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FRUIT TREE PHYTOPLASMA DISEASES DIFFUSED IN NATURE BY PSYLLIDS

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ABSTRACT

To compare the biological and genetical affinities between the phytoplasmas responsible for the most dangerous diseases of fruit trees transmitted in nature by psyllids, i.e. pear decline (PD), European stone fruit yellows (ESFY) and apple proliferation (AP), sources of inoculum, test plants used in the experimental transmissions and infectious psyllids were examined by PCR. Following digestion of the amplification products with restriction enzymes, each DNA extracted from plants and insects infected by the agent of the three diseases showed the same restriction profiles. Negative reactions were always obtained from the asymptomatic test plants and controls. DAPI confirmed the PCR results. No symptoms and positive PCR analyses were obtained in pear or apple seedlings exposed to ESFY-infected *Cacopsylla pruni* nor in plum test plants on which PD-infected *Cacopsylla pyri* were placed for transmission during feeding. The data obtained underline the biological similarities between the three diseases, although they appear to be distinct. No case of mixed infections either in the host plants or in the psylla vectors was detected. It has already been stated that the causal agents of PD, ESFY and AP are phytoplasmas that are closely related but distinguishable from each other and are placed within the AP group.

IZVLEČEK

FITOPLAZEMSKE BOLEZNI SADNEGA DREVJA, KI SE V NARAVI PRENAŠAJO Z BOLŠICAMI

Da bi primerjali biotične in genetske podobnosti med fitoplazmami, ki povzročajo najbolj nevarne bolezni sadnega drevja in se v naravi prenašajo z bolšicami, npr. propadanje hrušk (PD), evropska rumenica plodov koščičarjev (ESFY) in proliferacija jablan (AP) so s PCR raziskali vire inokuluma, testne rastline, uporabljene pri eksperimentalnem prenašanju in kužne bolšice. Po digestiji amplifikacijskih produktov z restrikcijskimi encimi, je vsaka DNA, ki so jo ekstrahirali iz rastlin in žuželk, okuženih s povzročitelji teh treh bolezni, imela isti restrikcijski profil. Negativne reakcije so vedno dobili s testnih rastlin brez simptomov in od kontrol. DAPI je potrdil rezultate PCR. Nikakršnih simptomov in pozitivnih analiz PCR niso dobili pri sejancih hrušk in jablan, ki so jih izpostavili napadu *Cacopsylla pruni*, okuženi z ESFY, niti na slivovih testnih rastlinah, na katere so dali s PD okužene *Cacopsylla pyri* za sesanje zaradi prenosa bolezni. Dobljeni podatki podlegajo biotičnim podobnostim med temi tremi boleznimi, čeprav je vsaka za sebe različna. Nobenega primera mešanih okužb niso ugotovili niti pri gostiteljskih rastlinah, niti pri bolšicah vektorjih. Poudarjeno je že bilo, da so povzročitelji PD, ESFY in AP fitoplazme, ki so si zelo sorodne, vendar med seboj razločljive in so razvrščene v skupino AP.

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1 INTRODUCTION

Pear decline (PD), European stone fruit yellows (ESFY) and apple proliferation (AP) are widely considered to be among the most dangerous diseases of fruit trees in Europe. In spite of the fact that their phytoplasma aetiology has been established for quite a long time, some problems concerning their epidemiology are still being discussed.

Pear decline or "moria" is a destructive disease that in the late 1950s and early 1960s 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") also in northern Italy (Refatti, 1948; Shalla *et al.*, 1961) and has been reported in other European and extra-European fruit areas. Lindner *et al.* (1962) proved that *Cacopsylla pyricola* Foerster, while feeding, introduced a systemic toxin that appeared to be the direct cause of pear decline. Shalla *et al.* (1963) reported that a transmissible agent, believed to be a virus, caused pathological effects at the graft union of pear trees identical to those associated with PD.

Jensen *et al.* (1964) provided evidence that pear psylla transmits a virus capable of causing PD disease. Hibino and Schneider (1970) - using the electron microscope examined leaf veins of PD-affected trees inoculated by psylla or by grafting - found mycoplasma-like bodies (MLBs) in functional mature sieve tubes but not in healthy material. Hibino *et al.* (1971) detected MLOs in the salivary glands and in the cephalic part of the foregut of pear psyllas collected from PD-affeted pear trees. Bodies were absent in psyllas from decline free orchards. The data acquired permitted the American scientists to conclude that MLBs may be the causal agent of PD.

