Plant Pathology (2001) **50**, 130–135

Presence of European stone fruit yellows (ESFY or 16SrX-B) phytoplasmas in apricots in Austria

M. Laimer Da Câmara Machado[a]*†, S. Paltrinieri[b], V. Hanzer[a], W. Arthofer[a], S. Strommer[a], M. Martini[b], M. Pondrelli[b] and A. Bertaccini[b]

[a]*Institute of Applied Microbiology, University of Agricultural Sciences, Nussdorfer Laende 11, 1190 Vienna, Austria; and* [b]*UCI-STAA-Patologia Vegetale, Università degli Studi di Bologna, Bologna, Italy*

A severe decline of apricot trees, present in Austria for several years, has recently reached notifiable levels. Initial symptoms on affected trees resemble deficiencies in water and nutrient supplies, expressed as leaf rolling, chlorosis and early reddening, leading to sudden dieback during the growing season. Small, wilted fruit and dried leaves may also persist during the autumn. Phytoplasmas belonging to the 16SrX-B subgroup of European stone fruit yellows (ESFY) were identified by nested polymerase chain reaction/restriction fragment length polymorphism analysis in a high percentage of affected trees from two different regions of Austria.

Keywords: apricot decline and dieback, phytoplasmas, polymerase chain reaction, restriction fragment length polymorphism

Introduction

Symptoms of apricot tree decline have been observed in France and Italy since the beginning of the twentieth century. Morvan (1977) named the disease, which is associated with leptonecrosis (Goidanich, 1934) or with new sprouting in winter, 'apricot chlorotic leaf roll' (ACLR). The association of these symptoms with a phytoplasma infection has been recognized for only 20 years, since phytoplasmas were detected as single cells in sieve tubes by electron or fluorescence microscopy, and transmission experiments to other stone fruits and indicator plants were successfully carried out (Morvan, 1977; Goidanich *et al*., 1980; Giunchedi *et al*., 1982; Pastore *et al*., 1995).

European stone fruit yellows (ESFY) has been proposed as the common name for phytoplasma-related diseases in European stone fruits (Kison *et al*., 1997). Amongst others it comprises the French 'enroulement chlorotique de l'abricotier' (ECA) or ACLR, which is a quarantine organism of EPPO (European Plant Protection Organisation) (OEPP/EPPO, 1986), included in the EPPO certification scheme for virus-tested fruit trees (OEPP/EPPO, 1991/1992).

Phytoplasmas may be detected in stone fruit plants by several methods, including fluorescence microscopy with 4,6-Diamidino-2-phenylindole (DAPI), Southern blotting with 4,6-Diamidino-2-phenylindole (DAPI), grafting and enzyme-linked immunosorbent assay (ELISA) (Kison *et al*., 1997; Desvignes, 1999). Because phytoplasmas are not completely biologically characterized, molecular tools based on restriction fragment length polymorphism (RFLP) analyses of 16S rDNA and/or the spacer region amplified by polymerase chain reaction (PCR) and/or nested PCR are helpful in distinguishing different groups and subgroups (Lee *et al*., 1998; Seemüller *et al*., 1998). In fruit trees, it appears advisable to differentiate phytoplasmas also according to host species (Seemüller, 1999).

This report refers to the increasing appearance of apricot dieback in commercial Austrian orchards. The degree of incidence varies between 5 and 40%, depending on rootstock, cultivar and the age of the plant at the first appearance of disease symptoms (Wurm, 1999; S. Rögner, Niederösterreichische Landeslandwirtschaftskammer, St. Pölten, personal communication, 1999). Initially, symptoms develop on single branches with a rolling of leaves that become chlorotic, indicating a disturbance of water and nutrient supply. These phenomena are followed by the sudden death of whole branches or the entire crown of trees as described by Pastore *et al*. (1997a). On the branches and trunks of affected trees phloem necrosis, corresponding to the symptoms initially described by Goidanich (1934), may also be observed. Fruits are small, shrunken and tasteless and may fall prematurely. Leaf coloration may occur earlier in autumn in affected trees than in

*To whom correspondence should be addressed.

†E-mail: m.laimer@iam.boku.ac.at

Accepted 10 August 2000.

healthy ones. Leaves having a paper-like appearance remain attached to the branches.

Affected plants were tested by PCR to confirm the presence and identity of phytoplasmas associated with this disease. Primers designed to amplify parts of the ribosomal region were employed in nested PCR to obtain two generic fragments and one group-specific DNA fragment, which were tested further by RFLP analyses.

