Phytopathologia Mediterranea (2012) 51, 3, 607–617

Research Papers

Axenic culture of plant pathogenic phytoplasmas

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Summary. Phytoplasmas are microorganisms associated with severe plant diseases affecting diverse agrarian activites worldwide. Since their discovery, conclusive proof of their pathogenicity is lacking due to failure to culture these organisms. This has also hindered studies on their biology, biochemistry and physiology, although significant taxonomic progress has been achieved from study of the 16S ribosomal gene and full genomic sequencing of four phytoplasma strains. The inability to fulfil Koch's postulates severely restricts the understanding of the roles of these organisms in plant diseases and in plant/insect/phytoplasma interactions. Here we show that specific commercial media support axenic growth of phytoplasmas under defined conditions; the identity of the organisms was confirmed by PCR/RFLP analyses and sequencing of phytoplasma-specific genes. We have demonstrated for the first time that phytoplasmas, similarly to mycoplasmas, can grow independently from their host(s). This should assist reduction of the socio-economic impact of phytoplasma diseases worldwide through improved pathogen detection and consequently better management of the diseases they cause. Knowledge of mechanisms underlying the autonomous life of phytoplasmas, that are among the smallest living organisms, should also provide important information about basic mechanisms of life.

Key words: PCR/RFLP analyses, sequencing.

Introduction

The first demonstration that mycoplasma-like organisms (MLOs) were associated with numerous diseases of plants was 45 years ago, and this caught the field of plant pathology by surprise (Doi *et al*., 1967). This discovery of a new group of plant pathogens related to bacteria led to the finding of pleomorphic, wall-less prokaryotes with diameters less than 1 μm (Figure 1), located in the phloem of many plant species affected by yellows-type diseases believed to be caused by viruses, identifying their infectious nature, and demonstrating their transmission by insects (Kunkel, 1926, 1931, 1955; McCoy *et al*., 1989; Lee and Davis, 1992; Maramorosch, 2011). The disappearance of symptoms in some cases after antibiotic (i.e. tetracycline) treatment provided additional evidence to support these prokaryotic micro-organisms as putative agents of several plant diseases (Ishiie *et al*., 1967).

In contrast to mycoplasmas, which cause an array of disorders in animals and humans, the phytopathogenic MLOs have resisted all attempts to culture them *in vitro* in cell free media. However, following the application of molecular technologies, the enigmatic status of MLOs amongst the prokaryotes was resolved, and led to the new trivial name of "phytoplasma", and eventually to the designation of a new taxon named '*Candidatus* phytoplasma' (IRPCM, 2004).

Phytoplasmas have diverged from Gram-positive bacteria in the *Bacillus/Clostridium* group, belonging to the Class *Mollicutes*, and similarly to mycoplasmas have very small genomes (680–1,600 kb). Molecular data have provided considerable insights into their molecular diversity and genetic interrelationships (Bai *et al*., 2006; Oshima *et al*., 2007, 2011; Kube *et al*., 2008; Tran-Nguyen *et al*., 2008; Hoshi *et al*., 2009; Sugio *et al*., 2011). Despite the small genome size when

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ISSN (print): 0031-9465
ISSN (online): 1593-2095

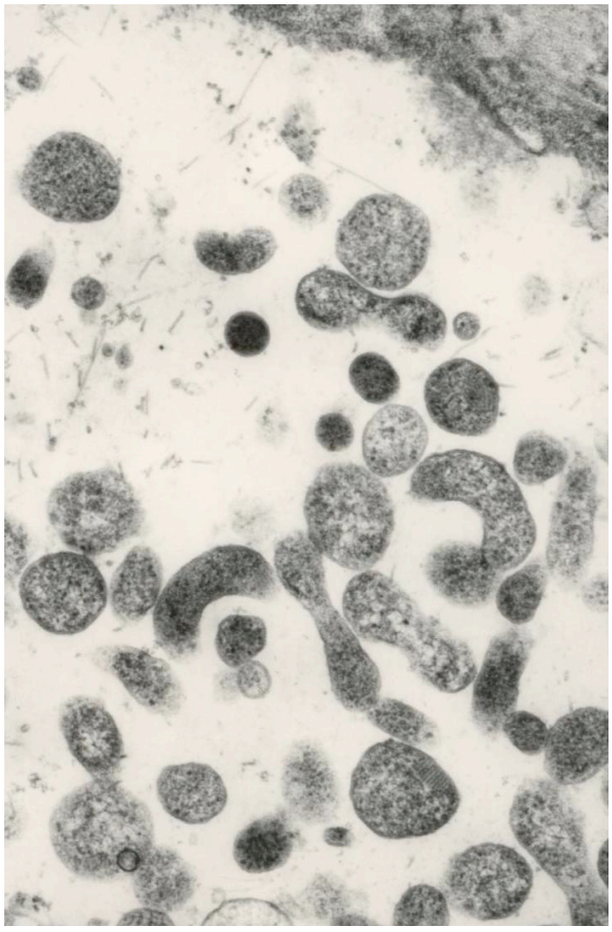

Figure 1. Electron micrograph of ultrathin section of phytoplasma in a sieve-tube of an infected plant. After embedding in Spurr resin the phloem of infected gladiolus plants were cross sectioned and stained with lead citrate and uranyl acetate then observed with a Philips electron microscope at 80 KV. Pleomorphic shape and lack of cell walls are clear, together with filaments that are DNA structures. (Magnification × 10,000).

compared with those of their ancestors, these organisms retain an independent metabolism that allows them to survive in trans kingdom environments, such as plant phloem and insect hemolymph. This is a property unique among microbes, only shared with some animal or plant infecting viruses.

Materials and methods

The methodology modified from preliminary work (Bertaccini *et al*., 2010) was patented under the application number PCT/IB2012/052965 on June 12, 2012.

