Detection and identification of phytoplasmas in peach based on woody indexing and molecular methods

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Németh M.[1], Ember I.[1], Krizbai L.[1], Kölber M.[1], Hangyál R.[2] and Bozsics Gy.[3]

[1]*Plant Health and Soil Conservation Station (PHSCS) of MARD, Budapest H-1118 Budapest, Budaörsi út 141–145.*

[2]*PHSCS of County Fejér H-2481 Velence, Ország út 232.*

[3]*PHSCS of County Somogy H-7401 Kaposvár, Guba S. u. 20.*

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Summary: Symptoms resembling phytoplasma disease have been observed on peach trees in a seed-source plantation of stone fruits in south Hungary quite recently. In this publication we report on the results of woody indexing of symptomatic peach trees on GF 305 indicator in the field and under greenhouse conditions as well as on molecular studies. Phytoplasma infection detected on GF 305 indicators in greenhouse and field indexing was confirmed by PCR. Nested PCR was conducted using universal primer pairs followed by group and subgroup specific primers for the second amplification. RFLP analysis of nested PCR products was performed using RsaI restriction enzyme. Based on the results of molecular studies it can be concluded that phytoplasmas, belonging to the European stone fruit yellows subgroup (16SrX-B) were identified in peach trees. Further studies on symptomatic peach trees originating from different parts of Hungary are in progress.

Introduction

Phytoplasmas (formerly called mycoplasma-like organisms = MLOs) can cause severe diseases on fruit tree species *(Németh* 1986). Detection of phytoplasmas has been performed with biological indexing on woody indicators for several decades, but identification of the causal agents became possible only with recent advances in laboratory methods. Molecular techniques such as Polymerase Chain Reaction (PCR) assays using specific primers based on cloned phytoplasma DNA sequences as well as Restriction Fragments Length Polymorphism (RFLP) analyses applying different restriction enzymes for digestion of PCR products (amplified 16S rDNA) and ribosomal gene sequences provided possibility to develop reliable and rapid means for differentiation and classification of phytoplasmas. So nowadays laboratory techniques became available not only for detection, but also for distinction between phytoplasmas, belonging to the same 16Sr group or subgroup and causing similar symptoms on different host species.

In the previous decades phytoplasma diseases of fruit trees were not widespread in Hungary. Apple proliferation was the first such disease to be detected in the 1960s. Indexing on woody indicators proved that the pathogen can be transmitted. A similar method was applied for the detection of apple rubbery wood in apple cultivars and rootstocks, and the causal agents were described as apple proliferation and rubbery wood viruses *(Németh,* 1960). Later on, according to literature data, they were classified among mycoplasma diseases *(Németh,* 1979). In Hungary *del Serrone* et al. *(1998)* detected apple proliferation and pear decline phytoplasmas using PCR and RFLP analysis, but the latter could not be confirmed by biological indexing *(Németh,* 2000, *pers. comm.)*. Until the middle of the '90s causal agents could not be detected in stone fruit trees with symptoms resembling phytoplasma infection in Hungarian orchards, though in the southern parts of Europe, especially

in the Mediterranean area it has been considered a severe disease since a half century.

In France a virus disease was described on apricot in 1957 *(Morvan,* 1957). Later it was found that its causal agent is a mycoplasma *(Morvan* et al., 1973). All the knowledge obtained until that time was summarised also in France under the name of apricot chlorotic leaf roll mycoplasma *(Morvan,* 1977). The current name of the phytoplasma is European stone fruit yellows = ESFY *(Lorenz* et al., 1994). Recently *Lee* et al. *(1998)* classified ESFY in subgroup 16SrX-B within their scheme containing 14 groups and 32 subgroups. The syndrome on apricot has already been known in other southern countries like Italy *(Goidanich,* 1934), Greece *(Agrios,* 1971) and Spain *(Sanchez-Capuchino* et al., 1976) for several decades.