Materials and methods

Samples

Samples were collected from March to September 1999 in different locations in two regions of Austria, i.e. Lower Austria and Burgenland, where stone fruits (mainly apricots) are grown commercially, by selecting bud wood from plants with and without symptoms (Table 1).

Three plantations were surveyed in Poysdorf, Lower Austria: P1 and P2 were, respectively, 9- and 6-year-old plantations of stone fruits maintained for demonstration purposes, containing many different cultivars, whilst P4 was a commercial plantation containing three cultivars, Hargrand, Rouge de Fournes and Bergeron, with severe dieback symptoms. B3 and B4 were commercial plantations in Pamhagen and Kroatisch-Minihof, in Burgenland. B4 was an especially critical case, as it was a 4-year-old plantation of modern cultivars (Aurora, Hargrand, Orangered) where diseased and dead trees had already been replaced during the preceding 2 years (3% of annual replanting).

Phytoplasma identification

DNA was extracted from fresh midribs of leaves with and without symptoms and from phloem of nonlignified branches of 29 apricot trees of 11 cultivars (Table 1), using a chloroform/phenol procedure (Prince *et al.*, 1993) that yielded clean DNA preparations.

Positive DNA control samples were extracted from periwinkle plants infected by phytoplasma strains

Table 1 Results of PCR analyses of stone fruit cultivars in different locations in Lower Austria (P1, P2, P3, P4, different plantations in Poysdorf) and Burgenland (B3, Pamhagen; B4, Kroatisch-Minihof)

			PCR results	
Site	Cultivar	Symptom observation	R16F2n/R2	R16(X)F1/R1
P1a	Hargrand 1	Earlier autumn coloration	+[a]	
P1b	Hargrand 2	Earlier autumn coloration	–[b]	+
P1c	Hargrand 3	Symptomless	–	–
P1d	Hargrand 4	Earlier autumn coloration	–	+
P1e	Hargrand 5	Symptomless	+	
P1f	Goldrich 1	Earlier autumn coloration	+	
P1g	Rubira	Reduced growth	–	–
P2a	Aurora 1	Earlier autumn coloration	–	+
P2b	Sundrop 1	Symptomless	–	–
P2c	Sundrop 2	Earlier autumn coloration	–	+
P2d	Sundrop 3	Earlier autumn coloration	–	–
P2e	Sundrop 4	Earlier autumn coloration	–	+
P2f	Sundrop 5	Earlier autumn coloration	+	
P2g	Harogem 1	Decreased vigour	–	–
P2h	Harogem 2	Decreased vigour	–	–
P4a	Hargrand	Healthy-looking control	–	+
P4b	Hargrand	Decreased vigour	–	+
P4c	Hargrand	Decreased vigour	+	
P4d	Rouge de Fournes	Decreased vigour	+	
P4e	Rouge de Fournes	Affected tree: branch with symptoms	+	
P4f	Rouge de Fournes	Affected tree: branch without symptoms	+	
P4g	Bergeron	Doubtful plant, branch A	+	
P4h	Bergeron	Doubtful plant, branch B	+	
P4i	Black Beauty	Sharka symptoms	+	
B3a	Ungarische Beste	Decreased vigour	–	+
B4a	Aurora R3/B5	Decreased vigour	–	+
B4b	Aurora R4/B31	Decreased vigour	–	+
B4c	Hargrand R3/B100	Decreased vigour	–	+
B4d	Orangered R3/B9	Decreased vigour	–	–

[a]Positive amplification.
[b]Negative amplification.

already identified: clover phyllody (CPh) obtained from I. M. Lee (MPPL, UDSA, Beltsville, MD, USA), peach X disease (CX) obtained from B. C. Kirkpatrick (University of California, Davis, CA, USA via I. M. Lee), elm yellows (EY) obtained from H. Griffith and W. A. Sinclair (Cornell University, NY, USA), apple proliferation (AP) obtained from L. Carraro (Università di Udine, Italy) and pear decline (PD) and ESFY obtained from E. Seemüller (BBA, Dossenheim, Germany).

Polymerase chain reaction experiments were carried out on the nucleic acid samples from apricots and 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}. The reaction mixtures for direct PCR (25 μL) contained 0·8 μL of nucleic acid, 200 μM of each dNTP, 0·625 U Taq polymerase (Perkin Elmer, Norwalk, CT, USA) and 0·4 μM of primers. The buffer and the PCR conditions were as described by Schaff *et al*. (1992). Nested PCR reactions were performed under the same conditions using as template the product of the previous amplification, diluted 1 : 40 with sterile water. Samples with the reaction mixture devoid of DNA templates or containing DNA from symptomless plants were included in each experiment as negative controls.

