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Detection and prophylaxis of Elm Yellows phytoplasma in France

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Abstract

Surveys conducted since 1998, showed that symptoms typical of Elm Yellows (EY) were quite frequent in the elm conservatories of Nogent-sur-Vernisson and Guémené-Penfao, France, and on forest trees in several locations in western Europe. Phytoplasmas were detected in symptomatic trees and plants using Polymerase Chain Reaction amplification followed by Restriction Fragment Length Polymorphism analyses of conserved regions of 16S rDNA of phytoplasmas, or of the EY group-specific non ribosomal DNA fragment FD9. Potential vectors were searched among leafhoppers and planthoppers trapped in conservatories and tested for the presence of phytoplasma in their body. Sanitation of multiplication material was experimented using soaking of dormant cuttings and branches in hot water, according to the method devised for destruction of phytoplasma in grapevine multiplication material. Phytoplasmas detected in a number of the elm clones held in the conservatories and in several forest or hedgerow elm trees, showed some diversity, according to restriction analyses of the FD9 fragment. Both American and European types were characterized. Four leaf- and planthopper species were found to carry an EY phytoplasma. Plants grown from cuttings taken from EY-infected clones and soaked in hot water (50 °C, 45 mn) never developed symptoms and tested phytoplasma negative. Most of untreated control plants were all symptomatic and positive. In the future, EY phytoplasma should be carefully checked, especially in sensitive cultivars. More studies of EY phytoplasma variability and potential vecteurs and the assessment of routine use of hot water therapy in multiplication of material should be conducted.

Key words: conservation, sanitation, hot water treatment, leafhoppers, planthoppers.

Resumen

Detección y profilaxis del fitoplasma del amarilleamiento del olmo en Francia

Las observaciones realizadas desde 1998 han mostrado que los síntomas típicos del amarilleamiento del olmo (EY) son muy frecuentes en los bancos de olmo de Nogent-sur-Vernisson y Guémené-Penfao, Francia, y en árboles en campo de varias localidades de Europa occidental. La presencia de fitoplasmas se detectó, mediante el uso de la reacción en cadena de la polimerasa seguida de análisis de polimorfismo de longitud de los fragmentos de restricción en regiones conservadas de rADN de16S de fitoplasmas, o del fragmento FD9 de ADN no ribosómico, específico del fitoplasma del EY, en árboles y plantas sintomáticos. Se buscó vectores potenciales entre homópteros cicadélidos y fulgóridos capturados en los invernáculos y estudiados con el fin de detectar la presencia de fitoplasmas en su cuerpo. Como tratamiento profiláctico del material reproductivo, se experimentó sumergiendo las estaquillas y ramas durmientes en agua caliente, de acuerdo con el método desarrollado para la destrucción de fitoplasmas en material de reproducción de la vid. Según el análisis de restricción del fragmento FD9, los fitoplasmas que fueron detectados en algunos clones de olmo conservados en los bancos clonales, así como en varios olmos de campo, mostraron una cierta diversidad genética. Tanto el tipo americano como el europeo fueron caracterizados. Se encontró que cuatro de las especies de cicadélidos y fulgóridos eran portadoras del fitoplasma del EY. Las plantas que se desarrollaron a partir de estaquillas de clones infectados por el EY y que habían sido sumergidas en agua caliente (50 ºC, 45 minutos) nunca presentaron síntomas. Igualmente, las pruebas para la detección del fitoplasma fueron negativas. Por el contrario, todas las plantas control que no habían sido tratadas presentaron síntomas, y los resultados de las pruebas fueron positivos. En el futuro, la presencia del fitoplasma del EY deberías ser comprobada sistemáticamente, especialmente en cultivares sensibles. Deberían desarrollarse más estudios sobre la variabilidad del fitoplasma del EY y vectores potenciales, y sobre la evaluación del uso continuo del tratamiento con agua caliente en la multiplicación del material.

Palabras clave: conservación, saneamiento, tratamiento con agua caliente, cicadélidos, fulgóridos.

