COST Action FA0807: Integrated Management of Phytoplasma Epidemics in Different Crop Systems

Short-term Scientific Mission (STSM) Report

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STSM Topic: Detection of apple proliferation phytoplasmas in samples from Romania

Background

Apple proliferation (AP), caused by `*Candidatus* Phytoplasma mali` and pear decline (PD) caused by '*Candidatus* Phytoplasma pyri' are important diseases of apple and pear trees grown in many European countries. The phytoplasma pathogens are spread in a persistent manner by the psyllids mainly *Cacopsylla melanoneura* and *C. picta* (AP) and pear psyllids, *C. pyricola*, *C. pyri*, respectively.
In Romania, symptoms of the apple proliferation diseases on apple trees have been observed since 1967 (Pop et al., 1967 cited by Gheorghiu, l975), when it has been supposed to be associated with viral diseases or some physiological problems. Further investigations proved that the causative agents of disease is a phytoplasma (formerly known as mycoplasma-like organism) (Ploaie, 1973; Gheorghiu, l975, 1981). The first pear decline symptoms was reported by Bălăşcuţă et al. in 1979, and serological studies on associated phytoplasmas were published by Ploaie et al. in 2006. Species compositions and population dynamics of psyllids associated with diseased apple and pear orchards have been done by Chireceanu et al. (1998, 2012). The spreading of the AP and PD diseases emphasized by specific symptoms is still observed in many fruit growing areas, but the phytoplasmas associated with these diseases have not yet been detected and characterised.

Purpose of visit

The main objectives of the work conducted in this STMS were detection and molecular characterization of the 16S rRNA gene of phytoplasma isolates infecting apple and pear trees as well as insects collected from orchards in Romania.

Description of the work

Material and methods

The field activities included orchards, surveying for apple proliferation and pear decline symptoms on affected apple and pear trees, respectively as well as collection of the samples from selected trees and the insects, potential vectors of the phytoplasmas.
Samples of leaves were collected in 2011 from symptomatic apple (35), pear (11), and hawthorn (1) plants growing in four regions of Romania (Dâmboviţa, Argeş, Bistriţa-Năsăud and Bucharest). The insects (11 samples) were captured on apple trees from the Bucharest

area using the beat tray method. Descriptions of the samples are shown in Table 1. Total DNA was extracted from leaf midribs of the trees exhibiting symptoms of phytoplasmal diseases using the modified CTAB-based protocol (Maixner et al., 1995) and was analyzed in the Virology Section of the Research Institute of Horticulture in Skierniewice, Poland. DNeasy Plant Mini (Qiagen, Germany) and InnuPrep Plant DNA (AnalytikJena, Germany) commercial kits were used for DNA extraction from ten batches of 4-5 insects, the symptomatic *Catharanthus roseus* (periwinkle) plant after transmission by *Cacopsylla melanoneura* trials as well as from phloem tissue and leaf midribs of plants infected with phytoplasma isolates kindly provided by the host institution.

DNA samples were amplified in direct PCR amplifying the 16S rDNA, the spacer region between 16S and 23S rDNA and the 5' portion of 23S rDNA. Fast Star Taq DNA Polymerase kit (Roche) and universal phytoplasma primers P1/P7 (Deng and Hiruki, 1991; Schneider et al., 1995) or fU5/rU3 (Lorenz et al., 1995), and primers fAT/rAS (Smart et al., 1993), specific for the apple proliferation phytoplasma group (16SrX) were used in PCRs.

Nested PCR with primer pairs F1/B6 (Davis and Lee, 1993; Padovan et al., 1995) or R16F2n/R16R2 (Gundersen and Lee, 1996) was conducted on the template of direct PCR products diluted (1:29) in sterile distilled water. DNAs from periwinkle infected with `*Ca.* Phytoplasma asteris` or apple infected with `*Ca*. Phytoplasma mali` were used as positive controls. Samples from healthy plants were included as the negative controls of the reactions. PCRs were performed with thermocycler PTC-200 (MJ Research, USA) and the amplification products (10 µl) were separated in 1.2% agarose gels followed by staining in ethidum bromide and visualization of DNA bands using an UVi-Tec transilluminator (Syngen, USA). The molecular weight of PCR products was estimated by comparison with a 100 bp DNA ladder (Fermentas, Lithuania).

Restriction Fragment Length Polymorphism (RFLP) analyses were conducted after digestion of F1/B6 products (~1, 65 kb) with *Mse*I, *Hpa*II and *Ssp*I enzymes (Fermentas, Vilnius, Lithuania). R16RF2n/R16R2 amplicons (~1, 24 kb) were restricted using *Mse*I, *Rsa*I and *Alu*I enzymes (Fermentas, Vilnius, Lithuania). The restriction fragments were separated on 5% polyacrylamide gels or 2% agarose gels and visualized by staining with ethidium bromide under UV transillumination. The RFLP profiles were compared to the patterns of the reference strains (Lee et al., 1998; Paltrinieri et al., 2010).