The disease causes severe losses in other *Prunus* species, too, thus in Japanese plum (*Prunus salicina*) in France *(Morvan,* 1977), Spain *(Sanchez-Capuchino* et al., 1976), Greece *(Syrgianidis* et al., 1976; *Rumbos* & *Bosalidis,* 1985) and Italy *(Giunchedi* et al., 1982).

Since the early 30s various symptoms on peach, caused by phytoplasmas presumably, were described under different disease names in the US such as X disease or peach yellow leaf roll (PYLR) etc. *(Gilmer* & *Blodgett,* 1976). The earliest record of the disease on peach in Europe is from Switzerland *(Bovey,* 1956). Significant damages in this fruit species were reported from France, *(Bernhard* et al. 1977), Greece *(Agrios,* 1971; *Syrgianidis,* 1974; *Tsialis* & *Rumbos,* 1980) and Spain *(Sanchez-Capuchino* et al., 1976), too. Based on molecular studies *Kison* et al.(1997) demonstrated that the causal agents of ESFY in peach from Germany and PYLR in peach from California are clearly distinguishable phytoplasmas. *Paltrinieri* et al. (2000) detected, besides ESFY (16SrX-B), phytoplasmas belonging to groups of aster yellows (16SrI-B), apple proliferation (16SrX-A) and stolbur (16Sr XII-A), furthermore, in one tree mixed infection of 16Sr I-B and X-A groups was also found in peach. *Navratil* et al. (2000) reported on the presence of ESFY and pear decline (16SrX-C) in peach trees.

In Hungary, ESFY phytoplasma was first described in apricot *(Lorenz* et al., 1994), confirmed by further studies of *Viczián* & *Süle* (1996) and *Viczián* et al. (1997/a, b). ESFY was detected also in almond *(Viczián* & *Süle,* 1996), Japanese plum and cherry *(Süle, 2000, pers. comm.),* and recently in *Prunus mahaleb,* too *(Varga* et al., 2000).

In the frame of indexing of peach trees from a seed-source plantation of stone fruit species during assessment on GF 305 indicators both in greenhouse and in the field, phytoplasma specific symptoms were found in 1999 and 2000. Based on these results, in the course of the inspection of the seed-source plantation in August 2000, appearance of symptoms, different phases of the disease and the caused damage were observed. In 2000 molecular investigations were conducted allowing identification of the pathogen. The present paper summarises the results of biological indexing and molecular studies.

Material and methods

Woody indexing

In the frame of the regular official phytosanitary control of seed-source mother trees, symptoms, characteristic for phytoplasma infection, were observed on woody indicator GF 305 peach seedling in the field for the first time in 1999.

The tested peach trees are in a seed-source plantation in the southern part of Hungary. The trees are 12 years old. Both in the greenhouse and the field GF 305 indicator was used in 4–5 replicates. Transmissions were done using chip budding (2 chips per plant) in late August of 1998 and 1999. Symptoms appeared on the indicator plants after 4–5 months in the greenhouse and in late June – early July of the next year in the nursery.

Visual observation

Seed-source mother trees, found positive for phytoplasma infection in greenhouse and field indexing in 1998–1999, were visually inspected for presence and severity of symptoms in August 2000. Furthermore, based on symptoms, the entire seed-source plantation was surveyed to determine the distribution of the disease.

Molecular studies

Mother trees providing budwoods for indexing in 1999 died by 2000, thus their molecular studies could not be conducted. Therefore, in the same plantation, samples were taken for the molecular studies from two trees showing phytoplasma symptoms, the field indexing of which was positive in 1999.

Leaf samples were collected twice in 2000: in April and August, from the seed-source mother trees as well as from GF 305 plants in the greenhouse and in the nursery, which were inoculated in late August 1999.

$R16F_{2n}/R_2$ nested PCR products of apple proliferation (AP), pear decline (PD), and European stone fruit yellows (ESFY) phytoplasmas, kindly provided by Milan Navratil (Czech Republic), were used as positive controls for RFLP analysis.

DNA extraction

Total DNA from infected and healthy peach leaf material was extracted in Doyle buffer *(Doyle* & *Doyle,* 1990).