Growth of phytoplasma colonies

Selected periwinkle shoots infected with phytoplasmas (Table 1) and maintained in micropropagation for at least 10 y were employed. All the experiments of growth and confirmation of phytoplasma identity were carried out in two separated laboratories, where phytoplasmas were not manipulated in any other way than cultural.

Two lengths of stem of each shoot were moistened with 0.5 mL of P*iv*L (Phytoplasma *in vitro* liquid medium, composition covered by patent) with phenol red and sliced along their axes by cutting the stems with sterile scalpels whilst held with sterile forceps. After slicing, the liquid plus plant pieces were transferred to a 4 mL vacuette tube (Greiner Bio-One, ref. 45001, K) and maintained under incubation at 25±1°C. Uninoculated tubes and tubes inoculated with healthy micropropagated periwinkle shoots were maintained as negative controls under the same conditions, at the Department of Agricultural Sciences (DipSA), University of Bologna (Italy) for all the experiments. The tubes were inspected for signs of a colour change from orange-red (pH above 7.0) to yellow (pH below 6.8) which could indicate growth of phytoplasmas. When acid colour changes occurred, 50 µL of acid broth cultures were inoculated onto 6 cm diameter plastic plates containing 8 mL of P*iv*S (Phytoplasma *in vitro* solid medium, composition covered by patent). P*iv*L and P*iv*S are the same formulations as Mycoplasma Experience (Reigate, UK) (MEX) liquid and solid medium, omitting thallium acetate and incubated in an atmosphere of 5% CO_2 and 95% N_2 for 5–7 days at 25±1°C. See Chalker *et al*. (2004), Hannan *et al*. (1997), Pitcher *et al*. (2005), Webster *et al*. (2003) and Windsor *et al*. (2010) for application of Mycoplasma Experience media.

All experiments were carried out twice in both MEX, Reigate, UK and DipSA - University of Bologna (UB) laboratories, under the same conditions using the same micropropagated shoot material for five strains (CH-1, CY-TO, TBB, STOL, and PD) (Table 1). Additional strains JR1 and AP were each grown only in one lab (Reigate and Bologna laboratory, respectively) and only from host shoots maintained in agar. Furthermore, growth trials were also carried out only

Table 1. Results of phytoplasma growth from infected micropropagated periwinkle shoots in agar medium at MEX and at UB.

Collection number*	Original host/ geographic location	Transmitted to periwinkle by/ from	16S rRNA classification	Name of the strain (acronym)	Culture No	Time (d) of acid change	Growth on plating**
Results of phytoplasma growth at MEX							
94.	*C. frutescens*/ Liguria, Italy	Leafhopper/ M. Conti	16SrI-B	Chrysanthemum yellows (CY-TO)	1	15	+
					2	20	+
61.	Tomato/ Australia	Unknown/ J.A. Osmelak	16SrII-D	Tomato big bud (TBB)	1	No change by day 138	
					2	44	+
44.	Pear/ Germany	Dodder/ E. Seemüller	16SrX-C	Pear decline (PD)	1	26	+
					2	46	+
99.	*Vitis vinifera*/ Bologna, Italy	Dodder/ R. Credi	16SrXII-A	Grapevine yellows (CH-1)	1	86	+
					2	52	+
59.	Pepper/ Serbia	Dodder/ D. Sutic	16SrXII-A	Stolbur (STOL)	1	33	+
					2	21	+
31.	Poinsettia/ USA	Dodder/ I-M. Lee	16SrIII-H	Poinsettia branching factor (JR)	1	10	-
					2	91	+
Results of phytoplasma growth at UB							
94.	*C. frutescens*/ Liguria, Italy	Leafhopper/ M. Conti	16SrI-B	Chrysanthemum yellows (CY-TO)	1	17	+
					2	14	+
61.	Tomato/ Australia	Unknown/ J.A. Osmelak	16SrII-D	Tomato big bud (TBB)	1	65	-
					2	67	-
44.	Pear/ Germany	Dodder/ E. Seemüller	16SrX-C	Pear decline (PD)	1	58	+
					2	60	+
99.	*Vitis vinifera*/ Bologna, Italy	Dodder/ R. Credi	16SrXII-A	Grapevine yellows (CH-1)	1	75	+
					2	80	+
59.	Pepper/ Serbia	Dodder/ D. Sutic	16SrXII-A	Stolbur (STOL)	1	41	+
					2	38	+
3a.	Apple/ Udine, Italy	Dodder/ L. Carraro	16SrX-A	Apple proliferation (AP-15)	1	65	+
					2	No change by day 102	

* Maintained at the UB phytoplasma collection (http://www.ipwgnet.org/collection).
**, + Colony formation; -, no colony formation.

at Mycoplasma Experience from shoots transferred in sterile water 10 days before isolation to verify the effectiveness of the isolation method after transportation under different conditions (Table 2).

Microscope observation of colonies

The colonies obtained (diameter between 0.1 and 0.2 mm at the initial stages) were observed under an bifocal microscope at ×20 magnification and compared in shape and dimensions to mycoplasma colo-

Table 2. Results of phytoplasma growth from infected micropropagated periwinkle shoots transferred in sterile water 10 d before isolation in liquid medium.

Acronyms*	Culture No	Time (d) of acid change	Growth on plating**
CY-TO	1	63	+
	2	50	+
TBB	1	No change by day 140	
	2	69	+
PD	1	48	+
	2	62	?+
CH-1	1	78	+
	2	70	+
STOL	1	86	+
	2	67	+

*, Acronyms as in table 1
**, + Colony formation; -, no colony formation

nies. Photographs were then taken at magnifications between ×12 and ×50.

