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Spreading of ESFY Phytoplasmas in Stone Fruit in Catalonia (Spain)

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Abstract

A survey was carried out in nine stone fruit commercial orchards located in Barcelona province where plum and apricot trees of different cultivars showing European stone fruit yellows (ESFY) symptoms were present. A 4-year survey with visual inspection of symptoms in one apricot orchard showed a rather high ESFY disease spread, also in a Japanese plum plantation newly infected plants were detected every year in a similar rate (about 2%). All the inspected symptomatic trees were polymerase chain reaction (PCR) tested and ESFY phytoplasma identity was confirmed by restriction fragment length polymorphism analyses and sequencing of ribosomal DNA amplification products. In apricot plantation the detection of ESFY phytoplasma was also tested on 69 asymptomatic trees sampled in summer 2002. The nested PCR with 16SrX group-specific primers allowed detection of ESFY phytoplasmas in 50% of the trees that indeed showed symptoms by the next winter (2003). The molecular detection of ESFY phytoplasma in asymptomatic apricot trees indicates the risk of maintaining phytoplasma foci in the fields where eradication is based only on visual inspection.

Introduction

Plant pathogenic phytoplasmas induce severe diseases of stone fruits in Europe for which different names were given, such as apricot chlorotic leaf roll (ACLR) on apricot trees (*Prunus armeniaca* L.) (Sanchez-Capuchino et al., 1976; Morvan, 1977) and leptonecrosis (LN) on Japanese plum (*Prunus salicina* Lindl.) (Goidanich, 1933; Giunchedi et al., 1982). Southern hybridization, as well as other molecular analyses on conserved genes revealed that the phytoplasmas associated with these diseases are genetically very closely related, and thus the name European stone fruit yellows (ESFY) phytoplasmas was proposed for them (Lorenz et al., 1994). According to classification

proposed by Lee et al. (1998) ESFY phytoplasmas belong to subgroup B of the apple proliferation group (16SrX).

The psyllid species *Cacopsylla pruni* Scopoli has been reported to be a vector of the pathogen in northern Italy (Carraro et al., 1998b), southern France (Jarausch et al., 2001) and northeast Spain (Laviña et al., in press). ESFY phytoplasma is widely present in apricot, Japanese plum, European plum (*P. domestica* L.) and peach (*P. persica* L.) in southern Europe (Poggi Pollini et al., 1993, 1995; Jarausch et al., 1998); *Prunus* rootstocks are also affected by this disease (Dosba et al., 1991; Jarausch et al., 1998). ESFY phytoplasma has been also detected in wild *Prunus* species such as *Prunus spinosa* L. and *P. cerasifera* Ehrh. (Carraro et al., 2002) and also in other wild plants such as *Rosa canina* L., *Celtis australis* L. and *Fraxinus excelsior* L. (Jarausch et al., 2001) as well as in grapevine (*Vitis vinifera* L.) in Hungary (Varga et al., 2000).

In Spain ACLR disease was first described in Catalonia by Sala (1935) on apricot. Avinent and Llàcer (1994) confirmed the presence of this disease on apricot and Japanese plum from Valencia, Murcia and Sevilla.

This report refers to the increasing appearance of the die-back associated with ESFY phytoplasma in apricot and Japanese plum of commercial Catalanian (Northeast Spain) orchards. Common symptoms are off-seasons growth in winter, yellowing and leaf roll in summer and decline.

The main objective of this paper was to test for the presence of phytoplasma by using polymerase chain reaction (PCR), from affected cultivars in infected orchards. To verify the relation between the presence of symptoms and molecular identity of the associated phytoplasmas, restriction fragment length polymorphism (RFLP) analyses and sequencing of amplification products were performed. Moreover, asymptomatic trees were tested in order to evaluate the possibility to

perform an early detection of ESFY phytoplasma presence and to relate the presence of infected asymptomatic trees with the spreading of the disease. To verify the sensitivity of the phytoplasma detection method two different PCR procedures were also evaluated.

