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Leek proliferation: A new phytoplasma disease in the Czech Republic and Italy

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Abstract

During the summer 1996, twelve of twenty-eight leek plants located in a garden near České Budějovice, South Bohemia exhibited symptoms typical of diseases associated with phytoplasmas. In summer 1998 similar symptoms were detected in leek plants in a field used for seed production located in Romagna, North Italy. In both cases the plants were established in the spring of the previous year. Plants showed flower abnormalities: stamen elongation, anther sterility, pistil proliferation, as well as poor, if any, seed production. Phytoplasma-like structures were detected by scanning and transmission electron microscopy in phloem sieve elements in the Czech diseased plants, but not in healthy ones. Nested-PCR amplifications of extracted DNA with phytoplasma-specific oligonucleotide primer pairs confirmed the presence of phytoplasmas in these plants at low concentrations. Restriction fragment length polymorphism analyses of amplified ribosomal sequences allowed the identification of detected phytoplasmas: all the samples from the Czech Republic contained aster yellows related phytoplasmas (16SrI-B) while in the Italian samples aster yellows related phytoplasmas (16SrI-B) together with stolbur related phytoplasmas (16SrXII-A) were identified. This is the first report of detection and identification of a phytoplasma disease of leek in the Czech Republic and Italy.

Introduction

Phytoplasmas (formerly mycoplasma-like organisms, MLO) are wall-less prokaryotes grouped in the class *Mollicutes* that are thought to be the causal agents of more than 300 diseases of higher plants (McCoy et al., 1989). Thus far none have been isolated in pure culture. The inability to isolate and culture phytoplasmas *in vitro* has impeded their identification and classification. Traditionally, diseases have been classified according to the specificity of vector transmission, host plant range and symptoms induced in infected plants. Transmission electron microscopy (TEM) allows the observation of characteristic phytoplasma morphology in sieve tubes of host plants (Doi et al., 1967). In recent years, phytoplasma-specific monoclonal antibodies (see review in Sarindu and Clark, 1993) and cloned DNA probes (see review in Gundersen et al., 1996; Chen and Lin, 1997) have been employed for phytoplasma detection and identification. The introduction of polymerase chain reaction (PCR) assays on conserved ribosomal genes has greatly advanced the capacity to detect and identify phytoplasmas. Through restriction fragment length polymorphism

(RFLP) analyses of PCR product phytoplasmas have been classified into distinct groups and subgroups (Lee et al., 1998; Seemüller et al., 1998).

Leek (*Allium ampeloprasum* L. var. *porrum*), a member of the family *Liliaceae*, is an important vegetable grown worldwide for its content of valuable proteins, vitamins, mineral substances, enzymes and ethereal oils. Several virus and fungal diseases occur in leek (Bos, 1983), but the only phytoplasma disease reported in this crop was in Romania (Ploaie et al., 1974).

Leek plants with stunted growth and flower abnormalities were found in the Czech Republic and Italy during 1996–1998. The combined application of electron microscopy and molecular techniques were used to verify phytoplasma presence and to identify them.

Materials and methods

Samples

During summer 1996, twelve of twenty-eight leek plants located in a garden near České Budějovice, South Bohemia, exhibited symptoms typical of diseases associated with phytoplasmas. In summer 1998 similar symptoms were detected in leek plants in a field for seed production located in Romagna, North Italy. In both cases the plants were established in the spring of the previous year and were showing symptoms of phyllody, reduced size and yellowing (Figure 1). Specimens were taken from phyllodied flower heads; symptomless leeks were used as healthy controls. Maryland aster yellows (AY); tomato big bud (BB) and clover phyllody (CPh) strains obtained from I.-M. Lee (USDA, Beltsville, MD, USA) were used as control for groups 16SrI-B, 16SrI-A and 16SrI-C respectively; peach X disease (CX) from B.C. Kirkpatrick (University of California, Davis, CA, USA via I.-M. Lee) for group 16SrIII, elm yellows (EY) from H. Griffith and W.A. Sinclair (Cornell University, NY, USA) for group 16SrV, apple proliferation (AP) from L. Carraro (Università di Udine, Italy) for group 16SrX-A, and Italian periwinkle virescence (IPVR) for group 16SrXII-A were employed as reference phytoplasma strains.

