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PCR Assay for specific detection of European stone fruit yellows phytoplasmas and its use for epidemiological studies in France

W. Jarausch[1,3], M. Lansac[1], C. Saillard[2], J.M. Broquaire[3] and F. Dosba[1,4]
[1] *Unité de Recherches sur les Espèces Fruitières et la Vigne (Fax: 556 843083);* [2] *Laboratoire de Biologie Cellulaire et Moléculaire, Institut National de la Recherche Agronomique, Bordeaux, BP 81, 33883 Villenave d'Ornon, France;* [3] *SICA Centrex , 66440 Torreilles, France;* [4] *Present address: ENSA.M - INRA, 2 place Viala, 34060 Montpellier, France*

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Abstract

DNA amplification by polymerase chain reaction was used to specifically detect phytoplasmas associated with severe decline diseases of European stone fruits. PCR primers were designed according to the partial sequence of a nonribosomal genomic fragment of European stone fruit yellows phytoplasmas obtained by direct sequencing of a specific PCR product. A PCR assay was developed which resulted in specific amplification of a 237 bp-DNA fragment from total DNA extracts derived from over 300 stone fruit samples. No PCR product was obtained with DNA from healthy controls or plants diseased with various other phytoplasmas, e.g. the closely related apple proliferation and pear decline phytoplasmas. Phytoplasma infection was checked in all samples by PCR amplification with universal ribosomal primers. Detection rate with specific and universal primers was correlated by 97%. European stone fruit yellows phytoplasmas were detected in samples of 114 out of 139 examined orchards which represent the major stone fruit growing regions of France. Typical symptoms like chlorotic leaf roll in summer and off-season growth in winter were correlated by 95% to the presence of phytoplasmas. However, phytoplasmas were also detected in 51% of samples derived from trees showing non-specific symptoms. A comparison study including 201 samples showed that 81% of the PCR-positive samples were also tested positive using fluorescence microscopy with DAPI staining.

Introduction

All stone fruit species in the southern half of Europe, are affected by severe decline diseases associated with phytoplasmas (formerly called mycoplasma-like organisms). Typical symptoms are yellowing and leaf roll in summer and off-season growth in winter. Dieback and a more or less rapid decline follow. The economically most important diseases are apricot chlorotic leaf roll (ACLR) on apricots (Morvan, 1977) and plum leptonecrosis (PLN) on Japanese plums (Giunchedi et al., 1982). Decline diseases of European plum are also wide-spread in southern Europe (Dosba et al., 1991; Poggi Pollini et al., 1995). A serious disorder has been reported for cherry in the southwest of France (Bernhard et al., 1977). Phytoplasmas have also been found on peach and almond showing yellowing and decline (Poggi Pollini et al., 1993; Lederer and Seemüller, 1992). *Prunus* rootstocks are also severely affected by the same disorders (Desvignes and Cornaggia, 1982; Dosba et al., 1991; Dosba, 1992).

Cross inoculation experiments with phytoplasma isolates of various *Prunus* species demonstrated that they are graft-transmissible within the *Prunus* genus and induce similar symptoms on different *Prunus* species (Dosba et al., 1991; Dosba, 1992; F. Dosba, unpublished results). Southern hybridization studies of *Prunus*-affecting phytoplasma isolates confirmed the biological observations and showed that phytoplasmas associated with diseases of *Prunus* species in Europe are genetically similar (Ahrens et al., 1993; Lorenz et al., 1994). The name European stone fruit yel-

lows (ESFY) phytoplasma has been proposed for these organisms (Lorenz et al., 1994). RFLP analysis results obtained with a PCR amplified nonribosomal fragment of different *Prunus*-affecting phytoplasma isolates supported these findings (Jarausch et al., 1994b; Jarausch et al., 1994c). Our studies further confirmed that ESFY phytoplasmas are closely related to apple proliferation (AP) and pear decline (PD) phytoplasmas. According to 16S rDNA restriction site and sequence analysis of various phytoplasma isolates these three phytoplasmas form together the AP strain cluster. They are only distantly related to most North American stone fruit-affecting phytoplasmas which are regrouped in the X-disease cluster (Schneider et al., 1993; Seemüller et al., 1994). However, a recent report indicates that the California peach yellow leaf roll phytoplasma is more closely related to phytoplasmas of the AP strain cluster than to those of the X-disease strain cluster (Kirkpatrick et al., 1994).

Routine diagnosis of stone fruit diseases associated with phytoplasmas still refers to symptomatology and biological indexing (Boyé and Desvignes, 1996). DAPI (4′,6′-diamidino-2-phenylindole, 2HCl) staining in conjunction with epifluorescence microscopy (Seemüller, 1976) is used to confirm the results. However, these methods are non-specific and do not allow the identification of the pathogen. As fruit tree-affecting phytoplasmas rank among quarantine organisms in Europe and North America, sensitive identification techniques for these pathogens are required. However, the phytoplasmas occur in relatively low titers in the tree and are often inconsistently distributed within the phloem of the different organs. Furthermore, their presence in the different parts of the tree is subject to environmental and seasonal fluctuations. Under these circumstances, only PCR technology meets the needs of sensitive and reliable routine diagnosis of phytoplasmas affecting stone fruits. PCR detection is more sensitive than microscopic, serological, and hybridization methods (Schaff et al., 1992; Chen et al., 1993) and proved to be very useful for phytoplasma-detection in fruit trees (Ahrens and Seemüller, 1992; Jarausch et al., 1994b; Jarausch et al., 1995; Lorenz et al., 1995).

