Eur J Plant Pathol (2007) 118:411–416
DOI 10.1007/s10658-007-9142-8

SHORT COMMUNICATION

Occurrence and distribution of '*Candidatus* Phytoplasma trifolii' associated with diseases of solanaceous crops in Lebanon

Elia Choueiri · Pascal Salar · Fouad Jreijiri · Souheir El Zammar · Randa Massaad · Hani Abdul-Nour · Joseph-Marie Bové · Jean-Luc Danet · Xavier Foissac

Received: 6 October 2006 / Accepted: 16 April 2007 / Published online: 5 June 2007

Abstract A survey for phytoplasma diseases in tomato and pepper fields in Lebanon was conducted during 2003 and 2004. Tomato plants with stunting, yellowing or purplish leaves, proliferation of laterals buds, hypertrophic calyxes and virescent flowers were found in 25% of the tomato fields surveyed, where they represented 2–8% of the plants. Pepper plants displaying stunting and yellowing of leaves, were found in 27% of the fields and 1–4% of the plants were affected. Phytoplasmas infecting tomato and pepper had identical 16S-rDNA RFLP profiles and sequences. A phytoplasma isolate named PTL was transmitted by dodder from a diseased tomato plant to a periwinkle (*Catharanthus roseus*) plant in which it induced leaf yellowing, virescence and phyllody. 16S-rDNA phylogenetic analysis classified PTL as a strain of '*Candidatus* Phytoplasma trifolii'.

E. Choueiri · F. Jreijiri · S. El Zammar · R. Massaad
Lebanese Agricultural Research Institute, Tal Amara, PO Box 287, Zahle, Lebanon

H. Abdul-Nour
Faculté des Sciences II, Université Libanaise, Fanar, Liban

P. Salar · J.-M. Bové · J.-L. Danet · X. Foissac (✉)
UMR Génomique Diversité et Pouvoir Pathogène, INRA Université Victor Ségalen Bordeaux 2, BP 81, 33883 Villenave d'Ornon cedex, France
e-mail: foissac@bordeaux.inra.fr

Keywords Tomato disease · Pepper disease · Lebanon · Phloem-restricted bacteria · Leafhopper · Psyllid

Tomato (*Lycopersicon esculentum*), and pepper (*Capsicum annuum*) are important vegetable crops in Lebanon. Solanaceous crops are planted all over Lebanon but in particular in the following regions: the Bekaa valley, the Byblos area north of Beirut, the Akkar valley in the north, Mount Lebanon and to a lesser extent in the south. The two crops are cultivated for local consumption, industry and export but their production is limited by many insect pests and diseases which cause heavy economic losses (Choueiri et al. 2004).

Phytoplasmas are phloem-limited bacteria associated with many plant diseases world-wide and are transmitted by phloem-feeding hemipteran insect-vectors (Lee et al. 2000; Weintraub and Beanland 2006). Infection of tomato, pepper and potato by phytoplasma have been reported from several areas in the Mediterranean basin (Avinent and Llacer 1995; Cousin et al. 1968; Cousin et al. 1989; Fos et al. 1992; Marcone and Ragozzino 1995; Zimmerman-Gries and Klein 1978). In Italy, phytoplasmas from phylogenetic groups 16SrI, III, V and XII were identified by PCR and RFLP analysis of 16S-rDNA amplicons (Del Serrone et al. 2001). In Australia, phytoplasmas from group 16SrII were demonstrated to be responsible for tomato big bud (Davis et al.

1997), whereas in California similar symptoms were associated with phytoplasmas of group 16SrVI (Shaw et al. 1993). Phytoplasmas in 16SrVI group, previously called Clover proliferation (CP) group, were described associated with a disease of alsike clover in Canada originally associated with a yellows-type virus (Chiykowski 1965). Subsequent investigations revealed that CP was in fact a phytoplasma also associated with phytoplasma diseases of tomato as well as potato and elm in North America (Chen and Hiruki 1975; Deng and Hiruki 1991; Jacobs et al. 2003; Lee et al. 1991; Shaw et al. 1993). CP is now taxonomically described as '*Candidatus* Phytoplasma trifolii'(Hiruki and Wang 2004). This phytoplasma was recently associated with diseases of pepper in Spain and tomato plants in Jordan (Anfoka et al. 2003; Castro and Romero 2002).

