Detection of phytoplasmal DNA in flowers and seeds from elm trees infected with Elm Yellows

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Detection of phytoplasmal DNA in flowers and seeds from elm trees infected with Elm Yellows

Emanuela Bertelli, Stefania Tegli, Alessandra Sfalanga and Giuseppe Surico

Dipartimento di Biotecnologie Agrarie, Sezione di Patologia vegetale, Università,
P.le delle Cascine 28, 50144 Firenze, Italy

Summary. The occurrence of phytoplasmas associated with Elm Yellows (EY) was investigated in the reproductive structures (flowers, unripe and ripe fruits) of two EY-infected trees of the hybrid elm clone Lobel and two healthy trees, an *Ulmus laevis* and an *U. japonica*. Phytoplasma group-specific Polymerase Chain Reaction (PCR), restriction fragment length polymorphism analysis and sequencing of amplified fragments were carried out using as template DNA extracted from these reproductive structures. The flowers and fruits were dissected into parts (the flowers into anthers and ovaries, the fruits into seeds and membranaceous wings), and then examined separately. A total of 350 seeds from infected trees were sown, producing 24 plantlets, which were sampled for EY phytoplasma DNA one and five months after germination. Both flowers and seeds from the EY-infected trees were good sources for the extraction and PCR-amplification of EY phytoplasmas, but no EY phytoplasmas were detected in either the flowers and seeds of the two healthy trees or in samples collected from the 24 plantlets grown from seed.

Key words: phytoplasmas, molecular detection, reproductive structures.

Introduction

Elm Yellows (EY), formerly known as elm phloem necrosis, is a systemic disease associated with phytoplasmas and found in North America and Europe (Swingle, 1938; Wilson *et al.*, 1972; Sinclair, 1972, 1981; Braun and Sinclair, 1979; Pisi *et al.*, 1981; Conti *et al.*, 1987; Mittempergher *et al.*, 1990). In North America, highly susceptible trees generally die within one growing season after the onset of symptoms, which include degeneration of the conductive phloem and of the cambium in the roots and in the lower trunk, followed by foliar epinasty, yellowing, and casting (Sinclair, 2000). Sometimes the leaves wilt rapidly, wither, and turn dark-brown, though they still remain attached for several weeks. The most obvious diagnostic symptom is the scent of oil of wintergreen (methyl salicylate) emanating from the inner bark, which has a butterscotch to dark-brown discoloration. Natural infections are known on American or white elm (*Ulmus americana* L.), cedar elm (*U. crassifolia* Nutt.), red or slippery elm (*U. rubra* Mühl.), September elm (*U. serotina* Sarg.), and winged elm (*U. alata* Michx.) (Sinclair, 1981). In Europe, elm species of European and Asiatic origin, and their interspecific hybrids between them, rarely die from EY: they more often show a general decline, becoming stunted, with chlorosis and witches' brooms. These Eurasian species suffer little damage from EY, even when they grow

Corresponding author: S. Tegli
Fax: +39 055 4573232
E-mail: stefania.tegli@unifi.it

in North America, but the reason for this is still unknown. In Europe, the elm species, whether native or introduced, that have been found affected by EY include: *U. americana, U. villosa, U. chenmouyi* (Mittempergher, 2000).

The phytoplasmas hitherto associated with EY are mostly members of the so-called 16SrV-A subgroup according to the classification based on molecular analysis of 16S rDNA and ribosomal protein sequences (Lee *et al.*, 2000). However, phytoplasmas belonging to other groups, e.g. 16SrI-B and 16SrXII-A, have also been identified on EY-infected elms (Lee *et al.*, 1993, 1994, 1995; Griffiths *et al.*, 1994, 1999; Marcone *et al.*, 1997).

Elm Yellows tends to reach epidemic proportions quickly (Sinclair, 2000), except in Europe, where epidemics generally do not spread rapidly and the disease can remain endemic for many years (Mittempergher, 2000). EY also does not spread uniformly along an unbroken front in a territory: infected trees can be bunched in clumps of various sizes among uninfected trees; or there can be single trees in orchards of otherwise healthy trees. So far phytoplasmas associated with EY are known to spread between closely spaced elms via root grafts. Over longer distances, EY phytoplasmas are transmitted by the elm leafhopper *Scaphoideus luteolus*, which up to now has not been found in Europe (CAB International, 1997).