16S rDNA sequence data has been widely used to design universal or cluster-specific PCR primers which allow detection of all known phytoplasmas or groups of phytoplasmas, respectively (Ahrens and Seemüller, 1992; Lee et al., 1993; Namba et al., 1993; Davis and Lee, 1993; Lorenz et al., 1995). However, RFLP analysis is required to identify the pathogen. Attempts to select pathogen-specific ribosomal primers for fruit tree-affecting phytoplasmas were not very satisfactory because of cross reaction problems (Lorenz et al., 1995). Therefore, sequence data derived from cloned nonconserved chromosomal fragments of apple proliferation phytoplasma were used to design AP-specific PCR primers (Jarausch et al., 1994b; Lorenz et al., 1995). In a next step cycle sequencing of PCR products has been successfully used to obtain PD-specific primers (Lorenz et al., 1995). Some of these pathogen-specific primers, however, were not able to detect all strains of the pathogen (Lorenz et al., 1995).

Up to now, no pathogen-specific primers for ESFY phytoplasmas have been obtained which would allow the one-step PCR diagnostic of stone fruit diseases. The objective of this study was therefore to develop a PCR-based assay for the direct identification of ESFY phytoplasmas suitable for routine diagnosis. The sensitivity of the test was compared to DNA amplification with universal primers and to phytoplasma detection by DAPI test or symptomatology. The developed PCR assay was used to evaluate the epidemiological situation in France based on the identification of the pathogen. We report results of a great number of samples collected in the field during different vegetation periods.

Materials and methods

Sources of naturally infected trees

Samples from naturally infected *Prunus* species have been collected or obtained from all major stone fruit growing regions in France. In total, 139 orchards were tested (see Figure 1). 400 samples were obtained from 8 different *Prunus* species and from 3 different interspecific hybrids (see Table 1). Field-collected samples of AP-diseased *Malus pumila* and PD-diseased *Pyrus communis* were used as controls.

Sources of experimentally maintained phytoplasma strains

Different strains of ESFY phytoplasmas have been maintained *in vitro* on micropropagated *Prunus marianna* GF 8-1 (Jarausch et al., 1994a; Lansac et al., 1995). These strains are ECA-G32 (origin *Prunus armeniaca*), DBT (origin *P. marianna* GF 8-1), RCL (origin *Prunus domestica* Reine-Claude), FOURM (origin *P. domestica*).

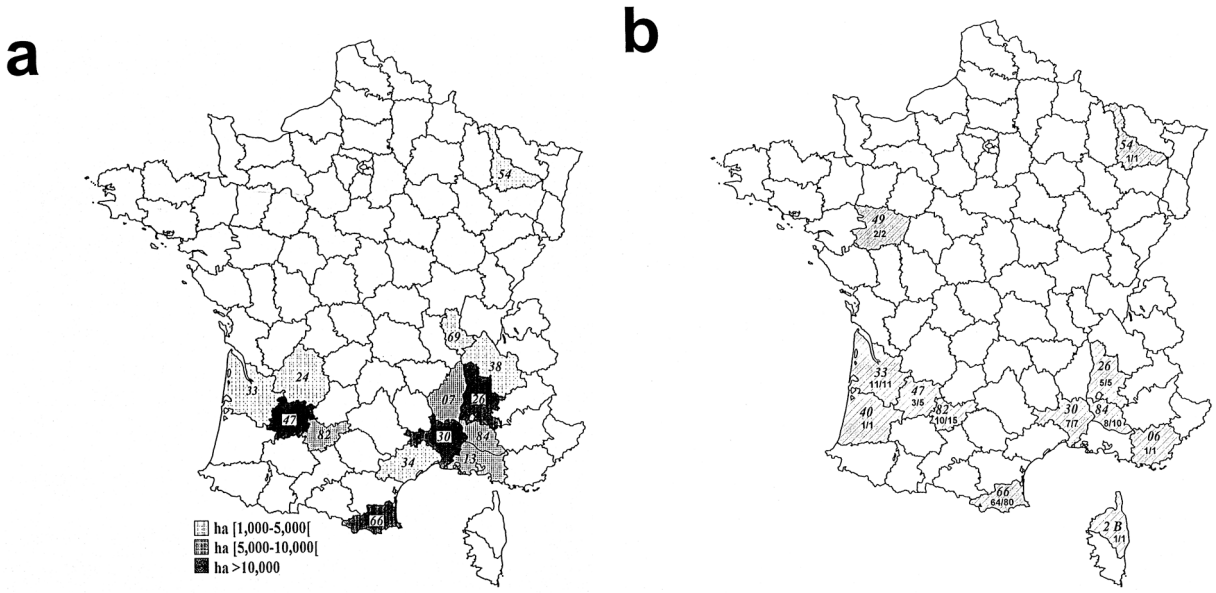

Figure 1. (a) Map of France showing the departments with a surface area higher than 1000 ha planted with *Prunus* fruit trees (source: Agreste, Données chiffrées - Agriculture N° 70, 1995, French minstry of agriculture, fishery and alimentation); (b) Map of France showing the departments prospected for phytoplasma infections of *Prunus* fruit trees, the number of diseased orchards versus the number of tested orchards is indicated. The departments tested were: Haute Corse (2B), Alpes-Maritimes (06), Drôme (26), Gard (30), Pyrénées-Orientales (66), Vaucluse (84), Gironde (33), Landes (40), Lot-et-Garonne (47), Tarn-et-Garonne (82), Maine-et-Loire (49) and Meurthe-et-Moselle (54).

Different strains of AP-phytoplasmas have been maintained on *in vitro* propagated *Malus pumila* (Jarausch et al., 1996). An English strain of PD-phytoplasmas has been obtained on micropropagated *Pyrus communis* from M.F. Clark (Davies and Clark, 1994).

