



MOLECULAR HYBRIDIZATION AND PCR AMPLIFICATION OF NON-RIBOSOMAL DNA TO DETECT AND DIFFERENTIATE STOLBUR PHYTOPLASMA ISOLATES FROM ITALY

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SUMMARY

Amplification of ribosomal phytoplasma DNA by direct or nested PCR and stolbur-specific primers, followed by restriction length polymorphism analysis were used to detect and identify stolbur phytoplasmas in symptomatic alfalfa, celery, field bindweed, grapevine, olive and tomato plant samples from different regions of Italy. Alfalfa appears to be a new host for this phytoplasma. All isolates showed RFLP patterns indistinguishable from one another and from the one of the Serbian reference isolate from pepper. In Southern hybridization experiments, *EcoRI* and *HindIII* restricted DNAs from stolbur-infected herbaceous hosts hybridized with two riboprobes from random-cloned chromosomal DNA fragments of a Sardinian stolbur isolate from tomato, yielding more or less complex patterns. None of the probes hybridized with DNAs from stolbur-unrelated phytoplasma infected or healthy plants or olives known to harbour stolbur phytoplasma. With one of the probes, two types of molecular hybridization profiles were detected among stolbur-infected samples. Based on the nucleotide sequence of this probe, a new non-ribosomal primer pair was designed. These primers amplified a stolbur-specific fragment of 720 bp from stolbur-infected samples, but not from symptomatic olives, healthy plants or plants infected with unrelated phytoplasmas. To verify whether absence of amplified fragments in gels was due to lack of priming or to undetectably low amplification of stolbur DNA, negative PCR products were tested in hybridization assays with the primers parental probe. Positive hybridization signals were obtained with such samples but not with products from healthy DNAs or negative PCR controls. The same probe was also used to detect stolbur DNA on membranes printed with tissues from field-infected tomato plants or from glasshouse-maintained stolbur-infected periwinkles or young asymptomatic tomato seedlings grafted with infected field material.

Key words: stolbur-specific PCR primers, molecular hybridization, Southern-hybridization, dot-blot, tissue printing.

INTRODUCTION

Based on restriction fragment length polymorphism (RFLP) analysis of 16S rRNA and ribosomal protein genes the monophyletic clade of the phytoplasmas was divided into 14 major 16S rDNA groups (I to XIV) and 32 subgroups within the class *Mollicutes* (Lee *et al.*, 1998).

In Italy, numerous plant diseases affecting trees, weeds, shrubs, vegetable, ornamentals and forage crops, characterized by symptoms of yellowing, witches'-broom, epinasty, slow growth and/or decline have been associated with phytoplasmas of groups I, II, III, V, X and XII (Lee *et al.*, 1993; Davis and Sinclair, 1998; Lee *et al.*, 1998; Seemüller *et al.*, 1998). Although phytoplasmas of group I appear to be the commonest in various parts of Italy (Bertaccini *et al.*, 1992; Vibio *et al.*, 1996; Marcone *et al.*, 1997b; Marzachi *et al.*, 1999), members of group XII have also been found in several naturally infected plant species. *Actinidia deliciosa* (*chinensis*) Planch. (kiwi), *Allium ampeloprasum* L. var. *porrum* (leek), *Apium graveolens* L. (celery), *Catharanthus roseus* (L.) G. Don (periwinkle), *Convolvulus arvensis* L. (field bindweed), *Hydrangea macrophylla* (Thunb.) Ser. (French hydrangea), *Jasminum officinale* L. (jasmine), *Lavandula officinalis* Chaix (lavender), *Lycopersicon esculentum* Mill. (tomato), *Olea europea* L. (olive tree), *Persica laevis* DC (= *Amygdalus persica nectarina* Maxim, *A. nuci-persica* Reich) (nectarine), *Pyrus communis* L. (pear), *Viola odorata* L. (violet) and *Vitis vinifera* L. (grapevine) have been reported to harbour stolbur phytoplasmas (Bertaccini *et al.*, 1995; Lee *et al.*, 1995; Albanese *et al.*, 1996; ; Marcone *et al.*, 1996; Vibio *et al.*, 1996; Boudon-Padieu *et al.*, 1997; Marcone *et al.*, 1997a, b; Minucci and Boccoardo, 1997; Schneider *et al.*, 1997; Albanese *et al.*, 1998; Bertaccini *et al.*, 1999; Marzachi *et al.*, 1999; Poggi Pollini *et al.*, 1999a, b). Group XII is divided into two subgroups (A and B),

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typified by Serbian stolbur from pepper (Stol) and Australian grapevine yellows (*Candidatus* phytoplasma australiense) (Lee *et al.*, 1998). However, with the exception of isolates of uncertain classification from field bindweed (Marcone *et al.*, 1997a; Seemüller *et al.*, 1998), all characterized stolbur phytoplasmas from Italy appear to be indistinguishable from Stol (Bertaccini *et al.*, 1995; Albanese *et al.*, 1996; Marcone *et al.*, 1996; Vibio *et al.*, 1996; Marcone *et al.*, 1997b; Minucci and Boccardo, 1997; Albanese *et al.*, 1998; Bertaccini *et al.*, 1999; Marzachi *et al.*, 1999; Poggi Pollini *et al.*, 1999b).