The pathogen occupies phloem sieve cell tissue responsible for the translocation of photosynthates and it probably tends, as phytoplasmas generally do (Siddique *et al.*, 1998), to move away from photoassimilate source organs (leaves) to photoassimilate sink organs: roots, shoot apices, flowers, immature leaves. It is therefore the roots as well as the leaf midribs and twigs that are usually examined for the DNA of EY phytoplasmas. However, PCR tests to specifically amplify the 16S rDNA of EY phytoplasmas have not infrequently been negative even when trees were infected (Sfalanga *et al.*, 2002), and conversely asymptomatic trees have sometimes given a positive reaction in these same tests. This study was therefore carried out with a two-fold aim: 1. to determine the reliability of the PCR method normally used to detect EY-related phytoplasmas in elms, and 2. to study the distribution of phytoplasmas in various plant organs with and without EY symptoms at various months in the year.

In this report we present part of the research performed, and in particular the occurrence of EY phytoplasmas in those organs (other than the roots, or the petioles and twigs of witches' brooms) where phytoplasmas are thought to be most common: the flowers and fruits. We also report on investigations into the occurrence of EY phytoplasmas in seedlings/plantlets grown from elm seeds infected with EY phytoplasmas. The results indicate that flowers and seeds are particularly useful for the extraction and amplification of EY phytoplasma DNA, and preliminary results suggest, as expected, that EY phytoplasmas are not transmitted from seeds to the plantlets grown from them.

Materials and methods

Site location

The elm species and hybrids examined were grown in an experimental nursery originally part of a breeding programme to investigate the resistance of selected elm clones to Dutch Elm Disease in the Mediterranean area. This nursery was part of the Estate of San Rossore in the park of the same name, at Pisa, Italy.

Selection of plants for study and tissue sampling

Two 15-year-old hybrid elm clones Lobel (*U. glabra* Huds. × *U. wallichiana* Planch.) × (*U. hollandica* Wredei self.), one plant of *U. laevis* Pall., and one of *U. japonica* (Rehd.) Sarg. were tested.

Natural phytoplasma infection was checked for two years before the present study both by visual examination of the typical external symptoms of EY, and by molecular analysis (Sfalanga, 1997). The two Lobel hybrids were found to be infected with EY phytoplasmas, while the other two elms were not infected and were therefore included in this study for comparison. For the study, flowers, unripe and ripe fruits (samaras), and seeds were collected from all four trees and tested for EY by molecular analysis. Sampling was carried out in March, April and May (Table 1) from healthy-looking branches. In addition, about 100 seeds from each tree were sown in pots (about 10 seeds/pot) containing a mixture of ground and sand (1:1, v:v), watered regularly and kept in a gauze tunnel to avoid infection by insects that are vectors of phytoplasmas and viruses. Thirty and 150 days after sowing, these plantlets and seedlings were

Table 1. Plant tissues examined in this study, sampled at different times of year, with the amounts used for DNA extraction. For each tissue, samples were collected from the two Lobel hybrid clones, and from *Ulmus laevis* and *U. japonica* trees.

Sample	Date of collection[a]	Amount of tissue used for DNA extraction (g)
Flowers (whole)	March	0.5 –1.66
Anthers		0.09–0.14
Ovaries		0.25–0.28
Samaras (unripe and ripe)	April–May[b]	1.13–1.16
Membranaceous wings		0.12–0.19
Seeds		0.91–1.15
Seedlings[c]	June–October	
Epigeal parts[d]		1.14–1.45
Roots		0.88–1.18

[a] Samples collected in 1999.
[b] Seeds from the two Lobel hybrid clone trees, from *U. laevis* and *U. japonica*, collected in April and again in May.
[c] Seedlings were dissected, and then processed and examined according to their age, as described in Materials and methods.
[d] Lower, upper and middle section.

checked for EY phytoplasmas by molecular analysis.

