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COMPARISON OF REAL-TIME PCR PROTOCOLS IN DETECTION AND QUANTIFICATION OF FRUIT TREE 16SRX GROUP PHYTOPLASMAS

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In this work, two real-time PCR protocols based on intercalating dye and two on hydrolysis probes were tested using field collected fruit tree samples infected by 16SrX group (AP, PD and ESFY) phytoplasmas. Specificity and sensitivity of protocols and amplification efficiency were the main testing parameters. Results of real-time PCR protocols were compared to nested PCR. All real-time PCR protocols confirmed their specificity of detection. All real-time PCR protocols were 10-100 times more sensitive than nested PCR. Afterall real-time PCR protocols based on hydrolysis probes were 10 times more sensitive than protocols based on intercalating dyes. Among protocols based on hydrolysis probes, slightly better detection characteristics were shown by protocol by CHRISTENSEN *et al*. (2004).

Keywords: AP; Bryt Green; ESFY; PD; *Malus*; *Prunus*; *Pyrus*; real-time PCR; TaqMan; TaqMan MGB

INTRODUCTION

Phytoplasmas, previously referred to as mycoplasma-like organisms (DOI *et al*., 1967), belonging to the class *Mollicutes* are plant pathogenic, cell wall-less prokaryotes. They are obligate intracellular parasites that inhabit phloem sieve cells, causing diseases in several hundred plant species (SEEMÜLLER *et al*., 2002).

The most common and severe fruit tree phytoplasmas are '*Ca*. P. mali', '*Ca*. P. prunorum' and '*Ca*. P. pyri' (SEEMÜLLER and SCHNEIDER, 2004), which belong to the 16SrX group (AP group) (WEI *et al*., 2007). These phytoplasmas are the causal agents of apple proliferation (AP), European stone fruit yellows (ESFY) and pear decline (PD), respectively. They are almost exclusively detected in genera *Malus*, *Prunus* and *Pyrus* (SEEMÜLLER and SCHNEIDER, 2004), respectively and can cause considerable economic loses by inducing decrease of size, quality and yield of fruit. '*Ca*. P. mali' and '*Ca*. P. pyri' are considered as quarantine and listed in the EPPO A2 list (EPPO/CABI 1997). AP is only known in Europe, ESFY is known mainly in Europe, but

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has also been reported in Turkey (JARAUSCH *et al*., 2000a) and PD was first reported in North America (MCLARTY, 1948) and seems to have been introduced from Europe (SEEMÜLLER, 1992). All three fruit tree phytoplasmas are transmitted by psyllids from genus *Cacopsylla*.

Sensitive and specific detection of fruit tree phytoplasmas is possible by PCR on the level of 16SrX (AP phytoplasma) group (SEEMÜLLER *et al.*, 1998b), but also on the level of individual phytoplasma species (LORENZ *et al*., 1995; YVON *et al.*, 2009). Since the content of phytoplasmas in woody plants is relatively low (LEE *et al*., 2000) their detection by PCR might be uneasy. This problem has been solved by using nested PCR (GUNDERSEN and LEE, 1996), which allows detection of small amount of phytoplasma in the sample. However, there is an increased risk of cross-contamination by introducing the second PCR run. When compared to real-time PCR, the possibility of cross-contamination is reduced by real-time PCR. Currently, real-time PCR protocols are available for species-specific (MARTINI *et al*., 2007b; PIGNATTA *et al.*, 2008; JARAUSCH *et al*., 2010) and group-specific (TORRES *et al*., 2005) detection and quantification of phytoplasmas based on the use of hydrolysis probes (PIGNATTA *et al.*, 2008) and intercalating dyes (TORRES *et al*., 2005; MARTINI *et al*., 2007b; JARAUSCH *et al*., 2010). Lately, protocols using TaqMan® MGB probes for detection and quantification of AP group phytoplasmas were designed. TaqMan® MGB deoxyribonucleotides are compared to TaqMan probes shorter, anneal stronger to the template DNA and are more specific (YAO *et al*., 2006).

In this publication, real-time PCR protocols based on intercalating dyes and hydrolysis probes for the detection and quantification of phytoplasmas from AP group with focus on ESFY were compared to nested PCR. Specificity, sensitivity and effectivity of each protocol was evaluated. Real-time PCR protocols using TaqMan and TaqMan® MGB probes were tested. Bryt^{TM} Green dye instead of SYBR Green dye was used in real-time PCR protocols based on intercalating dyes. Primers from the region of 16S rDNA and non-ribosomal region were tested by protocols using intercalating dyes.