Phytoplasma molecular identification

To confirm phytoplasma identity, single colonies after at least three passages on P*iv*S were collected and suspended in 10 µL sterile distilled deionised water. In each passage a single colony was picked and transferred into fresh liquid medium. After acid colour change, 50 µL of broth were inoculated onto a plate containing P*iv*S, and this was repeated three times. Phytoplasma identification was carried out using specific PCR assays on the 16S rRNA gene. One µL of solution was then employed as template for PCR amplification using R16F2n/R2 universal primers (Gundersen and Lee, 1996), followed by nested PCR with 16Sr group general primers 16R758f/16R1232r (=M1/M2) amplifying all described phytoplasma ribosomal groups (Gibb *et al*., 1995), 16Sr group specific primers R16(I)F1/R1 amplifying phytoplasmas in ribosomal groups 16SrI, -II, and -XII (Lee *et al*., 1994; Tolu *et al*., 2006) and R16(X)F1/R1 amplifying only phytoplasmas belonging to ribosomal group 16SrX (Lee *et al*., 1995) carried out as previously described (Schaff *et al*., 1992; Lee *et al*., 1998). In each PCR and nested-PCR experiment two negative control samples were added. These were one sample of sterile distilled water and the second was a suspension of uninoculated medium (liquid or solid according with the material employed in PCR assays) in sterile distilled water. Phytoplasma identification was completed using RFLP analyses and by direct sequencing of selected amplicons. Further analyses were carried out by PCR/sequencing analyses on the tuf gene as described (Schneider *et al*., 1997).

Tubes with acid colour changes and single colonies were both tested to confirm phytoplasma identity. From each tube 100 µL were collected and centrifuged at 14,000 rpm for 30 min, and pellets were then resuspended in 10 µL sterile distilled water. Each single colony was collected under sterile condition and suspended in 10 µL sterile distilled deionised water. Direct PCR assays were carried each out on 1 µL of this solution, and each nested-PCR assay was carried out using 1 µL of a 1:30 dilution of amplicons from direct PCR as template. Each 25 µL PCR reaction mix contained 5.0 µL of 5× PCR buffer, 0.8 U *Taq* polymerase (Promega, Madison, WI, USA), 0.2 mM dNTPs, 1.5 mM $MgCl_2$ and 0.4 mM of each primer. Six µL of PCR products were separated in 1% agarose gel, stained with ethidium bromide and visualized with UV transilluminator. Identification of detected phytoplasmas was carried out using RFLP analyses with *Tru1*I, *Rsa*I and *Ssp*I (Fermentas, Vilnius, Lithuania) restriction enzymes. RFLP products were separated

in a 7% polyacrylamide gel, stained with ethidium bromide and visualized under UV transilluminator. Direct sequencing of selected PCR products was also performed using the primer employed for amplification. Sequences were assembled using the Staden program package (Staden *et al*., 2000), aligned using Clustal X (Thompson *et al*., 1997), and deposited in GenBank.

Results

The described culturing method allowed the cultivation of phytoplasma colonies in axenic medium from up to seven micropropagated strains from the phytoplasma reference collection (Table 1), and also allowed the visualisation of the phytoplasma colony phenotype.

Colony shape and size were similar to those of human and animal mycoplasmas and the majority of strains cultured were successfully obtained first as broth cultures. The timing of colour changes (Figure 2) differed among genomically distinguishable phytoplasma strains; the results were repeated in the Bologna and Reigate laboratories as shown at Table 1 and were substantially consistent for phytoplasmas derived from the same micropropagated strain in periwinkle grown in agar.

The average time (UB and MEX laboratories) occurring before colour change was: for strain CY-TO 16.5 d, for STOL 33.3 d, for PD 47.5 d, and for CH-1 73.2 d, while TBB was only successfully cultivated in a very few cases and almost never reached the colony production step. Strains JR and AP showed colour changes at 91 and 65 d respectively, but were isolated, respectively, only at Reigate and Bologna laboratories. Uninoculated tubes did not show colour changes, while tubes inoculated with healthy micropropagated periwinkle shoots sometimes gave rapid acid colour changes (3–4 days) and resulted in a bacterial and/or fungal growth, possibly caused by plant endosymbionts (Hardoim *et al*., 2008). Further experiments carried out on the shoots listed in Table 2 that were transferred in sterile distilled water from agar about 10 d before isolation, showed differences in time of colour change of the medium. In particular strain CY-TO gave average timing of 56.5 d and STOL of 76.5 d (Table 2).

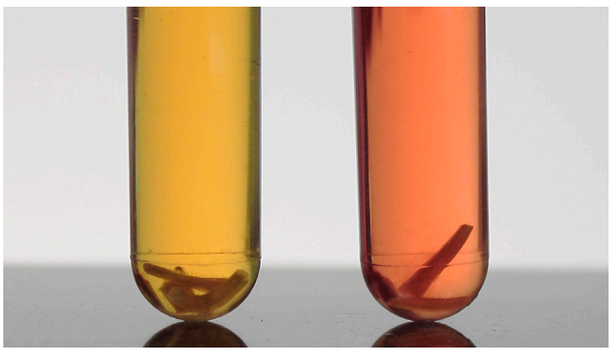

Figure 2. Acid colour change (lefthand tube), photographed 15 days after inoculation with materials from a micropropagated shoot infected with phytoplasma strain CY-TO. Righthand tube inoculated at the same time with healthy micropropagated periwinkle.

Colonial growth of phytoplasmas occurred in 2 to 5 d in most of the cases (Tables 1 and 2). Colonies were comparable in shape and dimensions to mycoplasma colonies, the most closely related microorganisms; they were often well-developed after incubation for 5 d at 25°C, and frequently showed reticulated surfaces, unlike mycoplasma colonies (Figure 3A, B, C, D, and E).

Amplification with R16F2n/R2 followed by nested-PCR with general and group specific primers, according with phytoplasma isolated ribosomal groups, confirmed their presence in a few acid tubes and in a number of tested colonies. All negative control samples gave no amplification in either direct or nested PCR assays.