Primers and PCR

Universal primer pairs $R16F_1/R16R_0$ (for the first nested PCR) and $R16F_{2n}/R_2$ (for the first nested PCR) were used for amplification of phytoplasma 16SrDNA. Second nested PCR was primed by group and subgroup specific primers: $R16F_{2n}/R_2$, $R16XF_1/R_1$, $R16IF_1/R_1$, $R16IIIF_1/R_1$, $R16VF_1/R_1$, as well as Stolbur and EY specific primers *(Lee* et al., 1994).

PCR was performed in mixtures of in 50 µl final volume containing 5 µl 10xPCR buffer (100mM Tris-HCl, pH=8.3,

500mM KCl, 0.01% gelatin), 1.5 mM $MgCl_2$, 250 µM each dNTP, 20pmol each primers, 2U Taq polimerase. About 2ng total DNA was amplified in the first PCR.

PCR products were diluted in 1/50 and 1 µl DNA was used for the second nested PCR.

The following amplification conditions were used: 94 °C for 3min, 94 °C for 1min (30sec for nested), 55 °C for 2min (30sec for nested), 72 °C for 3min (1min for nested), 35 cycles and 72 °C for 5min. PCR products were analysed in 1.5% ethidium-bromide stained agarose gel.

RFLP

$R16F_{2n}/R_2$ nested PCR products were digested with RsaI restriction enzyme (NBL Gene Sciences) according to the manufacturer's instructions, to distinguish the phytoplasmas belonging to different subgroups of the 16SrX group.

Results and discussion

Woody indexing

Field indexing

First symptoms on GF 305 indicators appeared in July 1999 and 2000, a year after inoculation.

At shooting stage GF 305 plants seemed to be normal. From the middle of June leaves began to turn yellow, then to bright red, leaves developed later became narrow, enrolled towards the midrib, bark of the shoot also turned red, in the middle of July leaf drop began from the bottom *(Fig. 1)*. The infected plants dried and died by September – October *(Fig. 2)*, next year they did not shoot in spring.

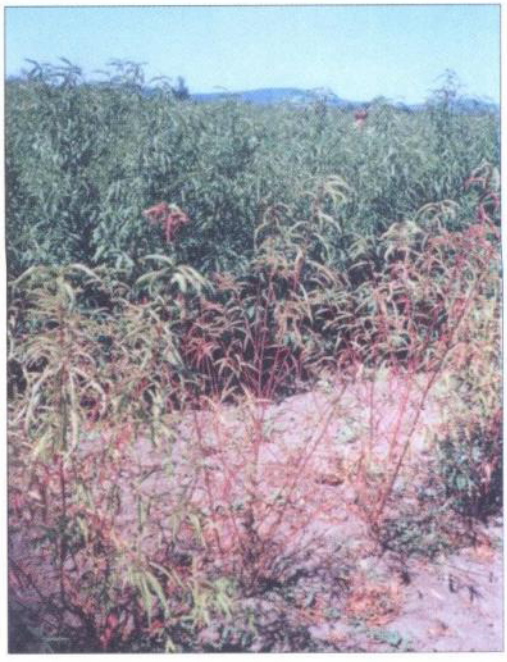

Figure 1 Red discoloration and rolling of leaves, reddening on the bark of the shoots on GF 305 indicators in the field in July of the following year after inoculation

Figure 2 Decline of GF 305 indicator plants in September of the following year after inoculation

Greenhouse indexing

In the greenhouse 4–5 months after transmission interveinal reddening was observed *(Fig. 3)*, terminal shoots formed rosettes with narrow leaves followed by wilting, finally the whole plant died *(Fig. 4)*.