Tomato crops have from time to time been the target of destructive epiphytotic caused by stolbur phytoplasmas (Martelli *et al.*, 1969; Lovisolo *et al.*, 1982) occurring in areas where the crop is grown on a large scale (Marcone *et al.*, 1997b; Minucci and Boccardo, 1997; Albanese *et al.*, 1998; Coghe *et al.*, 1999). Stolbur-specific molecular probes, generated by random cloning of chromosomal DNA fragments after *Hind*III and *Eco*RI restriction of a Sardinian Stol isolate from tomato, showed distinct hybridization profiles with stolbur isolates that gave identical ribosomal RFLP patterns (Minucci and Boccardo, 1997).

In this report, digoxigenin (DIG)-labelled RNA probes transcribed from 2 of these cloned DNA fragments (pTS₁224 and pTS₁318) have been used to investigate the possibility of discriminating various stolbur isolates obtained from herbaceous and woody host plants growing in different Italian regions. The nucleotide sequence of one of the cloned DNA fragments has been determined in order to design new non-ribosomal PCR primers and compare their reactivity versus commonly used stolbur-specific oligonucleotides (Maixner *et al.*, 1995).

MATERIALS AND METHODS

Plant samples and phytoplasma reference strains.

Samples of branches, leaves and buds when present were collected from symptomatic field alfalfa (*Medicago sativa* L.), celery, field bindweed, grapevine, olive and tomato plants from various regions of Italy (Table 1). Alfalfa and field bindweed samples were collected in the vicinity of symptomatic grapevines infected by Stol phytoplasmas. Healthy plants grown from seeds in the glasshouse were used as control.

Scions from symptomatic tomato plants collected in 1999 (Table 1: T3 and T7-9) were grafted onto healthy seedlings of the same species and tested in this study. Isolates of chrysanthemum yellows, European aster yellows and primula yellows (CY, EAY and PY, respectively; subgroup IB), clover phyllody (CPh; subgroup IC),

Table 1. Species and origins of symptomatic plant samples.

Sample	Species	Origin
A	alfalfa	Piemonte
T1	tomato	Piemonte
T2	tomato	Piemonte
T3	tomato	Piemonte
T4	tomato	Liguria
T5	tomato	Veneto
T6	tomato	Veneto
T7	tomato	Calabria
T8	tomato	Calabria
T9	tomato	Calabria
C1	celery	Piemonte
C2	celery	Piemonte
Fb	field bindweed	Piemonte
G1	grapevine	Piemonte
G2	grapevine	Piemonte
G3	grapevine	Piemonte
O1	olive	Trentino
O2	olive	Trentino
O3	olive	Trentino
O4	olive	Trentino
O5	olive	Campania
O6	olive	Marche
O7	olive	Marche
O8	olive	Marche

Australian tomato big bud (TBB; group II) and of the original stolbur isolate from the Sardinian tomato (TS₁) phytoplasmas, (Milne *et al.*, 1995; Bosco *et al.*, 1997; Minucci and Boccardo, 1997; Marzachi *et al.*, 1999), of this Institute's collection were used for comparison.

Isolates of American aster yellows (AAY; subgroup IB), faba bean phyllody, crotalaria witches'-broom and crotalaria phyllody (FBP, CrWB and CrP, respectively; group II), and Stol (subgroup XIA), phytoplasmas, kindly supplied by Dr. M.F. Clark (Horticulture Research International, East Malling, UK), were graft-propagated and maintained in periwinkle. DNAs from elm yellows (EY-1; group V), peach yellow leaf roll (PYRL; subgroup IIIA), apple proliferation and pear decline (AP and PD; subgroups XA and XC, respectively) phytoplasmas, gifts from Drs. E. Seemüller (Institut für Pflanzenschutz im Obstbau, Dossenheim, Germany), and M. Barba, (Istituto Sperimentale per la Patologia Vegetale, Roma, Italy), respectively, were also used. The taxonomic assignments of the various isolates are those proposed by Lee *et al.* (1998).

DNA extraction, PCR amplifications and RFLP analysis. DNA was extracted as detailed by Marzachi *et al.* (1999) from 1.5 g of plant material. Midribs of leaves from healthy-looking field plants, and greenhouse-grown reference phytoplasma-infected periwinkles or healthy seedlings were used as controls. The DNA was resuspended in 100 µl sterile double distilled water and aliquots used in the different experiments listed below.

In direct PCR assays, aside from the primers developed in this study, two primer pairs derived from highly conserved ribosomal sequences and designed to amplify DNA fragments of different specific lengths were used: the universal primer pair R16F2/R2 (Lee *et al.*, 1993; 1225 bp) and the stolbur-specific fStol/rStol (Maixner *et al.*, 1995; 570 bp). DNAs from symptomatic plants which did not respond positively to amplification with R16F2/R2 primer pair were diluted 1:40 and used as templates in nested PCR reactions driven by primer pair R16(I)F1/R1 (Lee *et al.*, 1994; 1100 bp). For ribosomal primer pairs, reactions and cycling conditions were as detailed in the original papers.