Ribosomal general primers R16F1/R0 and R16F2n/R2 (Lee *et al*., 1995), amplifying fragments internal to each other in the 16S ribosomal gene, were employed, respectively, in direct and nested PCR. Negative samples were further tested in nested PCR with primers specific for some phytoplasma groups: R16(I)F1/R1 for groups 16SrI (aster yellows) and 16SrXII (stolbur), R16(III)F2/R1 for group 16SrIII (X-disease), R16(V)F1/R1 for group 16SrV (elm yellows) and R16(X)F1/R1 for group 16SrX (apple proliferation) (Lee *et al*., 1994, 1995). Samples (6 μL) of each PCR product were subjected to electrophoresis in a 1% agarose gel and visualized by staining with ethidium bromide and UV illumination.

Samples (10 μL) of PCR product amplified with R16F2n/R2 or with phytoplasma group 16SrX-specific primers were digested using enzymes *Rsa*I and *Ssp*I at 37°C for at least 16 h, following the instructions of the manufacturer (Fermentas, Lithuania). The restriction patterns were then compared with those of control strains after electrophoresis through a 5% polyacrylamide gel in 1X TBE buffer, followed by staining with ethidium bromide and visualization under a UV transilluminator.

Virus detection by ELISA

To determine a possible involvement of viruses in the apparent decline symptoms observed in the orchard, collected mixed leaf samples from individual trees were screened for the presence of plum pox virus (PPV), prunus necrotic ringspot virus (PNRV), prune dwarf virus (PDV), apple mosaic virus (ApMV), apple chlorotic leafspot virus (ACLSV) and tobacco ringspot virus (ToRSV) by two-step ELISA (Clark & Adams, 1977; Clark *et al*., 1978). Polyclonal antisera were purchased from Loewe (Germany) and employed according to the manufacturer's instructions.

Results

There was no evidence of infection with the common fruit tree viruses, as all samples gave negative ELISA values when tested for PNRV, PDV, ApMV, ACLSV and ToRSV. There was also no detectable PPV infection with the exception of the cultivar Black Beauty, which was also showing clear Sharka symptoms.

However, it was possible to detect phytoplasmas in most of the trees with symptoms that were examined (Table 1). Using primer pairs R16F2n/R2, a 1200-bp product was obtained from a number of apricot samples showing symptoms and from phytoplasma control strains in periwinkle. RFLP analyses indicated that this product belonged to the 16SrX-B group (Figs 1 and 2). Nested PCR using phytoplasma group-specific primers with the samples that were negative when tested with R16F2n/R2 primers allowed the detection of phytoplasmas in the majority of the samples tested. In fact, an 1100-bp product was observed when primers R16(X)F1/R1 were employed (Fig. 3); with other phytoplasma group-specific primers, only the corresponding controls were amplified (not shown). RFLP analysis with R16(X)F1/R1-amplified sequences using *Rsa*I and *Ssp*I (Fig. 4) showed that all the apricot samples had an identical restriction pattern and were indistinguishable from the ESFY control phytoplasma.

Discussion

The presence of ESFY disease, previously described as ACLR, has been reported in France, Spain, Italy, Greece, Hungary, Romania, Switzerland, Germany

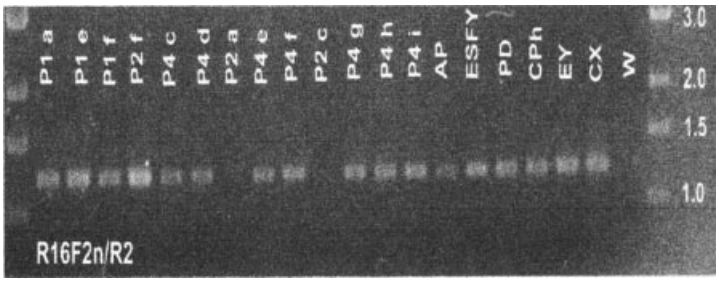

Figure 1 Agarose gel with 1200-bp PCR products of phytoplasma ribosomal DNA amplified with primers R16F2n/R2 from some of the apricot samples shown in Table 1. W, water control; AP, apple proliferation; ESFY, European stone fruit yellows; PD, pear decline; CPh, clover phyllody; EY, elm yellows; CX, peach X disease.