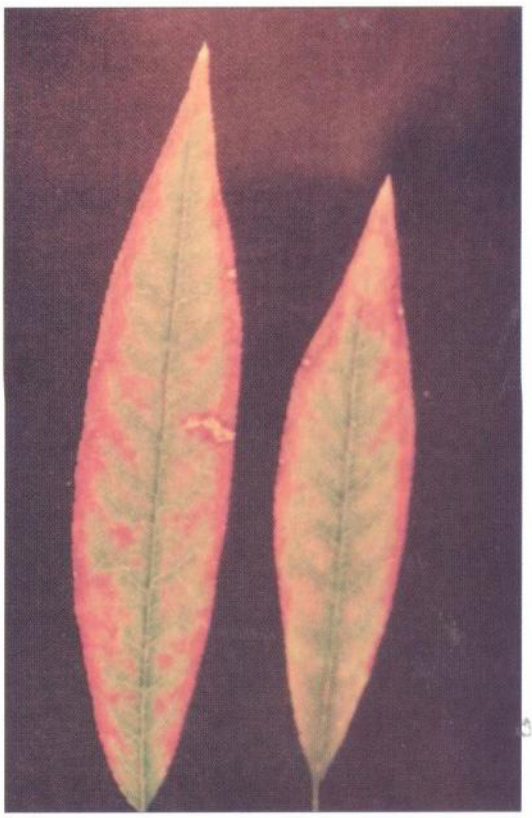

Figure 3 Interveinal red discoloration on the leaves of GF 305 indicator in the greenhouse in the 4th month after inoculation

Figure 4 Wilting and decline of GF 305 indicators in the greenhouse in the 4th-5th month after inoculation

Visual observation

In early August 2000, during visual observation of nine seed-source peach mother trees found infected with phytoplasma according to the results of indexing. It was observed that five trees completely died *(Fig. 5)*. Among the living trees, two ones showed partial decline *(Fig. 6)*, while leaf reddening, rolling of the leaves *(Fig. 7)* and yellowing *(Fig. 8)*, symptoms characteristic for phytoplasma infection also could be found. Symptomatic leaves were drooping as if the tree needed water. Shaking the diseased branches, dropping of the lower leaves already began.

In the survey to determine the distribution of the disease in the seed-source peach plantation, among the trees, not included in field indexing in 1998–1999, there were many plants showing similar symptoms or completely died. It was concluded that about 60% of the trees were infected. According to the observation of the phytosanitary inspector, in 1999 the infection rate was only around 25–30%. First leaf symptoms, characteristic for phytoplasma infection, such as reddening *(Fig. 7)*, yellowing and rolling *(Fig. 8)*,

Figure 6 Early stage of decline on ESFY infected seed-source peach mother tree

Figure 5 Seed-source peach mother tree killed by ESFY

Figure 7 Reddening and rolling of leaves on shoots of ESFY infected seed-source peach mother trees

Figure 8 Yellowing and rolling of leaves on ESFY infected peach seed-source mother trees in August

where shown up in 1998. This observation is supported by the fact that presence of phytoplasma could not be detected before 1998, during the regular official phytosanitary control of the plantation. Rapid spread of the disease is demonstrated by the high incidence of the infected trees. Based on several years' experience of the phytosanitary inspector, in the second half of the vegetation the leafhopper population significantly increased in the plantation after the sprayings against aphids were stopped in July.

Molecular studies

PCR

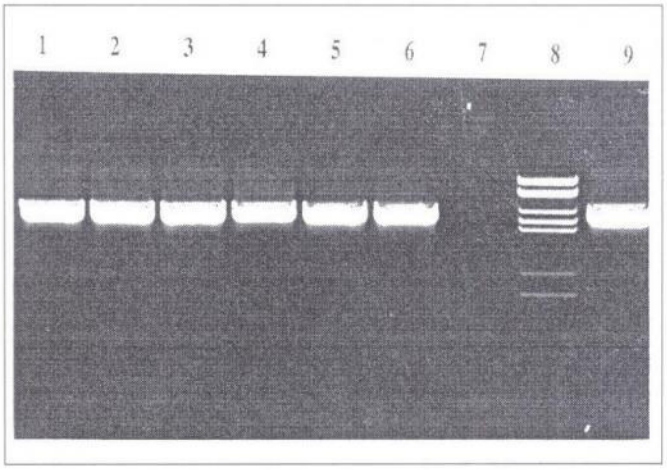

Figure 9 Results of nested PCR for detection of phytoplasmas in peach leaves with universal primer pair $R16F_1/R_0$ followed by amplification with primers $R16F_{2n}/R_2$ *(Lee et al.,* 1994)

Samples:

1–2 phytoplasma infected GF 305 indicator from the greenhouse
3–4 phytoplasma infected GF 305 indicator from the field
5–6 phytoplasma infected peach tree from the seed-source plantation
7 healthy GF 305 indicator
8 DNA marker
9 positive control

Molecular studies were conducted in 2000 for confirmation of woody indexing and identification of the pathogen.