With primer pair M1/P8 (see below), an initial denaturation step of 3 min at 95°C was followed by 10 cycles at 95°C for 2 min, 70°C (with a decrease of 1°C at each cycle) for 45 sec and 72°C for a further 45 sec, followed by 25 cycles at 95°C for 2 min, 55°C for 45 sec and 72°C for 45 sec (3 min in the last cycle). In addition to the DNA (1:50 of total extract), 25 µl reaction mixture contained 0.2 mM deoxynucleotide mixture, 2.5 mM MgCl₂, 0.56 µM of each primer and 1 unit of *Taq* polymerase (Polymed, Sambuca, Firenze, Italy).

Reaction products were analyzed in 1% agarose gels buffered in 0.5x TBE (TBE buffer: 90 mM Tris(hydroxymethyl)aminomethane (Tris), 90 mM boric acid, 3 mM ethylenediaminetetraacetate Na salt (EDTA), pH 8.3) along with 1 Kb DNA size marker (BRL-Life Technologies, Gaithersburg, MD, USA) and visualized by UV light after staining with EtBr.

Directly or nested-amplified 16S rDNA fragments (typically 8 µl aliquots) were separately digested for 2-16 h with 1-2 units of the restriction enzymes (Life Technologies) *AluI*, *DraI*, *HinfI*, *HbaI*, *RsaI*, *MseI* at 37°C or for 2.5 h with *TaqI* at 65°C. Digestion products were resolved in 5% polyacrylamide gels buffered in 1x TBE and visualized as above. Size marker was 1 Kb plus DNA ladder (Life Technologies).

Southern hybridization, tissue printing and dot-blot preparations. Aliquots (1:10 of the total DNA extract) of purified DNAs were double-digested with 5 units each of *EcoRI* and *HindIII* restriction enzymes (Life Technologies) for 2 h at 37°C in the presence of 25 µg RNase A (Sigma Chemical Co., St. Louis, MO, USA).

Restricted DNA fragments were separated in 1% agarose gels (see above). Nucleic acids were denatured, neutralized and transferred downwards onto nylon membranes (Roche Diagnostic GmbH, Mannheim, Germany) for 3 h at room temperature using a turboblotter rapid transfer system (Schleicher und Schuell, Dassel, Germany), following the manufacturer's instructions.

Young stems or midribs from field-collected symptomatic tomato plants, along with corresponding tissues from healthy, experimentally graft-infected seedlings or phytoplasma-infected greenhouse-grown periwinkles, were cut transversely and/or longitudinally with sterile razor blades and the cut edges lightly pressed onto nylon membranes (Accotto *et al.*, 1998). DNAs were then denatured and neutralized *in situ* by 5 min exposures to 500 mM NaOH and 1 M Tris-HCl, pH 8.0, respectively.

Products of M1/P8 primer-driven PCR reactions which did not yield visible bands were diluted 1:4 with TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and 25 µl aliquots denatured by boiling for 5 min and quenching on ice. Nucleic acids were then spotted *in vacuo* onto nylon membranes using a manifold apparatus (Minifold I, Schleicher und Schuell).

In all cases, DNAs were immobilized to the membranes by UV light cross-linking on both sides of the membranes prior to hybridization. Prior to further treatments, Southern-blot and tissue-printed membranes were washed at 65°C for 30 min in 1x SSC (150 mM NaCl, 15 mM sodium citrate, pH 7.0) containing 0.1% sodium dodecylsulfate (SDS) to minimize non specific background.

Probe synthesis and hybridization conditions. DIG-labelled riboprobes were transcribed from *HindIII* linearized pTS₁224 (803 bp insert) and/or pTS₁318 (~1700 bp insert) (Minucci and Boccardo, 1997) using T7 RNA polymerase and a commercially available transcription kit (DIG RNA labelling and detection kit, Roche Diagnostics) and stored at -80°C. Aliquots of the probes were diluted immediately before use to 10 ng ml⁻¹ in hybridization buffer [5x SSC, 0.02 % SDS, 0.1% N-lauroylsarcosine, 2% Blocking Reagent (Roche Diagnostics) containing 50% deionized formamide]. Synthesis of the probe, prehybridization membrane treatments, hybridization conditions and posthybridization washings were as recommended by the manufacturer. Chemiluminescent detection of hybridization signals was achieved by exposing the membranes to Hyperfilms (Amersham Italia, Milan, Italy) for 2-30 min.

Sequencing and primer selection. The complete nucleotide sequence of the cloned DNA pTS₁224 fragment was determined directly on the intact plasmid us-

ing the Sequenase (United States Biochemicals, Cleveland, OH, USA) dideoxynucleotide sequencing system and adenosine 5'-[α - 35 S] thiotriphosphate (>1000 Ci mmol⁻¹) (ICN Pharmaceuticals, Costa Mesa, CA, USA), with the help of external and internal commercial primers (Life Technologies). The sequence was assembled and computer analyzed (PC Gene, Intelligenetics Inc., Mountain View, CA, USA) and the most suitable PCR primers appeared to be M1: 5'-ACTTATTTTCA-CAACAACGG-3' and P8: 5'-TGTCTAATTCTC-CTTCAGGG-3'.