Materials and Methods

Selected cultivars tested

Nine stone fruit commercial orchards located in Barcelona province, with ESFY symptomatic as well as asymptomatic trees, were included in this study. Samples were collected from February till September 2001 in a selection of trees of six different plum and four different apricot cultivars showing symptoms (Table 1).

Disease spread

From two of these orchards (plantations 2 and 6) the disease spread was surveyed during the years 2001, 2002, 2003 and 2004. In plantation 2 only Japanese plum trees (122; 10 years old Japanese plums cv. Fortune) and in plantation 6 (Fig. 1) only apricot trees (323; 7 years old apricots, of the cvs. Kov, Moniqui, Traver and Modesto) were present. In these fields the

farmers removed all symptomatic trees every year and did not introduce new plants. All the trees were inspected for disease symptoms and those with symptoms were PCR tested (Table 2).

Detection in asymptomatic trees

In plantation 6 (Fig. 2) the detection of ESFY phytoplasma in asymptomatic trees was also tested. Samples were selected from the area of the field that showed ESFY affected trees in both winters 2001 and 2002. A total of 69 asymptomatic apricot trees were sampled in summer 2002 and tested by nested PCR with 16SrX group-specific primers. Trees that show growth in January or February of 2003 were also tested by nested PCR with 16SrX group-specific primers.

DNA extraction

Total DNA was isolated from fresh or frozen (-20°C) midrib tissues using the method described by Torres et al. (2003), resuspended in autoclaved Milli-Q water and stored at -20°C. DNA from control samples listed in Fig. 3 was extracted following the chloroform-phenol procedure described by Prince et al. (1993) and stored in TE buffer at -20°C.

Table 1

Localization of the commercial orchards and occurrence of symptomatic plants. Trees with symptoms of European stone fruit yellows sampled in 2001 were all positive for the presence of 16SrX-B group phytoplasma

Orchard	Symptomatic tree	Species	Cultivar	Town	Area (m ²)	% Symptomatic trees in 2001
1	377	<i>Prunus salicina</i>	Freedom	Piera	2.000	> 80%
	378	<i>P. salicina</i>	Songold			
	379	<i>P. salicina</i>	Fortune			
2	380	<i>P. salicina</i>	Fortune	Begues	2.000	< 10%
	719	<i>P. salicina</i>	Fortune			
3	381	<i>P. salicina</i>	Golden Japan	Sta. Coloma de Cervelló	5.000	10–20%
	382	<i>P. salicina</i>	Angeleno			
4	420	<i>Prunus armeniaca</i>	Canino	Sta. Coloma de Cervelló	300	< 10%
5	422	<i>P. armeniaca</i>	Torres	Cervelló	1.000	< 1%
6	1	<i>P. armeniaca</i>	Kov	Castellbisbal	10.000	< 10%
	142	<i>P. armeniaca</i>	Traver			
7	471	<i>P. salicina</i>	Golden Japan	Sta. Coloma de Cervelló	2.500	> 80%
8	711	<i>P. salicina</i>	Angeleno	Begues	1.000	> 80%
9	784	<i>P. salicina</i>	Sta. Rosa	St. Sadurní d'Anoia	2.000	< 1%

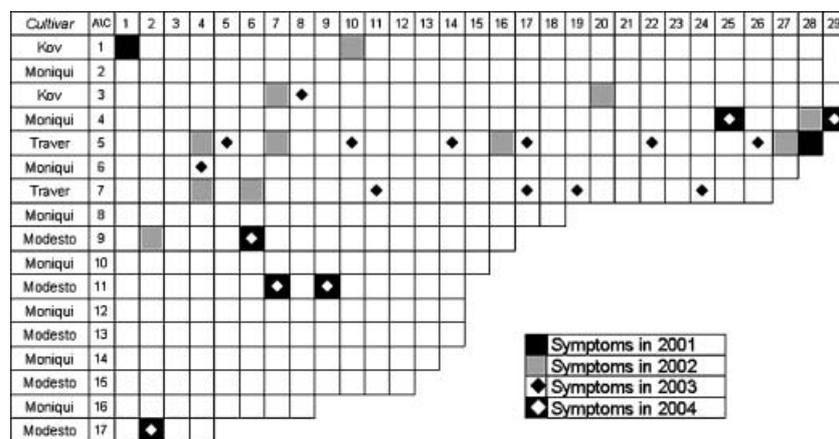


Fig. 1 Distribution of different cultivars of apricot trees, in plantation 6. The symptomatic trees detected during the 3 years survey are showed. The right border is flanked by a stream. (A\C = number of arrow and column)

