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TRANSMISSION OF EUROPEAN STONE FRUIT YELLOWS PHYTOPLASMA BY *CACOPSYLLA PRUNI*

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SUMMARY

Experimental transmission of European stone fruit yellows phytoplasma to Japanese plum cv. 'Ozark Premier' was achieved using *Cacopsylla pruni*, caught in the field or raised on infected plants under controlled conditions. Using different groups of infectious psyllas, 89% of the inoculated test plants were infected. Both nymphs and adults were able to transmit. Insects exposed to test plants immediately after capture or raised in cages transmitted the disease. After an incubation period of 4-5 months the first test plants showed typical symptoms. Sources of inoculum, test plants and vectors were examined by PCR for the presence of phytoplasmas. Following digestion of the amplification products with restriction enzymes, isolates from plants and insects showed the same restriction profiles. Interestingly, pear decline and European stone fruit yellows, members of the apple proliferation cluster, are both transmitted by *Cacopsylla* spp.

RIASSUNTO

TRASMISSIONE DEL FITOPLASMA ASSOCIATO AL GIALLUME EUROPEO DELLE DRUPACEE A MEZZO DI *CACOPSYLLA PRUNI*. Si riferisce su una serie di esperimenti condotti in ambiente controllato al fine di individuare il vettore del giallume europeo delle drupacee (ESFY). Attraverso una serie di esperienze condotte in passato ed utilizzando varie tecniche di trasmissione, non era stato possibile trasmettere la malattia con nessuna delle numerose specie di insetti, principalmente cicaline, prese in considerazione. Anche mediante tecniche di biologia molecolare (PCR) non era stata messa in evidenza la presenza del fitoplasma in insetti catturati in zone infette. In seguito, in una zona del nord-est Italia ove si pratica l'agricoltura biologica, si è potuta osservare, a inizio di primavera, la presenza di *Cacopsylla pruni* su impianti di susino giapponese con evidenti sintomi di ESFY. Nelle prove di trasmissione successivamente condotte si è potuto dimostrare che tale specie di psilla è vettore del fitoplasma associato a ESFY. Oltre agli adulti svernanti ed alle ninfe e adulti della nuova generazione raccolti direttamente in frutteti infetti, anche ninfe ed adulti allevati in cattività hanno trasmesso il patogeno in studio. Dopo inoculazioni multiple, 89% delle piante test (susino cv. 'Ozark Premier') sottoposte ad analisi mediante il test DAPI e PCR-RFLP è risultato infetto. Le stesse piante hanno anche manifestato dopo 4-5 mesi i tipici sintomi della malattia, fatta eccezione per la necrosi del floema. Sottoponendo a digestione enzimatica il DNA amplificato di fitoplasmi ottenuto dalle piante sorgente di inoculo, dalle piante test e dai vettori animali, sono stati ottenuti costantemente gli stessi profili di restrizione. Sulla base dei dati finora a disposizione, emerge che i fitoplasmi inclusi nel gruppo genetico degli scopazzi del melo (es. moria del pero e giallume europeo delle drupacee) sono trasmessi da psille.

Key words: psyllids, phytoplasmas, Japanese plum, PCR, detection.

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INTRODUCTION

Phytoplasmas are considered to be the causal agents of several stone fruit disorders, including plum leptonecrosis, decline of European plums, apricot chlorotic leaf roll, Molière's disease and other yellows and decline diseases of stone fruit trees (Sanchez-Capuchino and Forner, 1973; Morvan, 1977; Giunchedi *et al.,* 1982; Rumbos and Bosalidis, 1985; Dosba *et al.,* 1990; Poggi Pollini *et al.,* 1993; Lee *et al.,* 1995; Marcone *et al.,* 1996; Carraro *et al.,* 1998b). According to Lorenz *et al.,* (1994) such European stone fruit phytoplasma diseases are believed to have a common aetiology. The name 'European stone fruit yellows phytoplasma' (ESFY-P) has been proposed for the pathogen. On the basis of sequence and restriction fragment length polymorphism (RFLP) analysis of PCR-amplified 16S rDNA, the agent of ESFY is placed within the apple

proliferation (AP) group (Schneider *et al.,* 1993; Seemüller *et al.*, 1994; Lee *et al.,* 1995; Seemüller *et al.*, 1998). This cluster also includes phytoplasmas associated with several perennial fruit tree diseases present in Europe such as AP and pear decline (PD). These phytoplasmas are closely related but distinguishable from each other (Jarausch *et al.*, 1994; Malisano *et al.*, 1996). ESFY-P is also distinctly different from the agents of X-disease and peach yellow leaf roll, major phytoplasma diseases of stone fruit trees in North America which have not been shown to occur in Europe (Sinha and Chiykowsky, 1980; Kirkpatrick *et al.,* 1990; Kison *et al.,* 1997). The possible role of any secondary phytoplasmas in the decline of stone fruit trees is under discussion (Lee *et al.,* 1995).