RESULTS

Symptoms. All infected plants (Table 1) showed phytoplasma-like syndromes already described in the literature for the various species, with the exception of alfalfa (*Medicago sativa* L.), which is a new stolbur host. However, there was no correlation between a distinctive symptom and the presence of the phytoplasma.

For example, infected olive trees sampled in the north eastern Trentino region (O1 to O4) showed pronounced witches'-brooming but only slight yellowing, whereas those growing in southerner areas (O5 to O8) exhibited diffused yellowing and sickle leaves. Grapevine plants from a restricted area in southeastern Piemonte region, showed rolling and thickening of the leaves, discoloration limited to the veins, shortening of internodes and production of loose, sometimes dessicated clusters.

Symptoms in tomato consisted of stunting, chlorosis and reduction of leaf laminae, shortening and thickening of internodes, abnormal production of erect axillary shoots, fasciation of both the foliage and the fruits when present, flower malformation frequently including hypertrophy of the calix and leaf reddening at later stages.

Field bindweed showed diffuse yellowing, undersized leaves and stunting of the whole plant; similar symptoms, accompanied by leaf reddening were observed in alfalfa.

The infected celeries showed pronounced stunting and leaf reddening.

Southern hybridizations. Woody plant DNAs never hybridized with the riboprobes tested, whereas DNAs from herbaceous hosts, except A and T6, did react. DNAs from healthy control plants gave no signals with either probe (Fig. 1).

Riboprobe TS₁318 hybridization patterns were complex, highly variable, and consisted of multiple bands, which differed in individual isolates, with sizes other

than the cloned insert, clearly present in isolate TS₁ only (Fig. 1, upper panels). Irrespective of the geographical origin and/or of the botanical species infected, hybridization profiles showed only a few common bands, with the majority of signals, characteristic of individual isolates.

Riboprobe TS₁224 also yielded complex hybridization patterns with high molecular weight bands, but these were less numerous and variable than with TS₁318 probe. A band corresponding in size to the cloned DNA fragment was present in all profiles (Fig. 1, lower panels). In some cases, for example with TS₁ DNA, a slightly larger band not present in Stol DNA was visible (Fig. 1, lower panels). Hence, on the basis of reactivity with TS₁224 riboprobe, the analyzed stolbur phytoplasmas could be divided into two hybridization classes: TS₁- and Stol-like (7/11 and 4/11, respectively; Fig. 1, lower panels).

Sequencing and primer selection. The nucleotide sequence (803 bp, 35% G + C content; Fig. 2) of probe TS₁224 was determined and a restriction map predicted (not shown). No significant homologies were found with nucleotide sequences present in NCBI/Genbank database (<http://www.ncbi.nlm.nih.gov/taxonomy/>), thus providing no clue as to the nature and origin of the cloned DNA fragment. PCR primer sequences M1/P8 were selected: these primers allow the amplification of a 720 bp DNA fragment of unknown location within the phytoplasma genome.

PCR detection of 16S rRNA and RFLP analysis. None of the primer pairs used amplified target DNA from either asymptomatic samples collected in the field or healthy greenhouse-grown control plants (not shown).

Table 2 summarizes the results obtained with DNAs from symptomatic samples with different primer pairs. With the few exceptions listed in Table 2 (A, G3, T6 and T8), all tested herbaceous and grapevine samples, but none of the olive trees, possessed a phytoplasma titre high enough to yield amplified fragments of the expected sizes using primer pair R16F2/R2. Upon restriction with *AluI*, *MseI* and *TaqI*, all these amplicons yielded the same patterns as Stol and TS₁ (not shown). In nested amplifications driven by primer pair R16(I)F1/R1 phytoplasma DNA from all woody plant samples and G3, T6 and T8 was amplified (Table 2), and successive RFLP analysis showed that also these amplicons were indistinguishable from those of the reference Stol and TS₁ isolates (not shown). However, alfalfa DNA was not amplified even under these conditions.