The number of colonies on one plate differed among different samples. In general, a large number of colonies (between 10 and 20) were observed for STOL, between five and ten for CH1 and CY-TO, and three to six for all the other strains. Moreover, the percentage of colonies positive for phytoplasma after the molecular analyses differed among the samples. Generally, this was about 80% for STOL and CH1 and 50% for all the other strains. RFLP analyses with informative enzymes allowed the confirmation of phytoplasma identity in several of the amplicons obtained. The RFLP patterns obtained from the same strain colonies cultivated in the two different labs were indistinguishable (Figure 4A and B). Further PCR/RFLP analyses carried out on the phytoplasma tuf gene similarly allowed confirmation of these findings (data not shown). The 14 sequences obtained on 16S rRNA and tuf genes showed 99% to 100% homology with the cor-

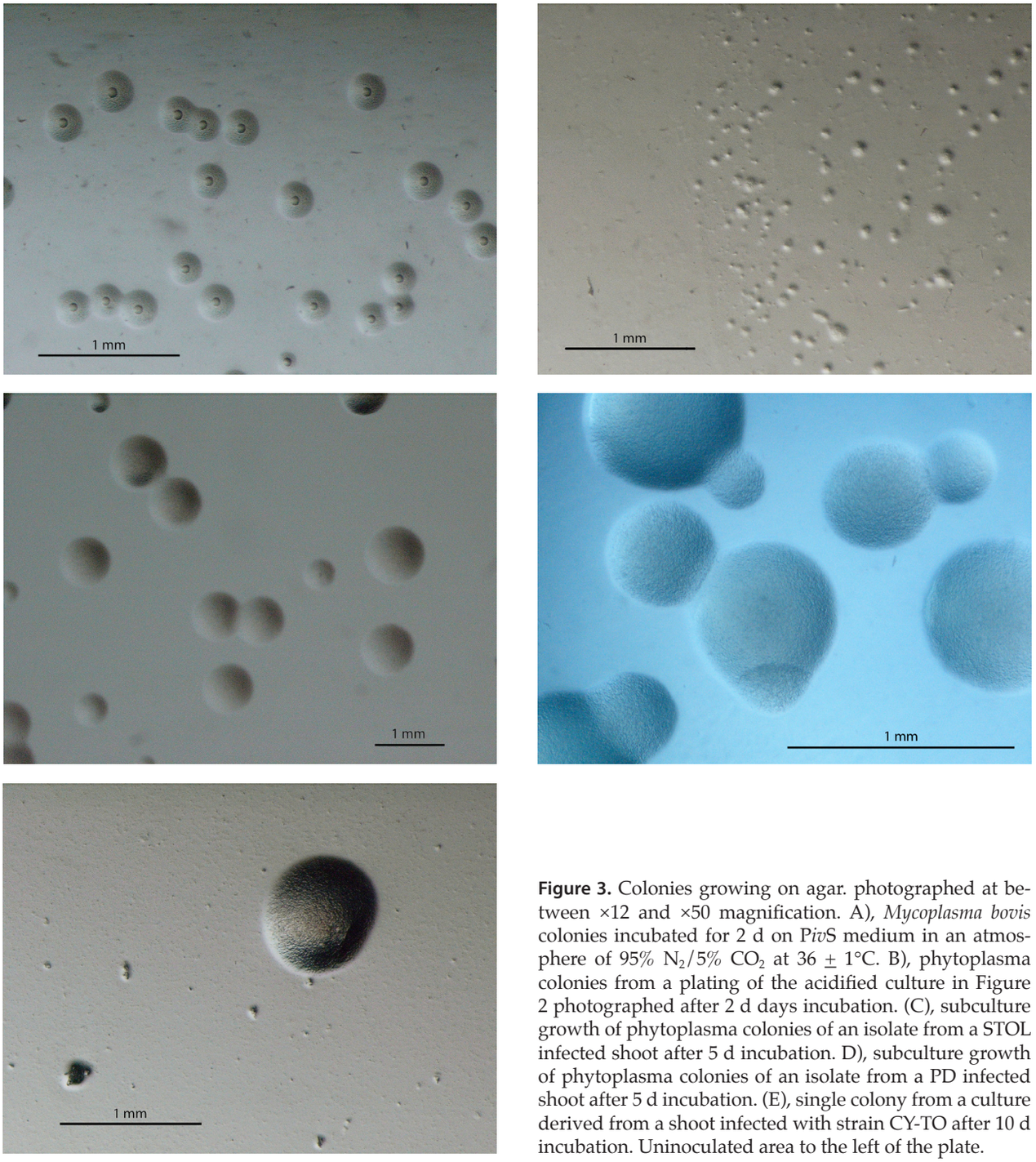

Figure 3. Colonies growing on agar. photographed at between ×12 and ×50 magnification. A), *Mycoplasma bovis* colonies incubated for 2 d on P*iv*S medium in an atmosphere of 95% N_2/5% CO_2 at 36 ± 1°C. B), phytoplasma colonies from a plating of the acidified culture in Figure 2 photographed after 2 d days incubation. (C), subculture growth of phytoplasma colonies of an isolate from a STOL infected shoot after 5 d incubation. D), subculture growth of phytoplasma colonies of an isolate from a PD infected shoot after 5 d incubation. (E), single colony from a culture derived from a shoot infected with strain CY-TO after 10 d incubation. Uninoculated area to the left of the plate.

responding genes of the original strains (data not shown). Selected sequences of both amplified genes were deposited under accession numbers JX469425, JX469426 and JX469427, and their comparisons with corresponding genes of original strains are reported in Table 3.