MATERIALS AND METHODS

Plant and DNA material

Samples for DNA extraction were collected from field grown fruit trees, under long term observation, with typical symptoms or suspected of 16SrX group phytoplasma ('*Ca*. P. prunorum', '*Ca*. P. mali' and '*Ca*. P. pyri') infection. For detection of '*Ca*. P. prunorum' 22 apricot trees (*Prunus armeniaca*), 7 peach trees (*Prunus persica*) and 1 almond tree (*Prunus amygdalus*) were selected. For detection of '*Ca*. P. mali' 6 apple trees (*Malus domestica*) were selected. For detection of '*Ca*. P. pyri' 4 samples of isolated total DNA from pears (*Pyrus communis*) were provided by RBIP (Research and Breeding Institute of Pomology) in Holovousy, Czech Republic. All trees, except for 4 apricot trees grown at production orchard in Kobyli, were grown in the experimental orchard of the Faculty of Horticulture in Lednice (MENDELU in Brno).

DNA extraction

Total DNA was extracted from ± 1g of phloem from two-year-old shoots according to the slightly modified method by AHRENS and SEEMÜLLER (1992). DNA pellets were dissolved in 100 µl TE buffer. For use in real-time PCR, DNA samples were diluted 10 times in sterile distilled water. DNA samples were stored at -20°C.

Nested PCR

All samples were tested for phytoplasma presence by PCR with primer pair P1/P7 (SCHNEIDER *et al*., 1995). For the presence of 16SrX group phytoplasmas subsequent nested PCR with primer pair fO1/rO1 (LORENZ *et al*., 1995) was carried out using 1 µl of P1/P7 PCR product. One reaction contained 0.25 µM of each primer (P1/P7 or fO1/rO1), 125 µM of each dNTP, 1 unit of GoTaq® G2 Hot Start polymerase (Promega), 1x Colorless GoTaq® Flexi buffer, 2 mM Mg^{2+}, sterile distilled water and 1 µl of DNA. Total volume of the reaction was 20 µl (19 µl Mix + 1 µl DNA sample). Thermal conditions of primary PCR and nested PCR were the same, starting with 2 min at 95°C and followed by 35 cycles of 30 s at 95°C, 60 s at 55°C and 90 s at 72°C with final extension for 5 min at 72°C. PCR was performed on TC-3000 cycler (Techne). PCR products were separated by electrophoresis on 1% agarose gel and visualized by GelRed (Biotium) on UV transilluminator.

Real-time PCR protocols

For real-time PCR quantification of phytoplasmal DNA, two protocols based on hydrolysis probes according to CHRISTENSEN *et al*. (2004) and NIKOLIĆ *et al*. (2010) and two protocols based on intercalating dyes according to YVON *et al*. (2009) and JARAUSCH *et al*. (2010) were selected for comparison.

Protocol by CHRISTENSEN *et al*. (2004) is a protocol for universal detection of phytoplasmas. For the project, TaqMan hydrolysis probes were supplied by Generi Biotech (Czech Republic), labeled with FAM reporter at the 5' end and BHQ1 (Black Hole Quencher) at the 3' end. Real-time PCR was performed in 10 µl reaction volumes containing 1 µl of DNA, 300 nM forward primer, 900 nM reverse primer, 200 nM TaqMan probe, 2 mM Mg^{2+}, 125 µM of each dNTP, 1 unit of GoTaq® G2 Hot Start polymerase (Promega), 1x Colorless GoTaq® Flexi buffer (Promega) and sterile distilled water.

Protocol according to NIKOLIĆ *et al*. (2010) uses universal primers for 16SrX phytoplasma group and specific probe for each fruit tree phytoplasma from the 16SrX group (probe ESFY for '*Ca*. P. prunorum', probe AP for '*Ca*. P. mali' and probe PD for '*Ca*. P. pyri'). For the project TaqMan® MGB hydrolysis probes were supplied by Life Technologies, labeled with FAM reporter at the 5' end and NFQ (Non Fluorescent Quencher) with MGB (Minor Groove Binder) at the 3' end. Real-time PCR was run in 10 µl reaction volumes containing 1 µl of DNA, 300 nM of each primer, 150 nM of TaqMan® MGB probe, 4 mM Mg^{2+}, 125 µM of each dNTP, 1 unit of GoTaq® G2 Hot Start polymerase (Promega), 1x Colorless GoTaq® Flexi buffer (Promega) and sterile distilled water.