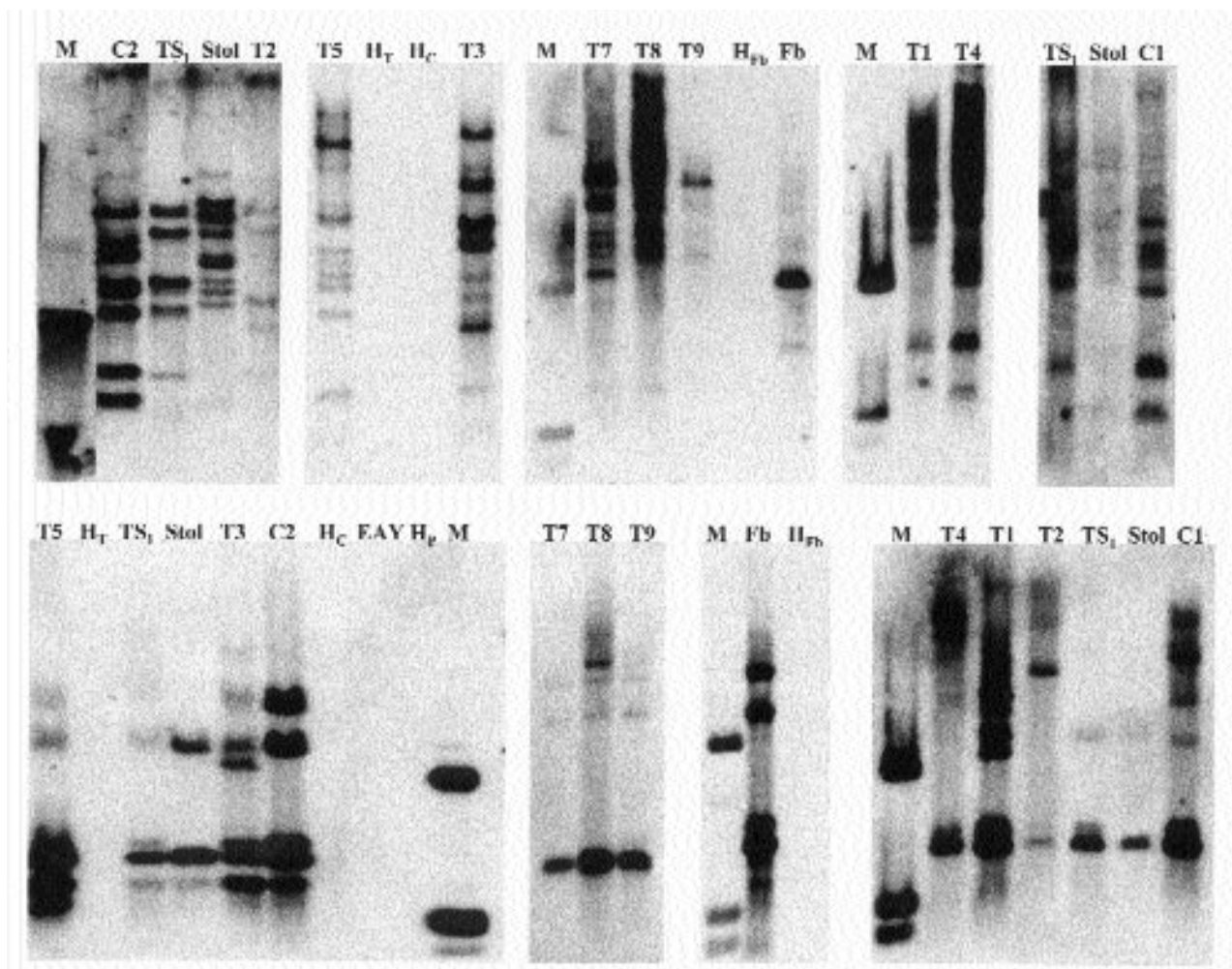


Fig. 1. Hybridization patterns of riboprobes TS₁₃₁₈ (upper panels) and TS₂₂₄ (lower panels) with *EcoRI/HindIII* double-digested DNAs from infected samples collected in the fields or corresponding healthy (H) controls. For host and reference isolates abbreviations see Table 1 and text, respectively. M is the 1kb DNA ladder (Life Technologies): the 1635, 500 and 400 bp fragments hybridize with the probe.

Using either of the stolbur-specific primer pairs, olive DNAs never yielded amplicons identifiable in gels (Table 2). As a rule, DNAs from both grapevine and herbaceous host samples were amplified by both pairs, although DNAs from three tomato plants (T1, T6 and T8), which were amplified by M1/P8, did not respond positively to primer fStol/rStol pair (Table 2 and Fig. 3, middle and lower panels, respectively). DNAs from healthy control plants or from plants infected by phytoplasmas other than those of subgroup 16Sr-XIIA were not amplified in PCRs using stolbur-specific primer pair M1/P8 (Fig. 3, upper panel). Irrespective of hybridization class, no differences were detected in RFLP patterns from M1/P8 amplicons digested with *AluI*, *DraI*, *HinfI* and *MseI* restriction enzymes (not shown).

Dot-Blots. Negative M1/P8 PCR reactions (samples O1-O8), positive and negative PCR controls were blotted onto membranes and probed with DIG-labelled TS₂₂₄ riboprobe. DNAs from symptomatic but PCR-negative samples and positive PCR controls yielded clear hybridization signals, whereas those from negative controls did not (Fig. 4), thus indicating that amplification was too low to be detected in gels.

Tissue printing. In order to assess whether TS₂₂₄ riboprobe could be routinely used to evaluate the sanitary status of tomato crops, its reactivity with tissue-printed membranes was tested. Freshly collected field-infected tomatoes possessed Stol DNA titres high enough to hybridize with the probe without the need to



Fig. 2. Nucleotide sequence (deposited at EMBL Nucleotide Sequence database under the accession number AJ272132) of the cloned pTS₂₂₄ insert showing in bold letters PCR primers M1/P8.

extract and process phytoplasma DNA (Fig. 5, left panel). Furthermore, prints from asymptomatic tomato seedlings kept in the glasshouse, one month after grafting with T7 tomato scions, hybridized with the probe, albeit more weakly than TS₁-infected glasshouse-maintained periwinkle (Fig. 5, right panel).