Scanning and transmission electron microscopy

Tissue specimens obtained from flowers, with and without phyllody symptoms, were cut into 2-mm sections and immediately fixed with 5% glutaraldehyde in 0.1 M potassium phosphate buffer pH 7.2, postfixed in 2% osmium tetraoxide in the same buffer, dehydrated through a graded acetone series and dried at the critical point. The samples were coated with gold under vacuum and examined with Jeol 6300 scanning electron microscope (SEM). Ultrathin sections for TEM were prepared as described earlier by Fránová et al. (1996).

Figure 1. Leek flowers with proliferation symptoms.

Phytoplasma identification

Nucleic acid samples from symptomless controls, from 6 leek plants showing symptoms collected in South Bohemia and from two collected in Romagna, were extracted from about 2 g of freshly cut phyllodied flowers and stems according to the method described by Lee et al. (1991). PCR experiments were carried out with nucleic acid samples from leek and with nucleic acid from periwinkle control samples diluted in TE buffer [10 mM Tris-HCl, 1 mM EDTA (pH 8.0)] to give a final concentration of 20 ng μl^{-1} (Schaff et al., 1992) in total 25 µl reaction mixtures containing 0.5 µl of nucleic acid, 200 µmol l^{-1} of each dNTP, 1.25 U Taq polymerase (Perkin Elmer, Norwalk, CT, USA) and 0.4 µmol l^{-1} of primers. General ribosomal primers R16F1/R0 and R16F2n/R2 (Gundersen et al., 1994; Lee et al., 1995) amplifying

fragments internal to each others were employed in nested-PCR. Further 16S rRNA group-specific primer pairs were used in nested-PCR assays to eliminate aspecific amplification and to verify the presence of mixed infection. In particular R16(I)F1/R1 primer pair was employed for phytoplasmas belonging to groups 16SrI and 16SrXII, R16(III)F2/R1 for group 16SrIII, R16(V)F1/R1 for group 16SrV, R16(X)F1/R1 for group 16SrX (Lee et al., 1994, 1995). Nested-PCR was also performed to indagate about sequence variability in the spacer region in the phytoplasmas infecting leek compared with those of control strains using primers 16R723f/P7 followed by primers 16R758f/m23SR amplifying from base 758 in the ribosomal gene to the beginning of the 23S ribosomal gene (Martini et al., 1998). Thirty-five PCR cycles were conducted in an automated thermal cycler following the procedure described by Schaff et al. (1992). Tubes with the reaction mixture devoid of DNA templates or containing DNA from symptomless samples were included in each experiment as negative controls.

Ten µl of PCR product amplified with R16F2n/R2, with 16R758f/m23SR and with phytoplasma group specific primers were digested using enzymes *Kpn*I, *Alu*I, *Hha*I and *Mse*I at 37 °C for at least 16 h and with *Taq*I at 65 °C for the same period following the instructions of the manufacturer and the restrictions patterns were compared with those of control strains after electrophoresis through a 5% polyacrylamide gel.

Results

Scanning and transmission electron microscopy

Numerous spherical bodies with sizes varying from 60 to 1500 nm were found by SEM only in the phloem of plants showing symptoms (Figure 2). In some cases, their concentrations were very high, mainly along the cell wall of the sieve elements or near the sieve plates. A few phytoplasmas were observed only in sieve elements of diseased plants also by TEM (Figure 3). No other pathogenic organisms, bacteria, virus or virus-like structures, were observed.

Phytoplasma identification

Using the general primer pairs R16F2n/R2 in nested-PCR on the R16F1/R0 product a 1200 bp band was obtained only from leek samples showing symptoms from both Czech Republic and Italy and from control strains (Figure 4a). RFLP analyses of these products with *Kpn*I, *Alu*I, *Hha*I (data not shown) and *Mse*I