Phytoplasmas were detected in all the six symptom-showing samples collected from peach mother trees of the seed-source plantation as well as from GF 305 indicators in the greenhouse and in the field in the nested PCR system using universal primers $R16F_1/R_0$ and $R16F_{2n}/R_2$ *(Fig.9).* ESFY was identified with group specific primer pair $R16(X)F_1R_1$ in a nested PCR in every tested tree *(Fig. 10).* No phytoplasma specific PCR products were detected using other group or subgroup specific primer pairs $R16(I)F_1/R_1$, $R16(III)F_1/R_1$, $R16(V)F_1/R_1$, with either Stolbur or EY primers *(Fig. 10).*

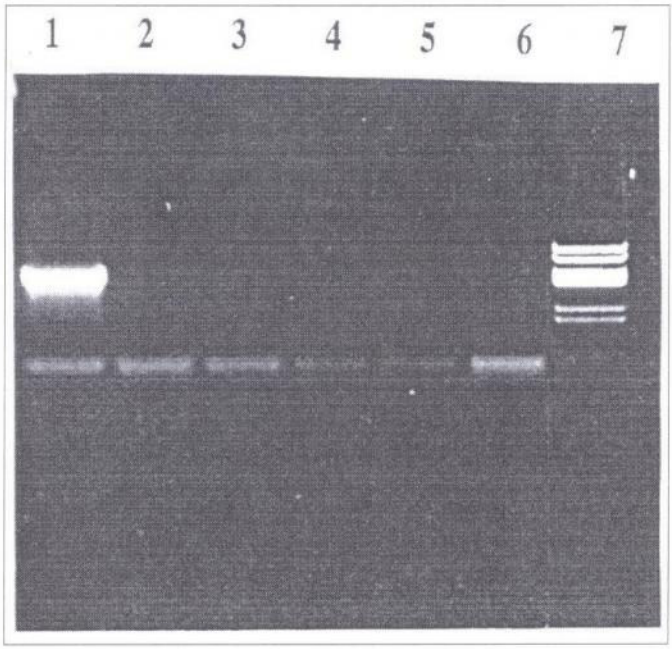

Figure 10 Result of nested PCR of one of the studied seed-source peach mother trees with different group and subgroup specific primers *(Lee et al.,* 1994)

1 R16(X)
2 Stolbur
3 R16(I)
4 R16(III)
5 R16(V)
6 EY
7 DNA marker

RFLP

RFLP analyses confirmed results of group specific nested PCR, when $F16R_{2n}/R_2$ nested PCR products were digested by RsaI enzyme. Only phytoplasmas belonging to the 16SrX group, subgroup of European stone fruit yellows (16SrX-B) were present in all the peach samples found infected in nested PCR *(Fig. 11).*

Based on the results of this study on six symptomatic peach trees we may assume that European stone fruit yellows is very likely to be one of the most common phytoplasmas in Hungarian peach orchards, as also in other European countries. However previous and recent surveys of higher number of trees in Europe demonstrated that several other phytoplasmas can occur in peach as apple proliferation (*Lorenz et al.* (1994), pear decline (*Navratil et al.*, 2000),