We reported from a preliminary investigation, the occurrence of phytoplasma diseases in two tomato and pepper fields in the Bekaa valley, which could be associated with infection by phytoplasmas related to the clover proliferation group (Choueiri et al. 2002). As a follow up, an extensive survey was undertaken to determine the disease incidence in Lebanon and to isolate and confirm the identify of the phytoplasma strain associated with the observed disorders. Surveys were conducted during the 2003 and 2004 growing seasons in the major tomato and pepper cultivation regions of Lebanon. In each region, a certain number of fields were arbitrarily selected and inspected from June to October. Fifty fields were selected in the Bekaa valley, 35 fields in Mount Lebanon, 25 fields in the north and 20 fields in the south. Incidence of phytoplasma infection (% of plants with phytoplasma symptoms) was estimated for each field by visual inspection of 1,000 plants following a W pattern (crossing the rows) as a sampling procedure. Symptoms of phytoplasma diseases were common in the fields surveyed particularly on tomato. Tomato plants generally showed stunting, yellowing or purplish leaves, intense proliferation of laterals buds, hypertrophic calixes, virescent flowers, and inhibition of anther and ovary formation. Plant apices generally lacked leaves and the youngest leaves were very small, thick and distorted (Fig. 1A and 1B). The occurrence, distribution and relative incidence of phytoplasma infection varied between the different growing regions (Table 1). Symptoms of phytoplasma infections were seen in 11 fields in 2003 and 14 fields in 2004, in three regions (Bekaa Valley, Mount Lebanon and North Lebanon). No symptoms of phytoplasma infection were detected in South Lebanon. Incidence of symptomatic plants varied greatly, not only between regions but also between fields. Symptomatic plants were seen in fields located in Ferzoul, Kab-Elias (central Bekaa) or in West Bekaa. Symptomatic plants in the fields surveyed in 2003 in the north of Lebanon and Mount of Lebanon were few (2–4%); however the incidence of symptomatic plants in the same regions slightly increased in 2004. The highest disease incidence was recorded in central Bekaa and amounted to 8% in 2004 (Table 1). In the case of pepper, plants suspected to be infected with phytoplasma showed stunting, yellowing and small leaves with wavy margins. Inspection fields showed that the central Bekaa was the most affected region and had a high incidence of phytoplasma infection up to 4% of infected plants in some fields. The Mount Lebanon area was less affected than the Bekaa valley. In 2004, the symptoms were seen in six of the pepper fields and were more severe than in 2003; no pepper fields were monitored in the south of Lebanon.

Nucleic acids were extracted from 0.5 g of leaf midvein of symptomatic and symptomless plants of tomato and pepper, using the CTAB (cethyltrimethyl-ammonium bromide) extraction protocol (Maixner et al. 1995). DNA from periwinkle plants infected with the StolC isolate of the stolbur phytoplasma was used as a control. The stolbur—infected periwinkle plants were from the phytoplasma collection at INRA Bordeaux. The universal phytoplasma PCR primer pair fU5/rU3 (Seemüller et al. 1994) was used for PCR amplification of a part of phytoplasma 16S-rDNA from plant samples. A PCR product of 0.9 kbp was amplified from DNA extracted from 35 symptomatic tomato plants and 16 pepper plants collected from 10 different fields in Bekaa valley, Mount Lebanon and North Lebanon. No PCR product was obtained in the case of symptomless plants. All fU5/rU3 PCR products amplified from peppers (Fig. 2, lanes 1–2) and tomato plants (Fig. 2, lanes 3–9) gave identical RFLP profiles with *Alu*I (Fig. 2A) and *Rsa*I (Fig. 2B), clearly different from the RFLP pattern of the stolbur phytoplasma (Fig. 2, lane St). The fU5/rU3 PCR products obtained from three tomatoes and two peppers had 100% sequence identity with the corresponding sequence of Washington Potato Purple Top

