Phytoplasma Occurrence in Apple Trees in the Czech Republic

Renata FIALOVÁ, Milan NAVRÁTIL and Pavla VÁLOVÁ

Department of Cell Biology and Genetics, Faculty of Science, Palacký University, Olomouc-Holice, Czech Republic

Abstract

Fialová R., Navrátil M., Válová P. (2003): **Phytoplasma occurrence in apple trees in the Czech Republic**. Plant Protect. Sci., **39**: 7–12.

The presence of phytoplasmas in apple trees with proliferation symptoms, rubbery wood symptoms and no symptoms was determined by using polymerase chain reaction assays with primers amplifying phytoplasma 16S rRNA gene. Phytoplasmas were detected in all trees with proliferation symptoms. Positive tests for phytoplasma in the group of trees with rubbery wood symptoms and of those without symptoms revealed a relatively high incidence of latent phytoplasma infection. Using restriction fragment length polymorphism analysis, phytoplasma of the same identity – apple proliferation phytoplasma (subgroup 16SrX-A) – was recorded in all positively tested trees.

Keywords: proliferation symptoms; rubbery wood symptoms; non-symptomatic infection; PCR; RFLP

Growing apples is an important part of fruit production in the Czech Republic. Apple trees are one of a wide spectrum of phytoplasma hosts. An important disease caused by phytoplasma is apple proliferation (Gianotti *et al*. 1968; Amici *et al*. 1972; Brčák *et al*. 1972; Cazelles 1973; Petzold & Marwitz 1976). It is one of the most severe apple diseases, as both quantity and quality of the yield are affected (Blattný *et al*. 1963; Seidl 1971; Seidl & Komárková 1977). Phytoplasmas associated with the disease belong to the quarantine organisms (EPPO/CABI 1997). The disease is present in most pome fruit areas of Europe, particularly in southern and Central Europe. The current situation of apple proliferation disease is known in detail in Germany, where investigations on the geographic distribution and epidemiology have been carried out (Frein & Baumann 1982; Bliefernich & Krczal 1995). Seemüller *et al*. (1998) revealed that in large areas of Germany a high percentage of trees in low-intensity orchards is infected.

Typical conspicuous symptoms of the disease are shoot proliferation (witches' brooms) and abnormally enlarged and distinctly dentate stipules. However, the symptom expression of infected apple trees can vary considerably over a period of several years. This variability is related to the colonisation behaviour of causal phytoplasmas (Seemüller *et al*. 1984), climate and growing conditions (Seemüller *et al*. 1998).

Phytoplasmas associated with apple proliferation disease could also occur in trees with symptoms other than typical proliferation, such as reddening of leaves and rubbery wood (Bertaccini *et al*. 2001). In addition to such non-specific symptoms, possible latency must be considered. Seemüller *et al*. (1984) proved a latent phytoplasma infection by successful transmission (grafting) of an apple proliferation agent from non-symptomatic apple trees.

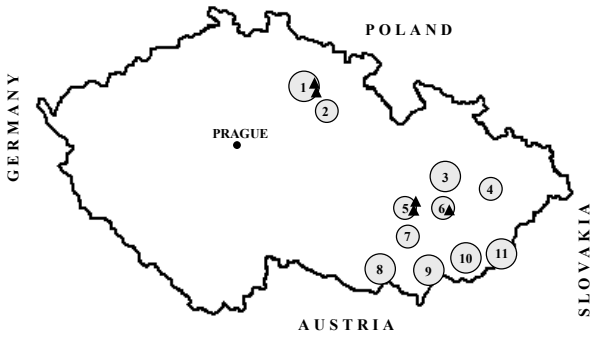
In this study, we report results of the screening for phytoplasma occurrence in samples of apple

Supported by the Grant Agency of the Czech Republic, Grant No. 522/98/P115.

trees collected from selected growing areas of the Czech Republic.