Orchard	Year	Cultivar	Symptomatic trees
2 <i>Prunus salicina</i>	2002	Fortune	1251, 1253, 1254
	2003	Fortune	2132, 2133
	2004	Fortune	2970, 2971, 2972
6 <i>Prunus armeniaca</i>	2002	Kov	10, 63, 76
		Modesto	215
		Moniqui	113,
	2003	Traver	118, 121, 130, 141, 173, 175
		Kov	64
		Moniqui	146
	2004	Traver	119, 124, 128, 131, 136, 140, 180, 186, 188, 193
		Modesto	219, 251, 253, 321
		Moniqui	110, 114

Table 2
Trees with symptoms of European stone fruit yellows collected and - tested during 2002 and 2003, all shown positive for the presence of 16SrX-B group phytoplasma

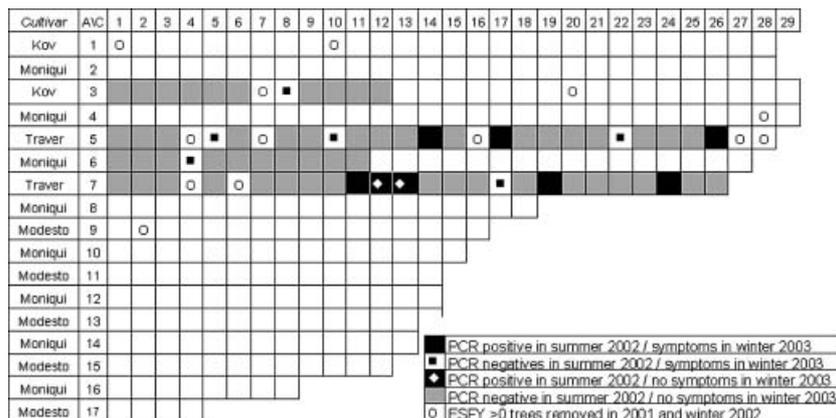


Fig. 2 Trees of plantation 6 analyzed in order to study the early detection of European stone fruit yellows phytoplasma. (A/C = number of arrow and column)

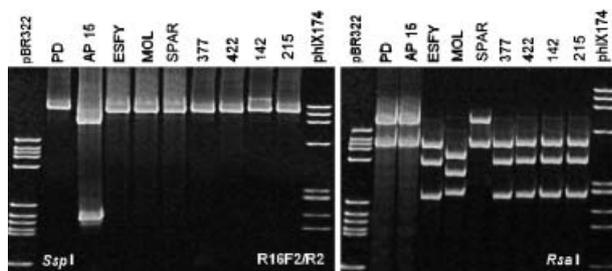


Fig. 3 Polyacrylamide gels (5%) showing the restriction fragment length polymorphism patterns of amplification products from four selected phytoplasmas detected in apricot and plum (numbers are as in Table 3) and phytoplasma control strains (16S rDNA fragments amplified with R16F2/R2 primers analyzed with restriction enzymes *SspI* and *RsaI*). Abbreviations are: pear decline, PD (group 16SrX-C); apple proliferation, AP15 (group 16SrX-A); European stone fruit yellows, ESFY (group 16SrX-B); Molière disease, MOL (group 16SrXII-A); spartium witches' broom, SPAR (group 16SrX-D). Markers ϕ X174 *HaeIII* digested; fragment sizes in base pairs (top to bottom): 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118 and 72; pBR322 *MspI* digested; fragment sizes in base pairs: 622, 527, 404, 307, 242, 238, 217, 201, 190, 180, 160, 147, 123, 110, 90, 76, 67, 34, 26, 15, 9