More recently transmissions of the PD agent by *C. pyricola*, in England (Davies *et al.*, 1992) and by *Cacopsilla pyri* L., in France (Lemoine, 1991) and in Italy (Carraro *et al.*, 1998) have been obtained.

European stone fruit yellows is the name proposed by Lorenz *et al.* (1994) for a group of stone fruit disorders - including plum leptonecrosis, apricot chlorotic leaf roll, Moliere's disease, peach vein enlargement and other yellows and decline diseases - believed to have a common aetiology.

The disease was first described in Italy by Goidanich (1933) under the name "plum leptonecrosis". Although several species of stone fruits are susceptible to ESFY (Giunchedi *et al.*, 1982; Dosba *et al.*, 1990), the disease, that spreads naturally, is particularly devastating for Japanese plum (*Prunus salicina* Lindl.) and apricot (*Prunus armeniaca L.*) (Desvignes and Cornaggia, 1982).The agent of the disease was transmitted by grafting from and to various species of stone fruits (Giunchedi *et al., l.c.;* Carraro *et al.*, 1992).

Morvan *et al.* (1973) succeeded in transmitting through dodder (*Cuscuta subinclusa* Dur.) the agent of ESFY from affected apricots to *Catharanthus roseus* L. In thin sections of tissues of the herbaceous test plant they found MLOs, believed to be the causal agent of the disease. Positive transmissions of MLOs from myrobalan (*Prunus cerasifera* Ehrh.), "Ozark Premier" and "Shiro" plums and peach GF 305 seedlings, both with natural and artificial infections of ESFY, to *C. roseus* by *Cuscuta campestis* Yuncker were obtained also by Loi *et al.* (1995).

In spite of the high infection pressure and the rapid spread of the disease, the natural vector(s) of ESFY remained unknown for a long time. In the early 1990s we began searching for these vectors. Initially we concentrated on leafhoppers, but with no success (Carraro *et al.*, 1992). Consequently, in 1996 we started to focus our attention on psyllids. In the past, several *Cacopsylla* spp. were found associated with pear, apple and stone fruit trees in the monitored orchards. *Cacopsylla pruni* Scopoli appeared to be the most common psyllid on stone fruit in

the areas investigated. Carraro *et al.* (1998) transmitted the ESFY agent from plum to plum by *C. pruni* and proved that such an insect is not only an experimental vector but a natural one, too.

Apple proliferation or apple witches' broom is a widespread disease of apple trees. It was first reported in Italy on adult apple trees and transmitted by top grafting from apple to apple and pear healthy scions (Rui, 1950). It was also detected on yearling trees in the nursery and transmitted by budding (Refatti and Ciferri, 1954). Nowadays AP occurs in most important European pome fruit growing areas. Apple seedlings and clonal rootstocks are sensitive, too.

At the beginning AP was believed to be a virus disease. Later, by means of electron and fluorescence microscopy (Seem,ller, 1976), MLOs were detected in sieve tubes of affected apple trees (Gianotti *et al., 1968*; Amici *et al.*, 1972; Schaper and Seem,ller, 1982). The agent of AP was also transmitted by dodder (*C. subinclusa)* from apple to *C. roseus* and back to apple (Petzold and Marwitz, 1976). Carraro *et al.*, (1988) obtained the transmission of AP from apple to *C. roseus* by *C. campestris* but not vice versa.

Research has been carried out to identify the vector of the AP-agent, having found that the disease spreads in nature (Schmid, 1975). Krczal *et al.* (1988) reported that in experiments under natural conditions they succeeded in transmitting the AP-agent from apple to apple using naturally infected *Fieberiella florii* Stal. leafhoppers.

Our recent results seem to indicate that psyllids could be vectors of AP, too. M. E. Vindimian and her group (personal communication) in trials carried out in the Trento province, using psyllas collected from AP-infected apple trees, obtained transmission of the causal phytoplasma by *Cacopsylla costalis* Flor.

This paper reports the results of an investigation to compare the biological and genetical affinities between PD, ESFY and AP diseases and their agents.