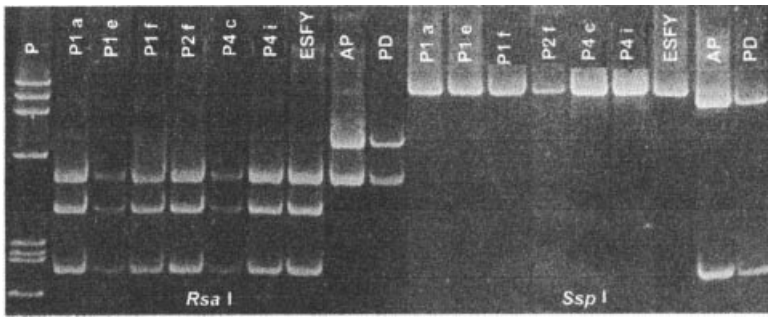

Figure 2 Polyacrylamide gel (5%) showing the RFLP patterns of phytoplasma 16S rDNA fragments of 1200 bp obtained with primers R16F2n/R2 from some of the apricot samples shown in Table 1 digested with *Rsa*I and *Ssp*I, and the control strains (ESFY, European stone fruit yellows; AP, apple proliferation; PD, pear decline). P, marker ΦX174 *Hae*III digested; fragment sizes in base pairs from top to bottom: 1353, 1078, 872, 603, 310, 281, 271 and 234.

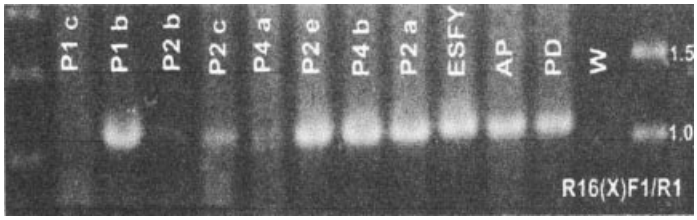

Figure 3 Agarose gel electrophoresis of PCR products amplified with phytoplasma-specific primers R16(X)F1/R1 from some of the apricot samples that yielded no visible amplification product with R16F2n/R2. W, water control; ESFY, AP and PD are positive controls as in Fig. 2.

and former Yugoslavia (Morvan, 1977; Nemeth, 1986), causing decline and death to apricot, Japanese plum, more rarely to peach (Llacer & Medina, 1988), almond, flowering cherry and European plum (Seemüller *et al.*, 1998). In Austria, the first observations of phytoplasmas in apricots with dieback symptoms were reported by the DAPI staining technique in 1998 and confirmed by PCR using the primers fU5/rU3 as universal and f01/r01 as specific for the apple proliferation (AP) group (Richter, 1999).

Lichou (1999) described ESFY as one of the factors that limit the culture of apricot and Japanese plum in France. About 5% of apricot trees die annually, but cultivars vary in sensitivity, e.g. for certain cultivars, such as Beliana, Priana and Ferriana, 20–30% of trees may die annually in high risk areas, whilst Rouge de Roussillon has emerged as one of the least sensitive cultivars in south-eastern France (Desvignes, 1999).

This is consistent with the present report, which shows a similar correlation to data obtained in a survey of 10 807 trees, carried out in 1998, where cultivar sensitivity ranged from low in cultivars Goldrich and Harlayne to moderate in Klosterneuburger and Ungarische Beste, to high in Rouge de Fournes, Polonaise, Bergeron and Orangered, being highest in Hargrand (Zull, 1999). This survey further revealed that orchards in the Wachau region, where locally adapted cultivars like Klosterneuburger and Ungarische Beste are grown without much pruning, appear to suffer less from the disease than orchards in the Poysdorf region, where modern plantations with new cultivars are more common (Zull, 1999).