PCR amplifications

Amplifications were done using Ready-to-Go PCR Beads (Amersham-Pharmacia Biotech, Uppsala,

Sweden), as previously described (Torres et al., 2003; Method A) or a 25 μ l reaction mixture containing 0.8 μ l of nucleic acid (20 ng/ μ l), 200 μ M of each dNTP, 1.25 U Taq polymerase (Polymed, Florence, Italy) and 0.4 μ M of primers was used as described by Schaff et al. (1992) (Method B).

Because of the possibility of the presence of low phytoplasma DNA concentration in the DNA preparations, nested PCR assays were performed. After a first amplification (direct PCR) done with primers P1/P7 (Deng and Hiruki, 1991; Schneider et al., 1995), aliquots of the amplimers thus obtained were used as template for a second amplification (nested PCR). For this, general primers R16F2/R2 or apple proliferation group specific primers R16(X)F1/R1 (Lee et al., 1995) were used (Fig. 4). After an initial denaturation at 94°C for 5 min, the cycling parameters were respectively 40 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 1 min for primers R16F2/R2, or 35 cycles of 1 min at 94°C, 2 min at 50°C and 3 min at 72°C for primers R16(X)F1/R1. A final extension at 72°C for 10 min was made in both cases.

Controls, without DNA, were run for each experiment to check for DNA carryover. PCR products were subjected to electrophoresis in a 1% agarose gel and visualized by staining with ethidium bromide and UV illumination.

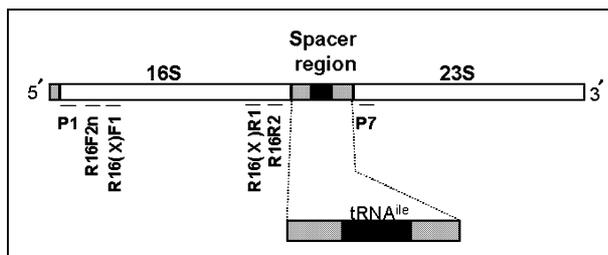


Fig. 4 Relative position of the primer pairs used in this study: P1/P7 (1800 bp), R16F2/R2 (1200 bp) and R16(X)F1/R1 (1100 bp)

Restriction fragment length polymorphism

RFLP analyses on 200 ng of DNA from R16F2/R2 and R16(X)F1/R1 amplimers obtained from samples collected in 2001 (Table 1) and the control samples indicated in Fig. 3 were carried out with *RsaI* and *SspI* (Fermentas, Vilnius, Lithuania) for at least 16 h following the instructions of the manufacturer. The restriction patterns were compared after electrophoresis on a 5% polyacrylamide gel followed by ethidium bromide staining, and photographed with Polaroid system under UV at 312 nm using a transilluminator.

Sequencing of PCR products and analyses

Seven amplification products (Table 3) from different plum and apricot cultivars were purified using the EZNA clean kit (Omega Biotech, Doraville, USA) and the nucleic acid concentration was quantified after electrophoresis on a 2% agarose gel or with GenQuant II RNA/DNA Calculator. Two samples gave enough direct P1/P7 amplification products to be sequenced. Two samples produced light bands from P1/P7 amplifications (<20 ng/ μ l), in this case the purified products were cloned using pGEM®-TEasy vector (Promega Corporation, Madison, WI, USA). For the other three samples the amplification product sequenced was the result of the nested PCR with R16F2/R2.

Both strands were sequenced separately, using primers specific to phytoplasma (P1, P7, R16F2 and/or R16R2) or using universal primers specific to vector sequences (T7 and SP6). The sequencing was performed in an ABI Prism 377 genetic Analyzer and the

ABI Prism™ BigDye™ terminator Cycle Sequencing Ready Reaction kit with AmpliTaq®DNAPolymerase (Perkin–Elmer Applied Biosystem, Foster City, USA).