2 MATERIALS AND METHODS

a) Testing for the presence of phytoplasmas in test plants and *C. pyri,* involving PD. Adults of *C. pyri,* montly captured from May to October 1995 in orchards with pear trees affected by PD, were used to inoculate 36 pear seedlings (*Pyrus communis* L.). Groups of 50 individuals of *C. pyri* were also collected from the same orchard in July, August and September and tested by PCR. In 1996 , after an adequate winter chilling period, the test plants and a representative number of controls were subjected to DAPI (4-6-diamino-2-phenylindole) fluorescence staining and to PCR analyses.

The procedures used for DAPI were those reported by Seem,ller (*l.c.*). Specimens (3-6 x 10-15 mm) of twigs and roots from symptomatic pear trees were fixed in glutaraldehyde (0.1 M phosphate buffer, pH 7.0) and stored at 4∞C for at least one day. After rinsing in buffer they were cut longitudinally using a freezing microtome (Leica 1205). The 20 µm thick sections were treated with DAPI (1µg/ml in 0.1 M phophate buffer, pH 7.0) and examined with a fluorescence microscope (Leitz Orthoplan with Pleomopack 2.1). The bark samples were taken from 3-10 cm long twigs. Root samples were avoided so as not to damage the plants and because the root growth was poor under pot growth conditions.

For DNA extraction, central veins of leaves were used and a modification of the phytoplasma enrichment procedure developed by Kirkpatrick was adopted (Malisano *et al.*, 1996). The DNA extraction from insects was according to Doyle's (1990) procedure. For the analysis, the two different pairs of ribosomal primers used were: fPD/rO1 (Lorenz *et al., 1995*) and AP3/AP5 (Firrao *et al.*, 1994).

b) Testing for the presence of phytoplasmas in sources of inoculum and inoculated plants and in *C. pruni,* involving ESFY. As reported by Carraro *et al.* (1998), the potted test plants used for the transmission trials of ESFY by *C. pruni* were micropropagated "Ozark Premier", derived from healthy mother trees, grown in the greenhouse. Except for the experiments performed with psyllas caught in infected orchards and then transferred to healthy test plants for direct transmission, all the

experimental sources of inoculum were potted young "Ozark Premier". The plants had been inoculated by grafting in the greenhouse using ESFY-infected budwood from trees of symtomatic "Ozark Premier" and asymptomatic but infected myrobalan. For comparison, groups of ESFY-infected *C. pruni* were exposed to either three pear or three apple seedlings, as test plants for PD and AP. In addition, three groups of PD-infected *C. pyri* were transferred to "Ozark Premier" test plants.
Twigs of test plants and sources of inoculum were examined using DAPI, adopting the procedures reported in a) for PD. Healthy "Ozark Premier" plants, grown in the greenhouse, were used as negative control.
For DNA amplification and RFLP analyses, DNA was extracted from approximately 1 g of leaf petiol and midrib tissues from each of the sources of inoculum and test plants and from individual groups of 30 *C. pruni*. Extraction of DNA from healthy and infected plants and from insects as well as PCR analyses were performed as described above. The presence of phytoplasmas was determined by PCR using the ribosomal primer pair f01/r01 (Lorenz *et al., l.c.*). Five µl of the PCR products were analyzed by electrophoresis in 1.5% agarose gel in TAE buffer (40 mM Tris-acetate, 1mM EDTA, pH 8.0) in the presence of 0.5 µg/ml ethidium bromide. Ten µl of the PCR products were digested separately with *Ssp* I and *Bsa* AI, according to the manifacturer'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 and PD-infected pear trees was used for comparison in RFLP analyses.
c) Preliminary testing for the presence of phytoplasmas in tissues of AP-infected trees and in psyllas captured in infected orchards. In 1998 adults of *Cacopsylla* spp. were captured in our sampling area in northern Italy, where organic agriculture is practiced and the insect populations are not influenced by insecticides and here different phytoplasmas associated with perennial fruit trees and weeds had been detected (Osler *et al.*, 1994). Twigs and roots cut from apple trees showing clear symptoms of AP were examined by DAPI, PCR, ELISA and immunoflorescence (IF) techniques.The insects were analyzed by PCR. The procedures adopted for DAPI and PCR are those reported above. PCR analyses were carried out using the primer pair f01/r01. ELISA and IF tests were conducted using the Mab 1F4/1E2 against AP, obtained by Loi *et al.* (1998).
Preliminary transmission trials with psyllids caught on AP-infected apple trees in the field were performed, too.