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Introduction

Elm Yellows (EY) is a disease known in North America (Sinclair, 1981, 2000) and in several countries of Europe (Mittempergher, 2000) with differences in severity between the two continents. It is epidemic and lethal in the American elm (*Ulmus americana* L.) grown in America and it is much less severe in the European elms. It is associated in both continents with related phytoplasmas. Phytoplasmas, formerly Mycoplasmalike organisms (MLO) are obligate parasitic phloem-restricted bacteria lacking a cell wall (Caudwell et al., 1982) which are transmitted by insect vectors (McCoy *et al*., 1989), and propagated by vegetative multiplication of plant material (Caudwell *et al*., 1994). All known insect vectors of phytoplasmas are phloem-feeding hemipters among leafhoppers, planthoppers and psyllids species. Because phytoplasma have not been cultivated and are difficult to purify from their host cells, knowledge on their biology and pathogenicity is scarce (Meignoz *et al*., 1992). For the last decade, sequencing and Restriction Fragment Length Polymorphism (RFLP) analyses of phytoplasma DNA, especially of ribosomal DNA, have permitted to recognize that they form a homogenous cluster in which about 20 groups or subgroups have been differentiated (Lee *et al*., 1998; Seemüller *et al*., 1998). Phytoplasmas associated to EY belong to the 16SrV or EY-group in the latter classifications (Lee *et al*., 1995; Marcone *et al*., 1997). To this group also belong phytoplasmas associated to diseases of several species of *Alnus* sp., *Olea* sp., *Rubus* sp., *Vitis* sp. and *Ziziphus* sp. (Daire, 1994; Daire *et al*., 1992, 1997 a; Griffiths, 1999; Lee *et al*., 1995, 1998; Mäurer *et al*., 1993; Mäurer and Seemüller, 1994; Pollini *et al*., 1996; Seemüller *et al*., 1998; Seddas *et al*.,1996).

In addition to propagation by infected plant material, natural diffusion of phytoplasma diseases depends on the ethology, abundance and ubiquity of insect vectors. In *Vitis*, the grapevine yellows known as Flavescence dorée in western Europe, is particularly severe because it is transmitted by a vine-feeding leafhopper, *Scaphoideus titanus* Ball (= *Scaphoideus littoralis* Ball), an insect introduced from North America (Schvester *et al*., 1963; Caudwell *et al*., 1970, 1971; Boudon-Padieu *et al*., 1989) which lives in abundant populations within the Mediterranean western Europe (Boudon-Padieu, 2002). In America, *Scaphoideus luteolus* is a vector of EY (Baker, 1949) but correlation between its presence and diffusion of the disease was not achieved and other insect species are suspected. It is remarkable that two leafhopper species in the same genus transmit closely related phytoplasmas. Other known vectors of EY-group phytoplasmas are Macropsinae. *Rubus* stunt is transmitted by *Macropsis fuscula* (McCoy *et al*., 1989). In Germany, alder yellows was shown to be transmitted by *Oncopsis alni* to alder (Maixner and Reinert, 1999) and occasionally to grapevine (*Vitis vinifera*), where it is associated to a Grapevine yellows (Maixner *et al*., 2000). Recently, *Macropsis mendax* was found to be a vector of EY in Italy (Carraro *et al*., 2004).

Several authors have pointed out the importance of EY in Europe (Lee *et al*., 1995; Marcone *et al*., 1994; Mäurer *et al*., 1993; Mittempergher *et al*., 1990; Mittempergher, 2000). Observations made in 1998 in the elm conservatories of Nogent-sur-Vernisson and Guémené-Penfao, France, revealed that about 30% of the clones were symptomatic of EY. Subsequently, the observers who had become familiar with EY symptoms started identifying symptomatic field elms (*Ulmus minor* Mill.) growing in hedgerows or forests in various places of France. The frequent occurrence of EY in Europe could be dangerous in trade exchanges of cuttings of clonal material, either of indigenous elms or of hybrids between European and Asian elm species which may be highly sensitive (Mittempergher, 2000). The present work evaluates the importance of EY in conservatories and on autochthonous elms in France, characterizes associated phytoplasmas and tests on elm material sanitation methods that have been developed to cure grapevine from Flavescence dorée phytoplasma (Caudwell *et al*., 1990, 1997). In addition, hemipters were trapped in conservatories and submitted to detection assays of phytoplasma in their body.