Fig. 1 Stunted tomato plants with heavy proliferations of small purplish leaves (**A**), hypertrophic calixes (big bud) on infected tomato plant (**B**). Periwinkle plant infected by '*Ca.* P. trifolii' strain PTL displaying yellows, virescence (**C**), and phyllody (**D**)

phytoplasma strain PPT (genbank accession number AY496004), classified as '*Ca.* P. trifolii' (Hiruki and Wang 2004). To obtain a reference strain of the Lebanese tomato/pepper agent, the phytoplasma was transmitted by dodder (*Cuscuta campestris*) from an infected tomato plant collected in central Bekaa to a *Catharanthus roseus* periwinkle plant. Following dodder inoculation, one of the periwinkle plants displayed pronounced greening of petals and eventually leaf yellowing, virescence and phyllody (Fig. 1C, D). The diseased periwinkle plant was diagnosed positively for phytoplasma using universal primers P1/P7 (Schneider et al. 1995) and the PCR product was sequenced (genbank accession number AM260486). This phytoplasma isolate was named PTL (for Phytoplasma Tomato Lebanon). Comparison and phylogenetic analysis by maximum parsimony of the 16S-rDNA and 16S-23S intergenic sequence (P1P7 PCR product) using MEGA2 software (Kumar et al. 2001), clearly identified the PTL isolate as a strain of '*Ca.* P. trifolii' in the 16SrVI phylogenetic group of the phytoplasma taxonomy (Hiruki and Wang 2004; Lee et al. 2000) (Fig. 3).

As a preliminary inventory of insects which could act as vectors of '*Ca.* P. trifolii' in Lebanon, a search was conducted for insect hosts. Hemipteran insects such as leafhoppers, planthoppers and psyllids were captured with an aspirator in different tomato fields and in non-cultivated areas from North Lebanon and the Bekaa Valley (localities: Kfar Habou, Koussaya and Safra) from June to September, and from 2002 to 2005. Nucleic acids were extracted from batches of 1–5 insects depending on insect size, with the method used for plants. Insects were tested by fU5/rU3 PCR and amplified products were sequenced to characterize the detected phytoplasma. Of 179 insect batches tested, 16 gave positive amplification reactions. Five different phytoplasmas could be identified by sequencing the PCR products. '*Ca.* P. asteris' (group 16SrI) was detected in 5 of 6 batches of *Euscelis*

Table 1 Occurrence of phytoplasma symptoms in tomato and pepper fields during 2003 and 2004 surveys in different regions of Lebanon

Crop	Tomato		Pepper	
	Affected fields[a]	Incidence[b] (% in positive fields)[c]	Affected fields[a]	Incidence[b] (% in positive fields)[c]
Bekaa Valley				
2003	5/15	2–4 (2.1, 2.2, 3, 2, 4)	2/10	2–3 (2, 3)
2004	6/15	5–8 (8, 6.4, 5, 5, 6.8, 6)	4/10	2–4 (2, 2, 2.1, 4)
Mount Lebanon				
2003	4/15	2–4 (3.1, 2.3, 4, 2)	0/3	–
2004	5/15	2–5 (2.2, 2, 3, 2.1, 5)	1/2	1.2
North Lebanon				
2003	2/10	2–3 (2, 3)	–	–
2004	3/10	2–4 (2.2, 4, 2)	1/5	2.3
South Lebanon				
2003	0/15	–	–	–
2004	0/5	–	–	–