MATERIALS AND METHODS

Material. In co-operation with the State Phytosanitary Administration, visual inspections were carried out in apple growing areas of east Bohemia, central and south Moravia from 1999 to 2002 (Figure 1). Apple trees from intensive orchards, nurseries, and private gardens were investigated for the presence of proliferation symptoms and enlarged stipules. Samples were taken during the vegetation season from all trees with symptoms. Several apple trees with rubbery wood symptoms and a few randomly selected non-symptomatic trees were also included in the study. Altogether, 49 trees (cultivars: Fiesta, Golden Delicious, James Grieve, Lord Lambourne, Ontario, Parména, Rubín, Šampion and unknown) were examined for the presence of phytoplasmas. Three shoot samples per tree were used for the analyses.



Regions: 1 – Jičín; 2 – Hradec Králové; 3 – Olomouc; 4 – Přerov; 5 – Blansko; 6 – Prostějov; 7 – Brno; 8 – Znojmo; 9 – Břeclav; 10 – Hodonín; 11 – Uherské Hradiště. Triangles indicate locations where apple proliferation phytoplasma was present

Figure 1. Apple growing areas inspected for apple proliferation in the Czech Republic. Larger circles represent growing areas over 200 ha of apple tree orchards; smaller circles represent growing areas up to 200 ha

DNA isolation. The isolation of DNA followed the procedure of Ahrens and Seemüller (1992), using the axial phloem tissue of apple trees. In addition, DNA extracts were obtained from plants of *Catharanthus roseus* L. infected with apple proliferation phytoplasma (AT isolate kindly provided by Dr. E. Seemüller, Biologische Bundesanstalt für Land- und Forstwirtschaft, Dossenheim, Germany) as a positive control.

Detection of phytoplasmas by PCR. Universal primers derived from 16S rRNA gene were employed for the detection of phytoplasmas in apple trees. Direct PCR assays were performed using fU5/rU3 (Lorenz *et al*. 1995). PCR assays were also carried out using the primer pair R16F1/R0 (Lee *et al*. 1995). Products were diluted (1:10) with sterile deionised water and re-amplified with the primer pair R16F2/R2 (Lee *et al*. 1995) in nested-PCR. Tubes with the reaction mixture devoid of DNA templates were included in PCR experiments as a negative control and DNA extracts from plants of *Catharanthus roseus* L. infected with AP phytoplasma (AT isolate) as a positive control. PCR products were analysed by electrophoresis through a 1.5% agarose gel and stained with ethidium bromide. DNA bands were then visualised with an UV transilluminator.

Identification of phytoplasmas by RFLP analysis. Phytoplasma identities were revealed by RFLP analysis of amplified fragments of 16S rDNA. Nested-PCR products obtained with the primer pair R16F2/R2 were subjected to digestion with selected restriction endonucleases. 15 µl of each PCR product was digested separately with RsaI and BfmI (Fermentas, Lithuania) according to the instruction of the manufacturer at 37°C overnight. The restriction products were separated by electrophoresis through 3% MetaPhor agarose (FMC, USA) gel stained with ethidium bromide and then visualised with an UV transilluminator. RFLP patterns of samples were compared with corresponding restriction profiles of phytoplasmas that are important in the Czech Republic, i.e. apple proliferation phytoplasma (AP), European stone fruit phytoplasma (ESFY), pear decline phytoplasma (PD) and aster yellows phytoplasma (AY).

RESULTS AND DISCUSSION

Using fU5/rU3 primer pair in direct-PCR, respectively R16F1/R0 and R16F2/R2 primer pairs in nested-PCR, phytoplasma infection was demonstrated in all trees with apparent proliferation symptoms. In addition, phytoplasmas were found in half the trees with rubbery wood symptoms, and in three of seven trees without symptoms (Table 1).