DISCUSSION

It is well established that different phytoplasmas can cause identical symptoms in certain plant species, whereas closely related phytoplasmas can in turn induce distinct syndromes on the same hosts (Davis and Sinclair, 1998). Therefore, little significance can be attributed to the slight differences in symptoms occasionally encountered in this study compared with those described in literature.

In olive trees from various regions of Italy, symptoms of yellowing, leaf chlorosis and proliferation of

reduced leaves have been associated with phytoplasmas of groups I (B and C subgroups), III, V, XII (Danielli *et al.*, 1996; Del Serrone *et al.*, 1996; Pollini *et al.*, 1996; Marzachi *et al.*, 1999; Poggi Pollini *et al.*, 1999a, b). Similar symptoms have been associated with subgroup XIII phytoplasmas in Spain (Font *et al.*, 1998).

Symptoms in diseased tomatoes similar to those described here have been reported under different disease names in various parts of the world for plants affected by phytoplasmas of groups I (A and B subgroups), II, III, VI and XII (Lee *et al.*, 1992; Shaw *et al.*, 1993; Marcone and Ragozzino, 1995; Boudon-Padieu *et al.*, 1996; Vibio *et al.*, 1996; Davis *et al.*, 1997a; Marcone *et al.*, 1997b; Minucci and Boccardo, 1997; Albanese *et al.*, 1998; Coghe *et al.*, 1999; Louro *et al.*, 1999).

In celery, the plants previously reported to harbour a phytoplasma with an RFLP pattern indistinguishable from that of Stol, did not show leaf reddening but yellows symptoms (Vibio *et al.*, 1996).

Table 2. Summary of stolbur detection by universal and specific primers-driven PCR reactions and hybridizations with riboprobe TS₁₂₂₄.

Sample	Primer pairs				Dot blot
	R16F2/R2	R16(I)F1/R1	fStol/rStol	M1/P8	
A	-	-	+	+	nt
T1	+	nt	-	+	nt
T2	+	nt	+	+	nt
T3	+	nt	+	+	nt
T4	+	nt	+	+	nt
T5	+	nt	+	+	nt
T6	-	+	-	+	nt
T7	+	nt	+	+	nt
T8	-	+	-	+	nt
T9	+	nt	+	+	nt
C1	+	nt	+	+	nt
C2	+	nt	+	+	nt
Fb	+	nt	+	+	nt
G1	+	+	+	+	nt
G2	+	+	+	+	nt
G3	-	+	+	+	nt
O1	-	+	-	-	+
O2	-	+	-	-	+
O3	-	+	-	-	+
O4	-	+	-	-	+
O5	-	+	-	-	+
O6	-	+	-	-	+
O7	-	+	-	-	+
O8	-	+	-	-	+

In Europe and elsewhere, syndromes similar to those we here report have been associated with diseased grapevines in which phytoplasmas belonging to different groups have been identified (Prince *et al.*, 1993; Bertaccini *et al.*, 1995; Maixner *et al.*, 1995; Albanese *et al.*, 1996; Bianco *et al.*, 1996; Padovan *et al.*, 1996; Daire *et al.*, 1997; Davis *et al.*, 1997b, c; Martini *et al.*, 1999).

While phytoplasmas of groups II and VI have been detected in diseased alfalfa with witches'-broom symptoms in southern Italy (Marcone *et al.*, 1997b) and Canada (Khadhair *et al.*, 1996), respectively, this is the first report of a subgroup XHIA phytoplasma naturally occurring in this plant.

The possible role of bindweed, among other weeds, as alternate host for stolbur phytoplasmas affecting grapevines has already been discussed (Fos *et al.*, 1992; Maixner *et al.*, 1995; Sforza *et al.*, 1998). We here confirm that a symptomatic plant showing symptoms similar to those described in the literature (Maixner *et al.*, 1995; Marcone *et al.*, 1997a; Schneider *et al.*, 1997),

collected in the vicinity of stolbur-affected grapes indeed carried this phytoplasma. However, experimental transmission of the phytoplasmas from the weeds to grapevines would be needed to corroborate this assumption also in the case of alfalfa, which, having been collected from the same location as bindweed, might constitute a further natural source of inoculum.

Identification of the phytoplasma isolates under examination has been also achieved using universal primer pairs in direct or nested amplification assays (Lee *et al.*, 1993, 1994) and subsequent RFLP analysis. All isolates showed identical RFLP patterns characteristic of subgroup XHIA (Lee *et al.*, 1998). The identity of all our isolates from herbaceous and/or woody plants is in line with previous reports in Europe (Bertaccini *et al.*, 1995; Maixner *et al.*, 1995; Albanese *et al.*, 1996; Vibio *et al.*, 1996; Marcone *et al.*, 1997b; Minucci and Boccardo, 1997; Schneider *et al.*, 1997; Albanese *et al.*, 1998; Marzachi *et al.*, 1999; Poggi Pollini *et al.*, 1999b). Stolbur isolates with an RFLP pattern different from that of the Stol reference strain have been described from field