[a] Number of affected fields / total number of fields surveyed

[b] Percentage of symptomatic plants for 1000 plants

[c] Percentage of symptomatic plants in positive fields

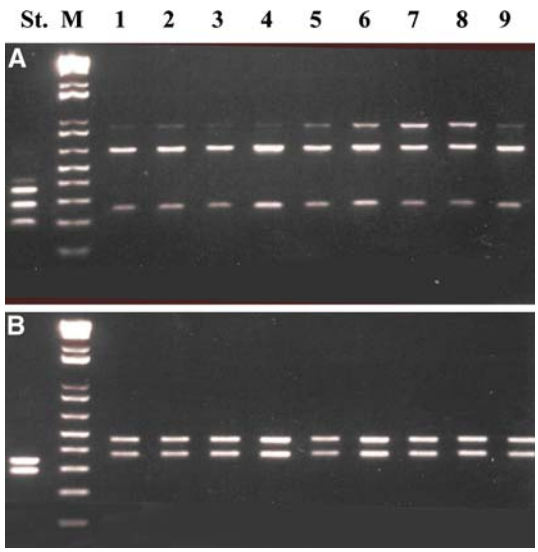

Fig. 2 RFLP analysis by *Alu*I (**A**) and *Rsa*I (**B**) digestion of fU5-rU3 16S-PCR products from diseased pepper (lanes 1–2) and tomato plants (lanes 3–9). Phytoplasma reference was stolbur phytoplasma (lane St). Lane M, 1 kb Plus DNA ladder from Invitrogen

incisus Kirschbaum and in 2 of 24 batches of *Psammotettix provincialis* Ribaut. The *Lactuca serriola* phytoplasma (group 16SrIX) (Verdin et al. 2003) was identified in 1 of 3 batches of *Circulifer* sp. Zachvatkin and in 4 of 6 batches of *Neoaliturus fenestratus* Herrich-Schäffer. The vigna little leaf phytoplasma of group 16SrIX (Schneider et al. 1999) and '*Ca.* P. rhamni' were detected in 1 of 2 batches of *Selenocephalus tapan* Dlabola and 1 of 13 batches of *Cacopsylla myrthi* Puton respectively. Finally, *Anaceratagallia laevis* Ribaut (1 of 17 batches) and *Balclutha* sp. Kirkaldy (1 of 3 batches) were found to carry '*Ca.* P. trifolii'. Whether these latter leafhoppers are able or not to transmit this phytoplasma, remains to be determined. The beet leafhopper *Circulifer tenellus* Baker was reported in the USA as the vector of the Columbia basin potato purple top and tomato big bud phytoplasmas, two strains of '*Ca.* P. trifolii' (Munyaneza et al. 2006; Shaw et al. 1993). However, no beet leafhoppers were captured in tomato fields and surroundings even though it is known to be present in the eastern Mediterranean basin (Bové et al. 1987; Rasooly et al. 1994). In conclusion, '*Ca.* P. trifolii' was confirmed to be associated with a severe disease of tomato and pepper in three regions of Lebanon out of four surveyed. A Lebanese phytoplasma strain (PTL) was transmitted to periwinkle and characterized. These diseases were consistently observed and had a low incidence, which is in agreement with the low number of hemipteran insects found to carry the associated phytoplasma.