Table 1. Plant and insect samples collected in four fruit growing regions of Romania

Sample ID	Plant species / cultivar / insect	Symptoms	Locality	PCR results
GD1.DB	apple/Golden Delicious	enlarged stipules	Dâmboviţa (DB)	+
J1.DB	apple/Jonathan	enlarged stipules	Dâmboviţa (DB)	-
I1.DB	apple/Idared	Leaf reddening	Dâmboviţa (DB)	-
GD2.DB	apple/Golden Delicious	Leaf reddening	Dâmboviţa (DB)	-
GD3.DB	apple/Golden Delicious	Leaf reddening	Dâmboviţa (DB)	-
I2.DB	apple/Idared	Leaf reddening	Dâmboviţa (DB)	-
Root.DB	rootstock	witches' broom	Dâmboviţa (DB)	+
GD4.DB	apple/Golden Delicious	witches' broom	Dâmboviţa (DB)	+
Id1.AG	apple/Idared	enlarged stipules	Argeş (AG)	-
W1.AG	apple/Wagner	enlarged stipules	Argeş (AG)	-
F1.AG	apple/Florina	leaf rosetting	Argeş (AG)	-
F2.AG	apple/Florina	leaf rosetting	Argeş (AG)	-
GD1.AG	apple/Golden Delicious	enlarged stipules	Argeş (AG)	-
Is1.AG	apple/Iris	enlarged stipules	Argeş (AG)	+
Id2.AG	apple/Idared	enlarged stipules	Argeş (AG)	-
Un1.Bu	Apple/unknown	Leaf reddening	Bucharest (Bu)	-
GD1.Bu	apple/Golden Delicious	enlarged stipules	Bucharest (Bu)	+
Un2.Bu	Apple/unknown	Leaf reddening	Bucharest (Bu)	-
GD2.Bu	apple/Golden Delicious	witches' broom	Bucharest (Bu)	+
A1.Bu	Apple/Aura	leaf reddening	Bucharest (Bu)	-
G1.Bu	apple/Generos	leaf reddening	Bucharest (Bu)	-
C1.Bu	apple/Ciprian	enlarged stipules	Bucharest (Bu)	-
GD3.Bu	apple/Golden Delicious	enlarged stipules	Bucharest (Bu)	-
Jp1.Bu	apple/Jonaprim	leaf reddening	Bucharest (Bu)	-
Id1.Bu	apple/Idared	leaf reddening	Bucharest (Bu)	-
R1.Bu	apple/Redix	leaf reddening	Bucharest (Bu)	-
E1.Bu	apple/Everest	leaf reddening	Bucharest (Bu)	-
GD4.Bu	apple/Golden Delicious	enlarged stipules	Bucharest (Bu)	-
F1.Bu	apple/Florina	leaf reddening	Bucharest (Bu)	-
Id2.Bu	apple/Idared	enlarged stipules	Bucharest (Bu)	-
F2.Bu	apple/Florina	leaf reddening	Bucharest (Bu)	-
GD5.Bu	apple/Golden Delicious	enlarged stipules	Bucharest (Bu)	-
GD6.Bu	apple/Golden Delicious	no symptoms	Bucharest (Bu)	-
G1.BN	apple/Generos	witches' broom	Bistriţa-Năsăud (BN)	+
G2.BN	apple/Generos	witches' broom	Bistriţa-Năsăud (BN)	+
Un1.DB	pear/unknown	leaf reddening	Dâmboviţa (DB)	-
L1.DB	pear/L12172P/BN	leaf reddening	Dâmboviţa (DB)	-
Un2.DB	pear/unknown	leaf reddening	Dâmboviţa (DB)	-
N1.AG	pear/Napoca	no symptoms	Argeş (AG)	-
N2.AG	pear/Napoca	leaf reddening	Argeş (AG)	-
E1.AG	pear/Euras	leaf reddening	Argeş (AG)	-
W1.Bu	pear/Williams	leaf reddening	Bucharest (Bu)	+
C1.Bu	pear/Conference	leaf reddening	Bucharest (Bu)	+
BH.Bu	pear/Beaure Hady	leaf reddening	Bucharest (Bu)	-
W2.Bu	pear/Williams	leaf reddening	Bucharest (Bu)	+
Un1.Bu	pear/unknown	leaf reddening	Bucharest (Bu)	+