Material and Methods

Plant material

The experiments described in this paper were carried out using clonal material of native European species of elms, generally *U. minor*, propagated and maintained *ex situ* in the frame of the French programme for the conservation of elm genetic resources (Collin, 2001). The plants and cuttings submitted to hot water treatment were obtained from cuttings harvested in the conservatories at Nogent-sur-Vernisson and Guémené-Penfao, France.

Detection of phytoplasma was undertaken using the same kind of clonal French material and also eleven clones of *U. glabra* and one clone of *U. minor* propagated in the frame of the Saxon programme for the conservation of elm genetic resources and held in the clone collection of the State Board for Forestry in Pirna (Saxony, Germany). Additional material consisted of *U. minor* twigs harvested in woods and hedgerows in different parts of France, and from one symptomatic plant of a hybrid cultivar found in a French nursery.

Phytoplasma detection and characterization

Leaf veins, stem shavings of phloem tissues or rootlets, were taken from suspected plants and healthy control. Positive control were phytoplasmas maintained in periwinkle (*Catharanthus roseus* L.): EY1 (isolated by W.A. Sinclair in New York) and ULW (isolated by G. Morvan in France). One gram of each tissue sample was homogenized at room temperature in separate disposable plastic sachets with a ball-bearing device (Tecam for Bioreba AG, Basel, Switzerland) and total nucleic acids were extracted as described elsewhere (Angelini *et al.*, 2001). Individual insects or groups of 2 to 10 insects, according to their size, were also ground with pestle in an Eppendorf tube and submitted to a similar DNA extraction procedure (Daire *et al*., 1997 a; Sforza *et al.*, 1998; Gatineau *et al*., 2001).

Ribosomal DNA was amplified by a nested-PCR assay (Daire *et al*., 1997 b) employing phytoplasma universal primers. The first pair of primers used was P1/P7 (Deng & Hiruki, 1991; Smart *et al.*, 1996) and the second pair was U5/U3 (Lorenz *et al*., 1995). Alternatively, the non ribosomal fragment FD9 specific of EY- (16SrV-) group phytoplasmas was amplified by a nested-PCR assay (Daire *et al.*, 1997 a; Angelini *et al.*, 2001). The first pair of primers used was FD9f2/r and the second pair was FD9f3/r2. PCR products (1-5 µl) were analyzed using 1% agarose gel electrophoresis, stained with ethidium bromide and visualized under a UV transilluminator. The length of the U5-U3 fragment is about 860 bp and the length of the final FD9f3/r2 product is about 1,160 bp.

For further characterization, PCR products were digested with *Tru*9I (Promega) restriction enzyme. Restriction fragments were subsequently separated by 10% polyacrylamide gel electrophoresis, stained with ethidium bromide and visualized under a UV transilluminator. Restriction patterns of the ribosomal fragment U5-U3 allow to distinguish EY-group phytoplasmas from phytoplasmas in other groups (Daire *et al*., 1997b). Restriction patterns of the non ribosomal DNA fragment allow to differentiate phytoplasmas within the EY-group (Daire *et al.*, 1997a; Griffiths *et al*., 1999; Clair *et al*., 2000; Angelini *et al*., 2001; Martini *et al*., 2002; Angelini *et al*., 2003).