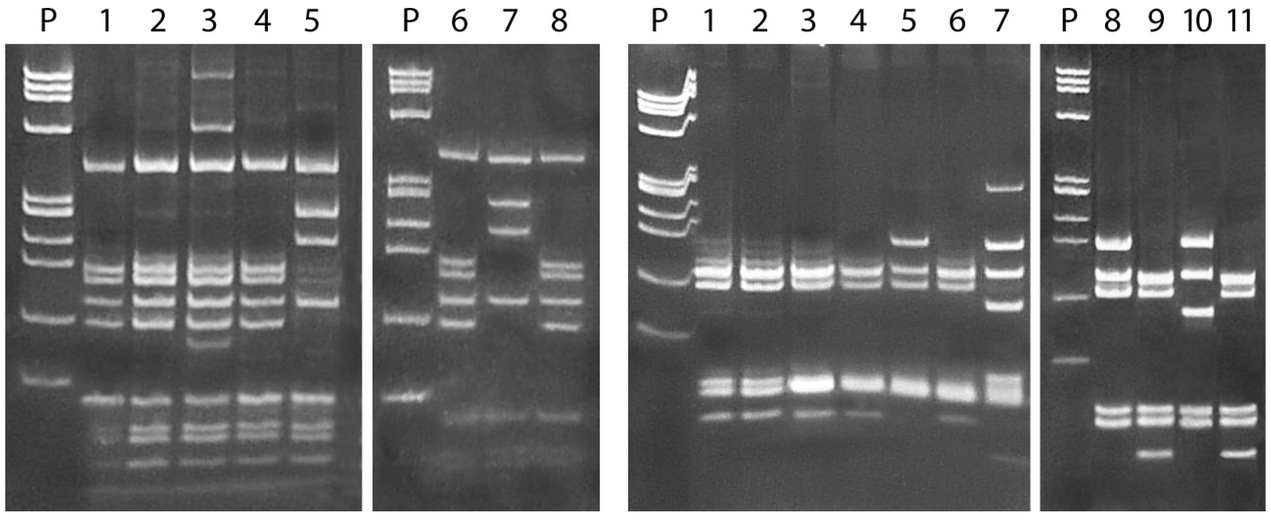

Figure 4. Results of RFLP analyses with *Tru1*I on R16(I)F1/R1 amplicons (1,100 bp) (**A**) and on M1/M2 amplicons (500 bp) (**B**) obtained from phytoplasma colonies. Samples in (**A**) 1, colony from strain CH-1 and 2, colony from strain STOL from Mycoplasma Experience laboratory (MEX); 3, colony from strain CH-1 and 4, colony from strain STOL from University of Bologna laboratory (UB); 5, colony from strain CY-TO at MEX; 6, strain CH-1 from micropropagated periwinkle; 7, strain CY-TO from micropropagated periwinkle; 8, strain STOL from micropropagated periwinkle. Samples in (**B**) 1, colony from strain CH-1 and 2, colony from strain STOL from MEX; 3, colony from strain CH-1 and 4, colony from strain STOL from UB; 5, colony from strain CY-TO and 6, colony from strain CH-1 at MEX (isolated from shoot in water, table 2); 7, colony from strain PD at UB; 8, strain CY-TO from micropropagated periwinkle; 9, strain CH-1 from micropropagated periwinkle; 10, strain PD from micropropagated periwinkle; 11, strain STOL from micropropagated periwinkle; P, marker *Φ*X174 *Hae*III digested, fragment sizes (from top to bottom): 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118, and 72.

Discussion

This is the first published demonstration of axenic cultivation of phytoplasmas.

Successful axenic culture was shown by the similarity of colonial size and morphology with mycoplasmas, and confirmed by the molecular evidence of their identity. This included the sequencing and the typical RFLP profiles. In some of the amplicons, high molecular weight bands were visible and were related to aspecific amplification provided in some cases by the primers employed, since they could occasionally produce aspecific products, visualized as high bands in polyacrylamide gels, that were clearly different from phytoplasma amplicons RFLP profiles. It is clear from these results that the isolation from plants is the first critical stage and that the time for this is quite variable according to the phytoplasma strain and also the growth condition of the starting material. In particular, micropropagated shoots maintained in agar gave less time for acid colour change than those maintained in sterile distilled water for 10 d prior to isolation.

Phytoplasmas have defied all attempts at culture since their recognition in 1967; hence this paper is concerned solely with successful growth obtained in parallel in Bologna (Italy) and Reigate (UK) laboratories using the same solid and liquid media, commercially available. The results of this study clearly demonstrate that phytoplasmas, similarly to mycoplasmas, can grow independently from their host(s). Phytoplasmas can now be studied using these methods, for fulfilment of Koch postulates, and eventually to demonstrate their role in plant diseases.

Successful axenic culture of phytoplasmas will also allow direct *in planta* verification of the recent molecular findings both in plant/phytoplasma and insect/phytoplasma interactions. Phytoplasmas encode very few metabolic functions, and the presence of two glycolytic gene clusters in some strains suggested that increased consumption of carbon sources together with specific proteins ('tengu') interacting with plant/insect hormone balance could be linked to pathogenicity (Oshima *et al*., 2004, 2007;

Table 3. Comparison of nucleotide sequences obtained from phytoplasma colonies with those of original phytoplasmas in plants.