Protocol according to YVON *et al*. (2009) detects specifically phytoplasma '*Ca*. P. prunorum'. Real-time PCR was run in 20 µl reaction volumes containing 1 µl of DNA, 150 nM forward primer, 300 nM reverse primer, aditional 0.5 mM Mg^{2+}, 1x GoTaq® Master qPCR Mix (Promega) containing Bryt™ Green intercalating dye and sterile distilled water.

Protocol according to JARAUSCH *et al*. (2010) detects specificaly phytoplasma '*Ca*. P. prunorum'. For the protocol the same GoTaq® qPCR Master Mix (Promega) containing Bryt™ Green intercalating dye was used as for the protocol according to YVON *et al*. (2009). Real-time PCR was run in 20 µl reaction volumes containing 1 µl of DNA, 200 nM forward primer, 400 nM reverse primer, 1 mM Mg^{2+}, 1x GoTaq® Master qPCR Mix (Promega) and sterile distilled water.

Temperature profiles of the real-time PCR protocols are placed in the Table 1. Compared to the original protocol according to JARAUSCH *et al*. (2010) annealing temperature was increased

from 54°C to 55°C because of the formation of double bands when annealing temperature 54°C was applied. All primers were synthesized by Generi Biotech (Czech Republic), dNTP's were supplied by Thermo Scientific. Real-time PCR assays were performed on ECO real-time PCR cycler (Illumina) using 48 cell microplates. For inter plate analysis a plate control was used in protocols detecting '*Ca*. P. prunorum'.

Tab. 1 Temperature profils of the real-time PCR protocols

Protocol	Polymerase activation temp./time	Denaturation temp./time	Annealing temp./time	Elongation temp./time	No. of cycles[a]
CHRISTENSEN *et al*. 2004	95°C / 120 s	95°C / 15 s	60°C / 60 s		40x
NIKOLIĆ *et al*. 2010	95°C / 120 s	95°C / 15 s	60°C / 60 s		40x
YVON *et al*. 2009	95°C / 120 s	95°C / 15 s	63°C / 30 s	72°C / 30 s	40x
JARAUSCH *et al*. 2010	95°C / 120 s	95°C / 15 s	55°C / 30 s	69°C / 30 s	40x

[a] 1 cycle: denaturation+annealing+elongation

Specificity of protocols

Specificity of protocols was tested as a cross-reactivity with other 16SrX group phytoplasmas using all DNA samples tested in this project. DNA samples were assayed in triplicates for each protocol. Each plate contained 2 positive controls, 2 negative controls and 2 NTC (no template control).

For the protocol according to CHRISTENSEN *et al*. (2004) plasmid with cloned PCR product was prepared by Generi Biotech (Czech Republic). Plasmid solution was diluted in sterile distilled water to produce a 10-fold serial dilution from 10^7 to 10^1 copies.μl^{-1}. By creating of standard curve from plasmid serial dilutions, absolute phytoplasma quantification in samples was performed. For other real-time PCR protocols Ct (threshold cycles) values were recorded. The Eco Real-Time PCR System Software (Illumina) was used for fluorescence acquisition and determination of Ct values.

Efficiency of amplification and sensitivity of protocols

One DNA sample from each tested 16SrX group phytoplasma species ('*Ca*. P. prunorum', '*Ca*. P. mali', '*Ca*. P. pyri') with the lowest Ct value, thus highest pathogen concentration measured by absolute quantification with protocol by CHRISTENSEN *et al*. (2004) (Tab. 4), was selected for testing of sensitivity, limit of detection ($LOD_{95\%}$) and efficiency (E) of real-time PCR protocols.

For each selected sample a 7 point 10-fold dilution series was prepared ranging from 10^6 to 10^0 copies/µl (Fig. 2). Dilution series of '*Ca*. P. prunorum' was used for all real-time PCR protocols, dilution series of '*Ca*. P. mali' and '*Ca*. P. pyri' were used for protocols by CHRISTENSEN *et al*. (2004) and NIKOLIĆ *et al*. (2010). Each dilution point was performed in 5 repeats.