DNA extraction from flowers

At each sampling date (4, 12 and 31 March 1999) 3 samples of flowers were harvested. Half the flowers of each sample were used for DNA extraction without prior treatment, while the remaining flowers were carefully dissected in a contaminant-free environment to separate the anthers and ovaries. Total DNA was extracted according to Lee *et al.* (1993) with some modifications. The amount of flowers, anthers and ovaries used is shown in Table 1, and ranged from 0.09 to 1.66 g. The flower material was ground to a fine powder in liquid nitrogen using a mortar and pestle, with 8 ml of precooled grinding buffer containing 95 mM K_2HPO_4, 30 mM KH_2PO_4, 10% sucrose, 0.15% BSA fraction V, 2% PVP-10 and 0.53% ascorbic acid (pH 7.6). The homogenate was centrifuged at 2×10^4 *g,* 4°C, for 20 min. The pellet was resuspended in 4 ml of extraction buffer (100 mM Tris-HCl, pH 8, 100 mM EDTA, 250 mM NaCl) and then treated with proteinase K (100 µg ml^{-1}) and 440 µl of 10% Sarkosyl, at 55°C for 1 h. After centrifuging at 8×10^3 *g,* 4°C, for 10 min, the supernatant was precipitated with isopropanol (1:1, v:v). The sample was centrifuged at 8×10^3 *g,* 4°C, for 15 min and the resulting pellet was resuspended in 3 ml of TE buffer (Sambrook *et al.*, 1989) containing 300 µg of proteinase K (100 µg ml^{-1}) and 0.5% sodium dodecyl sulphate (SDS) (v:v), and incubated at 37°C for 1 h. Then 525 µl of 5 M NaCl and 420 µl of 10% cetyl trimethylammonium bromide (CTAB) in 0.7 M NaCl were added and the mixture was incubated at 65°C for 10 min. The samples were extracted with phenol, chloroform and isoamyl alcohol, as described in Lee *et al.* (1993), and total DNA was resuspended in 100 µl of TE, after digestion with RNase A (100 µg ml^{-1}), at 37°C for 30 min.

DNA extraction from fruits, seeds and seedlings

DNA was extracted from both unripe and ripe samaras. Unripe samaras were used without treatment, ripe samaras were dissected, and the membranaceous wing separated from the seed, to extract DNA from the wings and the seeds separately. The amount of material collected ranged from 1.13 to 1.50 g (Table 1). In addition, a total of 350 seeds from infected trees were sown, producing 24 plantlets, which were used for DNA extraction 1 and 5 months after sowing. The manner of dissection depended on plant age: 1-month-old plantlets were separated into their epigean and hypogean parts, while five-month-old plantlets were dissected into the roots and the three main epigean sections (lower, upper and middle). On these older plantlets the stems and leaves of each of the three

epigean sections were examined separately. The amount of plantlet tissue available for DNA extraction ranged from 0.04 to 1.35 g. The procedure for DNA extraction was as described above.

PCR reactions

The molecular strategy adopted to test for EY phytoplasmas comprised a direct PCR, with the universal primers R16mF2/R1 (Gundersen and Lee, 1996), followed by two other sequential reactions (nested PCRs). In the first nested PCR the primer pair R16F2n/R2 was used (Gundersen and Lee, 1996); in the second nested reaction the primer pair R16(V)F1/R1 specifically amplified the DNA of EY phytoplasmas if they were present (Lee *et al.*, 1994). The reaction mixture had a final volume of 25 µl, containing 20 mM Tris-HCl (pH 8), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP) (dNTPs), 0.4 µM of each primer and 0.625 U *Taq* DNA Polymerase (Polymed, Florence, Italy). As regards DNA template, 30 ng was used in direct PCR, and the amplified DNA was then diluted 1:30 for use in the nested PCRs. For each plant sample, DNA from three different extractions was used as template, and each amplification was done in triplicate. As a positive control, DNA from phytoplasma strain SRL14 of EY subgroup 16SrV-A was used (Sfalanga, 1997). Negative controls consisting of sterile distilled water were included in all PCR amplifications to test for contaminants in the reagents. Amplification was carried out in an automated thermal cycler (Delphy 1000™, Oracle Biosystems™, MJ Researcher Inc., Watertown, MA, USA), with the same programme for both direct and nested reactions: 34 cycles of denaturation (1 min at 94°C), primer annealing (2 min at 50°C) and primer extension (3 min at 72°C) were performed, followed by a final extension at 72°C for 10 min. Aliquots (6 µl) of PCR products were analysed by electrophoresis in 1% (w:v) agarose gels, with TBE buffer (Sambrook *et al.*, 1989), stained with ethidium bromide (0.5 µg ml^{-1}) and photographed under UV light. The expected length of the amplified DNA fragment was estimated by comparison with a 1 kilobase Plus DNA Ladder (Life Technologies, Gibco BRL, Gaithersburg, MD, USA).