Detection of phytoplasma in different parts of elms

Six elm plants originally propagated in Guémené-Penfao and planted in the nursery of Peyrat-le-Château (France) were used in this experiment. One 3-year old plant (clone 92028; *U. glabra* Huds. from Franche-Comté) and five 2-year old plants (clone 87095; *U. minor* Mill. from Basse Normandie) showing symptoms, were used at the dormant stage (December 1999) for phytoplasma detection in different parts. On the 3-year-old plant, bark shavings were taken on distal branches grown in 1999 and within parts of the plant corresponding to the growth increments of 1997 and 1998. Additional samples were harvested at the base of the stem (collar), and on roots (big and medium-size) and rootlets. On the 2-year-old plants, phloem tissues were taken at the tip of younger branches, on medium-size roots and on rootlets. Altogether, 33 samples were submitted to DNA extraction and PCR-RFLP procedures.

Hot water treatment of dormant elm material

Hot water treatment (HWT) has been devised to cure grapevine planting material from phytoplasmas. Recommended HWT conditions for grapevine consist in soaking dormant canes or grafted plants into hot water (50 °C ± 0.5 °C) for 45 min (Caudwell *et al*., 1990, 1997; Groupe de travail national Flavescence dorée, 1999). HWT on elm was done in an experimental device at INRA Dijon. Fig. 1 shows a sketch of a prototype device (treatment tank capacity, 3.5 m^3) built in ENTAV (Le Grau du Roi, France), by RECS (Montélimar, France).

In a first experiment, two batches of 3-year-old elm plants were uprooted from a nursery in Peyrat-le-Château (France) in the Autumn of 1999. The first batch contained 18 plants of clone 87095 (11 symptomatic and 7 non symptomatic). Among the 11 symptomatic

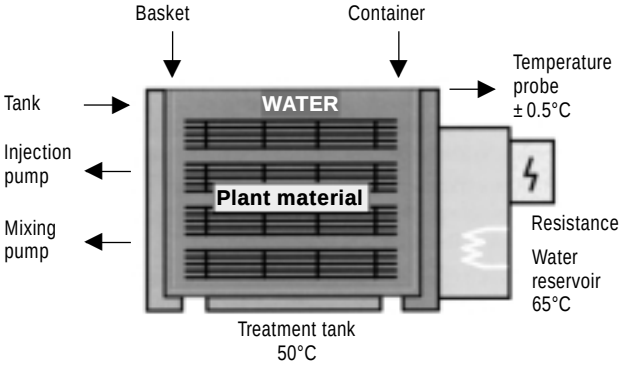

Figure 1. Sketch of the HWT device built at ENTAV (Le Grau du Roi, France) by RECS (Montélimar, France). With permission of ENTAV.

plants, 9 had tested positive for an EY-group phytoplasma in September 1999 and 2 had tested negative. The 7 asymptomatic plants had not been tested. The second batch of elms was taken from several clones and among its 37 plants, 21 were symptomatic. None of these plants had been formerly tested for the presence of phytoplasmas. HWT was undertaken on December 13, 1999, then plants (in pots) were forced in a climatic chamber until bud-burst (mid-February), transferred to a greenhouse and observed for symptom expression. In July 2000, 34 plants were then submitted to phytoplasma detection.

A second experiment was conducted on cuttings from clones 87074 and 87083 in the conservatory at Nogent-sur-Vernisson. A total of 105 cuttings were obtained on January 14, 2000, being 70 and 35 submitted to HWT or not, respectively. Then they were processed for rooting. Rooted plants (41) were grown in a greenhouse for two years and observed for symptom expression, then submitted to phytoplasma detection with PCR.

Insect trapping

Yellow sticky traps were placed at different heights over the ground (0 cm, 50 cm and 130 cm) on three sites from 8 July to 16 September, 1999, and replaced every two weeks. Two sites were plots containing elm clones of the national collection («Fond de la Montagne Conservatory» and «Nursery and mother plants»). The third site («Pond») was near an autochthonous elm showing symptoms of EY. Hemipters Auchenorrhyncha (cicadellids and cixiids) were sorted and determined, according to Ribaut (1952).