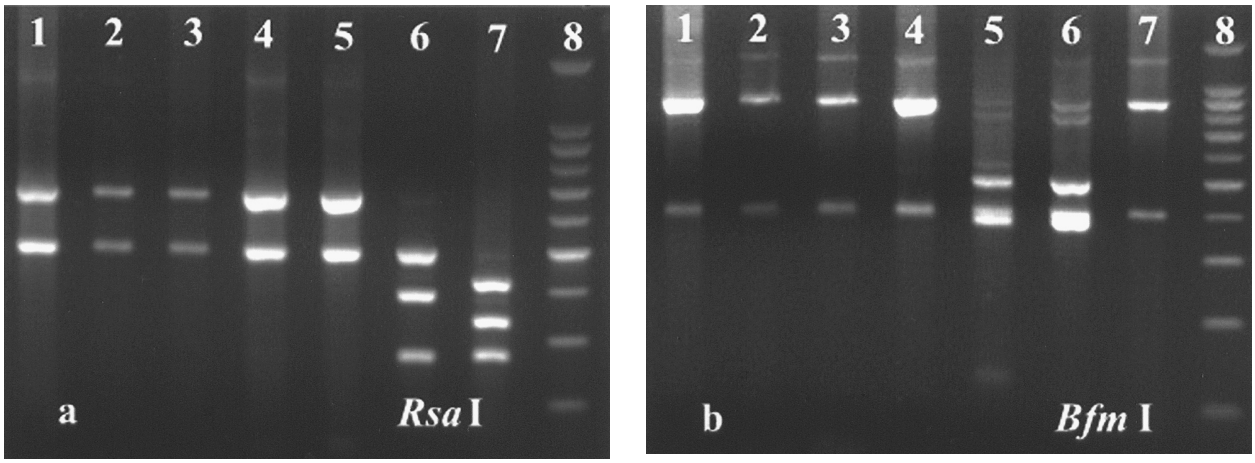
Table 1. Results of tests for phytoplasma in apple trees from selected growing areas in the Czech Republic during 1999–2002

Symptoms	Number of trees	
	tested	phytoplasmas present
Proliferation	24	24
Rubbery wood	18	9
None	7	3

Phytoplasmas infecting apple trees were distinguished by digesting R16F2/R2 PCR fragments. RFLP analysis revealed the same band patterns for products of amplification obtained from trees with proliferation symptoms, those with rubbery wood symptoms, and trees with no symptoms (Figure 2a,b). The patterns corresponded to the profile of apple proliferation phytoplasma – subgroup 16SrX-A (Lee *et al*. 1998).

Selected apple trees were repeatedly sampled and examined by nested-PCR employing R16F1/R0 and R16F2/R2 primer pairs. Such re-testing showed the reliable detection of phytoplasma in samples obtained from apple trees with shoot proliferation symptoms through the vegetative season of 2000 as well as during the other years. However, there was also a scattered distribution of a few PCR positive samples in the groups of trees with rubbery wood symptoms and of those without symptoms (Table 2).

Our results showed that phytoplasma diagnosis using PCR has been reliable in trees with sufficiently distinct symptoms of proliferation. Only a low number of trees with apparent proliferation symptoms were included in the study, and it should be noted that these were found mainly in private gardens. The scarcity of trees with proliferation symptoms would indicate a good health condition of intensive apple tree orchards of the tested growing areas. However, the positive detection of phytoplasma in some trees that appeared healthy shows the probability of a relatively high incidence of apple proliferation phytoplasma infection in a latent form. This, together with the gaps in our knowledge of the epidemiology of apple proliferation disease and the uncertain importance of vectors (Refatti *et al*. 1986; Seemüller 1990) present a phytosanitary risk for both production orchards and nurseries. In Germany, Seemüller *et al*. (1998) found a high percentage of phytoplasma positive samples in apple trees from low-intensity orchards. The authors discussed the reasons for the differences in incidence between low-intensity and high-intensity orchards, and attributed it to fewer insecticide applications in the former. In the Czech Republic, a change in the incidence of



Lane 1 – apple tree with proliferation symptoms; Lane 2 – tree with rubbery wood symptoms; Lane 3 – tree with no symptoms. Control isolates: Lane 4 – apple proliferation phytoplasma; Lane 5 – pear decline phytoplasma; Lane 6 – European stone fruit phytoplasma; Lane 7 – aster yellows phytoplasma; Lane 8 – marker 100 bp DNA ladder, fragment sizes in base pairs from top to bottom: 1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200

Figure 2. RsaI (a) and BfmI (b) restriction profiles of 16S rDNA amplified with primer pair R16F2/R2 from selected apple trees showing different symptoms

Table 2. Re-testing of selected apple trees by nested-PCR using R16F1/R0 and R16F2/R2 primer pairs

