Detection of Phytoplasma ESFY in Apricot Trees using Phloem and Petioles

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Abstract

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ESFY phytoplasma (European stone fruit yellows phytoplasma) is nowadays one of the most important plant diseases, especially on apricots and peaches, and it belongs to the list of organisms for which quarantine is required in the Czech Republic. The aim of this study was to determine the best period for tissue extraction and the best technique for ESFY detection. It was also to investigate the possibility of isolating DNA for use in ESFY detection from the leaf-stalks of randomly chosen symptomatic and asymptomatic apricot trees. Results of the amplification of DNA extracted from leaf-stalk and phloem sampled from 2-year old woody shoots during the years 2003 and 2004 were statistically analysed and compared, and visible disease symptoms were simultaneously evaluated and compared to the results of molecular detection. DNA isolation from leaf-stalks can be considered as less significant and reliable than isolation from phloem sampled from 2-year old woody shoots.

Keywords: ESFY; apricot; PCR; detectability; symptoms

At present, one of the economically most important diseases infecting stone-fruits, especially apricot and peach trees, is the ESFY phytoplasma (European stone fruit yellows phytoplasma). It has been confirmed to occur in most European countries (Davies & Adams 2000; Jarausch *et al.* 2001; Laimer Da Câmara Machado *et al.* 2001; Navrátil *et al.* 2001; Richter 2002; Topchiiska & Sakalieva 2002; Myrta *et al.* 2003; Torres *et al.* 2004).

The symptoms of this phytoplasma, which was earlier called ACLR (apricot chlorotic leaf roll), vary depending on the fruit species and on the virulence of the causal organism (Kison & Seemüller 2001). Leaf roll and discoloration (Figures 1 and 4) such as yellowing of all or part of the leaf blade, growth depresssion, premature leaf shedding and often even fruit dropping are the most common symptoms on apricot trees. On peach trees, leaf roll is often accompanied by leaf flushing with varying intensity, depression and premature leaf shedding starting from the base of the shoot etc. Symptoms such as leaf venation hypertrophy, premature flowering, and development of malformed fruits (Figures 3 and 4) can sometimes appear on stone-fruits. Affected plants usually die within a year or two (Carraro & Osler 2003).

Some studies indicate that apricot and peach trees as well as Japanese plums are among the most sensitive fruit tree species, whereas *Prunus cerasifera* and *P. domestica* are among the least sensitive species and varieties (Kison & Seemüller 2001).

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Figure 1. Leaf roll caused by ESFY phytoplasma on the variety Legolda

Figure 2. Left side – leaf discoloration caused by ESFY phytoplasma on the variety Velikij

Different modifications of molecular methods are now used for ESFY detection such as, e.g., "direct" PCR or "nested" PCR with different specific or non-specific primers such as the primer pairs R16F1/R0 and R16F2/R2 for nested PCR (Lee *et al*. 1995) or the universal primer pairs fU5/rU3 (positive signal: 874-bp) and specific primer pairs fAT/rPRUS (positive signal: 550-bp) (Lorenz *et al*. 1995; Richter 2002). Several techniques and methods of DNA isolation from phloem and leaf stalk have already been published (Doyle & Doyle 1990; Ahrens & Seemüller 1992; Prince *et al*. 1993; Carraro & Osler 2003, etc.).

Figure 3. Ripening irregularity caused by ESFY phytoplasma

MATERIALS AND METHODS

Plant material. Because the occurrence of this disease is most important in the species *Prunus armerica* L., only varieties belonging to this species were chosen for this experiment. Phloem samples were taken for DNA isolation from two different groups of trial trees. The first group included specimens that did not show any visual ESFY or other disease symptoms. The second group included specimens showing various ESFY symptoms of varying intensity.

An apricot tree of the variety Legolda where the presence of ESFY phytoplasma had earlier been proved by the nested PCR method, was chosen as a positive control. RFLP analysis of amplified frag-

Table 1. Isolation at weekly intervals in September (2003)