Sensitivity is the ability of the protocol to detect the lowest possible concentration within the dilution series. The sensitivity of real-time PCR protocols was compared to the sensitivity of nested PCR, where the same procedure for testing of sensitivity was performed as for real-time PCR protocols.

Subsequently $LOD_{95\%}$ was calculated to set a fixed point for analysis of sensitivities of real-time PCR protocols. $LOD_{95\%}$ reflects the 95% detection probability of each tested protocol with given sample. The statistical program R with drc package was used to compute the $LOD_{95\%}$. For each real-time PCR assay the best fitting curve with the lowest residual standard error according to MEHLE (2014) was chosen. Each $LOD_{95\%}$ was then compared to the lowest detected concentration of given phytoplasma species by nested PCR (Fig. 2).

Slope (k) of the linear regression line between the Ct values and log value of relative DNA concentration was used to calculate the amplification efficiency (E), $E = 10^{(-1 / k)} - 1$, where value of 1 equals 100% amplification. The squared regression coefficient (R^2) was determined after linear regression. The dynamic range represents the range of DNA concentration until the Ct values were linearly related to the log value of the DNA concentration. Detection range of real-time PCR protocols was also determined.

RESULTS

Detection of phytoplasmas from the 16SrX group by nested PCR (P1/P7 + fO1/rO1) resulted in 37 positive and 3 negative (1 pear and 2 peach) samples (Tab. 4). Phytoplasma concentration (according to the protocol by CHRISTENSEN *et al*., 2004) in the samples ranged from 1.21×10^4 to 6.93×10^5 copies/µl, with the Ct values ranging from 30.09 ± 0.01 to 23.52 ± 0.03 for protocol by CHRISTENSEN *et al*. (2004); from 30.93 ± 0.05 to $24.35 \pm 0:21$ for protocol by NIKOLIĆ *et al*. (2010); from 30.56 ± 0.10 to 22.44 ± 0.06 for protocol by YVON *et al*. (2009) and from 30.5 ± 0.16 to 22.75 ± 0.07 for protocol by JARAUSCH *et al*. (2010). Analysis of plate controls resulted in very homogenous inter plate performances of all real time PCR protocols detecting '*Ca*. P. prunorum' showing standard deviation values ranging from 0.16 – 0.21 (data not shown). The highest mean Ct values were obtained with protocol by NIKOLIĆ *et al*. (2010); followed by protocol by CHRISTENSEN *et al*. (2004); hereinafter by JARAUSCH *et al*. (2010), and by YVON *et al*. (2009) (Fig. 1)

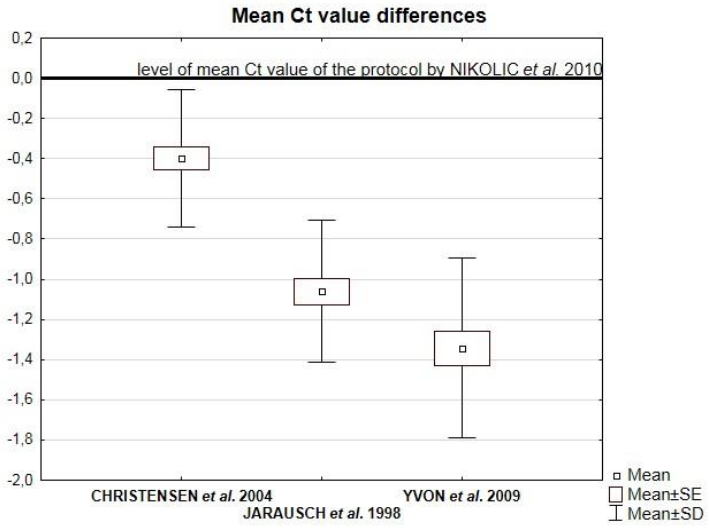

Fig. 1 Mean Ct value differences of real-time PCR protocols

Specificity of protocols

Specificity of all tested protocols was confirmed as reported by their authors (Tab. 4). In all PCR runs negative controls and NTCs were negative and positive controls were positive. No primer dimer formation was observed at negative controls and NTC's at analysis of melting curves of protocols based on intercalating dyes, however primer dimers were observed at positive controls and positive samples. All tested real-time PCR protocols have confirmed sample positivity/negativity results from nested PCR, no false positive/negative samples were observed.