Results

Detection of 16SrV-group phytoplasma in elm

During 1999 and 2000, in samples taken from adult trees and young plants of different native European elm species growing in France and Germany (Saxony), detection of phytoplasma was most of the time obtained from symptomatic elms. Non symptomatic elms taken for control always tested negative. Mainly, three «types» of EY-group phytoplasma could be distinguished in elm on the basis of *Tru*9I restriction pattern of the FD9 fragment (Fig. 2, lanes 14-21). One type resembled the American type EY1 (lanes 15 and 19, 20 and 21), another resembled the European type ULW (lanes 14 and 17). A third pattern (lanes 16 and 18) was slightly different from either of these two types. All elm phytoplasmas were clearly different from

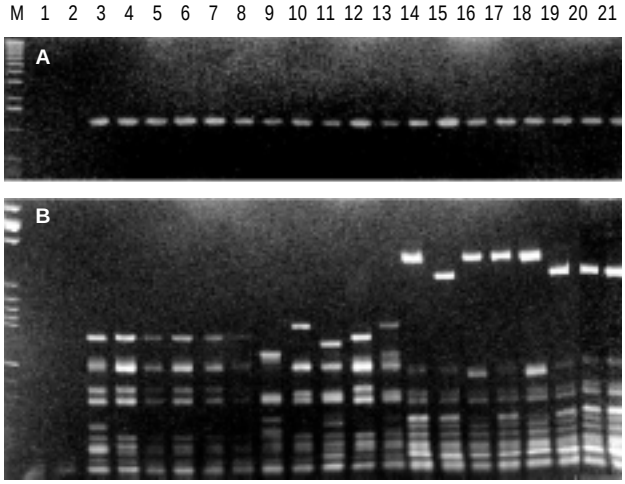

Figure 2. Gel electrophoresis of final FD9f3-FD9r2 products of nested-PCR amplification of non ribosomal DNA, from different symptomatic elms and grapevines. A) native amplimers. B) RFLP patterns of amplimers obtained in A after digestion with *Tru*9I. Lane M: Ladder (A: 1 kb, BRL; B: pBR322/HaeIII, Appligene). Lanes 1-2: DNA from healthy periwinkle and periwinkle infected with American Aster Yellows, respectively. Lanes 3-4: DNA from two Flavescence dorée (FD) strains maintained in periwinkle. Lanes 5-8: DNA from different FD-affected grapevines from French vineyards. Lanes 9-11: DNA from Palatinate grapevine yellows (PGY) – affected grapevines in Germany. Lanes 12-13: DNA from Alder yellows in Italy (ALY, maintained in periwinkle) and a symptomatic alder in Rhône valley, France, respectively. Lanes 14-15: DNA from European EY (ULW) and American EY (EY1), respectively, maintained in periwinkle. Lanes 16-21: DNA from samples taken on symptomatic elms in France; 16, *Ulmus minor* from Brittany; 17, authochtonous elm in Languedoc; 18-21, four different clones in nurseries in France.

Table 1. Effect of Hot Water Treatment (HWT) on rooting of cuttings taken on ramets of clones 87074 and 87084 in Nogent-sur-Vernisson. HWT was given on January 14. Rooting was checked on July 24

Clone	Rooted cuttings/total		Rooted cuttings/HW treated		Rooted cuttings/untreated	
	No	%	No	%	No	%
87074	27/45	60	20/29	68	7/16	44
87084	14/60	23	10/41	24	4/19	21

phytoplasmas associated to grapevine yellows and alder yellows (Fig.2, lanes 3-13).

Detection of phytoplasma in different parts of elms showing symptoms of EY

Three out of 16 tissue samples taken from the 3-year old plant of clone 92028, tested positive for the presence of an EY-group phytoplasma. One out of 4 samples taken from young wood, 1 out of 2 samples taken at the base of the stem and 1 out of 2 samples taken from medium size roots, tested positive. An European EY-type phytoplasma (ULW) was characterized in all the latter positive samples (results not shown, cf. Fig. 2B, lane 14 and 17). No presence was observed in the 3 samples taken from older wood, from the 2 samples taken from big roots, nor in the 2 samples taken from rootlets.