TBB gb | EF193359.1 - Tomato big bud phytoplasma 16S ribosomal RNA gene, complete sequence Length=511 Identities = 468/469 (99%), Gaps = 0/469 (0%)

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Query GTCTTTACTGACGCTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTA
      |||||| |||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct GTCTTAACTGACGCTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTA

Query GTCCACGCCGTAAACGATGAGTACTAAGTGTCGGGTTAAACCGGTACTGAAGTTAACACA
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct GTCCACGCCGTAAACGATGAGTACTAAGTGTCGGGTTAAACCGGTACTGAAGTTAACACA

Query TTAAGTACTCCGCCTGAGTAGTACGTACGCAAGTATGAAACTTAAAGGAATTGACGGGAC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct TTAAGTACTCCGCCTGAGTAGTACGTACGCAAGTATGAAACTTAAAGGAATTGACGGGAC

Query TCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAGATACCCGAAAAACCTTACCAGGT
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct TCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAGATACCCGAAAAACCTTACCAGGT

Query CTTGACATGTTTTTGCAAAATGATAGTAATATCGTGGAGGTTACCAGAAACACAGGTGGT
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct CTTGACATGTTTTTGCAAAATGATAGTAATATCGTGGAGGTTACCAGAAACACAGGTGGT

Query GCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTAGGTTAAGTCCTAAAACGAGCGAAAC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct GCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTAGGTTAAGTCCTAAAACGAGCGAAAC

Query CCTTATCGTTAGTTGCCAGCACGTTATGGTGGGGACTTTAACGAGACTGCCAATGATAAA
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct CCTTATCGTTAGTTGCCAGCACGTTATGGTGGGGACTTTAACGAGACTGCCAATGATAAA

Query TTGGAGGAAGGTGAGGATCACGTCAAATCAGCATGCCCCTTATGACCTG
      |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct TTGGAGGAAGGTGAGGATCACGTCAAATCAGCATGCCCCTTATGACCTG
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CH-1 gb | EU249337.1 | Stolbur clone 2 CH-1 16S ribosomal RNA gene, partial sequence Length=511 Identities = 466/466 (100%), Gaps = 0/466 (0%)

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      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct GATTAGATACCCTGGTAGTCCACGCCCTAAACGATGAGTACTAAACGTTGGATAAAACCA

Query GTGTTGAAGTTAACACATTAAGTACTCCGCCTGAGTAGTACGTACGCAAGTATGAAACTT
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct GTGTTGAAGTTAACACATTAAGTACTCCGCCTGAGTAGTACGTACGCAAGTATGAAACTT

Query AAAGGAATTGACGGGACTCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAGGTACCC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct AAAGGAATTGACGGGACTCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAGGTACCC

Query GAAAAACCTCACCAGGTCTTGACATGCTTTTGCAAAGCTGTAGAAATACAGTGGAGGTTA
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct GAAAAACCTCACCAGGTCTTGACATGCTTTTGCAAAGCTGTAGAAATACAGTGGAGGTTA
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(*Continued*)

Table 3. *Continues.*

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Query ACTGCCAATGATAAATTGGAGGAAGGTGGGGACGACGTCAAATCATCATGCCCCTTATGA
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct ACTGCCAATGATAAATTGGAGGAAGGTGGGGACGACGTCAAATCATCATGCCCCTTATGA

Query CCTGGGCTACAAACGTGATACAATGGCTGTTACAAAGGGTAGCTGA
      |||||||||||||||||||||||||||||||||||||||||||||
Sbjct CCTGGGCTACAAACGTGATACAATGGCTGTTACAAAGGGTAGCTGA
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STOL gb|JQ797670.1| Phytoplasma stolbur elongation factor Tu (tuf) gene, partial cds
Length=946 Identities = 763/764 (99%), Gaps = 1/764 (0%)

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Query AAAAAAGACACTATGCTCACGTTGATTGTCCAGGACACGCCGATTATATTAAAAACATGA
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct -AAAAAGACACTATGCTCACGTTGATTGTCCAGGACACGCCGATTATATTAAAAACATGA

Query TTACTGGTGCTGCTCAAATGGATGCCGCTATTTTAGTAGTTTCTGGCGCAGATAGTGTTA
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct TTACTGGTGCTGCTCAAATGGATGCCGCTATTTTAGTAGTTTCTGGCGCAGATAGTGTTA

Query TGCCTCAAACTAGAGAACATATTTTGTTAGCTCGCCAAGTAGGGGTTCCTAAAATTGTTG
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Sbjct TGCCTCAAACTAGAGAACATATTTTGTTAGCTCGCCAAGTAGGGGTTCCTAAAATTGTTG

Query TTTTCTTAAACAAATGTGACCTTTGTCCGGATGAAGAAATTTTAGAATTAGTTGAAATGG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct TTTTCTTAAACAAATGTGACCTTTGTCCGGATGAAGAAATTTTAGAATTAGTTGAAATGG

Query AAGTCCGTGAATTATTATCTAAATATGATTTTCCGGGCGATGATACCCCTATTATTAGAG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query GTTCTGCGTTAAAAGCTTTAGAAGGTGACAAACATTACATTGCACAAGTTAACGAATTAA
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Query TCAACGCTTTAGATTCTTACATTGAAGATCCAGTGCGTGAAGTTGATAAACCTTTCTTAA
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct TCAACGCTTTAGATTCTTACATTGAAGATCCAGTGCGTGAAGTTGATAAACCTTTCTTAA

Query TGCCAGTCGAAGATGTTTTCACTATCACTGGTAGAGGAACAGTAGTTACTGGTAGAGTTG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct TGCCAGTCGAAGATGTTTTCACTATCACTGGTAGAGGAACAGTAGTTACTGGTAGAGTTG
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(*Continued*)

Table 3. *Continues.*

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Query AAAGAGGACAAGTTAAAGCTGGTGATGAAATAGAAATCATAGGTCTTAAAGACACTAGAA
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct AAAGAGGACAAGTTAAAGCTGGTGATGAAATAGAAATCATAGGTCTTAAAGACACTAGAA

Query AAACCATCGTAACAGCAATTGAAATGTTTAAAAAAGATTTAGATTTTGCTCAAGCAGGCG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct AAACCATCGTAACAGCAATTGAAATGTTTAAAAAAGATTTAGATTTTGCTCAAGCAGGCG

Query ATAACGTTGGAGCTTTGTTGCGTGGAATTAACCGTGAAGATGTTCAACGTGGTCAAGTGT
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct ATAACGTTGGAGCTTTGTTGCGTGGAATTAACCGTGAAGATGTTCAACGTGGTCAAGTGT

Query TAGCCAAACCAGGTTCTGTGAAACCTCATTTTCAATTTGTGGCG
      |||||||||||||||||||||||||||||||||||||||||||
Sbjct TAGCCAAACCAGGTTCTGTGAAACCTCATTTTCAATTTGTGGCG
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Hogenhout *et al*., 2008; Hoshi *et al*., 2009). Moreover, the phytoplasma surface membrane protein Amp produces, in some strains, a complex with insect microfilament proteins correlated with insect transmissibility (Suzuki *et al*., 2006; Kakizawa *et al*., 2009). Phytoplasma SAP11 protein increases insect fecundity and also induces plant modification, suggesting its role in regulation of plant defence responses to pathogens and pests (Sugio and Hogenhout, 2012).