3 RESULTS

a) Pear decline. In 1996, 3 out of the 36 pear seedlings inoculated in 1995 by *C. pyri* showed PD symptoms. They reacted positively to DAPI as well as to PCR analyses using both pairs of primers; no positive reaction was obtained when the analyses were applied to asymptomatic test plants or controls. In 1997, two test plants showed again clear PD symptoms and reacted positively to DAPI test; the third one died in the greenhouse in 1997, after winter chilling. PCR analyses were positive when using the fPD/r01 primer pair and applied to all three groups of psyllid reared in July, August and September 1995 in the infected orchard.
b) European stone fruit yellows. DAPI tests and PCR analyses gave the same results. Using primer pair f01/r01, ESFY-phytoplasma DNA was amplified from all samples from sources of inoculum and from the symptomatic test plants that reacted positively to inoculative feeding (89%), as well as from the 8 groups of infectious psylla tested. After the digestion with *Ssp* I and *Bsa* AI, the PCR products obtained from samples of psylla and Japanese plum trees always showed the same restriction profiles, distinguishable from AP and PD profiles. No symptoms and positive results for the PCR analyses were obtained from pear or apple seedlings exposed to infectious *C. pruni* . Similarly negative results were obtained when PD-infected *C. pyri* were transferred to "Ozark Premier" test plants.
Apple proliferation. DAPI tests and PCR analyses detected phytoplasmas both in twigs and roots of AP-symptomatic apple trees. The diagnosis was confirmed by ELISA and IF tests

with the specific Mab 1FA/1E2. PCR analyses enabled the detection of AP-agent in adults of *Cacopsylla* spp. captured in the infected orchards. Up-to-now our first attempts to transmit the AP-agent to apple seedlings using the psyllids collected in the field have had no success.

4 DISCUSSION

The test plants showing clear PD-symptoms obtained in the 1995 transmission trials reacted posively to DAPI fluorescent staining for phytoplasmas as well as to PCR, when using the primer pair fPD/r01. The same was true for PCR when analyzing the PD-infected *C. pyri*. DAPI test on ESFY sources of inoculum plants and on test plants inoculated by *C. pruni* was positive for phytoplasmas. Using the primer pair f01/r01, PCR reactions of DNA extracted from infected tissues and infectious psyllas were positive, too. Also the restriction profiles of the PCR products obtained from samples of infectious *C. pruni* and from Japanese plum, both sources of inoculum and test plants, were always in agreement. No infection was obtained in pear and apple test plants exposed to ESFY-infected *C. pruni* or in "Ozark Premier" plants on which PD-infected *C. pyri* were placed to feed for transmission.

By DAPI test phytoplasmas were detected in twigs collected in the field from apple trees showing AP-symptoms. Also the PCR analyses using the f01/r01 primer pair for the DNA extracted from psyllids captured in infected orchards were positive. The Trento group (Grando *et al.*, 1998) reported that the DNA extracted from groups of 20-30 psyllas, fed for two months in summer on AP-infected apple trees, reacted posively to PCR analyses. On the basis of sequence and RFLP analysis of PCR-amplified 16S rDNA, they identified the phytoplasma as a member of the 16S rDNA X-A group. Our preliminary results, supported by those obtained by the researches of the Trento group, confirm that also the AP-agent has a psyllid as vector.

The data obtained in our research underline the biological similarities between PD, ESFY and AP: natural spread mediated by *Cacopsylla* spp.; host species-specificity; spontaneous recovery of a variable percentage of infected trees; presence of latent infections in woody hosts; uselessness of roguing the affected trees in the orchards to prevent natural spread of the disease; common induction of small flowers but not virescence or phyllody in *C. roseus* test plants; decline of woody hosts not necessarily leading to death of the plant. In spite of these similarities, we have not found cases of mixed infection, either in the host plants or in the psylla vectors, in the rather typical area studied, where PD, ESFY and AP spread naturally and are frequently present in the same orchard. Actually the three diseases appear to be clearly distinct.

It must be emphasizes that at present PD, ESFY and AP-agents are all phytoplasmas of woody plants mediated by psyllids. It is also pointed out that, on the basis of sequence and RFLP analysis of PCR-amplified 16Sr DNA, the agents of PD, ESFY and AP are placed within the AP group of phytoplasmas (Seemüller *et al.*, 1998). These three phytoplasmas are closely related, AP and PD more closely, but distinguishable from each other (Jarausch *et al.*, 1994; Malisano *et al.*, *l.c.*). They are also distinctly different from the agent 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).

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