Cultivar	Leaf-stalk	Phloem	Symptoms shown by tree
Lebona	–	+	leaf roll, growth depression
Legolda PP	+	+	leaf roll, growth depression
NJA35	–	–	without any symptoms
Ledana	–	–	without any symptoms
Velikij	+	–	discoloration – local yellowing
Goldrich	–	–	weak yellowing
Lerosa	–	–	nanism
Veselka	+	+	premature nutlet drop, growth depression
Curtis	–	+	nanism, weak yellowing
Luizet	+	+	yellowing, leaf roll, growth depression, dying off
P. Brigantina × Olimp	–	–	without any symptoms
SE041	–	–	without any symptoms
Legolda A	+	+	leaf roll, weak yellowing
Legolda B	–	–	without any symptoms
Lerosa A	.	–	without any symptoms
Lerosa B	–	–	without any symptoms
Vesprima	.	+	yellowing, growth depression
Leskora	–	–	strong yellowing
Lebona	+	+	leaf roll, growth depression
Velikij A	–	+	discoloration, local yellowing
Velikij B	–	–	part without symptoms
Lejuna	–	–	growth depression
Curtis	+	–	without any symptoms
Curtis symp	+	+	growth depression, weak yellowing
LE-995	–	–	yellowing
Polonais	–	+	yellowing, growth depression
Legolda	–	+	leaf roll, weak yellowing
Lerosa C	–	–	without any symptoms
Leskora	–	–	strong yellowing
Velikij	–	–	part without symptoms

ments obtained from nested PCR with the primer pair R16F2/R2 using restriction endonuclease Sfe I (BfmI), had been identified as ESFY phytoplasma, belonging to the group apple proliferation (16SrX) subgroup B (Lee *et al*. 1993; Lorenz *et al*. 1994). The last phylogenetic analysis revealed that the apple proliferation (AP), pear decline (PD) and European stone fruit yellows (ESFY) are closely related, differing by only 16–19 nucleotide positions in their 16S rDNA, which corresponds to 98.6–99.1% sequence similarity (Seemüller & Schneider 2004). On the basis of these results, these authors propose new species for AP, PD, and ESFY phytoplasmas as '*Candidatus*' ('*Candidatus* Phytoplasma prunorum', '*Candidatus* Phytoplasma mali' and '*Candidatus* Phytoplasma pyri').

The experiment was designed so that the leaves intended for DNA isolation were always taken from about the same part of the shoot as the phloem was (1–2 year old woody shoots). Samples were prepared from an approximately one meter long shoot that was divided into five equal lengths from which average samples of phloem were taken. All leaf-stalks, including central venations, were cut out from the leaves collected in about the same area as the phloem destined for DNA isolation. The technique used for DNA isolation itself was the same in both cases, as was the DNA amplification by the nested PCR method.

Samples were chosen so that they could show on the widest possible scale the problems and inaccuracies linked to the collecting period, the use of plant phloem and the intensity of visual symptoms.

Statistical analyses of the results was done using the computer software Unistat version 4.5. For analyses of the data two methods were used: Kruskal-Wallis one-way analysis of variance, and the method of Multiple comparison test for *t*-interval (95% *t*-interval).

DNA extraction, PCR amplification. The total DNA was isolated from 1 to 2 g of plant tissue by the method described by (Doyle & Doyle 1990). DNA obtained this way from both leaf-stalk and phloem sampled from 2-year old woody shoots was dissolved in 50 µl of TE buffer. The nested PCR method was used for phytoplasma detection: universal primers R16F1/R0 for the first amplification and R16F2/R2 for the second amplification reaction (Lee *et al*. 1995) were used with a dosage of 0.25µM for each reaction. The polymerase used was DyNAzyme™ II 2.0 U (firm Finnzymes) in a dosage of 1.0 U for each reaction, next 10× buffer (10mM Tris-HCl, pH 8.8, 1.5mM $MgCl_2$, 50mM KCl, 0.1% Triton® X-100) 1× for each reaction, dNTP mix in a dosage of 100µM for each reaction. In each 0.2 ml PCR Eppendorf tubes, 2 µl of dissolved DNA isolated in the TE buffer were added to the 18 µl of reaction mix. The amplification itself took place in a thermocycler T Gradient (Biometra) at 94°C (initial denaturation 2 min, 1 cycle), then 35 cycles including denaturation, 1 min at 94°C, annealing 2 min at 50°C and extension 3 min at 72°C. The final extension took place at 72°C during 10 min. Products from the first amplification were diluted (5×) for use in a second amplification with primer pairs R16F2/R2. The final products of the amplification were evaluated on a 1.5% agarose gel stained with ethidium bromide using electrophoresis and visualised on a UV transilluminator.