Phytoplasma strains representing the primary phytoplasma groups defined by Schneider et al. (1993) have been maintained on the experimental host *Catharanthus roseus* (periwinkle) by periodic graft-transmission. These strains are: aster yellows (AY) from France, the Stolbur-like strain of Molières disease (MOL-P) from France, an aster yellows-like strain named ACLR-L and transmitted in Spain by G. Llacer, ash yellows (ASHY) from the USA, elm yellows (EY) from France, a X-disease-like strain of flavescence dorée of grapevine (FD-I) from Italy, and western X-disease strain GVX from the USA. Further information and references about the periwinkle-maintained isolats used at Bordeaux is given in Jarausch et al. (1994b); their classification has been studied and published by Schneider et al. (1993).

Detection of phytoplasmas by DAPI test

DNA-specific DAPI (4′,6′-diamidino-2-phenylindole, 2HCl) staining in conjunction with epifluorescence microscopy was used as described by Seemüller (1976) to detect phytoplasmas in the sieve tubes of petioles of symptomatic leaves. In total, 201 field-collected samples were analyzed (see Table 2).

Nucleic acid extraction

Field-collected samples consisted of branches from symptomatic parts of the trees. From these branches crude phloem-enriched tissue fractions were prepared. DNA from phytoplasma strains used as controls was obtained from petioles of diseased *Catharanthus roseus* or from diseased *in vitro* propagated plants. For total DNA extraction approximately 1.0 g of this freshly prepared plant material was used. Nucleic acids were isolated either according to the phytoplasma enrichment procedure described by Ahrens and Seemüller (1992) or to the simplified protocol published by Maixner et al. (1995).

Table 1. Origin and sample number of the different *Prunus* species tested

	N° of French department [1]												
	South						Southwest				Nord		
Botanic species	2B	06	26	30	66	84	33	40	47	82	49	54	total
scion cultivars													
P. armeniaca (apricot)		1	7	6	224	12	1			4			255
P. salicina (Japanese plum)	1		4	1		2	1	5	4	29	4		51
P. domestica (European plum)							6		2	4			12
P. persica (peach)					6		4			1			11
P. avium (sweet cherry)			12							12			24
P. amygdalus (almond)					2	1				1			4
rootstock cultivars													
P. marianna (GF 8-1)					12	3	9			1		1	26
P. domestica				1	4							5	
P. cerasifera (Myrobolan)					2					3			5
P. domestica x *P. cerasifera*							1						1
P. salicina x *P. spinosa*							2		1				3
P. persica x *P. cerasifera*							3						3
total	1	1	23	8	250	18	27	5	7	55	4	1	400

[1] French departments prospected are: Haute Corse (2B), Alpes-Maritimes (06), Drôme (26), Gard (30), Pyrénées-Orientales (66), Vaucluse (84), Gironde (33), Landes (40), Lot-et-Garonne (47), Tarn-et-Garonne (82), Maine-et-Loire (49) and Meurthe-et-Moselle (54).

Sequencing and primer selection

To obtain primers specific for ESFY phytoplasmas, an intergenic region of a homologous chromosomal fragment of AP and ESFY phytoplasma was examined. The sequence of AP phytoplasma has been previously obtained in our laboratory (Jarausch et al., 1994b; GenBank data library accession number L22217). Two primers selected in this AP phytoplasma sequence which cross-react with the homologous chromosomal fragment of ESFY phytoplasmas were used for PCR amplification of the corresponding intergenic region of ESFY phytoplasma. Thus, primers AP3 (Jarausch et al., 1994b) and AP6 (Jarausch et al., 1995) were employed to amplify a 450 bp DNA fragment from total DNA extracts derived from ACLR-affected plants. PCR was carried out as described below; cycle conditions were 15 s at 95 °C, 15 s at 54 °C and 45 s at 72 °C. Prior to sequencing PCR products were purified by WIZARD PCR-Prep columns (Promega, Charbonnières, France). The ESFY phytoplasma-specific PCR products were partially sequenced by cycle sequencing using the *fmol* DNA sequencing system (Promega, Charbonnières, France) according to the manufacturer's instructions. Primers AP3 or AP6 were end-labelled with ^{32}P-γ-ATP and used in asymmetric PCR reactions under the above mentioned conditions. Sequence reactions were analyzed on 6% Long Ranger (Bioprobe Systems, Montreuil, France) sequence gel electrophoresis. Sequence gels were exposed to X-ray film at room temperature.

Sequence data obtained for different ESFY phytoplasma isolates were compared to the homologous sequence of AP phytoplasma. ESFY phytoplasma-specific primers ECA1 and ECA2 were selected in regions which exhibited a maximum of sequence divergence.

Detection of phytoplasmas by PCR

PCR amplification was carried out either with universal ribosomal primers or with primers ECA1 and ECA2 synthesized on the basis of the sequence data obtained in this study. From the ribosomal primers published by Ahrens and Seemüller (1992) and Lorenz et al. (1995) two combinations were used: fU5/rU3 and fU5/rU4. Both primer pairs amplify a similar internal fragment of the phytoplasma 16S rRNA gene.

With either primer pair 40 cycles were performed in a GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk, USA), preceded by a 1-min denaturation step at 95 °C and followed by an elongation step for 4 min

Table 2. Results of PCR detection of phytoplasmas in different *Prunus* samples

Botanic species	Typical symptoms	Atypical symptoms	Positive universal PCR	Positive specific PCR	Positive DAPI test
scion cultivars					
P. armeniaca	149/255[1]	106/255[1]	209/255[2]	208/255[2]	85/136[3]
P. salicina	45/51	6/51	46/51	46/51	26/26
P. domestica	4/12	8/12	6/12	4/12	0/6
P. persica	6/11	5/11	6/11	4/11	1/6
P. avium	0/24	24/24	0/24	0/24	0/10
P. amygdalus	2/4	2/4	2/4	1/4	0/1
rootstock cultivars					
P. marianna	17/26	9/26	26/26	24/26	6/9
P. domestica	1/5	4/5	3/5	3/5	1/3
P. cerasifera	2/5	3/5	2/5	2/5	1/4
P. domestica x *P. cerasifera*	1/1	0/1	1/1	1/1	nt
P. salicina x *P. spinosa*	3/3	0/3	3/3	3/3	nt
P. persica x *P. cerasifera*	3/3	0/3	3/3	3/3	nt
total	233/400	167/400	307/400	299/400	120/201
positive PCR	221/233[4] 95%	86/167[4] 51%			