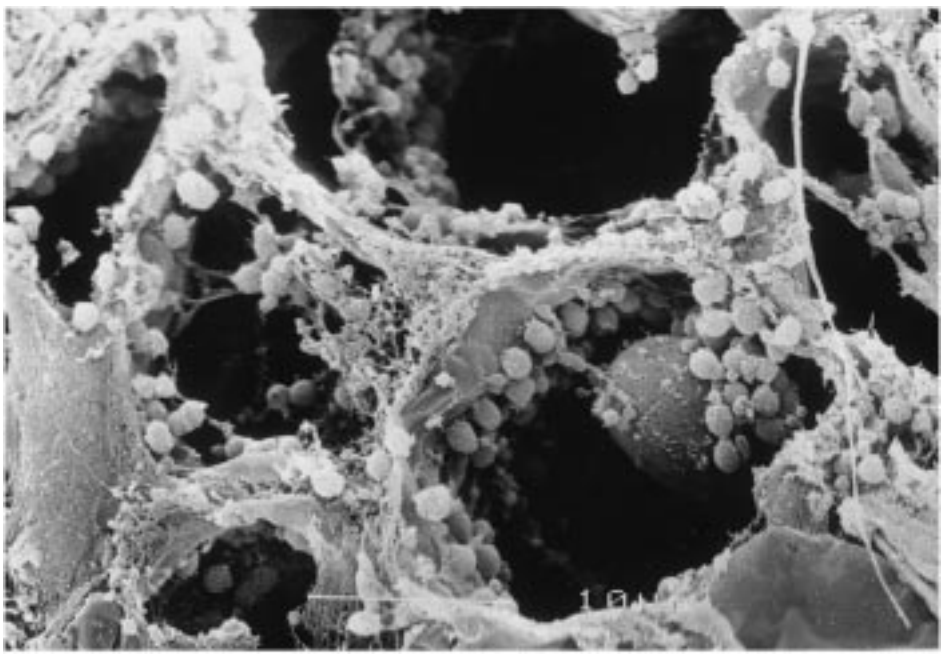

Figure 2. Electron micrograph (SEM): round structures in cross sections of sieve tube elements of phyllodied leek flower.

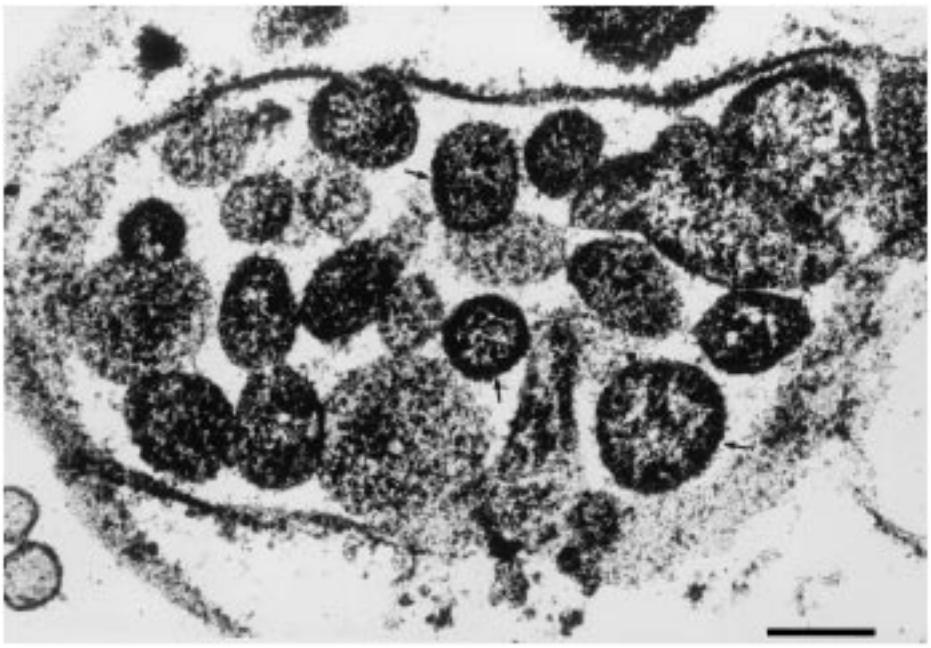

Figure 3. Electron micrograph (TEM) of phytoplasmas of different sizes and shapes in a sieve tube of symptomatic leek (arrows indicate the phytoplasma membranes) (bar = 200 nm).