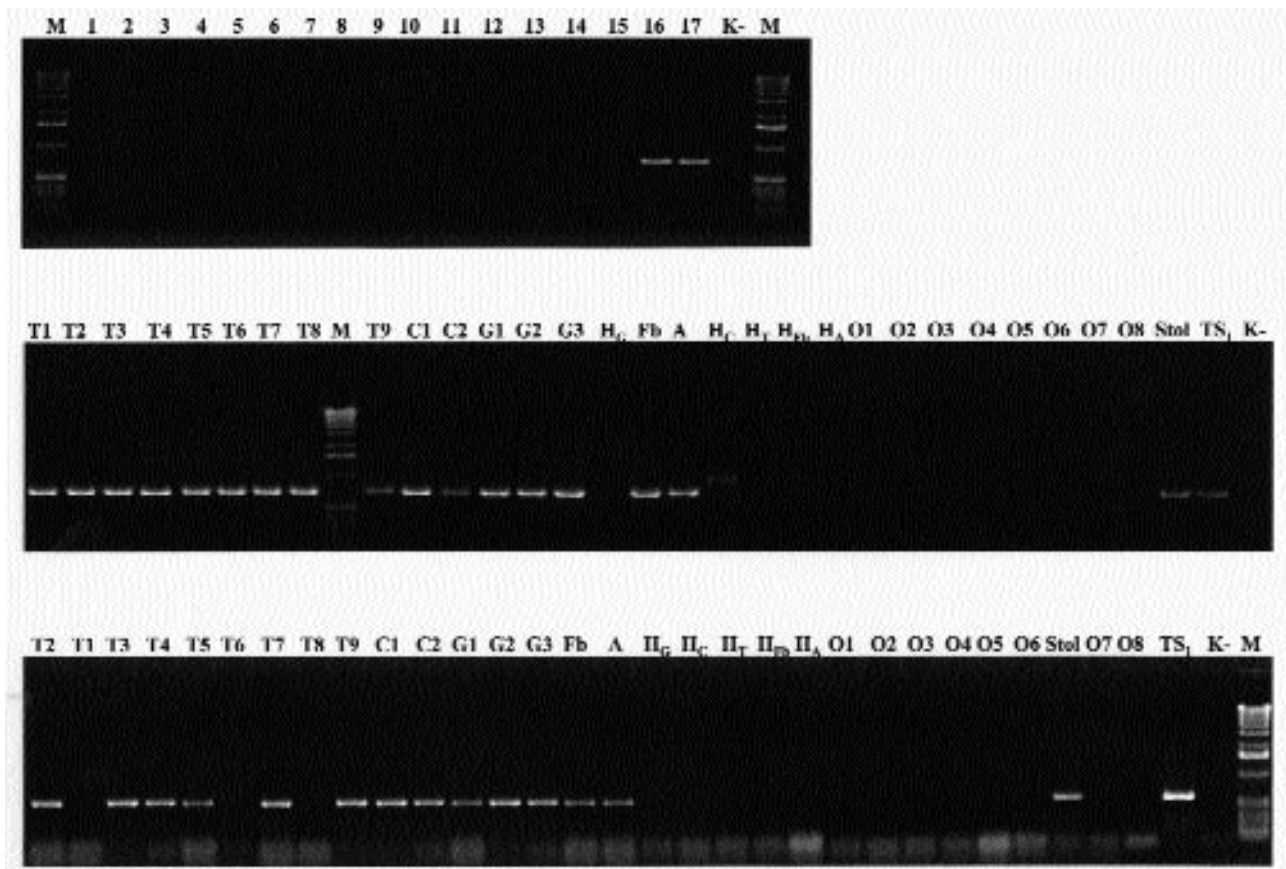


Fig. 3. Agarose gel electrophoresis of amplicons obtained with primers M1/P8 (upper and middle panels) and fStol/rStol (lowest panel). Upper panel, from left to right (1-17): EAY, CPh, CrWB, TBB, CY, PYLR, FBP, PY, CrP, AAY, EY-1, AP, PD, healthy periwinkle, healthy tomato, Stol, TS₁. Middle and lowest panels: infected samples collected in the fields or corresponding healthy (H) controls. In all cases M is the 1kb DNA ladder (Life Technologies) and K: the reaction mixture devoided of DNA. For host and reference isolates abbreviations see Table 1 and text, respectively.

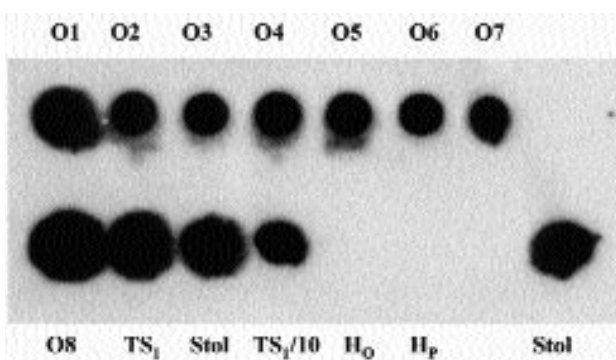


Fig. 4. Dot-blot hybridization of riboprobe TS₁224 with M1/P8-driven amplification products apparently negative upon gel analysis. For abbreviations see Table 1; TS₁/10: one tenth dilution of the amplicon obtained with TS₁ DNA in the same PCR; H_c and H_p: amplicons obtained with healthy DNAs from olive and periwinkle, respectively.

bindweed with symptoms similar to those here outlined from southern Italy (Marcone *et al.*, 1997a) and Canada thistle [*Cirsium arvense* (L.) Scop.] from southwestern Germany (Schneider *et al.*, 1997).