[1] number of symptomatic trees per total number of trees examined.
[2] number of PCR-positive trees per total number of trees tested.
[3] number of DAPI-positive trees per total number of trees tested.
[4] number of PCR-positive trees per total number of symptomatic trees.

at 72 °C. Cycle conditions were as follows: ribosomal primers, 15 s at 95 °C, 15 s at 55 °C and 60 s at 72 °C; primer pair ECA1/ECA2, 15 s at 95 °C, 15 s at 55 °C and 30 s at 72 °C. Reaction mixtures of 20 μl contained 10-100 ng total DNA, 0.5 μM each primer, 125 μM dNTP, 1.5 mM $MmgCl_2$, and 0.5 U Taq polymerase (BRL-Life Technologies, Cergy Pontoise, France) in the reaction buffer supplied by the manufacturer.

PCR amplification products (10 μl) were analyzed by 2% agarose gel electrophoresis. DNA was stained with ethidium bromide and visualized on a UV transilluminator.

Results

Development of PCR primers specific for European stone fruit yellows phytoplasmas

The cross reaction specificity of primers derived from the sequence of a non-conserved chromosomal fragment of AP phytoplasma was used to specifically amplify by PCR the homologous chromosomal fragment of ESFY phytoplasmas. Direct sequencing of these PCR products yielded partial sequence data for two isolates of ESFY phytoplasma: the reference strain ECA-G32 and an isolate obtained from a naturally infected apricot tree in the Pyrénées-Orientales department in the south of France. No differences were observed between these sequences. A comparison with the corresponding sequence of AP phytoplasma (Jarausch et al., 1994b) revealed two major regions of genetic variation. Two primers were selected in these regions: ECA1 (5′-AAT AAT CAA GAA CAA GAA GT-3′) and ECA2 (5′-GTT TAT AAA AAT TAA TGA CTC-3′). With regard to the AP phytoplasma sequence primer ECA1 exhibits 50% mismatches and primer ECA2 19%. Total DNA extracts from ESFY phytoplasma strains ECA-G32, RCL, DBT and FOURM, maintained in their micropropagated host plant *Prunus marianna* GF 8-1 (Jarausch et al., 1994a; Lansac et al., 1995), were used to test these primers in PCR reactions. After 40 cycles with an annealing temperature of 55 °C a PCR product of the expected size (237bp) was consistently obtained with template DNA of these reference strains but not with DNA isolated from healthy plants (data not shown). The specificity of the primers was tested with DNA extracts obtained from plants natu-

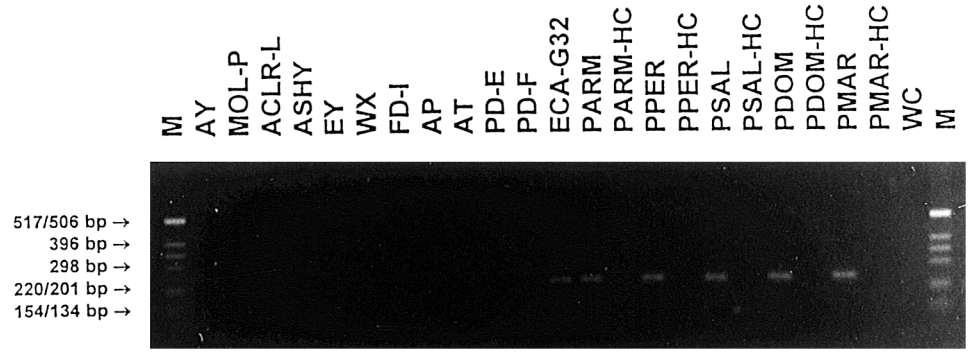

Figure 2. Results of DNA amplification with primers ECA1/ECA2 and template DNA prepared from periwinkle, *in vitro* propagated plants and from different *Prunus* species. The phytoplasma isolates represent major phytoplasma groups and subgroups according to the classification of Schneider et al. (1993). AY = Aster yellows, MOL-P = stolbur-like strain of Molières disease, ACLR-L = aster yellows-like strain of Apricot chlorotic leaf roll, ASHY = Ash yellows, EY = Elm yellows, WX = Western X-disease, FD-I = X-disease-like strain of Flavescence dorée, AP = Apple proliferation, type AP from *Malus*, AT = Apple proliferation, type AT from *Malus*, PD-E = Pear decline from *Pyrus*/England, PD-F = Pear decline from *Pyrus*/France, ECA-G32 = reference strain of Apricot chlorotic leaf roll, PARM = *Prunus armeniaca*, PARM-HC = *P. armeniaca* healthy control, PPER = *Prunus persica*, PPER-HC = *P. persica* healthy control, PSAL = *Prunus salicina*, PSAL-HC = *P. salicina* healthy control, PDOM = *Prunus domestica*, PDOM-HC = *P. domestica* healthy control, PMAR = *Prunus marianna*, PMAR-HC = *P. marianna* healthy control, WC = water control, M = Molecular size marker (1kb ladder, Life Technologies, Cergy Pontoise, France).