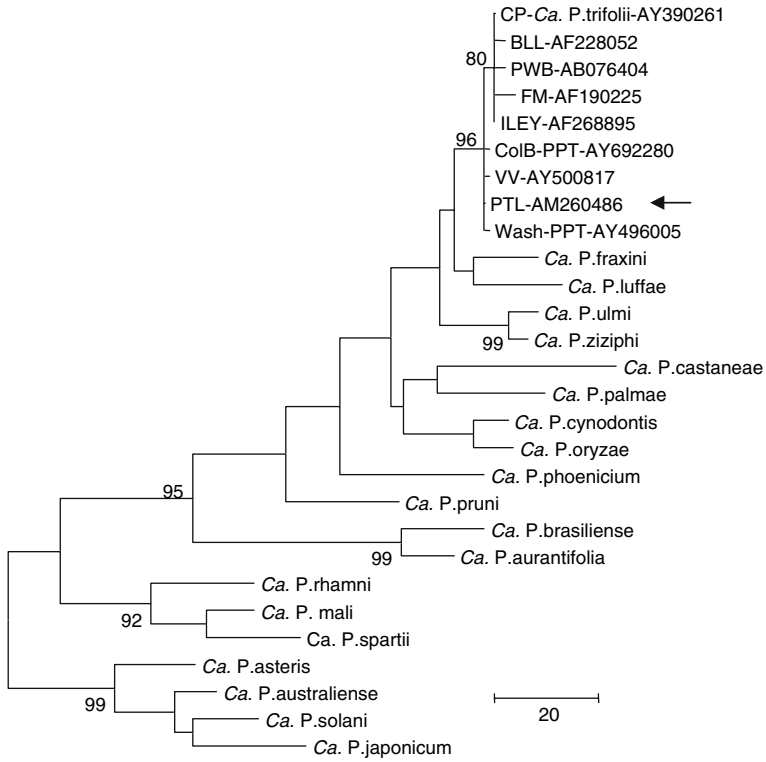

Fig. 3 Phylogenetic tree constructed with parsimony analysis of 16Sr-DNA sequences. '*Ca.* P. asteris', '*Ca.* P. australiense', '*Ca.* P. solani' and '*Ca.* P. japonicum' were taken as the outgroup. Branch lengths are proportional to the number of inferred character state transformation. Bootstrap values for 100 replicates are shown on branches. Black arrow indicates the position of the Lebanese isolate PTL. Sequences for '*Candidatus* Phytoplasma' species were downloaded from TreeBase (accession M1788) and group 16SrVI phytoplasma accessions from genbank

Finally, our data are in agreement with the presence of '*Ca.* P. trifolii' in the region after its detection in Jordan (Anfoka et al. 2003).

Acknowledgement This work was supported by the French Ministère des Affaires Étrangères and by the European Union (Projet CEDRE).

References

Anfoka, G. H. A., Khalil, A. B., & Fattash, I. (2003). Detection and molecular characterization of a phytoplasma associated with big bud disease of tomatoes in Jordan. *Journal of Phytopathology, 151*, 223–227.

Avinent, L., & Llacer, G (1995). Stolbur detection in Spain by polymerase chain reaction (PCR). *Plagas, 21*, 417–423.

Bové, J. M., Fos, A., & Lallemand, J. (1987). Epidemiology of *Spiroplasma citri* in the Oldworld. *Israel Journal of Medical Sciences, 23*, 663–666.

Castro, S., & Romero, J. (2002). The association of clover proliferation phytoplasma with stolbur disease of pepper in Spain. *Journal of Phytopathology, 150*, 25–29.

Chen, M. H., & Hiruki, C. (1975). Electron microscopy of mycoplasma-like bodies associated with clover proliferation disease. *Proceedings of the American Phytopathological Society, 2*, 52.

Chiykowski, L. N. (1965). A yellows-type virus of alsike clover in Alberta. *Canadian Journal of Botany, 43*, 527–536.

Choueiri, E., Jreijiri, F., El-Zammar, S., Verdin, E., Salar, P., Danet, J. L., Bové, J., & Garnier, M. (2002). First report of grapevine ''bois noir'' disease and a new phytoplasma infecting solanaceous plants in Lebanon. *Plant Disease, 86*, 697.

Choueiri, E., El-Zammar, S., Jreijiri, F., Mnayer, D., Massaad, R., Saad, A. T., Hanna, L., & Varveri, C. (2004). Phytosanitary status of potato in the Bekaa valley in Lebanon. *EPPO Bulletin, 34*, 117–122.

Cousin, M. T., Maillet, P. L., Gourret, J. P., Grison, C., & Staron, T. (1968). Présence de particules de type mycoplasme dans les plantes atteintes de ''jaunisse européenne'' Le stolbur de la tomate, l'aster yellow du glaïeul, la phyllodie du trèfle. Etudes cytologiques et ultrastructurales, Premiers essais de lutte chimique. *Compte rendu de l'Académie des Sciences série D Paris, 54*, 887–895.

Cousin, M. T., Dafalla, G., Demazeau, E., Theveu, E., & Grosclaude, J. (1989). In situ detection of MLOs for Solanaceae stolbur and faba bean phyllody by indirect immunofluorescence. *Journal of Phytopathology, 124*, 71–79.

Davis, R. I., Schneider, B., & Gibb, K. S. (1997). Detection and differentiation of phytoplasmas in Australia. *Australian Journal of Agricultural Research, 48*, 535–544.

Del Serrone, P., Marzachi, C., Bragaloni, M., & Galeffi, P. (2001). Phytoplasma infection of tomato in central Italy. *Phytopathologia Mediterranea, 40*, 137–142.