The demonstration of axenic growth of plant pathogenic phytoplasmas will give capability of studying biology and pathogenicity mechanisms of these important pathogens. Knowledge of the mechanisms underlying the autonomous life of phytoplasmas, that are among the smallest self-replicating organisms, should also provide important information about basic mechanisms of life.

The methods described here could introduce a new micro world, showing both the simplicity and complexity of life. Studying the mechanisms used by phytoplasmas to survive in two kingdoms (plants and animals) may help the understanding of the origin of life. Research with phytoplasma axenic cultures offers the prospect to further study the interrelationship between plants and these pathogens and plants and insect vectors, ultimately leading to reductions in the yield and quality losses due to phytoplasma diseases in important agricultural crops.

Acknowledgements

The authors dedicate this paper to the memory of R.F. Whitcomb, L. Carraro and M. Garnier who greatly contributed to phytoplasma research.

Literature cited

Bai X., J. Zhang, A. Ewing, S.A. Miller, A.J. Radek, D.V. Shevchenko, K. Tsukerman, T. Walunas, A. Lapidus, J.W. Campbell and S.A. Hogenhout, 2006. Living with genome instability: the adaptation of phytoplasmas to diverse environments of their insect and plant hosts. *Journal of Bacteriology* 188, 3682–3696.

Bertaccini A., N. Contaldo, A. Calari, S. Paltrinieri, H.M. Windsor and D. Windsor, 2010. Preliminary results of axenic growth of phytoplasmas from micropropagated infected periwinkle shoots. *18th Congress IOM*, Chianciano Terme, Italy, Abstract P-147, p. 153.

Chalker V.J., W.M.A. Owen, C. Paterson, E. Barker, H. Brooks, A.N. Rycroft and J. Brownlee, 2004. Mycoplasma associated with canine infectious respiratory disease. *Microbiology* 150, 3491–3497.

Doi Y., M. Teranaka, K. Yora and H. Asuyama, 1967. Mycoplasma or PLT grouplike microrganisms found in the phloem elements of plants infected with mulberry dwarf, potato witches' broom, aster yellows or pawlonia witches' broom. *Annals Phytopathological Society Japan* 33, 259–266.

Gibb K.S., A.C. Padovan and B.D. Mogen, 1995. Studies on sweet potato little-leaf phytoplasma detected in sweet potato and other plant species growing in Northern Australia. *Phytopathology* 85, 169–174.

Gundersen D.E. and I-M. Lee, 1996. Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. *Phytopathologia Mediterranea* 35, 144–151.

Hannan P.C.T., G.D. Windsor, A.D.E. Jong, N. Schmeer and M. Stegemann, 1997. Comparative susceptibilities of various animal-pathogenic mycoplasmas to fluoroquinolones. *Antimicrobic Agents and Chemotherapy* 41, 2037–2040.

Hardoim P.R., L.S. van Overbeek and van Elsas JD, 2008. Properties of bacterial endophytes and their proposed role in plant growth. *Trends in Microbiology* 16, 463–471.

Hogenhout S.A., K. Oshima, E.-D. Ammar, S. Kakizawa, H.N. Kingdom and S. Namba, 2008. Phytoplasmas: bacteria that manipulate plants and insects. *Molecular Plant Pathology* 9, 403–423.

Hoshi A., K. Oshima, S. Kakizawa, Y. Ishii, J. Ozeki, M. Hashimoto, K. Komatsu, S. Kagiwada, Y. Yamaji and S. Namba, 2009. A unique virulence factor for proliferation and dwarfism in plants identified from a phytopathogenic bacterium. *Proceedings of National Academy of Science U.S.A.* 106, 6416–6421.

IRPCM, 2004. '*Candidatus* Phytoplasma', a taxon for the wall-less, non-helical prokaryotes that colonize plant phloem and insects. *International Journal of Systematic and Evolutionary Microbiology* 54, 1243–1255.

Ishiie T., Y. Doi, K. Yora and H. Asuyama, 1967. Suppressive effects of antibiotics of tetracycline group on symptom development of mulberry dwarf disease. *Annals of Phytopathological Society of Japan* 33, 267–275.

Kakizawa S., K. Oshima, Y. Ishii, A. Hoshi, K. Maejima, H.Y. Jung, Y. Yamaji and S. Namba, 2009. Cloning of immunodominant membrane protein genes of phytoplasmas and their *in planta* expression. *FEMS Microbiological Letters* 293, 92–101.

Kube M., B. Schneider, H. Kuhl, T. Dandekar, K. Heitmann, A.M. Migdo, R. Reinhardt and E. Seemüller, 2008. The linear chromosome of the plant-pathogenic mycoplasma '*Candidatus* Phytoplasma mali'. *BMC Genomics* 9, 306.

Kunkel L.O. 1926. Studies on aster yellows. *American Journal of Botany* 13, 646–705.

Kunkel L.O. 1931. Celery yellows of California not identical with the aster yellows of New York. *Boyce Thompson Institute* 4, 405–414.

Kunkel L.O. 1955. Cross protection between strains of aster yellow-type viruses. *Advances in Virus Research* 3, 251–273.