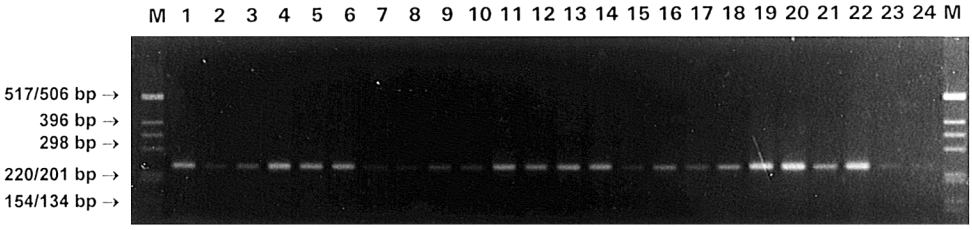

Figure 3. Results of DNA amplification with primers ECA1/ECA2 and template DNA prepared from different field-collected *Prunus* species showing various symptoms. Lane 1 = ECA-G32, reference strain of Apricot chlorotic leaf roll, lanes 2-6 = *Prunus salicina*, lanes 7-9 = *Prunus domestica*, lanes 10-13 = *Prunus marianna*, lanes 14-15 = *Prunus persica*, lanes 16-23 = *Prunus armeniaca*, lane 24 = *Prunus amygdalus*, M = Molecular size marker (1kb ladder, Life Technologies, Cergy Pontoise, France).

rally or experimentally infected with the closely related AP and PD phytoplasmas. For AP phytoplasmas, in total 8 different isolates originating from France, Germany and Italy which cover all AP phytoplasma subgroups defined by Jarausch et al. (1994c) were tested. Three PD-phytoplasma isolates from France and England as well as 7 other phytoplasma strains, representative for the genetic diversity of all known phytoplasmas, were included in the study. PCR amplification was only achieved from samples derived from plants infected with ESFY phytoplasmas (Figure 2 and data not shown). No PCR product was obtained with any other sample although PCR amplification with universal primers readily gave rise to phytoplasma-specific PCR products with the diseased samples.

Detection of European stone fruit yellows phytoplasmas in field-collected samples of different Prunus *species*

During several vegetation periods 400 field-collected samples have been obtained from different *Prunus* species. These samples represent 139 orchards covering a great part of the French stone fruit production area (Figure 1). Six different stone fruit species grown in France were tested as well as a range of *Prunus* rootstocks (Table 1). Samples were taken according to visual diagnosis. In winter, premature break of leaf buds and off-season growth are the most typical symptoms of stone fruit diseases associated with phytoplasmas. In summer, leaf chlorosis accompagnied with leaf roll is considered as typical symptom whereas chlorosis whithout leaf roll or leaf roll without leaf chlorosis

Table 3. Correlation between symptom expression and DAPI or PCR detection of phytoplasmas infecting *Prunus* species

	DAPI+ PCR+	DAPI- PCR+	DAPI+ PCR-	DAPI- PCR-	n° total	DAPI+ total	PCR+ total
Typical symptoms	97/201[1]	17/201	0/201	6/201	120/201	97/120[2] 81%	114/120 95%
Atypical symptoms	19/201[1]	10/201	4/201	48/201	81/201	23/81[2] 28%	29/81 36%

[1] number of trees per total number of trees tested.
[2] number of positive trees per total number of trees with typical or atypical symptoms, respectively.

are non-specific symptoms. Generally, diseased trees show also die-back, limb necrosis, growth reduction and decline, depending on the stage of disease. Typical symptoms like off-season growth and chlorotic leaf roll were found on all *Prunus* species except sweet cherries (Table 2). Cherry trees presumed to be affected by Molières disease (Bernhard et al., 1977) showed die-back and decline.

Primer pair ECA1/ECA2 readily directed PCR amplification of a specific DNA fragment with total DNA extracts of trees showing typical symptoms. Weaker signals were obtained in general from samples showing atypical symptoms or no symptoms at all (Figure 3). Table 2 summarizes the results of PCR detection of ESFY phytoplasmas in different *Prunus* species in France. Phytoplasmas were detected in all *Prunus* species except cherries. Positive results of PCR detection with primer pair ECA1/ECA2 enabled the direct identification of these phytoplasmas as ESFY phytoplasmas. Phytoplasma infections have been acertained for all French departments studied (Figure 1). In total, 114 out of 139 orchards were found to be contaminated by ESFY phytoplasmas.

Sensitivity of detection

Sensitivity of phytoplasma detection in field-collected samples is difficult to evaluate because the concentration of phytoplasma template DNA in total plant DNA extracts is unknown. Therefore, in practical terms, sensitivity of phytoplasma detection with primer pair ECA1/ECA2 was compared to phytoplasma detection with universal ribosomal primers using the same total DNA extracts. As shown in Table 2, 307 out of 400 samples reacted positive in universal PCR whereas 299 of the same samples were found positive in specific PCR. This represents a correlation of 97% and proves the equal sensitivity of both PCR assays.

Furthermore, these results were compared to phytoplasma detection using DAPI test. Among the 201 samples tested with both techniques, 143 samples reacted positive in specific PCR detection and 120 were considered to be positive by DAPI test (Table 2). That means the sensitivity of DAPI test was only 81% of that of PCR detection.

Correlation of symptom expression and presence of phytoplasmas in the tree

At different seasons samples were preferentially taken from symptomatic parts of a tree. So, the symptom expression could be correlated to phytoplasma detection by DAPI test or PCR. The results presented in Table 3 were essentially obtained with samples from apricot and Japanese plum (see Table 2). In these species typical symptom expression is very clearly marked by premature bud break in winter and chlorotic leaf roll in summer. As already mentioned, other symptoms were classified as atypical. A correlation of 95% was observed between the presence of phytoplasmas, as revealed by PCR detection, and the expression of typical symptoms. Again, 81% of these samples were also tested positive with DAPI test. However, only 36% of the samples showing atypical symptoms were found to be infected by phytoplasmas. This value decreases to 28% when DAPI test is employed.

An analysis of the PCR results obtained for all the 400 samples tested in this study gave nearly the same result (Table 2): 95% of the samples showing typical symptoms were tested positive by PCR. Nevertheless, phytoplasmas were detected in 51% of samples showing atypical symptoms.