Restriction analysis and sequencing

In order to confirm the occurrence of EY phytoplasmas specific to elm, which are those belonging to the EY-A subgroup, restriction analysis and sequencing were performed on PCR products obtained with the R16(V)F1/R1 primers.

Approximately 100 ng of R16(V)F1/R1 PCR products were treated with 3U of *Bfa*I (New England BioLabs GmbH, Germany) according to manufacturer's instructions, and incubated at 37°C for 3 h. Then the enzyme was inactivated at 65°C for 10 min. Enzyme-digested PCR products were resolved on a 2.5% (w:v) agarose gel, stained with ethidium bromide, visualised and photographed under UV light. As a size marker, a 1 kb Plus DNA Ladder (Life Technologies) was used. As positive control, 16SrV-A phytoplasma strain SRL14 was used for comparison.

The amplified R16(V)F1/R1 products to be sequenced were purified using "Qiaquick-spin" purification columns (Qiagen, Chatsworth, CA, USA), following manufacturer's recommendations. The purified products were sequenced by the Plant Genome Laboratory (ENEA, Rome, Italy). Both strands were sequenced directly using an ABI PRISM Ready Reaction Dye Terminator Cycle Sequencing Kit (Perkin Elmer, Norwalk, CT, USA), with the same amplification primer pair R16(V)F1/R1, on an Applied Biosystems model 373A Automated DNA Sequencer Stretch (Perkin Elmer). Multiple alignments were performed on the sequence data using the computer package Clustal W (Version1.7) (Thompson *et al.*, 1994), and compared with the 16SrV-A phytoplasma sequences present in the main databases (GenBank, EMBL, etc.).

Results and discussion

Elm Yellows is one of those phytoplasmal diseases that have been known for a long time and is therefore one of the most studied. Phytoplasmas were first observed in elm tissues by transmission electron microscopy (TEM) and they have since been confirmed by PCR amplification using specific primers for DNA sequences conserved at different taxonomic levels (Braun and Sinclair, 1976, 1979; Lee *et al.*, 1993, 1995; Bertaccini *et al.*, 1995; Smart *et al.*, 1996; Marcone *et al*, 1997; Griffiths *et al.*, 1999; Sfalanga *et al.*, 2002). In all these studies phytoplasmas were always looked for in the leaf midribs, roots and stems, of which at least the roots and stems are available all the year round.

By contrast reproductive structures such as flowers and seeds, perhaps because they are available only for a short period of time in the year, have not hitherto been used for molecular detection of EY phytoplasmas. In the present study it was found that EY phytoplasmas occurred also in the reproductive structures of elm, and were detected by PCR reactions followed by restriction/sequencing of the amplification products. When DNA was extracted separately from the flowers, anthers and ovaries of the hybrids Lobel, and each DNA sample was then used as template in PCR reactions, a fragment of 1,100 bp specific to EY phytoplasmas was in all cases clearly visible after the nested PCR with primers R16(V)F1/R1 (Table 2). By contrast, no DNA amplification was obtained with flower or seed samples from the healthy-looking *U. japonica* and *U. laevis* trees. The same results were obtained with unripe samaras, seeds and membranaceous wings.

In this study phytoplasmas belonging to the subgroup 16SrV-A (Lee *et al.*, 2000) were detected by restriction analysis using enzyme *Bfa*I and by sequencing the amplification product of the second nested PCR. Restriction patterns obtained after digestion of R16(V)F1/R1 amplification products with enzyme *Bfa*I were characterised by two fragments 450 and 650 base pairs in length characteristic of the 16SrV-A subgroup (Fig. 1), and this result was confirmed when the same samples were sequenced.

The occurrence of EY phytoplasmas in the seeds of EY-infected elms may shed light on the mechanisms by which these micro-organisms are transmitted and are spread in nature. For this reason, and also because the seed-borne transmission of phytoplasmas - and of phloem viruses - is generally considered impossible (probably because the phloem of the mother plant and the embryo within the seed are not immediately connected and because a positive PCR does not always signify the occurrence of viable phytoplasmas), a study was undertaken to ascertain whether phytoplasmas

Table 2. Presence of Elm Yellows (EY) phytoplasmas detected by PCR experiments on different organs of *Ulmus laevis*, *U. japonica*, and the two hybrid elm Lobel trees.