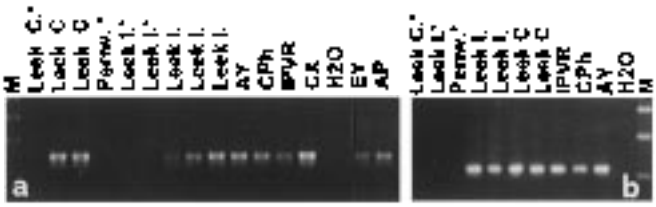

Figure 4. Agarose gel electrophoresis photographs of nested-PCR products of phytoplasma ribosomal DNA from Italian and Czech leeks and phytoplasma controls. In (a) 1200 bp products amplified with ribosomal general primers R16F2n/R2 and in (b) 1100 bp products amplified with specific primers R16(I)F1/R1. H_2O – water control, * – symptomless plants, Periw. – Periwinkle, C – samples from Czech republic, I – samples from Italy; AY – Maryland aster yellows, CPh – clover phyllody, IPVR – Italian periwinkle virescence, CX – peach X disease, EY – elm yellows, AP – apple proliferation. M, markers 1 kb DNA ladder; fragment sizes in kilobase pairs from top to bottom: 2.0, 1.6 and 1.0.

(Figure 5) allow the identification of phytoplasmas in the 16SrRNA groups I and XII. In particular all the phytoplasmas detected in the leek samples from the Czech Republic belonged to 16SrI-B subgroup. In the samples from Italy phytoplasmas of the 16SrXII-A group (or stolbur) formerly designated 16SrI-G were identified together with phytoplasmas of the subgroup 16SrI-B.

The nested-PCR with phytoplasma group-specific primers on products obtained with general primers confirmed that all the phytoplasmas detected in leek from both countries belonged to the groups 16SrI and 16SrXII. In fact the only positive results were obtained with R16(I)F1/R1 primer pair and with the corresponding controls (Figure 4b). The other phytoplasma-specific primer pairs only amplify the phytoplasma control strains of the corresponding group. No amplification was observed with symptomless leeks or water controls.

The restriction analyses of 16SrDNA products from base 758 to base 1804 in the spacer region (primers 16R758f/m23SR in nested PCR as described) confirmed the phytoplasma classification achieved by RFLP of the 16SrDNA from base 149 to base 1397 (region amplified with R16F2n/R2) (Figure 6a and b). No RFLP differences were found with all the enzymes employed in the leek sequences compared with those of the correspondent controls i.e. AY for the Czech isolates and AY and IPVR for the Italian isolates.

Discussion

The symptoms observed on leek during the present survey were very similar in plants from both the Czech

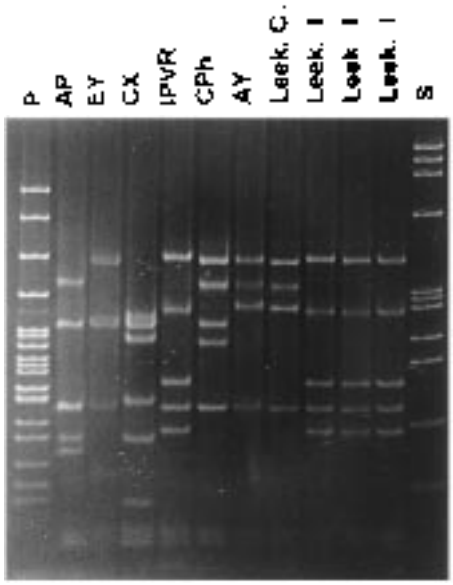

Figure 5. Polyacrylamide 5% gel showing the restriction fragment length polymorphism patterns of phytoplasma 16S rDNA fragments of 1200 bp obtained after digestion with *Mse*I from infected leek from Italy and Czech Republic. S, marker phiX174 *Hae*III digested, fragment sizes in base pairs from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72. P, marker pBR322 *Msp*I digested; fragment sizes in base pairs from top to bottom: 622, 527, 404, 307, 242, 238, 217, 201, 190, 180, 160, 147, 123, 110, 90, 76, 67, 34, 26, 15 and 9. Other abbreviations are as in Figure 4.

Republic and Italy. Moreover, very recently similar symptoms were observed also in *Allium altyncolicum* plants in the Czech Republic (M. Navratil, unpublished data). According to our knowledge, this is the first report of a phytoplasma-associated disease in leek in these countries. The presence of phytoplasmas in sieve tubes of diseased leek and their absence and the absence of other pathogens in the symptomless ones, supports a phytoplasma aetiology of the disease. In TEM observations of leek tissues from the Czech Republic the phytoplasmas detected were not very concentrated as reported in other plants such as rape and strawberry or as described in onion by Cousin et al. (1971) or by Petre and Ploaie (1973). A lot of mitochondria-like structures were detected by TEM in sieve tubes of infected cells. These structures could represent the majority of the numerous particles resembling phytoplasmas observed by SEM in comparable tissues. However this phenomenon indicates a strong sieve cell degeneration in the infected plants. The unusual low concentration of phytoplasmas in infected tissues of an herbaceous host is confirmed by the need of use the nested PCR technique in order to achieve the detection and identification of pathogens also when specific primers were employed [R16(I)F1/R1].