Deng, S. J., & Hiruki, C. (1991). Genetic relatedness between two nonculturable mycoplasmalike organisms revealed by nucleic acid hybridization and polymerase chain reaction. *Phytopathology, 81*, 1475–1479.

Fos, A., Danet, J. L., Zreik, L., Garnier, M., & Bové, J. M (1992). Use of a monoclonal antibody to detect the stolbur mycoplasma-like organism in plants and insects and to identify a vector in France. *Plant Disease, 76*, 1092–1096.

Hiruki, C., & Wang, K. (2004). Clover proliferation phytoplasma: '*Candidatus* Phytoplasma trifolii'. *International Journal of Systematic and Evolutionary Microbiology, 54*, 1349–1353.

Jacobs, K. A., Lee, I. M., Griffiths, H. M., Miller, F. D., & Bottner, K. D. (2003). A new member of the clover proliferation phytoplasma group (16SrVI) associated with elm yellows in Illinois. *Plant Disease, 87*, 241–246.

Kumar, S., Tamura, K., Jakobsen, I. B., & Nei, M. (2001). MEGA2: Molecular Evolutionary Genetics Analysis (MEGA) software. *Bioinformatics, 17*, 1244–1245.

Lee, I. M., Davis, R. E., & Gundersen-Rindal, D. E. (2000). Phytoplasma: phytopathogenic mollicutes. *Annual Review of Microbiology, 54*, 221–255.

Lee, I. M., Davis, R. E., & Hiruki, C. (1991). Genetic interrelatedness among clover proliferation mycoplasmalike organisms (MLOs) and other MLOs investigated by nucleic acid hybridization and restriction fragment length polymorphism analyses. *Applied & Environmental Microbiology, 57*, 3565–3569.

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

Marcone, C., & Ragozzino, A. (1995). Tomato big bud disease in southern Italy and characterization of its causal agent by RFLP analysis. *Petria, 5*, 195–205.

Munyaneza, J. E., Crosslin, J. M., & Upton, J. E. (2006). Beet leafhopper (*Hemiptera*:*Cicadellidae*) transmits the Columbia basin potato purple top phytoplasma to potatoes, beets and weeds. *Journal of Economic Entomology, 99*, 268–272.

Rasooly, R., Raccah, B., & Klein, M. (1994). Laboratory transmission of the citrus stubborn disease agent by a leafhopper from the *Circulifer tenellus* complex from the Jordan valley. *Phytoparasitica, 22*, 209–218.

Schneider, B., Seemüller, E., Smart, C. D., Kirkpatrick, B. C. (1995). Phylogenetic classification of plant pathogenic mycoplasmalike organisms or phytoplasmas. In *Molecular and diagnostic procedures in mycoplasmology* (Vol. 1, pp. 369–380). San Diego CA: Academic Press.

Schneider, B., Padovan, A., De la Rue, S., Eichner, R., Davis, R., Bernuetz, A., & Gibb K. (1999). Detection and differentiation of phytoplasmas in Australia: an update. *Australian Journal of Agricultural Research, 50*, 333–342.

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

Shaw, M. E., Kirkpatrick, B. C., & Golino, D. A. (1993). The beet leafhopper-transmitted virescence agent causes tomato big bud disease in California. *Plant Disease, 77*, 290–295.

Verdin, E., Salar, P., Danet, J. L., Choueiri, E., Jreijiri, F., El Zammar, S., Gélie, B., Bové, J. M., & Garnier, M. (2003). '*Candidatus* Phytoplasma phoenicium' sp. nov., a novel phytoplasma associated with an emerging lethal disease of almond trees in Lebanon and Iran. *International Journal of Systematic and Evolutionary Microbiology, 53*, 833–838.

Weintraub, P. G., & Beanland, L. (2006). Insect vectors of phytoplasmas. *Annual Review of Entomology, 51*, 91–111.

Zimmerman-Gries, S., & Klein, M. (1978). A tomato big bud-like disease of tomatoes in Israel and its association with mycoplasma like organisms. *Plant Disease Reporter, 62*, 590–594.