Tree organ	EY phytoplasmas in elm trees[a]			
	L1	L2	UL	UJ
Flowers	+	+	-	-
Anthers	+	+	-	-
Ovaries	+	+	-	-
Unripe samaras	+	+	-	-
Membranaceous wings	+	+	-	-
Seeds	+	+	-	-

[a] L1, tree No. 1 of Lobel hybrid clone; L2, tree No. 2 of Lobel hybrid clone; UL, *U. laevis*; UJ, *U. japonica*. Number of samples was 3 for each tree organ. All the samples from L1 and L2 were positive; all the samples from UL and UJ were negative.
+/-, presence or absence of a PCR amplification product 1.100 bp in length, obtained with R16(V)F1/R1 primers and specific for EY phytoplasmas.

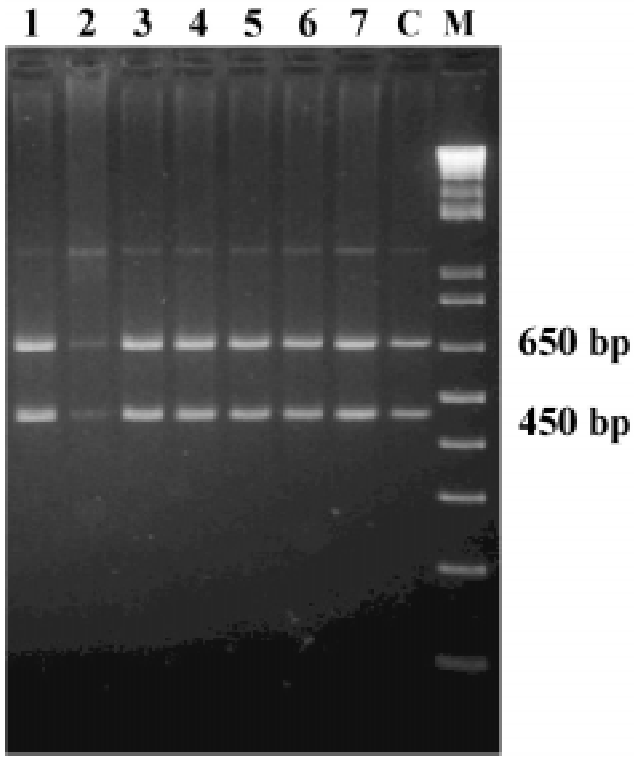

Fig. 1. Restriction fragment polymorphism analysis of PCR-amplified 16S rDNA from Elm Yellows phytoplasmas extracted from various organs of hybrid Lobel elm trees. After amplification with primers R16(V)F1/R1 the PCR-products were digested with endonuclease *Bfa*I and resolved by agarose gel electrophoresis. Lanes: 1, flowers; 2, anthers; 3, ovaries; 4, unripe seeds; 5, ripe seeds; 6, membranaceous wings; 7, ripe seeds. Lane C, positive control, using as template the DNA from phytoplasma strain SRL14. Lane M, 1 kb Plus DNA ladder (Gibco-BRL, Life Technologies).

occurred in seedlings/plantlets grown from infected seeds of the Lobel hybrids. No PCR amplification was ever obtained with such plant material 30 and 150 days after sowing. However, this does not prove conclusively that phytoplasmas causing EY are not transmitted by seeds to the plants grown from them, as is generally thought. The tests that were carried out examined only 24 plantlets, and did so only a relatively short period of time after sowing (considering the nature of this plant). When phytoplasmas are experimentally transmitted between host plants, whether by insect, grafting or dodder, they become detecTable by PCR only some time after the transmission is done and only after they reach a threshold concentration, and TEM observations reveal that the EY phytoplasma titre is generally low, and that the distribution of these micro-organisms is erratic throughout the tree (Braun and Sinclair, 1976). So far, there is no substantial evidence that phytoplasmal diseases are seed-borne. The finding reported here is therefore either simply a new *datum* on EY, or it may serve as the starting point for a new and interesting line of study on the transmission of EY in nature.

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