There have been numerous reports of phytoplasma diseases in the family *Liliaceae* (Ushiyama et al., 1969; Cousin et al., 1971; Hooper et al., 1971a, b; Slogteren

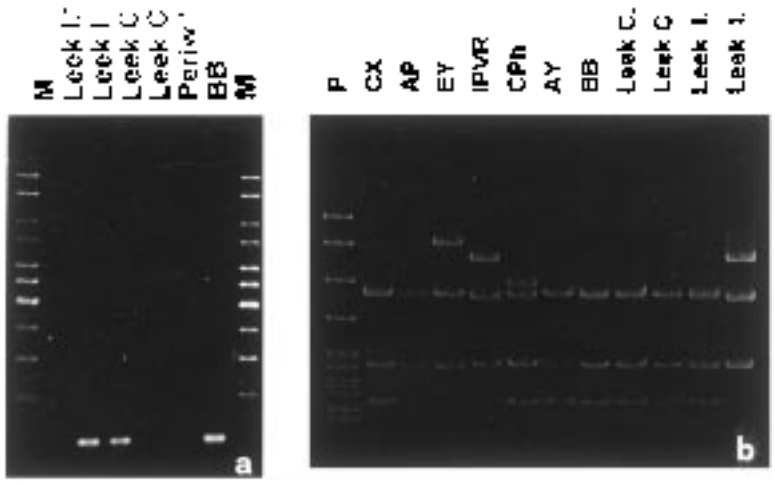

Figure 6. In (a) agarose gel with PCR products of 1100 bp obtained in nested-PCR with primers 16R758f/m23SR from leek and from control BB (tomato big bud). M, marker 1 kb DNA ladder; fragment sizes in kilobase pairs from top to bottom: 11.1, 10.1, 9.1, 8.1, 7.1, 6.1, 5.0, 4.0, 3.0, 2.0, 1.6 and 1.0. In (b) polyacrylamide 5% gel showing RFLP with *Taq*I of DNA fragments amplified in nested-PCR with 16R758f/m23SR. Marker and abbreviations are as in previous figures.

and Muller, 1972; Petre and Ploaie, 1973; Smookler and Dabush, 1974; Bertaccini and Marani, 1982; Bellardi et al., 1990; Vibio et al., 1995; Liefting et al., 1996; Kaminska et al., 1998; Poncarová-Voráckova et al., 1998), but this is the first confirmed report of phytoplasmas occurring in leek. Phytoplasmas infecting some of these plants such as onion, lily, and New Zealand flax have been characterised by PCR/RFLP analyses on 16SrDNA gene. Phytoplasmas from onion in Italy were classified in the aster yellows or 16SrRNA group I subgroup I-B (Vibio et al., 1995); phytoplasmas from Turks-cap lily in the Czech Republic belong to the subgroup 16SrI-C (Poncarová-Voráckova et al., 1998), and those from Casablanca lily in Poland were assigned to aster yellows group (16SrI) (Kaminska et al., 1998). The *Phormium* yellow leaf phytoplasma from New Zealand was classified in the stolbur group (Liefting et al., 1996, 1997), now 16SrXII, and is closely related to Australian grapevine yellows phytoplasma (Padovan et al., 1996).

The phytoplasmas infecting leek in Italy and in the Czech Republic belong to the 16SrI-B and 16SrXII-A groups. Phytoplasmas of the 16SrI-B group have also recently been identified in rape samples from the Czech Republic (Bertaccini et al., 1998b) and in various vegetable and ornamental plants in Italy (Lee et al., 1998). The detection of stolbur-related phytoplasmas in a monocotyledon species is new, but phytoplasmas related to this group have already been reported in Italy in diverse herbaceous and woody crops such as tomato (Vibio et al., 1996; Albanese et al., 1998) and grapevine (Bertaccini et al., 1995, 1996, 1998a; Albanese et al., 1996; Bianco et al., 1996), very often in mixed infection with phytoplasmas of other groups.

It will be important to continue monitoring the phytoplasmas in the two countries to identify the vector(s) and to prevent further spread of phytoplasma infections on the same or other agronomically important species.