European stone fruit yellows was first described in Italy by Goidanich (1933) under the name of 'plum leptonecrosis'. Although several species of stone fruits are susceptible to ESFY (Giunchedi *et al.,* 1982; Poggi Pollini *et al.*, 1995), the disease is particularly devastating for Japanese plum (*Prunus salicina* Lindl.) and apricot (*P. armeniaca* L.) (Desvignes and Cornaggia, 1982). The Japanese plum cultivar 'Ozark Premier' is highly susceptible to ESFY and was thus used as a test plant in our experiments. ESFY has caused epidemics in several areas of northeastern Italy where 50-70% of the plum trees became infected within the first three or four years of planting (Carraro *et al.,* 1992). In spite of the high infection pressure and the rapid spread of the disease, the natural vector(s) of ESFY remained unknown. In the early 1990s we began investigations to identify these vector(s). Initially we examined leafhoppers, but with no success (Carraro *et al.*, 1992). More recently, many different species of leafhopper from several infected orchards in the sampling area were analyzed by PCR using AP group-specific primers. All were found to be negative for this phytoplasma (G. Firrao, personal communication). Consequently, in 1996 we started to focus attention on psyllids. In the past, several *Cacopsylla* spp. were found associated with pear, apple and stone fruit trees in the orchards monitored. Recent molecular work has shown that ESFY-P is affiliated to the AP group and is closely related to PD (Seemüller *et al.*, 1998), which is known to be transmitted by at least two species of *Cacopsylla* (Jensen *et al.,* 1964; Lemoine, 1991; Carraro *et al.,* 1998a). *C. pruni* appeared to be the most common psyllid on stone fruit in the areas investigated.

This paper reports the transmission of the ESFY-P by *C. pruni* Scopoli, either caught in an infected area and immediately used for inoculative feeding on test plants, or raised in cages under controlled conditions for transmission experiments.

MATERIALS AND METHODS

All insects tested and inoculum sources were obtained from an area where different phytoplasmas are associated with perennial fruit trees and weeds (Osler *et al.,* 1994). Furthermore the three most prominent diseases of the AP group, *i.e.* AP, PD and ESFY have frequently been detected in the last 15 years in this region (Refatti *et al.,* 1986; Carraro *et al.,* 1992; Carraro *et al.,* 1998a, b). Except for the experiments performed with caught insects (reported in Table 1), all procedures involving *C. pruni* feeding were carried out in an environmentally controlled greenhouse with supplementary light to 16 h/day at 23-25°C. After inoculation, the test plants were held in separate sectors of the greenhouse.

Test insects and phytoplasma transmissions. Starting from March to April, groups of overwintering adults of *C. pruni* were periodically captured in the sampling area, with a moving net. They were then confined in groups on the test plants. Five groups of nymphs and young adults of *C. pruni*, hatched on infected stone fruit trees from eggs laid in spring by the overwintering females, were also used to directly inoculate test plants (Table 1). Eight groups of 30 insects from the transmission trials were analyzed by PCR using the primers f01/r01 (Lorenz *et al.*, 1995). The amplification products were then subjected to RFLP analysis after restriction with *Ssp*I and *Bsa*AI.

In a second experiment, hibernating insects were confined for a few days for ovideposition on two young infected 'Ozark Premier' plants (sources of inoculum), protected in ventilated plastic net cages. Nymphs of the third/fourth instar or young adults born in captivity on infected hosts were grouped and transferred for inoculative feeding to test plants for 13 to 20 days (Table 2).

For comparison, groups of *C. pruni* recovered at the end of the inoculation period reported in Table 2 were exposed to either three pear or three apple seedlings, as tests plants for PD and AP. In addition, three groups of infectious *C. pyri* L. were transferred to 'Ozark Premier' test plants. After inoculative feeding, all test plants were sprayed with insecticide and held in the greenhouse for symptom development and for phytoplasma detection tests.