Phytoplasma identification of the PCR products was done by RFLP analysis using a restriction endonuclease Sfe I (BfmI) (Navrátil *et al*. 2001). The incubation of 10 µl of product with the restriction enzyme took place at 37°C for 16 h, the obtained product was separated on a 2% agarose gel using a TBE buffer. The gel was stained with ethidium bromide and visualised on a UV transilluminator.

RESULTS

In 2003, thirty isolations from leaf-stalk and 30 from phloem were made from trees showing various visual symptoms or no symptoms (Table 1). Phytoplasma was detected in altogether 16 cases (53.3%). Of these, phytoplasma was confirmed in 50% of the isolations from leaf-stalk and in 85.7% of DNA isolations from phloem. The global ratio of positive reactions for leaf-stalk/phloem of evaluated trees reached 0.58 (23.3%, 40%) (Figure 5). The ratio of leaf-stalk/phloem for samples presenting different detection results reached 0.29. Symptoms on trees from which samples had been taken are given in Table 3. With the diagnostical detection of phytoplasma in DNA, a statistically highly significant difference was found between isolations from leaf-stalks and those from phloem sampled from 2-year old woody shoots.

In 2004, altogether 120 isolations were obtained from 57 leaf-stalks and 58 phloems (Table 3). This difference in the number of isolations was due to premature leaf shedding (in the period from 22. 7. to 17. 8.) and to the consequent death of one of the tested trees (Gvardejskij). Consequently it was not possible to isolate leaf-stalk DNA, and the diagnostics done on phloem were already negative in that period. ESFY phytoplasma was confirmed in 44 samples (75.9%) and of these, 59% of the DNA samples obtained from leaf-stalks and 93.2% of those from phloem were positive. The global ratio of positive reactions for leaf-stalk/phloem was in this group 0.63 (45.6%, 70.7%) (Figure 6). The ratio of leaf-stalk/phloem for samples presenting different detection results reached 0.18 in favour of phloem. Symptoms recorded in this variant are again presented in Table 3. Analysis of data of the diagnostic detection of phytoplasma in DNA proved a statistically highly conclusive

Figure 4. Left side: fruits from a tree infected by ESFY

Right side: same variety without ESFY – variety Veselka

difference between isolations from leaf-stalks and those from phloem.

Table 1 presents the characterisation (with numerical coding) and frequency of monitored symptoms during 2003–2004, and the number of positive tests from leaf-stalks or phloem sampled from 2-year old woody shoots by nested PCR.

Mathematical analysis of the data from Table 2 divided the tested collection into two homogeneous subgroups between which statistically conclusive differences were confirmed. The first subgroup contained specimens both without any visual symptoms and specimens with leaf yellowing symptom No. 2; the second subgroup contained specimens with symptoms Nos. 3, 4, 5, 6, 7, 8 and 9 (Table 1). A similar analysis of data from Table 3 did not show any statistically significant difference in detection between analysed symptoms. Table 1 also shows that ESFY phytoplasma was best detected by the nested PCR method if samples came from trees showing growth depression together with other symptoms such as leaf roll, leaf yellowing and eventually premature leaf shedding (symptom No. 7 with 87.5%). A rather good level of detection (58%) was obtained with trees showing leaf roll and yellowing, i.e. symptom No. 6. A detection

Table 2. Frequence of symptoms of ESFY phytoplasma and detection by PCR method

Recorded manifestation – ESFY symptoms	Frequence of symptoms on tested trees			PCR detection 2003		PCR detection 2004		Frequence of ESFY detection by PCR	
	2003	2004	sum	leaf-stalk	phloem	leaf-stalk	phloem	leaf-stalk	phloem
0 – Without any symptoms	11	6*	11	1	0	0	1*	1	1
1 – Yellowing – discoloration	6	1	7	1	1	1	4	2	5
2 – Pronounced wilting of leaves	–	–	–	–	–	–	–	–	–
3 – Local leaf roll	–	–	–	–	–	–	–	–	–
4 – Surface leaf roll (total)	–	3	3	–	–	6	8	6	8
5 – Premature fruitlet drop	1	–	1	1	1	–	–	1	1
6 – Yellowing + surface leaf roll	2	3	5	1	2	7	8	8	10
7 – Yellowing + surface leaf roll + growth depression	9	3	12	2	7	6	14	8	21
8 – Yellowing + surface leaf roll + wilting + leaf shedding	–	1	1	–	–		4	5	4
9 – Premature leaf shedding + dying off	1	1	2	1	1	2	2	3	3