Among the 2-year-old plants of clone 87095, 3 plants tested positive for EY1 type in all the samples taken (results not shown, cf. Fig. 2B, lanes 15, 19, 20 and 21). The other two plants tested negative for all samples.

Efficiency of hot water treatment

When plants of the first trial were forced in a climatic chamber, none of the treated plants died. However, they showed an earlier bud-burst compared to untreated control plants. Growth was also very advanced, since 71% of treated plants had more than 20 shoots measuring more than 20 cm in the end of January, and no untreated plant showed such a fast growth rate. None of the treated plants tested positive (i.e., not even the five plants which had tested positive before HWT). PCR detection was positive for an EY-group phytoplasma in 3 of untreated plants formerly symptomatic. However, several of the formerly symptomatic plants that had been left untreated also tested negative.

In the second experiment, HWT did not show any negative effect on rooting (Table 1). None of the plants grown from treated cuttings showed symptoms and 10 out of 11 plants grown from the untreated cuttings showed symptoms of EY (Table 2). The latter results were confirmed with PCR analyses. All treated plants tested negative, while all untreated symptomatic plants tested positive.

Trapping and EY detection in insects

Over 200 leafhoppers and planthoppers were captured on yellow traps on the three sites and were examined for taxonomic identification based on morphology and male genitalia. Of these individuals, 112 belonged to 16 different species (Table 3). Other incomplete or female specimen (n = 37) could be sorted into three groups representing three distinct species not formally identified and named.

Using universal primer pairs for phytoplasma DNA, 7 batches of insects tested positive for the presence of phytoplasma in their body (Fig. 3, lanes 1-7). According to the RFLP patterns obtained (Fig. 3B), 5 batches contained a EY-group phytoplasma (reference patterns on Fig. 3B, lanes 9 and 10) and 2 batches contained a Stolbur phytoplasma or a Clover phyllody phytoplasma, respectively (Fig. 3B, lanes 1 and 3, reference patterns not shown). Stolbur and Clover-phyllody are ubiquituous

Table 2. Effect of hot water treatment (HWT) on the rate of symptomatic plants grown from cuttings of clones 87074 and 87084. HWT was given on January 14, 2000 and symptoms were checked for 2 years

Clone	Symptomatic plants/ HW treated cuttings		Symptomatic plants/ untreated cuttings	
	No	%	No	%
87074	0/20	0	7/7	100
87084	0/10	0	3/4	75

Table 3. Leafhopper and planthopper species identified during Summer 1999 at Cemagref, Nogent-sur-Vernisson, France. Species observed phytoplasma positive are printed in bold

Trapping period	Name	Site of capture*	N	Phytoplasma detected
July 8-20	*Euscelis incisus*	C, N	2	—
N = 20	***Iassus scutellaris***	N	6	EY
	Iassus scutellaris	N	6	—
	Iassus scutellaris	C	5	—
	Philaenus spumarius	C	1	—
July 21- August 4	*Euscelis incisus*	C	2	—
N = 18	*Philaenus spumarius*	C	1	—
	Euscelidius variegatus	C, N	4	—
	Dicranotropis amata	C	1	—
	Eupterix heydeni	C	1	—
	Lamprotettix octopunctatus	P	1	—
	Circulifer fenestratus	C	1	Stolbur
	Arboridia parvula	C, P	6	—
	Euscelidius schencki	P	1	—
August 5-16	***Cixius sp.***	P	1	EY
N = 25	*Euscelis incisus*	N	1	—
	Eupterix heydeni	N	1	—
	Euscelidius schencki	P	2	—
	Philaenus spumarius	P	2	—
	Fieberiella flori	C, N	5	—
	Psammotettix sp.	C	1	—
	Allygidius furcatus	C	1	—
	Euscelidius variegatus	C	1	—
	Psammotettix confinis	C	2	—
	Arboridia parvula	C, N, P	8	—
August 17-30	*Fieberiella flori*	C, N	15	—
N = 35	*Euscelidius variegatus*	C, N	8	—
	Cicadella viridis	C	3	—
	Philaenus spumarius	C	1	—
	Allygidius furcatus	C	1	EY
	Psammotettix sp.	N	4	—
	Psammotettix sp.	C	2	—
	Adarrus multinotatus	C, N	1	Clover phyllody
August 31-	*Arboridia parvula*	C	1	—
Sept 16	*Cicadella viridis*	C	4	—
N = 15	*Philaenus spumarius*	C	2	—
	Fieberiella flori	C	3	—
	Eupteryx heydeni	C	1	—
	Adarrus multinotatus	C	1	—
	Psammotettix confinis	C	3	—