BIOEDIT™ software was used to identify the consensus sequence from the two strands of each amplification product. Nucleotide BLAST searches with the option Standard nucleotide-nucleotide BLAST of BLASTN 2.2.6 were used to compare the sequences obtained in this study against other nucleotides of the National Center for Biotechnology Information (NCBI) nucleotide databases (Altschul et al., 1997). Pair wise comparisons of the consensus sequences were obtained by optimal global alignment with BIOEDIT 5.0.6 software.

Results

Phytoplasma detection and characterization

All 51 symptomatic trees, 14 sampled in 2001 from the nine orchards under investigation (Table 1) and 37 sampled in 2002, 2003 and 2004 in the plantations 2 and 6 (Table 2) yielded, following PCR amplification, DNA bands of the expected size, when analyzed by general-nested PCR with P1/P7 and R16F2/R2 (1200 bp) and specific-nested PCR with P1/P7 and R16(X)F1/R1 (1100 bp) primers.

Samples listed in Table 1, were amplified both with PCR-beads (method A) or a regular PCR mix (method B). With both methods amplimers were visualized using the different primers pair combination of the general-nested PCR and the specific-nested PCR. The RFLP analysis of amplicons from phytoplasmas detected in these 14 trees confirms that only phytoplasmas belonging to 16SrX-B group were present in these samples. Position of restriction sites for *RsaI* is in agreement with virtual digestion of the sequences of ESFY phytoplasmas providing fragments of about 600, 400 and 300 bp as expected, *SspI* did not cut the amplicons since restriction sites are out of the analyzed PCR product (Fig. 3). Comparison of sequences obtained from samples 377 and 379 (plantation 1), 422 (plantation 5) and 142 (plantation 6), collected in 2001, and those obtained in 2002 in orchard 6 from samples 63, 173 and 215 (Table 3), shows the absence of significant polymorphisms among detected phytoplasmas in the DNA region sequenced. The Blast search of sequences AJ575105 and AJ575106

Table 3

Samples sequenced. Position and nature of the divergent nucleotides of the seven amplification products. Positions are numbered in relation to the sequence retrieved from the GenBank AJ542545 (ND, no data)

GenBank accession no.	Tree	Amplification product	Number nucleotides	Initial position	Position of divergent nucleotides				
					24	316	826	893	907
AJ542545			1784	1	G	C	T	T	A
AJ575105	215	Cloned P1/P7	1736	1	T	C	T	T	A
AJ575106	173	Cloned P1/P7	1736	1	T	G	T	T	A
AJ575107	63	P1/P7	1688	50	ND	C	–	–	A
AJ575108	142	P1/P7	1556	109	ND	C	T	T	A
AJ575109	422	R16F2/R2	1180	167	ND	C	T	T	A
AJ575110	379	R16F2/R2	1183	162	ND	C	T	T	C
AJ575111	377	R16F2/R2	1183	165	ND	C	T	T	C

obtained in this study from cloned P1–P7 products shows the most significant alignment with the sequence AJ542545 (*Candidatus* Phytoplasma prunorum) corresponding to the 16S rRNA gene, tRNA-Ile gene and 23S rRNA gene (partial) of strain ESFY-G2 submitted by Schneider and Seemüller (Institut fuer Pflanzenschutz im Obstbau, Dossenheim). The alignment of these two sequences with that of the reference isolate ESFY-G2, for 1736 nucleotides, shows only one difference (nt 24) for sequence AJ575105 (tree 215) and two differences (nt 24 and 316) for sequence AJ575106 (tree 173); one or two differences were also observed for some of the other sequences (Table 3).

Disease spread

The 4-years survey in plantation 2 and 6 showed a spread of ESFY phytoplasma. In plantation 2, the average of new affected trees by year was 2.1%: among the 122 Japanese plum cv. Fortune there were two trees with symptoms in 2001, both located at the border line of the field, as in plantation 6 each symptomatic tree was quickly cut down. Three new cases were detected in 2002, two new cases in 2003 and in last year's survey three more plum showed ESFY symptoms. In plantation 6 (Fig. 1) the percentage of new symptomatic trees by year was variable: two trees on 323 in 2001 (0.6%); 11 trees on 321 in 2002 (3.4%); 12 trees on 310 in 2003 (3.9%) and six trees on 298 in 2004 (2%).