Lee I-M. and R.E. Davis, 1992. Mycoplasmas which infect plants and insects, p 379-390. In: *Mycoplasmas - Molecular Biology and Pathogenesis*, (Maniloff J., McElhaney R.N., Finch L.R., Baseman J.B., eds.), Washington, DC, USA.

Lee I-M., D.E. Gundersen R.W., R.W. Hammond and R.E. Davis, 1994. Use of mycoplasmalike organism (MLO) group-specific oligonucleotide primers for nested-PCR assays to detect mixed-MLO infections in a single host plant. *Phytopathology* 84, 559–566.

Lee I-M., A. Bertaccini, M. Vibio and D.E. Gundersen, 1995. Detection of multiple phytoplasmas in perennial fruit trees with decline symptoms in Italy. *Phytopathology* 85, 728–735.

Lee I-M., D.E. Gundersen-Rindal and A. Bertaccini, 1998. Phytoplasma: ecology and genomic diversity. *Phytopathology* 88, 1359–1366.

Maramorosch K, 2011. Historical reminiscences of phytoplasma discovery. *Bulletin of Insectology* 64 (Suppl.), S5–S8.

McCoy R.E., A. Caudwell, C.J. Chang, T.A. Chen, L.N. Chiykowskyi, M.T. Cousin, G.T.N. Dale de Leeuw, D.A. Golino, K.J. Hackett, B.C. Kirkptrick, R. Marwitz, H. Petzold, R.H. Shina, M. Sugiura, R.F. Whitcomb, I.L. Yang, B.M. Zhu and E. Seemüller, 1989. Plant Diseases Associated with Mycoplasmalike Organisms. In: *The Mycoplasmas*, (Whitcomb R.F., Tully J.G., eds.), Vol. 5, New York, NY, USA.

Oshima K., S. Kakizawa, H. Nishigawa, H-Y. Jung, W. Wei, S. Suzuki, R. Arashida, D. Nakata, S. Miyata, M. Ugaki and S. Namba, 2004. Reductive evolution suggested from the complete genome sequence of a plant-pathogenic phytoplasma. *Nature Genetics* 36, 27–29.

Oshima K., S. Kakizawa, R. Arashida, Y. Ishii, A. Hoshi, Y. Hayashi, S. Kawagida and S. Namba, 2007. Presence of two glycolityc gene clusters in a severe pathogenic line of '*Candidatus* Phytoplasma asteris'. *Molecular Plant Pathology* 8, 481–489.

Oshima K., Y. Ishii, S. Kakizawa, K. Sugawara, Y. Neriya, M. Himeno, N. Minato, C. Miura, T. Shiraishi, Y. Yamaji and S. Namba, 2011. Dramatic transcriptional changes in an intracellular parasite enable host switching between plant and insect. *PLoS One* 6, e23242.

Pitcher D.G., D. Windsor, H. Windsor, J.M. Bradbury, C. Yavari, J.S. Jensen, C. Ling and D. Webster. 2005. *Mycoplasma amphoriforme* sp. nov., isolated from a patient with chronic bronchopneumoniae. *International Journal of Systematic and Evolutionary Microbiology* 55, 2589–2594.

Schaff D.A., I-M. Lee and R.E. Davis, 1992. Sensitive detection and identification of mycoplasmalike organisms by polymerase chain reactions. *Biochemic Biophysic Research Communication* 186, 1503–1509.

Schneider B., K.S. Gibb and E. Seemüller, 1997. Sequence and RFLP analysis of the elongation factor Tu gene used in differentiation and classification of phytoplasmas. *Microbiology* 143, 3381–3389.

Staden R., K.F. Beal and J.K. Bonfield, 2000. The Staden package. *Methods in Molecular Biology* 132, 115–130.

Sugio A. and S.A. Hogenhout, 2012. The genome biology of phytoplasma: modulators of plants and insects. *Current Opinion in Microbiology* 15, 247–254.

Sugio A., A.M. MacLean, H.N. Kingdom, V.M. Grieve, R. Manimekalai and S.A. Hogenhout, 2011. Diverse targets of phytoplasma effectors: from plant development to defense against insects. *Annual Revue of Phytopathology* 49, 175–195.

Suzuki S., K. Oshima, S. Kakizawa, R. Arashida, H.Y. Jung, Y. Yamaji, H. Nishigawa, M. Ugaki and S. Namba, 2006. Interaction between the membrane protein of a pathogen and insect microfilament complex determines insect-vector specificity. *Proceedings of National Academy of Science U.S.A.* 103, 4252–4257.

Thompson J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin and D.G. Higgins, 1997. The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acid Research* 24, 4876–4882.

Tolu G., S. Botti, R. Garau, V.A. Prota, A. Sechi, U. Prota and A. Bertaccini, 2006. Identification of 16SrII-E phytoplasmas in *Calendula arvensis* L., *Solanum nigrum* L. and *Chenopodium* spp. *Plant Disease* 90, 325–330.

Tran-Nguyen L.T., M. Kube, B. Schneider, R. Reinhardt and K.S. Gibb, 2008. Comparative genome analysis of '*Candidatus* Phytoplasma australiense' (subgroup tuf-Australia I; rp-A) and '*Ca.* Phytoplasma asteris' strains OY-M and AY-WB. *Journal of Bacteriology* 190, 3979–3991.

Webster D., H. Windsor, C. Ling, D. Windsor and D. Pitcher, 2003. Chronic bronchitis in immunocompromised patients: association with a novel *Mycoplasma* species. *European Journal of Clinic Microbiology Infectious Diseases* 22, 530–534.

Windsor G.D., J.M. Bradbury and C.M. Dare, 2010. A putative *Mycoplasma* species isolated from a salt-water fish. *18th Congress of the International Organization for Mycoplasmology*. Abstract 190, p. 180.

Accepted for publication: December 12, 2012