* Sites of capture: C, conservatory. N, nursery. P, pond.

phytoplasmas, that are hosted in many weeds and may be carried by several insect species (Sforza, 1998; Gatineau *et al.*, 2001). An EY-group phytoplasma was found in *Cixius sp.* (n = 1), in *Iassus scutellaris* (n = 6) trapped in the middle of July at the «Nursery» site and in *Allygidius furcatus* (n = 1) trapped in the second half of August at the «Conservatory» site (Table 3). An EY-group phytoplasma was also detected in 2 specimens individually tested of an undetermined species trapped between the beginning of July and the beginning of August at the «Nursery» and the «Conservatory», respectively (Fig. 3B, lanes 6 and 7). Four other batches of *Iassus scutellaris* trapped at the same period as the others in the «Conservatory» (n = 2; n = 3) and in the «Nursery» (n = 6)

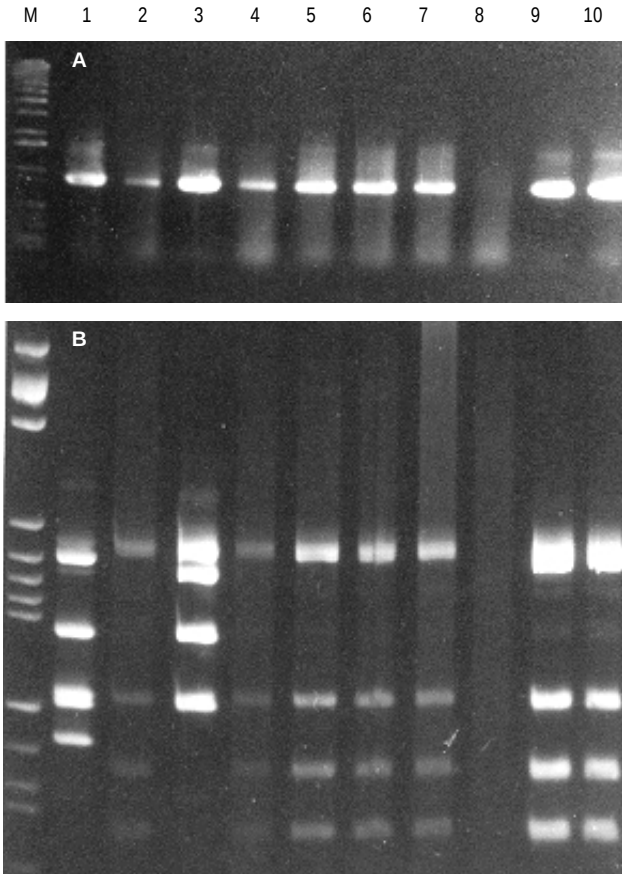

Figure 3. Gel electrophoresis of final U5-U3 products of nested PCR amplification of ribosomal phytoplasma DNA, from different specimen of auchenorrhynchas trapped at Nogent-sur-Vernisson, France. A) native amplimers. B) RFLP patterns of amplimers shown in A after digestion with *Tru*9I. Lane M : Ladder (A: 1 kb, BRL; B: pBR322/HaeIII, Appligene). Lane 1: *Circulifer fenestratus.* Lane 2: *Cixius sp.* Lane 3: *Adarrus multinotatus.* Lane 4: *Iassus scutellaris.* Lane 5: *Allygidius furcatus.* Lane 6-7: two specimens of an undetermined species. Lanes 9-10: DNA from American EY (EY1) and European EY (ULW), respectively, maintained in periwinkle.