All the symptomatic trees were confirmed to be infected by ESFY phytoplasma by nested PCR with group-specific R16(X)F1/R1 primers.

Detection in asymptomatic trees

During summer 2002, 69 asymptomatic apricot trees from field 6 were analyzed in order to verify ESFY phytoplasma detection before the symptom manifestation (Fig. 2). Eight samples were shown positives by nested PCR with 16SrX group specific primers. In winter 2003, six of these positive trees showed symptoms; the other two remained asymptomatic. However, in the survey conducted during winter 2003 six more trees showed symptoms of ESFY phytoplasma although the phytoplasma was not detected by PCR during summer 2002. The results show that 50% of the samples with early growth in January and February 2003 were already detected PCR positives during the previous summer, without showing any symptom. The two samples that yielded positive PCR results in summer 2002 and remained asymptomatic until 2004 were tested PCR positives when analyzed in January 2004.

Discussion

Since 1999 high incidence and widespread presence of phytoplasma associated symptoms in apricot and Japanese plum was detected in the orchards under investigation. All cultivars of Japanese plum and apricot from the fields selected in this study showed a close relationship between presence of symptoms and ESFY phytoplasma detection. The two nested PCR methods

A and B tested with P1/P7 plus R16F2/R2 and with P1/P7 plus R16(X)F1/R1 allow similarly reliable detection of phytoplasmas in symptomatic *Prunus* trees.

The similarity between ESFY phytoplasma strains in apricot from Spain and the strain ESFY-G2 from apricot in Germany is almost complete and it seems reasonable to conclude that there is not relevant taxonomic difference.

In the two surveyed orchards, the newly affected trees showed a quite random distribution. The disease spread could be explained by the transmission of ESFY phytoplasma by *Cacopsylla pruni* but the possible existence of latent infections in the trees from the nursery may also had an influence on distribution of the disease. In Catalonia, there is wide distribution of phytoplasmas of the 16SrX group: 16SrX-C in pear (Avinent et al., 1997; Garcia-Chapa et al., 2003), 16SrX-B in plum and apricot and 16SrX-D in *Spartium* (Torres et al., 2003). The prevalence of potential insect vectors (psyllids for groups 16SrX-B and C or unknown for 16SrX-D) is probably responsible for it together with the sanitary status of propagation material.

Some studies have been done in order to investigate the susceptibility of stone fruit trees on various rootstocks to phytoplasmas (Kison and Seemüller, 2001). In the apricot field, the rootstock used was rustic apricot for all the trees. However the disease expansion was different among the four cultivars: cv. Kov and Traver showed more phytoplasma-infected plants than cv. Moniqui and Modesto. Moniqui and Modesto cultivars have been grown in this commercial area for a long time whereas Kov and Traver are newly introduced. These differences may be explained by a difference of sensitivity to ESFY disease. More work is needed in order to confirm this and discard the hypothesis of the existence of a latent infection from nursery.

As a result of the ESFY expansion, growers are replacing Japanese plums by some cultivars of European plums less sensitive to ESFY phytoplasma (Carraro et al., 1998a). More study is in progress to determine the role of *C. pruni* and other possible vectors in the spread of the disease in this area. This knowledge together with specific research about possible presence of cultivars less susceptible to the phytoplasmas can help to plan defense measures to reduce and try to control ESFY disease in the area.

The molecular detection of ESFY phytoplasma in asymptomatic apricot trees shows the risk of the existence of focuses of phytoplasma disease in the fields that are not possible to eradicate on basis of visual inspection. These focuses together with the presence of affected wild plants in the surroundings of the commercial fields are an important factor in the spreading of this disease. The use of specific-nested PCR that allows the detection of the phytoplasma in some asymptomatic trees can be proposed as preventive practice to produce healthy mother plants for propagation material.

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