CatR1.Bu	*Catharanthus roseus*	leaf yellowing, shortened internodes	Bucharest (Bu)	+
Haw1.Bu	hawthorn	leaf rolling	Bucharest (Bu)	-
C.m.1.Bu	*Cacopsylla melanoneura*	-	Bucharest (Bu)	-
C.m.2.Bu	*Cacopsylla melanoneura*	-	Bucharest (Bu)	-
C.m.3.Bu	*Cacopsylla melanoneura*	-	Bucharest (Bu)	-
C.m.4.Bu	*Cacopsylla melanoneura*	-	Bucharest (Bu)	-
C.m.5.Bu	*Cacopsylla melanoneura*	-	Bucharest (Bu)	-
C.m.6.Bu	*Cacopsylla melanoneura*	-	Bucharest (Bu)	-
F.f.1.Bu	*Fieberiella florii*	-	Bucharest (Bu)	-
F.f.1.Bu	*Fieberiella florii*	-	Bucharest (Bu)	-
M.p.1.Bu	*Metcalfa pruinosa*	-	Bucharest (Bu)	-
M.p.1.Bu	*Metcalfa pruinosa*	-	Bucharest (Bu)	-

Results

PCR/RFLP results for identification of phytoplasmas from tested plants are summarised in Table 2. PCR with primers fAT/rAS showed that eight out of 35 apple and four out of 11 pear samples were infected with phytoplasmas from apple proliferation phytoplasma group (16SrX). *C. roseus* plant after experimental transmission trial by *C. melanoneura* was also positively tested by P1/P7 followed by nested reactions primed by R16F2n/R16R2 primers. No phytoplasmas were detected in any insect samples.

Nested PCR amplification with F1/B6 primers followed by RFLP analyses with *Mse*I and *Ssp*I enabled confirmation that all analyzed apple samples were infected with `*Ca*. P. mali` (16SrX-A), the causal agents of apple proliferation. The pattern for digested PCR products obtained after amplification of DNA from pear trees was different (Fig. 1). After the digestion with *Hpa*II enzyme of PCR products from apple and pear samples amplified with F1/B6 primers, the RFLP analyses showed two different profiles (P-I and P-II). The P-I profile was detected in the majority of apple isolates and all pear isolates, then P-II profile was detected only in three apple isolates.

Positive results were obtained after nested PCR with these primer pairs applied for amplification of 16S rDNA fragment of phytoplasma infecting periwinkle after transmission by *Cacopsylla melanoneura* trial as well as reference isolates of phytoplasmas kindly provided by the host institution. RFLP analyses of the R16F2n/R16R2 amplicons digested by *Rsa*I, *Mse*I and *Alu*I restriction enzymes showed profile characteristic for *Ca*. P. mali' from three apple samples from Poland. *Mse*I and *Alu*I patterns for *Catharanthus roseus* inoculated by infected *C. melanoneura* was undistinguised from profile for CelY isolate of stolbur phytoplasma, 16SrXII-A (Lee et al, 1998) (Fig. 2). DNA extracted from insects was negatively tested by PCR with P1/P7 followed nested R16F2/R16R2 primers.

*Hpa*II *Mse*I *Ssp*I

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 M 1 2 3 4 5 6 7 8 9 10 11 12 13 14