Sources of inoculum. Experimental sources of inoculum were potted young 'Ozark Premier' plants inoculated by grafting in the greenhouse using ESFY-infected budwood derived from trees of symptomatic 'Ozark Premier' and asymptomatic but infected 'Myrobalan' (*P. cerasifera* Ehrh.).

Table 1. Transmission patterns of groups of *C. pruni* Scopoli caught in infected orchards and then transferred to healthy micropropagated 'Ozark Premier' test plants for transmission experiments (a). The insects of groups 1 to 16 were overwintering adults captured in March and April on plum, apricot and 'Myrobalan' trees, and those of groups 17 to 21 were nymphs and young adults of the spring generation born on infected stone fruit trees. After inoculative feeding the test plants were treated with insecticide and kept in the greenhouse for symptom development and DAPI and PCR analysis (b).

Insect group	Psyllas/test plant	Inoculative feeding (days) from - to	Test plant reaction		
			Symptoms	DAPI	PCR
1	100	13/3 - 3/4	–	–	–
2	100	16/3 - 3/4	+	+	+
3	100	16/3 - 3/4	+	+	+
4	100	16/3 - 3/4	+	+	+
5	100	18/3 - 3/4	+	+	+
6	100	18/3 - 3/4	+	+	+
7	100	18/3 - 3/4	+	+	+
8	50	20/3 - 3/4	+	+	+
9	50	20/3 - 3/4	+	+	+
10	50	20/3 - 3/4	+	+	+
11	50	20/3 - 3/4	+	+	+
12	100	27/3 - 8/4	+	+	+
13	100	27/3 - 8/4	+	+	+
14	100	27/3 - 8/4	–	–	–
15	20	8/4 - 28/4	+	+	+
16	20	8/4 - 28/4	–	–	–
17	200	25/5 - 10/7	+	+	+
18	200	25/5 - 10/7	+	+	+
19	200	25/5 - 10/7	+	+	+
20	200	25/5 - 10/7	+	+	+
21	200	25/5 - 10/7	–	–	–
22 - 31 (c)	0	0	–	–	–
32 - 33 (d)	0	0	+	+	+
34 - 37 (e)	0	0	0	0	+

(a) Insects used to inoculate test plants immediately after collection from naturally infected stone fruit trees.
(b) +: positive reaction; –: negative reaction; 0: not tested.
(c) Test plants not inoculated: negative controls.
(d) Positive controls, respectively: one plant of *Catharanthus roseus* infected with ESFY-P; one plant of 'Ozark Premier' graft-inoculated by ESFY-P.
(e) Groups of 30 overwintering adults of *C. pruni* analyzed by PCR-RFLP.

Test plants. All test plants were micropropagated 'Ozark Premier' derived from healthy mother plants. The potted test plants were grown in the greenhouse until used when about 30 cm high.

Fluorescence microscopy. All test plants and sources of inoculum were examined using DAPI (4',6-diamidino-2-phenyl indole) (Seemüller, 1976). Specimens (3-5 x 8-10 mm) of twigs were placed in 5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0 and kept at 4°C for one day. After rinsing in buffer, they were cut longitudinally with a freezing microtome (Leica Jung CM 1500). The 20 µm thick sections were treated with DAPI (1 µg ml^{-1} in 0.1 M phosphate buffer, pH 7.0) and examined with a fluorescence microscope (Leitz Orthoplan with Pleomopack 2.1). Healthy 'Ozark Premier' plants, grown in the greenhouse, were used as negative controls.

Table 2. Transmission pattern of groups of *C. pruni* Scopoli nymphs and young adults hatched from eggs laid by overwintering females and reared on ESFY-infected 'Ozark Premier' plants in controlled greenhouse conditions. After acquisition infectious groups of insects were transferred to healthy micropropagated 'Ozark Premier' test plants for transmission experiments. The plants where then kept in the greenhouse for symptom development, PCR and DAPI analyses (a).