Non-isotopically labelled cloned probes have been successfully used for diagnosis and characterization of phytoplasmas (Lee and Davis, 1988; Bertaccini *et al.*, 1990). We have used single-stranded RNA probes derived from cloned DNA fragments of the genome of a Sardinian Stol isolate from tomato. The minor differences observed in individual hybridization patterns obtained with probes TS₁318 and 224 from those originally reported with reference strains (Minucci and Boccardo, 1997), could be explained by the different experimental conditions adopted. Probe TS₁318 showed almost individual different reactivity with the various stolbur isolates. Given their chromosomal origin (Minucci and Boccardo, 1997), the cloned insert

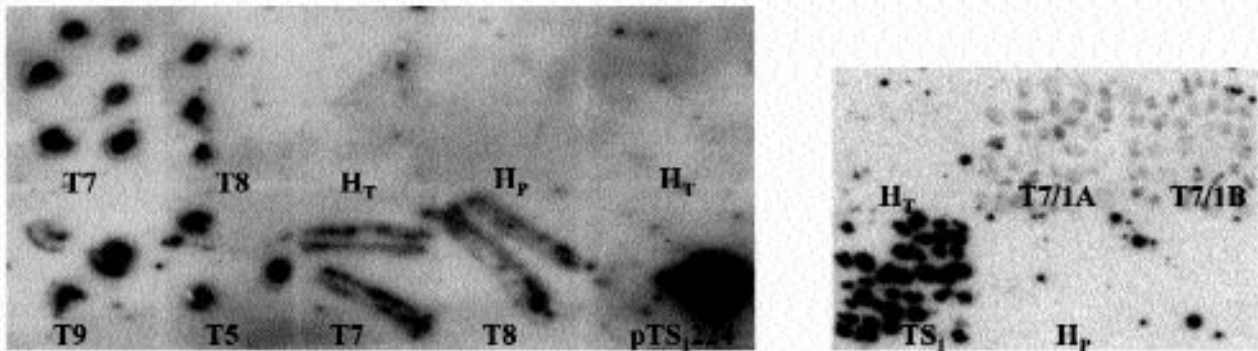


Fig. 5. Transverse and longitudinal tissue prints of tomato plants collected in the fields (left panel) and of symptomless tomato seedlings grafted with T7 scions (right panel; T7/1A and /1B) hybridized with riboprobe TS₁224. Left panel: pTS₁224 represents 10 ng of plasmid containing the cloned probe. Right panel: TS₁ is a print from glasshouse-maintained periwinkle-infected with the Sardinian reference isolate. For abbreviations see Table 1; H_T and H_P: prints from healthy tomatoes and periwinkles.

probably represents reiterative nucleotide sequences differently encompassed within variable regions of the stolbur phytoplasma genome. Probe TS₁224 allowed us to divide isolates XIIIa examined into two hybridization classes (TS₁- and Stol-like), distinguished by the presence in TS₁-like of a hybridization element absent in the Stol-like group. Both probes appear to detect minute differences among stolbur isolates, whose significance remains to be assessed. However, all hybridization patterns with probe TS₁224 contained a band corresponding in size to the cloned chromosomal fragment (803 bp). This, together with the slightly larger and distinctive TS₁ hybridization band were the only constant elements that could be reliably used to discriminate among isolates.

This prompted us to explore the possibility of using the TS₁224 probe sequence to design PCR primers of general validity for quick and easy stolbur diagnosis. This primer pair proved to be highly specific and able to amplify stolbur DNA from three extracts of tomato plants which did not react with other stolbur-specific ribosomal primers (Maixner *et al.*, 1995). It would therefore appear that M1/P8 primer pair may flank nucleotide sequences represented in higher number than those of the 16S rRNA gene in the stolbur phytoplasma genome. Notwithstanding specificity and sensitivity, no DNA has been amplified with either stolbur-specific primer pair from stolbur-affected olive trees. It appears that the negative results obtained after gel analysis of direct M1/P8-driven PCR assays with such DNA extracts were due to minimal levels of amplicons undetectable in gels and not to lack of specific priming. Hence, the sensitivity level attainable by group-specific nested PCR, might be equalled by combining M1/P8-driven amplifications and hybridization with

the primers' parental probe. Such an approach would minimize contamination risks and be perhaps less laborious.

Tissue-printed membranes have been used for detecting stolbur antigens in infected tomato tissue sections (Louro *et al.*, 1999). Results obtained with probe TS₁224 and membranes printed with tissues of young symptomless seedlings grafted with infected scions, appear interesting in the light of the possible use of such methodology in field mass diagnosis. It should be mentioned, however, that, to obtain reliable results, it is mandatory that the starting material be fresh and succulent in order to yield enough template for the probe to hybridize with. In fact, when months old and/or partly dehydrated tissues from infected tomato plants kept in the glasshouse were used for membrane printing, the response obtained were rather erratic (not shown).

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