Apple tree cultivar / Symptoms	Date of collection				
	September 1999	May 2000	August 2000	October 2000	September 2001
Ontario 1 / P	+	+	+	+	+
Unknown 1 / P	+	+	+	nt	+
Unknown 2 / P	+	+	+	nt	+
Unknown 3 / P	nt	+	+	+	+
Parména / P	+	nt	–	nt	+
James Grieve / P	nt	nt	nt	+	+
Rubín 1 / RW	+	+	–	–	nt
Rubín 2 / RW	+	+	–	–	+
Rubín 3 / RW	+	–	–	–	nt
Rubín 4 / RW	–	–	–	+	nt
Rubín 5 / RW	–	–	–	–	+
Šampion 1 / RW	–	–	–	–	–
Šampion 2 / RW	–	–	–	–	–
Šampion 3 / RW	–	–	–	–	–
Rubín 6 / no	+	–	–	–	–
Rubín 7 / no	–	–	+	–	+
Lord Lambourne 5 / no	–	–	nt	nt	–
Šampion 5 / no	–	–	–	–	–
Ontario 2 / no	–	–	–	–	–

P = proliferation symptoms; RW = rubbery wood symptoms; no = non-symptomatic trees; + = PCR positive; – = PCR negative; nt = not tested

apple proliferation can be expected if there is an increase in the number of low-intensity orchards in marginal apple growing areas and of those with organic production.

Apple trees with symptoms of rubbery wood disease were also included in the study. The present data do not confirm an association between the incidence of rubbery wood symptoms and infection with apple proliferation phytoplasma. However, positive phytoplasma tests within the group show that apple trees with rubbery wood symptoms may be a further source of apple proliferation phytoplasma. There have been some indications that trees with those symptoms may be affected by phytoplasma (Beakbane *et al*. 1971). More recently, the molecular evidence for phytoplasmas in apple trees with rubbery wood symptoms has been demonstrated by Bertaccini *et al*. (1998). The authors classified the phytoplasmas in trees with rubbery wood as members of the aster yellows phytoplasma group (Lee *et al*. 1998), with the only exception of a mixed infection of aster yellows phytoplasma and apple proliferation phytoplasma.

In this study, phytoplasma of the same identity – apple proliferation phytoplasma (subgroup 16SrX-A) – was detected in all positively tested trees. Bertaccini *et al*. (2001) found that apple trees with different symptoms (including apple proliferation symptoms and rubbery wood symptoms) can harbour a relatively wide spectrum of phytoplasmas. However, they also pointed out that the association between recorded symptoms and the presence or type of phytoplasma is not very clear.

References

Ahrens U., Seemüller E. (1992): Detection of DNA of plant pathogenic mycoplasmalike organisms by a polymerase chain reaction that amplifies a sequence of the 16S rRNA gene. Phytopathology, **82**: 828–832.

Amici A., Refatti E., Osler R., Pellegrini S. (1972): Bodies related to mycoplasmas in apple plants in-

fected with witches' broom disease. Rivista Patol. Veget., **8**: 3–19.

Beakbane A.B., Mishra M.D., Posnette A.F., Slater G.C. (1971): Mycoplasma-like organisms associated with chat fruit and rubbery wood diseases of apple, *Malus domestica* Borkh., compared with those in strawberry with green petal disease. J. Gen. Microbiol., **66**: 55–62.

Bertaccini A., Vibio M., Janečková M., Fránová-Honešlegrová J. (1998): Molecular detection of phytoplasmas in apple with rubbery wood symptoms. Acta Hortic., **472**: 693–700.

Bertaccini A., Davies D., Fialová R., Fránová J., Karešová R., Martini M., Navrátil M., Paltrinieri S., Brigheti M. (2001): A molecular survey to identify phytoplasmas associated with apple trees showing different diseases symptoms. Acta Hortic., **550:** 371–376.

Blattný C., Seidl V., Erbenová M. (1963): The apple proliferation of various sorts and possible strain differentiation of the virus. Phytopath. Medit., **2**: 116–123.

Bliefernich K., Krczal G. (1995): Epidemiological studies on apple proliferation disease in southern Germany. Acta Hortic., **386**: 444–447.