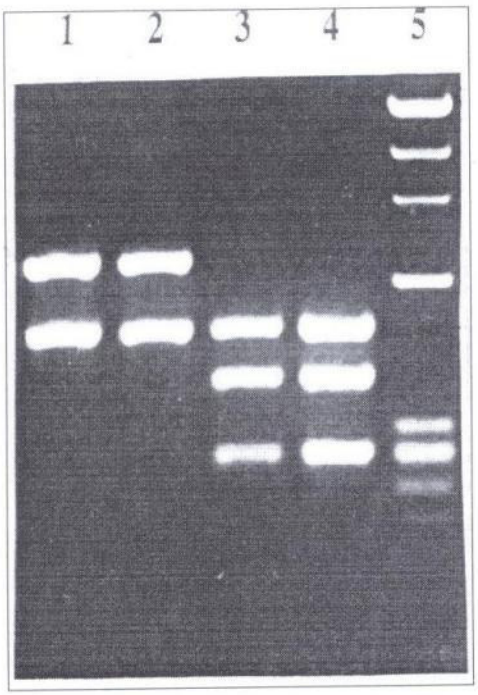

Figure 11 Results of RFLP analyses of the $R16F_{2n}/R_2$ nested PCR products from one of the studied seed-source peach mother trees using RsaI restriction enzyme.

Controls 1–3:
1 Apple proliferation
2 Pear decline
3 European stone fruit yellows
4 peach sample
5 DNA marker

stolbur, aster yellows and related strains, very often in mixed infections (*Paltrinieri et al.*, 2000).

This wide range of phytoplasmas found on peach in other countries, the rapid spread, as well as the great economic importance of the disease inspire us to start a national survey of stone fruit orchards for determining distribution of phytoplasma diseases, planned for the near future. Molecular studies of symptomatic peach trees originating from other parts of the country are already in progress.

References

Agrios, G.N. (1971): Premature foliation, cambial zone discoluration and stem pitting of peach and apricot in Greece. – Pl. Dis. Reptr. 55, 1049–1053.

Bernhard, R., Marenaud, C., Eymet, J., Sechet, J., Fos, A. & Moutous, G. (1977): Une maladie complex de certain *Prunus:* "Le dépérissement de Moliéres". – C.R. Acad. Agric. France, Séance 2.2. 1977, 178–189,

Bovey, R. (1956): L'enrulement chlorotique, une nouvelle maladie á virus du pécher. Rev. Romande Agric. Vitic. Arboric. 12(5):42–43.

Del Serrone, P., La Starza, S., Krizbai, L., Kölber, M. & Barba, M. (1998): Occurrence of apple proliferation and pear decline phytoplasmas in diseased pear trees in Hungary. Journal of Plant Pathology, 80 (1), 53–58.

Desvignes, J.-C. (1999): Apricot chlorotic leaf roll. *In:* Virus Diseases of Fruit Trees. Ctifl: 120–124.

Dosba, F., Lansac, M., Mazy, K., Garnier, M. & Eyquard, P. (1991): Incidence of different diseases associated with mycoplasmalike organisms in different species of *Prunus*. Acta Hort. 283, 311–320.

Doyle, J.J. & Doyle, J.L. (1990): Isolation of plant DNA from fresh tissue. Focus (BRL-Life Technologies) 12, 13–15.

Gilmer, R.M. & Blodgett, E.C. (1976): X disease. *In:* Virus Diseases and Noninfectious Disorders of Stone Fruits in North America. Washington, D.C.:USDA; Agricultural Handbook No. 437, 145–155.

Giunchedi, L., Poggi-Pollini, C. & Credi, R. (1982): Susceptibility of stone fruit trees to Japanese plum-tree decline causal agent. – Acta Hort. 130, 285–290.

Goidanich, G. (1934): La leptonecrosi dei ciliegi e degli albicocchi. – Boll. Staz. Patol. Veg. Roma 14, 531–540.

Kison H., Kirkpatrick, BC & Seemüller, E. (1997): Genetic comparison of the peach yellow leaf roll agent with European fruit tree phytoplasmas of the apple proliferation group. Plant Pathology 46: 538–544.

Lee, I.-M., Dawn, E., Gundersen-Rindal, Davis, R.E. & Bartoszyk, M. (1998): Revised classification scheme of phytoplasmas based on RFLP analyses of rRNA and ribosomal protein gene sequences. International Journal of Systematic Bacteriology 48, 1153–1169.