*DNA isolation from samples coming from trees without visual symptoms

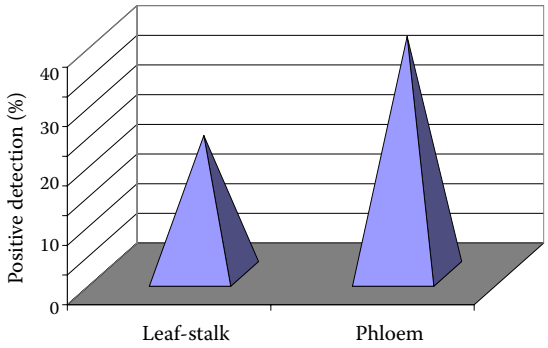

Figure 5. Ratio between number of positive tests from leaf-stalks and phloem (only different PCR detection)

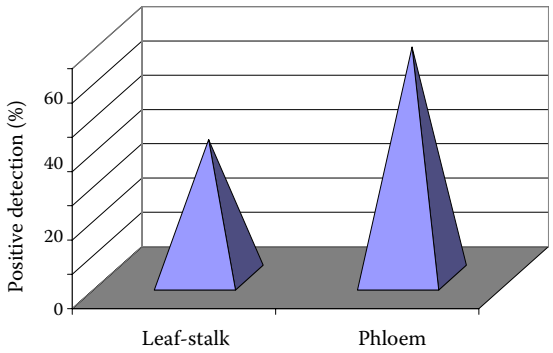

Figure 6. Ratio between number of positive tests from leaf-stalks and phloem (only different PCR detection)

rate of 50 to 53% was reached with samples having the classic symptoms Nos. 4 and 9. In contrast, the lowest level of positive detections (at 45.4%) occurred if symptom No. 1, leaf yellowing, was shown. DNA samples extracted from specimens without any symptoms gave a positive reaction

Table 3. DNA isolation at monthly intervals during the growth period 2004

Cultivar	Plant tissue	10. 6. 04	22. 7. 04	17. 8. 04	20. 9. 04	7. 10. 04	Symptoms shown by tree
Bronzovij	leaf-stalk	+	+	–	+	+	yellowing, premature leaf shedding
	phloem	+	+	+	+	+	
Gvardejskij	leaf-stalk	+	+	n	n	n	strong yellowing, growth deppression – dying off
	phloem	+	+	–	n	n	
Hargrand	leaf-stalk	+	+	+	+	+	leaf roll
	phloem	+	–	+	+	+	
Legolda	leaf-stalk	+	–	+	+	+	leaf roll, weak yellowing
	phloem	+	+	+	+	+	
Lejuna	leaf-stalk	–	–	–	–	–	weak leaf roll
	phloem	+	–	–	–	–	
Murfatlar	leaf-stalk	+	+	–	–	–	yellowing, leaf roll
	phloem	–	+	–	+	–	
Narjadnyj	leaf-stalk	+	–	–	–	–	yellowing, leaf roll
	phloem	+	–	–	–	–	
Saldcot	leaf-stalk	–	–	+	+	–	yellowing, growth depression
	phloem	+	+	+	+	+	
Velikij	leaf-stalk	–	–	–	+	–	discoloration – local yellowing
	phloem	+	+	–	+	+	
Veselka	leaf-stalk	–	+	+	+	+	premature fruitlet drop, growth depression
	phloem	+	+	+	+	+	
Vestar	leaf-stalk	–	–	–	–	–	leaf roll, growth depression
	phloem	+	+	–	+	+	
Velkopavlovická	leaf-stalk	–	–	–	+	–	leaf roll
	phloem	+	+	–	+	+	

in 4.1% of the cases. For symptoms No. 8 and 5 only one specimen was analysed, precluding an objective comment on the basis of such a result (100%) on the efficiency of diagnostics done by the nested PCR method.

The analysis of samples of DNA isolated from leaf-stalks and phloem of trees that showed no symptoms of ESFY phytoplasma detected the presence of phytoplasma only in one case after repeated isolations from the phloem (variety Neptun).