The present results confirm that the appearance of a tree in the field cannot be used as a reliable indicator of its health status (Seemüller, 1999). Hargrand trees (samples P1c, P1e and P4a) were judged symptomless and therefore regarded as healthy controls in this study. Nevertheless, two of them yielded a positive signal for the presence of ESFY either in direct or in nested PCR and in RFLP analysis. On the other hand, the reduced growth habit of Rubira and Harogem (samples P1g, P2g and P2h) could not be attributed to a phytoplasma effect,

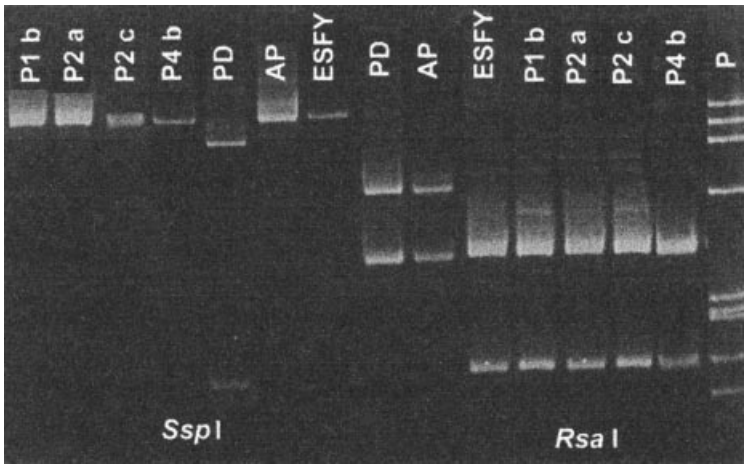

Figure 4 *Ssp*I and *Rsa*I RFLP profiles of phytoplasma 16S rDNA amplified in nested PCR with primers R16(X)F1/R1 from some of the apricot samples listed in Table 1, and control strains ESFY, AP, PD as in Fig. 2. P, marker ΦX174 *Hae*III digested.

whilst reduced growth was clearly correlated with the presence of ESFY in Hargrand and Rouge de Fournes (samples P4c and P4d). The apparent distribution within a tree canopy is somewhat misleading: both an affected and a healthy-looking branch of Rouge de Fournes were positive in direct PCR.

Before an overall conclusion can be drawn on the distribution within a tree canopy and the reliability of field detection, statistical sampling and grafting experiments, as well as the establishment of tissue culture, are required.

The use of nested PCR provided an increase in sensitivity and thereby the detection of ESFY in most plants with early autumn coloration in the Poysdorf area, and decreased vigour in Burgenland, indicating that the reliability of the system is satisfactory.

Certain isolates induce only a light rolling of chlorotic leaves, without promoting early autumn coloration (Desvignes, 1999). As a consequence, infected trees may not be damaged by winter frosts, as was described in some cases in Italy (Bertaccini *et al*., 1993), and may survive/die slowly over several years, although with a significant decrease in fruit production. In Austria, no cases of early sprouting of leaves in spring or premature bud burst in orchards, as reported by Pastore *et al*. (1997b) and Desvignes (1999), were observed.

European stone fruit yellows phytoplasmas have been reported to be transmitted by *Cacopsylla pruni* and by propagating material (Carraro *et al*., 1998; Süle, 1999). *C. pruni* is present in Austria only in very rare cases (F. Polesny, BFL, Vienna, personal communication, 2000), and therefore other modes of spread need to be considered, together with alternative natural sources of inoculum.

Acknowledgements

This work was supported by the EU-Project FAIR5 CT 3889 (Health Certification of Rosaceous Species Based on Disease Indexing of *In Vitro* Plants: Validation of Diagnostics and Diagnostic Strategies). We thank S. Rögner, T. Vertes, R. Schreiber and fruit tree growers in Lower Austria and Burgenland for providing infected plant material and valuable discussions.

References

Bertaccini A, Vibio M, Lee I-M, Lugaresi C, Benni A, 1993. Individuazione della presenza di micoplasmi in albicocchi mediante amplificazione genica. *Informatore Fitopatologico* **4**, 44–6.

Carraro L, Osler R, Loi N, Ermacora P, Refatti E, 1998. Transmission of European stone fruit yellows phytoplasma by *Cacopsylla pruni*. *Journal of Phytopathology* **80**, 233–9.

Clark MD, Adams AN, 1977. Characteristics of enzyme-linked immunosorbent assay for the detection of the microplate method of plant viruses. *Journal of General Virology* **34**, 475–83.

Clark MD, Flegg CL, Bar-Joseph M, Rottem S, 1978. The detection of *Spiroplasma citri* by enzyme-linked immunosorbent assay (ELISA). *Phytopathologische Zeitschrift* **92**, 332–7.

Desvignes JC, 1999. *Maladies à Virus des Arbres Fruitiers*. Paris, France: CTIFL.

Giunchedi L, Poggi Pollini C, Credi R, 1982. Susceptibility of stone fruit trees to the Japanese plum-tree decline causal agent. *Acta Horticulturae* **130**, 285–90.