Another aspect has to be emphasized: 108 samples derived from different *Prunus* species have been sampled and directly tested in the winter season. 105 samples, all showing typical off-season growth, were

tested positive. This is the first report for a large number of different *Prunus* samples which demonstrates that ESFY phytoplasmas are present in sufficient concentrations in the aerial parts of the trees in winter to be easily detectable by PCR.

Discussion

All cultivated stone fruit species in Europe suffer from severe decline diseases associated with phytoplasmas which are considered as quarantine organisms in Europe and North America. Nevertheless, reliable diagnostic tools for direct pathogen identification in routine diagnosis are missing. There are several reports which demonstrate that specific primers are best chosen in the sequence of nonconserved chromosomal fragments of phytoplasmas (Deng and Hiruki, 1990; Schaff et al., 1992; Jarausch et al., 1994b; Lorenz et al., 1995). However, selection of pathogen-specific primers which exhibit no cross reaction properties proved to be very difficult for the closely related phytoplasmas of the AP strain cluster (Jarausch et al., 1994b; Lorenz et al., 1995; Firrao et al., 1994). In the present study the cross reaction property of AP-primers was used to obtain partial sequence data of a non-coding region of ESFY phytoplasmas. Primers ECA1 and ECA2, selected in regions of maximum sequence heterogeneity among ESFY and AP phytoplasmas, proved to be very specific for ESFY phytoplasmas. No cross reactivity with various AP and PD phytoplasma target sequences could be observed. DNA from seven phytoplasma strains representing all other important 16S rDNA RFLP groups and subgroups defined by Schneider et al. (1993) was likewise not recognized by the primers.

As claimed by Lorenz et al. (1995), extensive testing of specific primers is necessary for successful application of PCR for diagnosis and substantial numbers of field-collected samples need to be included when assessing the usefulness of putative pathogen-specific PCR primers. We feel our study fulfils this important postulate. 299 out of 307 field collected, phytoplasma-diseased stone fruit samples were tested positive by ESFY-specific PCR. Furthermore, target DNA from all reference strains maintained in *in vitro* propagated plants at Bordeaux was readily detected in specific PCR. Thus, apparently all strains of ESFY phytoplasmas can be detected by PCR amplification with primers ECA1/ECA2. This strongly supports the hypothesis that all European stone fruit diseases with presumed phytoplasma aetiology are associated with the same pathogen which is genetically distinct from phytoplasmas belonging to the western X-disease strain cluster.

For routine diagnosis not only specificity but also sensitivity of PCR detection is required. Compared to the most sensitive detection of phytoplasmas in fruit trees achieved by universal ribosomal primers (Ahrens and Seemüller, 1992; Lorenz et al., 1995; Jarausch, unpublished results) ESFY-specific PCR yielded a similar sensitvity. Using DAPI test phytoplasmas were detected in 81% of PCR-positive samples whereas symptomatology allowed disease diagnosis of only 76% of these samples. Although these data demonstrate already the higher sensitivity of PCR detection it has to be kept in mind that samples have been chosen after visual diagnosis and sampling has been directed to diseased or suspicous trees. Nevertheless, only specific PCR enables the direct identification of the pathogen.

In practice, samples to be tested in routine diagnosis by PCR will still be taken according to symptomatology. In this regard, our study confirms for the first time for a large number of field-collected samples that typical symptoms of phytoplasma-associated diseases in European stone fruits like off-season growth in winter and chlorotic leaf roll in summer are highly correlated to the presence of ESFY phytoplasmas in the tree. These symptoms are especially well expressed by susceptible species like apricots, Japanese plums and peaches. In more tolerant or more resistant cultivars of *P. domestica* and *P. cerasifera* premature bud break in winter may be absent and summer symptoms may be solely leaf chlorosis together with a general growth reduction. In these cases, as well as in revealing early infections, symptomatology remains uncertain and PCR technology is necessary to confirm the visual diagnosis. Among the 400 field-collected samples tested in this study phytoplasmas were detected in over 50% of samples with doubtful symptoms. It can be supposed that the percentage of phytoplasma-diseased fruit trees in French orchards is much higher than previously thought.

Our study demonstrates for a large number of *Prunus* samples (105) the detectability of phytoplasmas in the aerial parts of the trees during winter. This is in contrast to the situation in apple and pear where the phytoplasmas seem to disappear from the aerial parts of the trees in winter (Seemüller et al., 1984; Seemüller et al., 1984b). The possibility to detect ESFY phytoplasmas in dormant wood in winter is of great importance for routine detection procedures which will have to be

used in quarantine, certification and clean stock programs as the vegetatively propagated fruit trees are sold throughout the world in form of dormant budwood.

A second aim of our study was to clarify the epidemiological situation of phytoplasma-associated diseases affecting stone fruits in France. Since the discovery by electron microscopy of phytoplasmas in AP-diseased apple by Giannotti et al. (1968) several decline disorders of stone fruits in France have been attributed to phytoplasmas. Using electron microscopy phytoplasma aetiology has only been confirmed for ACLR on apricot and on rootstock *P. marianna* GF 8-1 (Morvan et al., 1973), for peach decline on peach and for isolate DBT on *P. marianna* GF 8-1 (Dosba, 1992; Lansac, unpublished results). Therefore, most reports concerning phytoplasma-associated diseases of stone fruits in France are based on symptomatology, detection via biological indexing or non-specific DAPI staining (Morvan, 1977; Desvignes and Cornaggia, 1982; Morvan, 1988; Dosba et al., 1991; Cornaggia et al., 1994). Only recently, molecular DNA technology enabled the identification of ESFY phytoplasmas in a relatively few number of samples (Saillard et al., 1993; Lorenz et al., 1994; Jarausch et al., 1994b; Jarausch et al., 1995). However, the epidemiology of stone fruit diseases with presumed ESFY phytoplasma aetiology remains poorly understood. Alternative hosts of the pathogen and vectors are still unknown.