During the statistical analysis of the influence of the time of isolation on the detection efficiency of the nested PCR method, a statistically highly significant difference was found in the experiment (2004) between isolations done 10. 6. and 17. 8. In the period around 10. 6., 71% of the samples were detected as positive without any difference between origin of DNA, whereas in the period around 17. 8. only 37.5% of the samples earlier found to be positive were detected as positive. In other periods of a given year, time of isolation did not present any significant quantitative differences in the number of detected positive samples. Analysis of the differences between times when samples were extracted did not show any statistically significant differences when isolating from leave-stalks. In contrast, when isolating from the phloem, the month of June was shown to be the optimal period. In the year 2003, DNA isolation was conducted during one month and no statistically significant differences were found between the different dates.

In the Table 3 the influence of varieties was statistically analysed. Phytoplasma was most often detected in samples collected from the varieties Velikij, Narjadnyj, Murfatlar and Bronzovij, but less often in the varieties Velkopavlovická and Vestar. Analysis of the data showed statistically highly significant differences between the frequencies of detection in the varieties.

DISCUSSION AND CONCLUSION

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 (Lamier Da Câmara Machado *et al.* 2001). On the base of the results of the present study on detection and diagnosis of ESFY phytoplasma, DNA isolation from leaf-stalks can be considered as less significant and reliable than isolation from phloem sampled from 2-year old woody shoots. Jarausch *et al.* (1999) showed that phytoplasma detection is more reliable in phloem tissue, especially when the phytoplasma concentration is low. It can be said that the number of positive cases detected through isolations from leaf-stalks increases with the intensity of visual symptoms of ESFY phytoplasma. These observations confirm Torres *et al.* (2004) who described that in Japanese plum and apricot there is a close relationship between the presence of symptoms and ESFY phytoplasma detection. Phytoplasma is less detectable at the stage with leaf yellowing symptoms. The effect on vigour, foliar symptoms and phloem necrosis was less pronounced than the lethal effect of infection (Kison & Seemüller 2001). It was not demonstrated by the PCR test that plants that are affected by this disease are very often asymptomatic, it was more the opposite. Jarausch *et al.* (1998) observed that 95% of the trees with typical symptoms also tested positive using PCR. Nevertheless, phytoplasma was detected in 51% of samples showing atypical symptoms. Torres *et al.* (2004) described eight samples from 69 asymptomatic apricot trees that gave a positive reaction by nested PCR with 16SrX group specific primers. In winter, six of these positive trees showed symptoms, the other two remained asymptomatic. An influence of the timing of tissue sample extraction for DNA isolation at weekly intervals in September (the second optimal timing for extraction) was not proved. Instead, there was an influence if the extractions were spaced at monthly intervals. Of the five analysed months (June, July, August, September and October), the optimal month for phloem extraction was determined to be June. Another interesting observation was that August is the least suitable month for molecular genetic detection, whereas detection in September was found to be second best. Jarausch *et al.* (1998) report that, for practical reasons, leaf petioles are preferred to phloem preparations for large scale screening, and that sampling has to be done between July and September in order to obtain reliable results. Using phloem preparations of the branches, PCR detection can also be carried out during winter until March. The influence of varieties on the repeatability of detection was statistically confirmed in the present study. Together with the described symptomatology this aspect could be useful when searching for suitable woody indica-

tors for rapid detection of ESFY phytoplasma in cooperation with EPPO standards.

Further studies of the symptomatology of ESFY phytoplasma and its molecular genetic detection should probably consider symptom evaluation to begin with the onset of the vegetative growth period.

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Abstrakt

Nečas T., Krška B. (2005): **Detekce fytoplazmy ESFY v meruňkách s využitím lýka a řapíku**. Plant Protect. Sci., **41**: 132–140.

Fytoplazma ESFY (European stone fruit yellows phytoplasma), nově označovaná jako „*Candidatus* Phytoplasma prunorum", je stále významným škodlivým činitelem v sadech jak meruňkových, tak broskvoňových. Způsoby rostlinolékařské diagnostiky fytoplazem jsou různé. Molekulárně genetickou diagnostikou s použitím metod PCR byl prokázán významný vliv termínu odběru vzorku na výsledek testování analyzovaného rostlinného materiálu. Současně byl prokázán rozdíl v přesnosti molekulární diagnostiky pro použití odlišných rostlinných pletiv (respektive lýka zdřevnatělého výhonu a u řapíku listu). Z výsledků je zřejmé, že DNA izolovaná z lýka poskytuje vyšší procento pozitivních výsledků testů na fytoplazmu ESFY než DNA izolovaná z listového řapíku.

Klíčová slova: ESFY; meruňka; PCR; detekovatelnost; symptomy

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