Goidanich G, 1934. La leptonecrosi dei ciliegi e degli albicocchi. *Bollettino della Stazione di Patologia Vegetale, Rome* **14**, 531–40.

Goidanich G, Canova A, Bertaccini A, Marani F, 1980. Stone fruit decline associated with mycoplasma-like bodies and its natural diffusion. *5th Proceedings of the Mediterranean Phytopathological Union Congress, Patras, Greece*. Athens, Greece: Hellenic Phytopathological Society, 16.

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**, 538–44.

Lee I-M, Bertaccini A, Vibio M, Gundersen DE, 1995. Detection of multiple phytoplasmas in perennial fruit trees with decline symptoms in Italy. *Phytopathology* **85**, 728–35.

Lee I-M, Gundersen DE, Davis RE, Bartoszyk IM, 1998. Revised classification scheme of phytoplasmas based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences. *International Journal of Systematic Bacteriology* **48**, 1153–69.

Lee I-M, Gundersen DE, Hammond RW, 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**, 559–66.

Lichou J, 1999. Main reasons for the decline of apricot. *Klosterneuburger Mitteilungen* **49**, 209–10.

Llacer G, Medina V, 1988. A survey of potential vectors of apricot chlorotic leaf roll. *Agronomie* **8**, 79–83.

Morvan G, 1977. Apricot chlorotic leaf roll. *EPPO Bulletin* **7**, 37–55.

Nemeth M, 1986. *Virus, Mycoplasma and Rickettsia Diseases of Fruit Trees*. Dordrecht, The Netherlands: Martinus-Nijhoff.

OEPP/EPPO, 1986. Fiches informatives sur les organismes de quarantaine Nr. 146. Apricot chlorotic leafroll MLO. *EPPO Bulletin* **16**, 43–5.

OEPP/EPPO, 1991/1992. Schémas de certification no. 1 Arbres fruitiers et porte-greffe 'virus-free' ou 'virus-tested'. Parties I–IV. *EPPO Bulletin* **21**, 267–78;22, 255–84.

Pastore M, Bertaccini A, Vibio M, Izzo PP, Genovese MR, Santonastaso M, 1997b. Identificazione mediante tecniche molecolari di fitoplasmi associati all'arrotolamento fogliare dell'albicocco in Italia. *Italus Hortus* **4**, 76–7.

Pastore M, Marcone C, Pennone F, Cristinzio G, Ragozzino A, 1995. Detection of mycoplasma-like organisms (MLOs) in apricot by fluorescence microscopy (DAPI) in South Italy. *Acta Horticulturae* **386**, 506–10.

Pastore M, Vibio M, Murari E, Bertaccini A, 1997a. Incidenza, diagnosi e prospettive di difesa relative alla fitoplasmosi dell'accartocciamento clorotico fogliare dell'albicocco. *Frutticoltura* **7/8**, 67–71.

Prince JP, Davis RE, Wolf TK, Lee I-M, Mogen BD, Dally EL,

Bertaccini A, Credi R, Barba M, 1993. Molecular detection of diverse mycoplasmalike organisms (MLOs) associated with grapevine yellows and their classification with aster yellows, X-disease, and elm yellows MLOs. *Phytopathology* **83**, 1130–7.

Richter S, 1999. Chlorotisches Blattrollen der Marille — Erstauftreten in Österreich, Diagnose und Epidemiologie einer Quarantänekrankheit. *Klosterneuburger Mitteilungen* **49**, 245–9.

Schaff DA, Lee I-M, Davis RE, 1992. Sensitive detection and identification of mycoplasma-like organisms by polymerase chain reactions. *Biochemical and Biophysical Research Communications* **186**, 1503–9.

Seemüller E, 1999. On some problems in temperate fruit tree phytoplasma research. [*http://www.uniud.it/phytoplasma/pap/seem2700.html*].

Seemüller E, Marcone C, Lauer U, Ragozzino A, Göschl M, 1998. Current status of molecular classification of the phytoplasmas. *Journal of Plant Pathology* **80**, 3–26.

Süle S, 1999. Strategies for the control of apricot decline. *Klosterneuburger Mitteilungen* **49**, 250–2.

Wurm L, 1999. Marillenanbau in Österreich. *Klosterneuburger Mitteilungen* **49**, 255–8.

Zull P, 1999. *Bestandsaufnahme von Marillenanlagen in Hinblick auf Baumausfälle durch Apoplexie*. Austria: Diplomprojekt HBLVA Klosterneuburg.