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References

Albanese G, Davis RE, Granata G, Dally EL, Santuccio T and Tessitori M (1996) DNA-based analysis to detect and identify phytoplasmas in yellows-diseased grapevines in Sicily. Petria 6: 65–76

Albanese G, Polizzi G, Grimaldi V, Collororo S, Tessitori M and Davis RE (1998) Detection and molecular identification of a phytoplasma in diseased tomato plants in Calabria (Italy). Phytopath Medit 37: 83–87

Bellardi MG, Pisi A and Vicchi V (1990) Mycoplasma-like organisms in *Narcissus* sp. J Phytopathol 128: 288–292

Bertaccini A and Marani F (1982) Electron microscopy of two viruses and mycoplasma-like organisms in lilies with deformed flowers. Phytopath Medit 21: 8–14

Bertaccini A, Vibio M and Stefani E (1995) Detection and molecular characterization of phytoplasmas infecting grapevine in Liguria (Italy). Phytopath Medit 34: 137–141

Bertaccini A, Murari E, Vibio M, Danielli A, Davis RE, Borgo M, Consolaro R and Sancassani P (1996) Identificazione molecolare dei fitoplasmi presenti in viti affette da giallumi nel Veneto. L'Inf.tore Agrario 20: 55–59

Bertaccini A, Borgo M, Martini M, Mori N, Murari E, Posenato G, Sancassani P, Sartori S and Vibio M (1998a) Nei vigneti del Veneto continuano le epidemie di giallumi. L'Inf.tore Agrario 54: 85–90

Bertaccini A, Vorackova Z, Vibio M, Fránová J, Navratil M, Spak J and Nebesarova J (1998b) Comparison of phytoplasmas infecting winter oilseed rape in the Czech Republic with Italian *Brassica* phytoplasmas and their relationship to the aster yellows group. Plant Pathol 47: 317–324

Bianco PA, Davis RE, Casati P and Fortusini A (1996) Prevalence of aster yellows (AY) and elm yellows (EY) group phytoplasmas in symptomatic grapevines in three areas of northern Italy. Vitis 4: 195–199

Bos L (1983) Viruses and virus diseases of *Allium* species. Acta Hortic 127: 11–29

Chen M-F and Lin C-P (1997) DNA probes and PCR primers for the detection of a phytoplasma associated with peanut witches' broom. Eur J Plant Pathol 103: 137–145

Cousin M-T, Schweisguth MMB, Faivre-Amiot A, Kartha KK, Staron T, Moreau J-P and Lépine MP (1971) Contribution á l'étude du cycle des mycoplasmes chez les végétaux á l'aide d'observations effectuées sur Oignon atteint d''Aster yellows'. CR Acad Sc Paris, t 272, Série D: 830–833

Doi Y, Teranaka M, Yora K and Asuyama H (1967) Mycoplasma or PLK grouplike microorganisms found in the phloem elements of plants infected with mulberry dwarf, potato witches' broom, aster yellows or paulownia witches' broom. Ann Phytopath Soc Japan 33: 259–266

Fránová-Honetšlegrová J, Vibio M and Bertaccini A (1996) Electron microscopy and molecular identification of phytoplasmas associated with strawberry green petals in the Czech Republic. Eur J Plant Pathol 102: 831–835

Gundersen DE, Lee I-M, Rehner SA, Davis RE and Kingsbury DT (1994) Phylogeny of mycoplasmalike organisms (phytoplasmas): a basis for their classification. J Bacteriol 176: 5244–5254

Gundersen DE, Lee I-M, Schaff DA, Harrison NA, Chang CJ, Davis RE and Kingsbury DT (1996) Genomic diversity and differentiation among phytoplasma strains in 16S rRNA groups I (aster yellows and related phytoplasmas) and III (X-disease and related phytoplasmas). Int J Syst Bacteriol 46: 64–75

Hooper GR, Case JFW and Myers R (1971a) Mycoplasma-like bodies associated with a flower greening disorder of a wild flower *Trillium grandiflorum*. Plant Dis Rptr 55: 1108–1110

Hooper GR, Lacy ML and Vest G (1971b) Mycoplasmalike bodies associated with onion bulbs sprouting in storage. Plant Dis Rptr 55: 824–828

Kaminska M, Korbin M, Komorowska B and Pulawska J (1998) Stunting and flower bulb deficiency of *Lilium* sp.: a new phytoplasma associated disease. Acta Physiologiae Plantarum 20: 49–53