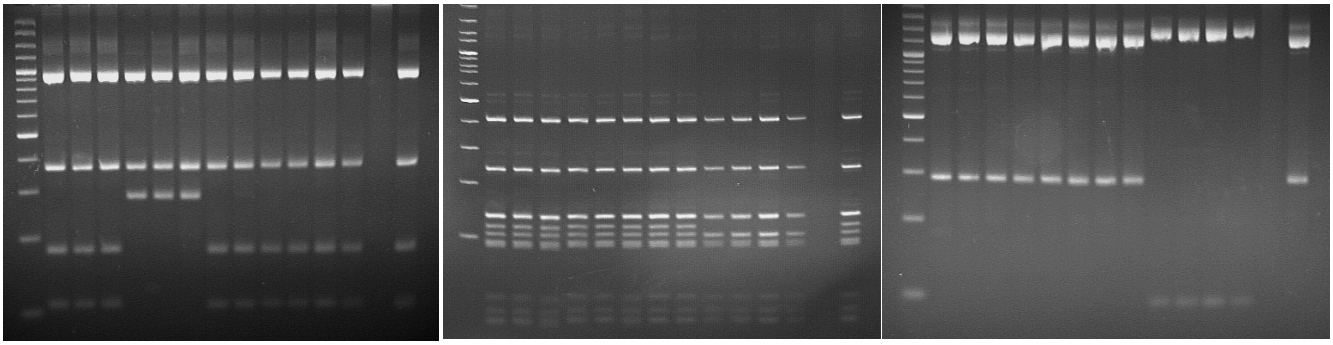

Fig. 1. RFLP patterns with and *Hpa*II, *Mse*I and *Ssp*I of 16 rDNA fragments amplified with primers F1/B6. M- molecular marker 100 bp. Apple:1- GD1.DB, 2- Root1.DB, 3- GD2.DB, 4- GD1.Bu, 5- GD2.Bu, 6- G1.BN, 7- G2.BN, 8- I1.AG, Pear:9- W1.Bu, 10- C1.Bu, 11- W2.Bu, 12- Un1.Bu, 13- healthy plant, 14- apple infected with `*Ca*. P. mali', P-I type. Phytoplasma isolates acronyms are listed in Table 1.

Table 2. Results of RFLP analyses of 16S rDNA fragments of phytoplasmas infecting apple and pear trees

Isolate ID	primers F1/B6			Group
	*Mse*I	*Hpa*II	*Ssp*I	
Apple				
GD1.DB	A	A	A	PI
Root.DB	A	A	A	PI
GD2.DB	A	A	A	PI
GD1.Bu	A	B	A	PII
GD2.Bu	A	B	A	PII
G1.BN	A	B	A	PII
G2.BN	A	A	A	PI
I1.AG	A	A	A	PI
Pear				
W1.Bu	B	A	B	PI
C1.Bu.	B	A	B	PI
W2.Bu	B	A	B	PI
Un1.Bu	B	A	B	PI

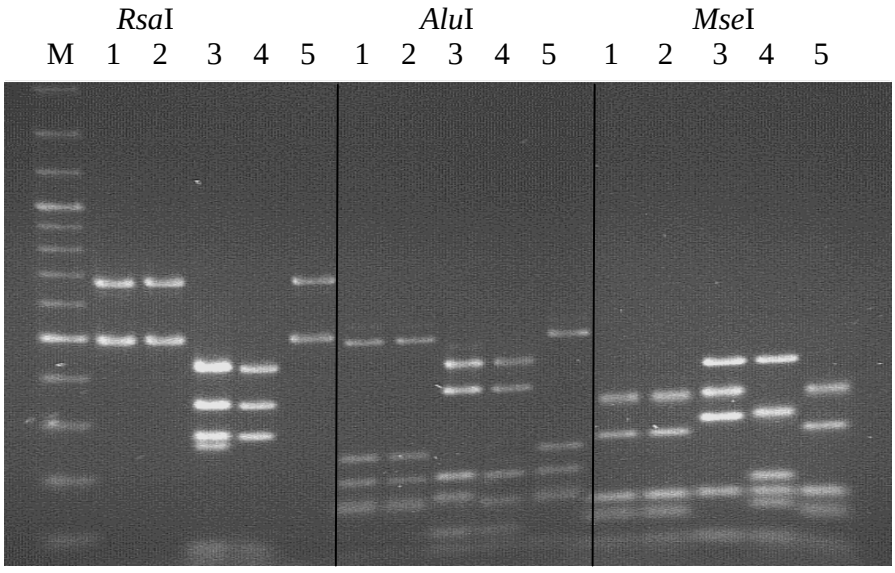

Fig. 2. RFLP profiles of 1.24 kb nested PCR amplified with primers R16F2n/R16R2 from samples of apple infected with reference isolates of apple proliferation phytoplasma (1, 2, and 5), periwinkle infected with reference strain of aster yellows phytoplasma (3) and periwinkle inoculated by infected *C. melanoneura* with stolbur phytoplasma (4).
M - 100 bp DNA ladder (Fermentas, Lithuania)

Conclusion and future collaboration

This STSM allowed improvemnet of knowledge concerning apple proliferation and pear decline diseases and their causal agents `*Ca*. P. mali` and `*Ca*. P. pyri`, to learn how to recognize the disease specific symptoms, to use the different methods of DNA extraction from plant material and insects using commercial kits, to select the suitable primers and enzymes for PCR/RFLP analysis and to interpret the obtained results.

I would like to express my thanks to Prof. Mirosława Cieślińska, for providing me the chance to work in fruit trees phytoplasma field, and her collaborators group for the support and assistance, lab space and equipment used for conducting our research at the Virology Section of the Research Institute of Horticulture in Skierniewice, Poland.
This collaboration will be very useful for us to continue our investigation on isolates of the phytoplasmas from apple proliferation group and it will open new oportunities with regard to other topics concerning the fruit trees phytoplasmas, eg. ESFY phytoplasmas in stone fruit trees. Host laboratory team accepted the future collaboration with us in further research topics.

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