Insect group (b)	Psyllas/test plant	Inoculative feeding (days) from - to	Test plant reaction		
			Symptoms	DAPI	PCR
1	50	22/4 - 12/5	+	+	+
2	50	22/4 - 12/5	+	+	+
3	50	22/4 - 12/5	+	+	+
4	50	22/4 - 12/5	+	+	+
5	50	22/4 - 12/5	+	+	+
6	30	22/4 - 12/5	+	+	+
7	30	28/4 - 12/5	+	+	+
8	30	4/5 - 17/5	+	+	+
9	100	4/5 - 17/5	+	+	+
10	30	4/5 - 17/5	+	+	+
11	50	11/5 - 27/5	+	+	+
12	100	11/5 - 27/5	+	+	+
13	150	11/5 - 27/5	+	+	+
14	150	11/5 - 27/5	+	+	+
15	100	11/5 - 27/5	+	+	+
16	100	11/5 - 27/5	+	+	+
17	100	11/5 - 27/5	+	+	+
18-27 (c)	0	0	–	–	–
28-29 (d)	0	0	+	+	+
30-33 (e)	0	0	0	0	+

(a) +: positive reaction; –: negative reaction; 0: not tested.
(b) Groups 1-8 were still nymphs when transferred to test plants; 9-17 groups were young adults.
(c) Test plants not inoculated: negative controls.
(d) Positive controls (see Table 1).
(e) Groups of 30 *C. pruni* analyzed by PCR-RFLP: nymphs (30-31); young adults (32-33).

DNA amplification and RFLP analyses. DNA was isolated from approximately 1 g of leaf petiole and midrib tissues from each of the test plants, the sources of inoculum and from individual groups of 30 *C. pruni.* A modification of the phytoplasma enrichment procedure developed by Kirkpatrick (Malisano *et al*., 1996) was used for extraction of DNA from healthy and infected plants and the method of Doyle and Doyle (1990) for extraction of insect DNA. The presence of 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 8.0) in presence of 0.5 µg ml^{-1} ethidium bromide. Ten µl of the PCR products were digested separately with *Ssp*I and *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. Amplified DNA obtained from AP-infected apple trees and PD-infected pear trees was used for comparison in RFLP analyses.

RESULTS

Natural host/sources of *C. pruni*. During March and April, overwintering adults of *C. pruni* were mostly

captured on 'Myrobalan' suckers and plum trees. Some were also captured from apricot trees, but none were found on cherry, peach, pear or apple trees. The first captures of hibernating adults from stone fruit trees were made at the beginning of March. In May large populations of nymphs were present, expecially on 'Myrobalan'.

Test insects and phytoplasma transmission. Overwintering adults captured in spring survived in captivity for up to 2 months. The average survival during the inoculation access periods was above 90%. Judging from the large colonies of offspring, the females were very fertile and the general rearing conditions were suitable.

In the first trial 2240 adults of *C. pruni* were caught in the field and used to inoculate 21 test plants. When analyzed by DAPI and PCR, 17 of these plants were phytoplasma-infected (Table 1). More particularly, 13 out of 16 and 4 out of 5 test plants became infected after being inoculated in spring by overwintering adults of *C. pruni* or nymphs and young adults born from eggs (new generation), respectively.

All 17 test plants of the second experiment (Table 2) became infected after exposure to infectious psyllas, either adults or nymphs hatched in cages. Transmission efficiency of adults and nymphs was similar.

The negative results of PCR analyses indicated that none of the six groups of the ESFY-P infected psyllas transmitted to pear or apple test plants. Similarly, negative results were obtained when PD-infected *C. pyri* were transferred to 'Ozark Premier' test plants.

Phytoplasma detection. DAPI tests and PCR analyses gave the same results. Using primer pair fO1/rO1, ESFY-P DNA was amplified from all samples from sources of inoculum as well as from 89% of the test plants used in inoculative feeding, and from the 8 groups of infectious psyllas tested. After the digestion with *Ssp*I and *Bsa*AI, the PCR products obtained from samples of psylla and Japanese plum always showed the same restriction profiles, distinguishable from AP and PD profiles (Fig. 1).

Disease development. Four to five months after inoculation, the first symptoms of ESFY appeared on the 'Ozark Premier' test plants. They consisted of faint but typical leaf reddening, small leaves, irregular leafing, leafroll and thickening of the leaf blade. The partial defoliation of several plants was attributable to insect feeding damage rather than to the disease.

