials. The psyllids continued to transmit the agent in a persistent manner up to their death. We also proved that the three groups of *C. pruni* transmitted the ESFY-phytoplasma retained over the winter and not newly acquired in the spring from the stone fruit trees, on which they were captured. Daily inspections eliminated this possibility as they could not have been present on these primary hosts from more than one day. Moreover, at this time plum trees were in winter dormancy, without leaves. Finally, no latency in the transmission phase was noticed, indicating that acquisition had occurred a long time before.

In conclusion, the results obtained in this research show that the vector *C. pruni* transmits the ESFY-phytoplasma in a typical persistent manner, with a minimum acquisition period of 2–4 days, a minimum latency of 2–3 weeks and a minimum inoculation period of 1–2 days. The vectors retain the infectivity up to their death, both in the greenhouse and in nature, as already proved for *C. pyricola* and pear decline (Davies et al., 1998). In the spring, the retained agent can be transmitted immediately by the overwintering insects, without any previous recharge feeding, as demonstrated also for *C. pyri* and pear decline (Carraro et al., 2001). These results, besides clarifying the transmission characteristics of phytoplasmas by psyllids, are also useful for planing possible defence measures: control of the overwintering generations of *C. pruni* seems to be of fundamental importance. It is important to know that the active presence of *C. pruni* on the primary hosts does not persist after the end of June to beginning of July, under our conditions.

Further investigations are necessary to determine other important aspects of the epidemiology of ESFY such as the exact nature of the persistent transmission: circulative or propagative. More precise information about the groups of 25 psyllids resulted ESFY-positive by PCR and unable to transmit the disease is also expected from planned experiments using single or small groups of insects.

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