did not give an amplification signal, nor 2 other individuals of *Allygidius furcatus* trapped with the first one. RFLP analyses of the U5-U3 rDNA fragment shown in Fig. 3 do not differentiate the two EY reference types, EY1 and ULW (Fig. 3B, lanes 9 and 10). However, RFLP analyses of the non ribosomal DNA fragment FD9 with *Tru*9I, showed that all EY phytoplasmas detected in insects resembled the American EY type (EY1) (data not shown).

Discussion

The present work confirmed that the frequency of EY is underestimated in Europe. Substantial data were obtained on detection and characterization of several EY-group phytoplasma isolates in elms in France and Germany. The examination of the EY-group specific DNA fragment FD9 showed some variability within phytoplasmas detected in these trees. Other studies have shown the relevance of the latter fragment for the analysis of diversity within this group (Daire *et al.*, 1997 a; Angelini *et al.*, 2001, 2003; Martini *et al.*, 2002) and analyses of ribosomal protein genes have been shown to display conclusions in accordance with the latter methods (Martini *et al.*, 2002). Indeed, the meaning of presence of phytoplasma strains resembling the reference American or European EY strains based on RFLP patterns of FD9 fragment, is not clear. However, this study was the first to be conducted in France. More phytoplasma strains isolated from elm in Europe should be identified and compared as has been done in North America (Griffiths *et al.*, 1999), using methods already described (Angelini *et al.*, 2001, 2003). Such studies would aim at a better understanding of the diffusion, vectors and pathogenicity of EY phytoplasmas.

Results on detection of phytoplasma in different parts of dormant young elms suggest that medium-size roots and brand-tips might be good material for control tests in winter; more work should be conducted in this field.

Under the conditions used in the first HWT experiment (Autumn treatment and then forcing), the technique was found a simple and secure way of producing phytoplasma-free plant material for planting and long distance exchange. Routine treatments carried out in a commercial nursery in 2000 and 2003 indicated that HWT may decrease plant survival in the case of a Spring treatment followed by harsh planting conditions in forest (unpublished observations). Similar observations have been obtained with HWT of grapevine material, where treatment should better be given either in the beginning or at the end of the cold conservation period to assure a good survival of treated material (Boudon-Padieu and Grenan, 2002, Tassart-Subirats *et al.*, 2003). Further trials should be done to compare the effect of the date of treatment on survival of elm plants.

Four hemipter species were found to carry EY phytoplasma. Two of these species, namely *Cixius* sp. and *Allygidius furcatus*, are well known polyphagous insects. The spread of a disease is usually reinforced by the ability of vectors to feed on a wide range of plant species. It is a way to maintain the inoculum in the en-

vironment. However, phytoplasmas have been detected in non-vector insect species (Vega *et al*., 1993; Sforza, 1998). This is a critical issue in vector studies, and future work on EY should focus the vectoring ability of each species collected. A starting point, for a better and faster approach to the problem, would be to focus on mono- and oligophagous insects, represented by *Iassus scutellaris* in our study. This elm sap-sucker leafhopper should be first targeted and widely collected for the estimation of population infection in the vicinity of elm nurseries, and mass-reared for artificial acquisition on contaminated elm cuttings. In addition, species among Macropsinae should be researched, owing to data on transmission of *Rubus* stunt and Alder yellows in Europe (Maixner and Reinert, 1999; McCoy *et al.*, 1989) and to recent findings of a vector of EY in Italy (Carraro *et al*., 2004). As a whole, the present work is a first attempt towards the assessment of the actual importance of EY in France, its vectors and the control of the disease.

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