Lee I-M, Davis RE and Hiruki C (1991) Genetic relatedness among clover proliferation mycoplasmalike organisms (MLOs) and other MLOs investigated by nucleic acid hybridization and restriction fragment length polymorphism analyses. Appl Environ Microbiol 57: 3565–3569

Lee I-M, Gundersen DE, Hammond RW and Davis RE (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, Bertaccini A, Vibio M and Gundersen DE (1995) Detection of multiple phytoplasmas in perennial fruit trees with decline symptoms in Italy. Phytopathology 85: 728–735

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

Liefting LW, Andersen MT, Beever RE, Gardner RC and Forster RLS (1996) Sequence heterogeneity in the two 16S rRNA genes of *Phormium* yellow leaf phytoplasma. Appl Environ Microbiol 62: 3133–3139

Liefting LW, Beever RE, Winks CJ, Pearson MN and Forster RLS (1997) Planthopper transmission of *Phormium* yellow phytoplasma. Australasian Plant Pathol 26: 148–154

Martini M, Vibio M, Sfalanga A and Bertaccini A (1998) Molecular and ecological diversity of phytoplasmas belonging to the elm yellows group in Italy towards their tentative epidemiology. 12th IOM Conference, Sydney Australia. (p 130)

McCoy RE, Caudwell A, Chang CJ, Chen TA, Chiykowskyi LN, Cousin MT, Dale de Leeuw GTN, Golino DA, Hackett KJ, Kirkpatrick BC, Martwitz R, Petzold H, Sinha RH, Sugiura M, Whitcomb RF, Yang IL, Zhu BM and Seemuller E (1989) Plant disease associated with mycoplasmalike organisms. In: Whitcomb RF and Tully JG (eds) The Mycoplasmas, Vol 5 (pp 545–640) Acad Press, New York, NY, USA

Padovan AC, Gibb KS, Daire X and Boudon Padieu E (1996) A comparison of the phytoplasma associated with Australian grapevine yellows to other phytoplasmas in grapevine. Vitis 35: 189–194

Petre Z and Ploaie PG (1973) Onion proliferation and aspermy, a new disease in Romania, caused by mycoplasma. Anelele Istitutului de Cercetari pentru Protectia Pantelor 9: 13–17

Ploaie PG, Petre Z and Ionica M (1974) Identification of some plant diseases with symptoms of proliferation, chlorosis and dwarfing associated with mycoplasma in Romania. Anelele Istitutului de Cercetari pentru Protectia Pantelor 12: 17–25

Poncarová-Voráckova Z, Fránová J, Válová P, Mertelík J, Navrátil M and Nebesárová J (1998) Identification of phytoplasma infecting *Lilium martagon* in the Czech Republic. J Phytopathology 146: 609–612

Sarindu N and Clark MF (1993) Antibody production and identity of MLOs associated with sugar-cane whiteleaf disease and bermunda-grass whiteleaf disease from Thailand. Plant Pathol 42: 396–402

Schaff DA, Lee I-M and Davis RE (1992) Sensitive detection and identification of mycoplasmalike organisms by polymerase chain reactions. Biochem Biophysics Research Comm 186: 1503–1509

Seemüller E, Marcone C, Lauer U, Ragozzino A and Goschl M (1998) Current status of molecular classification of the phytoplasmas. J Plant Pathol 80(1): 3–26

Slogteren DHM Van and Muller JP (1972) 'Lissers', an old disease in hyacinths, probably caused by a mycoplasma. Intern Symp On Crop Protec, Gent, 9.5.1972

Smookler M and Dabush S (1974) Mycoplasmalike bodies in tulips. Plant Dis Rptr 58: 1142

Ushiyama R, Bullivant S and Mattews REF (1969) A mycoplasma-like organism associated with *Phormium* yellow leaf disease. New Zealand J Bot 7: 363–371

Vibio M, Bertaccini A, Lee I-M, Davis RE and Clark MF (1996) Differentiation and classification of aster yellows and related European phytoplasmas. Phytopath Medit 35: 33–42

Vibio M, Camele I, Bertaccini A and Rana GL (1995) Molecular identification of phytoplasmas infecting onion in Italy. Advances in vegetable virus research. Proc 8th Conf Virus Diseases of Vegetables (pp 176–179) July 9–15, 1995, Prague