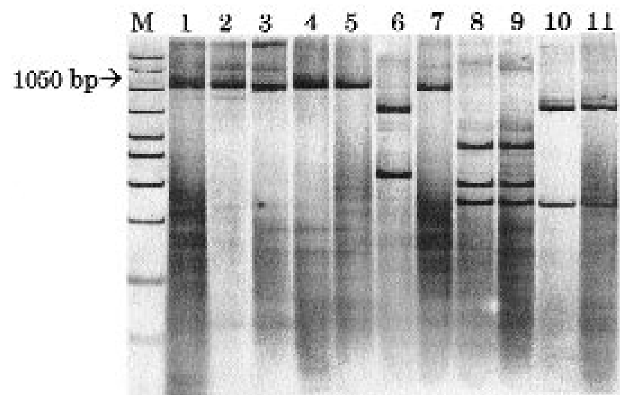

Fig. 1. *Ssp*I (lanes 4-7) and *Bsa*AI (lanes 8-11) restriction profiles of 16S rDNA amplified with primer pair f01/r01. The template DNA was from: ESFYp infected Japanese plum (lanes 4 and 8); ESFYp infected *C. pruni* (lanes 5 and 9); AP infected apple tree (lanes 6 and 10); PD infected pear tree (lanes 7 and 11). The lanes 1-3 are undigested DNA respectively from ESFYp infected Japanese plum (1), AP infected apple tree (2) and PD infected pear tree (3). M: marker (Bio Rad).

DISCUSSION

The results show that *C. pruni* is a vector of the European stone fruit yellows agent. Following digestion with *Ssp*I and *Bsa*AI, all samples (symptomatic inoculum sources, groups of psylla, and symptomatic test plants) consistently showed the same restriction profiles, which were identical to those of ESFY isolated on *Catharanthus roseus* by dodder (Loi *et al.,* 1995).

The data in Table 2 show the ability of *C. pruni* to transmit ESFY from infected to healthy plum trees under controlled conditions. In fact the vectors used for transmission were raised from eggs layed on the experimentally infected plum trees. The young adults and nymphs were then removed from these trees and used for separate inoculation experiments. It is largely accepted that vertical (=transovarial) transmission of phytoplasmas in their vector is an exception rather than the rule (Purcell, 1982).

The transmission efficiency of the groups of *C. pruni* caught in nature and transferred to infect the test plants (Table 1) was comparable to that of insects reared on ESFY-infected 'Ozark Premier' plants in controlled greenhouse conditions (Table 2). This indicates that *C. pruni* is not only an experimental vector of ESFY-P but also a natural vector. Furthermore, *C. pruni* did not transmit ESFY to apple or pear test plants specific for AP and PD, nor did *C. pyri* transmit PD to *Prunus*. These data suggest that the three diseases are caused by different phytoplasmas. In fact the agents of AP, PD, and ESFY are considered to be closely related but dis-

tinct, all belonging to the apple proliferation cluster (Seemüller *et al*., 1998).

The data obtained underline the biological similarities between ESFY and PD and AP: the natural spread of ESFY and PD are both mediated by *Cacopsylla* spp.; recently in our laboratory the AP agent was also detected in *Cacopsylla* spp. (Carraro *et al*., unpublished results). ESFY and PD are host species-specific and we have found no cases of mixed infection, either in the host plants or in the psylla vectors. In conclusion, in the rather typical area studied, where AP, PD and ESFY spread naturally and are present in the same orchard, the three diseases are clearly distinct.

Our results show that both nymphs and adults of *C. pruni* can transmit ESFY but we do not know yet whether in nature the overwintering adults found in spring can carry over infection from the previous year. The important question remains, whether infectivity may persist from spring to spring in psyllas, without intermediate reacquisition (recharging) (Sylvester and Osler, 1977). The potential influence of alternative host plants is also unknown.

On the basis of the positive transmissions by the groups of psylla born in spring on stone fruit trees (Table 1, groups 17-21) we can assume that in nature *C. pruni* can acquire infectivity from infected stone fruit trees before abandoning these principal hosts. Our results show that in spring all insects can be infectious, including those found on the principal host after overwintering and those of the spring generation on infected stone fruit trees.

We emphasize that the vector of ESFY-P was found in an area of organic farming where insect populations are not influenced by use of insecticides.

Further investigations are necessary to study the main transmission parameters of ESFY and the life cycle of the vector. It will be necessary to rear a phytoplasma-free colony of *C. pruni*, taking into account that this psylla is oligofagous on *Prunus*, overwinters on wild plants such as conifers and has a single generation per year (Conci *et al*., 1992).

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