To study the distribution of ESFY phytoplasmas in French orchards samples have been obtained from all major stone fruit growing regions of France and represent all stone fruit species grown in France. Because sampling has been directed by visual diagnosis and only few typical symptoms were found on peach, almond and European plum, these species are underrepresentated in our study. Although phytoplasmas have been detected in these species the economic impact of phytoplasma-associated diseases in these cultures seems to be actually less important in France. To our knowledge, this is the first report of phytoplasma infection of almonds in France. Phytoplasmas have already been detected in declining almond trees in Germany (Lederer and Seemüller, 1992).

No phytoplasmas have been detected in sweet cherry trees although 12 trees were sampled in the Molières region in the southwest of France where 20 years ago a severe outbreak of a decline disease has been reported (Bernhard et al., 1977). In the early 80es phytoplasma-like particles have been observed by DAPI test in cherry samples, but the phytoplasma aetiology of this Molières disease has been only indirectly demonstrated by transmission experiments to *Prunus marianna* GF 8-1 and *Catharanthus roseus* (Dosba et al., 1984). Recent genetic analyses of these isolates revealed, however, that the *C. roseus*-maintained isolate is of Stolbur-type (Schneider et al., 1993; Jarausch unpublished results) but the *P. marianna*-maintained isolate is a member of ESFY phytoplasmas (Jarausch unpublished results). Thus, more extensive sampling at different vegetation periods has to be done to confirm, firstly, the association of phytoplasmas with Molières disease and, secondly, to characterize the implicated phytoplasma isolates or possible mixed infections.

Apricot and Japanese plum production, on the other hand, is severely affected by phytoplasma-associated diseases. In the Pyrénées-Orientales department about 80% of the mortality and decline observed on apricot is due to ACLR (Cornaggia et al., 1994). The percentage of infected trees in old orchards varies between 60 and 96% depending on the cultivar (Broquaire, unpublished data). Our study confirm these results based on symptomatology: ESFY phytoplasmas associated with ACLR have been found in 64 out of 80 orchards. The presence of ESFY phytoplasmas in ACLR-diseased trees was also easily revealed in all other apricot production areas. Japanese plums are preferentially grown in the Tarn-et-Garonne department. ESFY phytoplasmas were detected in samples from all 8 Japanese plum orchards tested and the epidemiological situation in this region is very similar to that in the Pyrénées-Orientales department. As for the scion cultivars ESFY phytoplasmas were also detected in the various rootstock cultivars tested.

In conclusion, ESFY phytoplasmas are present in all French stone fruit growing areas. There economic importance depends on the susceptibility of the cultivars. The analysis of 400 field-collected samples proved that the specific PCR assay developed in this study is a reliable method for routine diagnosis. The PCR assay is a prerequisite to detect early infections in mother stocks and propagated material to limit the spread of ESFY phytoplasma-associated diseases by infected planting material. In searches for the unkown vector of ESFY phytoplasmas as well as for the unknown alternative host plants, pathogen-specific PCR should provide a practical means to survey substantial number of individuals.

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References

Ahrens U and Seemüller E (1992) Detection of DNA of plant pathogenic mycoplasmalike organisms by a polymerase chain reaction that amplifies a sequence of the 16S rRNA gene. Phytopathology 82: 828–832

Ahrens U, K-H Lorenz and Seemüller E (1993) Genetic diversity among mycoplasmalike organisms associated with stone fruit diseases. Molecular Plant-Microbe Interactions 6: 686–691

Bernhard R, Marénaud C, Eymet J, Séchet J, Fos A and Moutous G (1977) Une maladie complexe de certains Prunus: le dépérissement de Molières. Compte Rendus Academie Agricole France 1977, 178–189

Boyé R and Desvignes JC (1996) Détection des maladies de dégénérescence des arbres fruitiers sur le semis de pêcher GF 305 en serre. INFOS – CTIFL 124: 30–35

Chen KH, Guo JR, Wu XY, Loi N, Carraro L, Guo YH, Chen YD, Osler R, Pearson R and Chen TA (1993) Comparison of monoclonal antibodies, DNA probes, and PCR for detection of the grapevine yellows disease agent. Phytopathology 83: 915–922

Cornaggia D, Audubert A and Desvignes JC (1994) Enroulement chlorotique de l'abricotier en Roussilon. Phytoma 460: 20–23

Davies DI and Clark MF (1994) Maintenance of mycoplasma-like organisms occuring in Pyrus species by micropropagation and their elimination by tetracycline therapy. Plant Pathology 43: 819–823

Davis RE and Lee I-M (1993) Cluster-specific polymerase chain reaction amplification of 16S rDNA sequences for detection and identification of mycoplasmalike organisms. Phytopathology 83: 1008–1011

Deng S and Hiruki C (1990) Enhanced detection of a plant pathogenic mycoplasma-like organism by polymerase chain reaction. Proceedings Japanese Academie 66: 140–144

Desvignes JC and Cornaggia D (1982) Observations on apricot chlorotic leaf roll (ACLR): sensitiveness of different *Prunus* species, detection, spread in plum orchards. Acta Horticulturae 130: 249–256

Dosba F, Cassiau F, Mazy K, Crossa-Raynaud P (1984) Le dépérissement de Molières: étiologie et comportement de différents *Prunus*. pp. 99–107. In: 4ème Colloque sur les Recherches Fruitères, Bordeaux, CTIFL-INRA, Paris

Dosba F (1992) Les maladies à mycoplasmes. Le point sur les recherches. Arboriculture fruitière 454: 24–28

Dosba F, Lansac M, Mazy K, Garnier M and Eyquard JP (1991) Incidence of different diseases associated with mycoplasmalike organisms in different species of *Prunus.* Acta Horticulturae 283: 331–320

Firrao G, Gobbi E and Locci R (1994) Rapid diagnosis of apple proliferation mycoplasma-like organism using a polymerase chain reaction procedure. Plant Pathology 43: 669–674