Brčák J., Králík O., Seidl V. (1972): Mycoplasmas in apple trees infected with the proliferation disease. Biol. Plantarum, **14**: 427–429.

Cazelles O. (1973): Confirmation of the occurrence of mycoplasma-like micro-organisms in the phloem of apple trees with witches' broom disease. Phytopath. Z., **76**: 30–32.

EPPO/CABI (1997): Apple proliferation *Phytoplasma*. In: Smith I.M., McNamara D.G., Scott P.R., Holderness M. (eds): Quarantine Pests for Europe. 2nd ed. CAB Int., Wallingford, UK.

Frein H., Baumann G. (1982): Investigation on the spread of witches' broom disease of apples in west and south German orchards. Erwerbsobstbau, **11**: 261–265.

Gianotti J., Morvan G., Vago C. (1968): Microorganismes de type mycoplasme dans les cellules libérienne de *Malus sylvestris* L. atteint de la maladie des proliferations. C. R. Acad. Sc. Paris, **267**: 76–77.

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

Lee, I.M., Gundersen-Rindal D.E., Davis R.E., Bartoszyk I.M. (1998): Revised classification scheme of phytoplasmas based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences. Int. J. Syst. Bacter., **48**: 1153–1169.

Lorenz, K.H., Schneider B., Ahrens U., Seemüller E. (1995): Detection of the apple proliferation and pear decline phytoplasmas by PCR amplification of ribosomal and nonribosomal DNA. Phytopathology, **85**: 771–776.

Petzold H., Marwitz R. (1976): Versuche zur Infection von Apfelbäumen mit dem möglichen Erreger der Triebsucht des Apfels. Phytopath. Z., **86**: 365–369.

Refatti E., Osler R., Loi N., Roggero P. (1986): Research on transmission of apple proliferation. Acta Hortic., **193**: 345–355.

Seemüller E. (1990): Apple Proliferation. In: Compendium of Apple and Pear Diseases. Am. Phytopathol. Soc., St. Paul, Minnesota, USA: 67–68.

Seemüller E., Kison H., Lorenz K.H. (1998): On the geographic distribution and prevalence of apple proliferation phytoplasma in low-intensity orchards in Germany. Z. Pflkrankh. Pflschutz., **105**: 404–410.

Seemüller E., Kunze L., Schaper U. (1984): Colonization behaviour of MLO, and symptom expression of proliferation-diseased apple trees and decline-diseased pear trees over a period of several years. Z. Pflkrankh. Pflschutz., **91**: 525–532.

Seidl V. (1971): Contribution to the problems of apple proliferation disease. Ann. Phytopath., No. Hors. Ser.: 391–403.

Seidl V., Komárková V. (1977): Citlivost odrůd jabloně k mykoplazmózní proliferaci. Ochr. Rostl., **13**: 231–235.

Received for publication January 16, 2003
Accepted after corrections March 28, 2003

Souhrn

Fialová R., Navrátil M., Válová P. (2003): **Přítomnost fytoplazem u jabloní v České republice**. Plant Protect. Sci., **39**: 7–12.

Pomocí polymerázové řetězové reakce za použití primerů amplifikujících oblast genu pro 16S rRNA byla sledována přítomnost fytoplazem u jabloní s příznaky proliferace, gumovitosti a u bezpříznakových jabloní. Fytoplazmy byly detekovány u všech stromů vykazujících příznaky proliferace. Pozitivní testy na přítomnost fytoplazem

u jabloní gumovitých a bezpříznakových naznačují možnost poměrně vysokého výskytu latentní infekce. Analýzou délkového polymorfismu restrikčních fragmentů byla u všech infikovaných stromů zjištěna stejná fytoplazma – fytoplazma proliferace jabloně (podskupina 16SrX-A).

Klíčová slova: příznaky proliferace; bezpříznaková infekce; příznak gumovitosti; PCR; RFLP

Corresponding author:

RNDr. Renata Fialová, Ph.D., Přírodovědecká fakulta Univerzity Palackého, katedra buněčné biologie a genetiky, Šlechtitelů 11, 783 71 Olomouc-Holice, Česká republika
tel.: + 420 585 634 902, fax: + 420 587 444 902, e-mail: fialova@prfholnt.upol.cz