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

Llácer, G. (1974): El "Dépérissement" del alboricoquero. Estudio de una possible forma viral en la region del Ebro. Doct. Thesis, Zaragoza (Spain), March 1972. Sumary in An. Inst. nac. Invest agrar. 4:27–74.

Lorenz, K.-H., Dosba, F., Poggi-Pollini, C., Llácer, G. & Seemüller, E. (1994): Phytoplasma diseases of *Prunus* species in Europe are caused by genetically similar organisms. Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz 101 (6):567–575.

Morvan, G. (1957): Mise en evidence de l'action d'un virus dans le dépérissement de l'abricotier. G.R. Acad. D'Agric. Fr. 43:613–614.

Morvan, G. (1977): Apricot chlorotic leaf roll. – Eppo Bull. 7, 37–55.

Morvan, G., Gianotti, J. & Marchoux, G. (1973): Untersuchungen über die Aetiology des chlorotischen Blattrolls des Aprikosenbaums: Nachweiss von Mycoplasmen. Phytopath. Z. 76:33–38.

Nemeth M. (1960): Distribution of virus diseases of fruit trees in Hungary. Virus diseases of apple and pear. Kertészet és Szőlészet 9(12):25–26. (in Hungarian)

Nemeth M. (1979): Virus, Mycoplasma and Rickettsia Diseases of Fruit Trees. Mezőgazdasági Kiadó, Budapest (in Hungarian): 1–629.

Nemeth M. (1986): Virus, Mycoplasma and Rickettsia Diseases of Fruit Tees. Martinus Nijhoff, Dordrecht (NL): 1–841.

Paltrinieri, S., Martini, M., Stefani, E., Pondrelli, M., Fideghelli, C. & Bertaccini, A. (2000): Phytoplasma infection in peach and cherry in Italy. Acta Hort. (in press)

Rumbos, I.C. & Bosalidis, A.M. (1985): Mycoplasmalike organisms associated with declined plum trees in Greece. – Z. PflKrankh. PflSchutz 92, 47–54.

Sánchez-Capuchino, J.A., Llácer, R., Casanova, J., Forner, B. & Bono, R. (1976): Epidemiological studies on fruit tree mycoplasma diseases in the eastern region of Spain. – Acta Hort. 67, 129–136.

Süle S., Viczián O. & Panzer, B., (1997): Apricot decline caused by phytoplasma. Kertészet és Szőlészet 1997/45:8–9. (in Hungarian)

Syrgianidis, G.D. (1974): Transmission par gerftage d'une maladie du pecher en Gréce qui ressemble á celle d'enrulement chlorotique. Proc. XI[th] Inter. Hort. Cong., Warsaw. Sept. 1974. Vol. IA.,:255.

Syrgianidis, G.D., Mainou, A.C. & Mouchtouri, E.C. (1976): Appearance of a disease of Japanese plum varieties in Greece resembling that of chlorotic leaf roll: Transmission of the disease by grafting. – Mitt. Biol. Bund. Anst. Ld-Forstw. H. 170, 117–120.

Tsialis, D. & Rumbos, I.C. (1980): New observations on peach decline in Greece and its aetiology. – Acta Phytopath. Acad. Sci. Hung. 15, 279–283.

Varga K., Paltrinieri, S., Kölber, M., Ember I., Erdős Z. & Bertaccini, A. (2000): Identification of phytoplasmas infecting *Prunus mahaleb* in Hungary. Acta Hort. (in press)

Viczián O. & Süle S. (1996): Identification of phytoplasmas in stone fruits species using PCR. 42. Növényvédelmi Tudományos Napok (42[th]Plant Protection Days), 1996. február 27–28. Abstracts: 140. (in Hungarian)

Viczián O., Süle S., Pénzes B. & Seemüller, E. (1997): Apricot decline caused by a phytoplasma in Hungary. Új Kertgazdaság 3 (1):48–51. (in Hungarian)