Giannotti JG, Morvan G and Vago C (1968) Micro-organismes de type mycoplasme dans le cellules libériennes de *Malus sylvestris*L. atteint de la maladie des proliférations. Comptes Rendus Academie Science Paris, Série D, 276: 76–77

Guinchedi L, Poggi-Pollini C and Credi R (1982) Susceptibility of stone fruit trees to the Japanese plum-tree decline causal agent. Acta Horticulturae 130: 285–290

Jarausch W, Lansac M and Dosba F (1994a) Micropropagation for maintenance of mycoplasma-like organisms in infected Prunus marianna GF 8-1. Acta Horticulturae 359: 169–176

Jarausch W, Lansac M and Dosba F (1996) Long-term maintenance of non-culturable apple proliferation phytoplasmas in their micropropagated natural host plant. Plant Pathology 45: 778–786

Jarausch W, Saillard C, Dosba F and Bové JM (1994b) Differentation of mycoplasmalike organisms (MLOs) in European fruit trees by PCR using specific primers derived from the sequence of a chromosomal fragment of the apple proliferation MLO. Applied and Environmental Microbiology 60: 2916–2923

Jarausch W, Saillard C, Dosba F and Bové JM (1994c) Classification of phytoplasmas infecting European fruit trees. IOM Letters 3: 232–233

Jarausch W, Saillard C, Dosba F and Bové JM (1995) Specific detection of mycoplasmalike organisms in European fruit trees by polymerase chain reaction. EPPO Bulletin 25: 219–225

Kirkpatrick BC, Smart CD, Gardner S, Gao J-L, Ahrens U, Mäurer R, Schneider B, Lorenz K-H, Seemüller E, Harrison N, Namba S, Daire X (1994) Phylogenetic relationships of plant pathogenic MLOs established by 16/23S rDNA spacer sequences. IOM Letters 3: 228–229

Lansac M, Jarausch W and Dosba F (1995) In vitro behaviour of different hosts infected by different fruit tree mycoplasma-like organisms. Acta Horticulturae 386: 454–464

Lederer M and Seemüller E (1992) Demonstration of mycoplasmas in *Prunus* species in Germany. Journal of Phytopathology 134: 89-96

Lee I-M, Hammond RW, Davis RE and Gundersen DE (1993) Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasmalike organisms. Phytopathology 83: 834–842

Lorenz K-H, Schneider B, Ahrens U and Seemüller E (1995) Detection of the apple proliferation and pear decline phytoplasmas by PCR amplification of ribosomal and nonribosomal DNA. Phytopathology 85: 771–776

Lorenz K-H, Dosba F, Poggi-Pollini C, Llacer G and Seemüller E (1994) Phytoplasma diseases on *Prunus* species in Europe are caused by genetically similar organisms. Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz 101: 567–575

Maixner M, Ahrens U and Seemüller E (1995) Detection of the German grapevine yellows (Vergilbungskrankheit) MLO in grapevine, alternative hosts and a vector by a specific PCR procedure. European Journal of Plant Pathology 101: 241–250

Morvan G (1977) Apricot chlorotic leaf roll. In: 'Apricot apoplexy'. EPPO Bulletin 7: 37–55

Morvan G (1988) Present status of apricot chlorotic leaf roll in France and surrounding countries. Acta Horticulturae 209: 169–174

Morvan G, Giannotti J and Marchoux G (1973) Untersuchungen über die Aetiologie des chlorotischen Blattrolls des Aprikosenbaums: Nachweis von Mykoplasmen. Phytopathologische Zeitschrift 76: 33–38

Namba S, Kato S, Iwanami S, Oyaizu H, Shiozawa H and Tsuchizaki T (1993) Detection and differentiation of plant-pathogenic mycoplasmalike organisms using polymerase chain reaction. Phytopathology 83: 786–791

Poggi Pollini C, Giunchedi L and Gambin E (1993) Prescence of mycoplasma-like organisms in peach trees in Northern-Central Italy. Phytopathologia mediterrana 32: 188–192

Poggi Pollini C, Bissani R, Guinchedi L and Vindimian E (1995) Occurence of phytoplasma infection in European plums (*Prunus domestica*). Journal of Phytopathology 143: 701–703

Saillard C, Bonnet F, Bouneau L, Dosba F, Seemüller E and Bové JM (1993) DNA probes for detection of apple proliferation and chlorotic leaf roll MLOs. Phytopathologia mediterrana 32: 80

Schaff D, Lee IM and Davis RE (1992) Sensitive detection and identification of mycoplasmalike organisms by polymerase chain reactions. Biochemical and Biophysical Research Communications 186: 1503–1509

Schneider B, Ahrens U, Kirkpatrick B and Seemüller E (1993) Classification of plant-pathogenic mycoplasmalike organisms using restriction-site analysis of PCR amplified 16S rDNA. Journal of General Microbiology 139: 519–527

Seemüller E (1976) Investigations to demonstrate mycoplasmalike organisms in diseased plants by fluorescence microscopy. Acta Horticulturae 67: 109–12

Seemüller E, Schneider B, Mäurer R, Ahrens U, Daire X, Kison H, Lorenz K-H, Firrao G, Avinent L, Sears BB and Stackebrandt E (1994) Phylogenetic classification of phytopathogenic mollicutes by sequence analysis of 16S ribosomal DNA. International Journal of Systematic Bacteriology 44: 440–446

Seemüller E, Kunze L and Schaper U (1984) Colonization behaviour of MLO, and symptom expression of proliferation diseased apple trees and decline diseased pear trees over a period of several years. Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz 91: 525–532

Seemüller E, Schaper U and Zimbelmann F (1984b) Seasonal variation in the colonization patterns of mycoplasmalike organisms associated with apple proliferation and pear decline. Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz 91: 371–382