Efficiency of amplification and protocol sensitivity

Analysis of sensitivity showed that not all protocols are equally sensitive in detection of low amounts of target DNA in the sample. Furthermore at lower concentrations ($\leq 10^2$ copies/µl) all real-time PCR protocols exhibited higher standard deviation (≥ 0.5, data not shown) and at the lowest concentrations often only 1-3 repeats out of 5 were detected only. Subsequently $LOD_{95\%}$ was calculated. In concentrations lower than $LOD_{95\%}$, it might happen that not all sample repeats will give signal and, thus, aditional analysis will be needed to proove the positivity.

A Detection of '*Ca.* P. prunorum'

Dilution (x-fold)	10^0	10^1	10^2	10^3	10^4	10^5	10^6
Concentration (copies/µl)	$6.93x10^6$	$6.93x10^5$	$6.93x10^4$	$6.93x10^3$	$6.93x10^2$	$6.93x10^1$	$6.93x10^0$
CHRISTENSEN *et al.* 2004 (ct)	19.81	23.29	27.02	30.52	36.95	38.03	38.77
NIKOLIC *et al.* 2010 (ct)[a]	21.34	24.69	28.13	31.71	37.47	38.77	39.59
YVON *et al.* 2009 (ct)	18.27	21.83	25.39	29.72	36.21	36.80	ND
JARAUSCH *et al.* 2010 (ct)	19.52	22.71	26.46	30.50	36.88	39.21	ND

Nested PCR (P1/P7 +fO1/rO1)

1000bp

B Detection of '*Ca.* P. mali'

Dilution (x-fold)	10^0	10^1	10^2	10^3	10^4	10^5	10^6
Concentration (copies/µl)	$2.67x10^6$	$2.67x10^5$	$2.67x10^4$	$2.67x10^3$	$2.67x10^2$	$2.67x10^1$	$2.67x10^0$
CHRISTENSEN *et al.* 2004 (ct)	21.23	24.53	28.17	33.22	36.47	38.54	ND
NIKOLIC *et al.* 2010 (ct)[a]	21.89	25.31	28.82	34.55	37.24	ND	ND

Nested PCR (P1/P7 +fO1/rO1)

1000bp

C Detection of '*Ca.* P. pyri'

Dilution (x-fold)	10^0	10^1	10^2	10^3	10^4	10^5	10^6
Concentration (copies/µl)	$2.96x10^6$	$2.96x10^5$	$2.96x10^4$	$2.96x10^3$	$2.96x10^2$	$2.96x10^1$	$2.96x10^0$
CHRISTENSEN *et al.* 2004 (ct)	21.03	24.43	28.03	32.07	37.09	37.92	ND
NIKOLIC *et al.* 2010 (ct)[a]	22.18	25.53	29.06	33.64	38.09	39.05	ND

Nested PCR (P1/P7 +fO1/rO1)

1000bp

Fig. 2 Detection sensitivities of real-time PCR protocols and nested PCR

[a] ESFY probe was used

[b] AP probe was used

[c] PD probe was used

Analysis of protocol sensitivities at detection of '*Ca*. P. prunorum' (Fig. 2) showed that protocols by CHRISTENSEN *et al*. (2004) and NIKOLIC *et al*. (2010) were capable to detect less than 10 target copies (Tab. 3), resulting in 10 times higher detection sensitivity than protocols by YVON *et al*. (2009) and JARAUSCH *et al*. (2010). $LOD_{95\%}$-s (Tab. 2) of all real-time PCR protocols were 100 times higher than their sensitivities. When comparing real-time PCR protocols to nested PCR, sensitivities of protocols based on hydrolysis probes and intercalating dyes were 10 and 100 times, respectively, higher but performed same and 5-10 times, respectively, higher $LOD_{95\%}$ than the detection limit of nested PCR.

Analysis of protocol sensitivities at detection of '*Ca*. P. mali' (Fig. 2) showed that protocol by CHRISTENSEN *et al*. (2004) is capable to detect orders of 10^1 target copies (Tab. 3), resulting in 10 times higher detection sensitivity than protocol by NIKOLIĆ *et al*. (2010). $LOD_{95\%}$ (Tab. 2) of both real-time PCR protocols laid in the same orders of target copies as their sensitivities. When comparing real-time PCR protocols to nested PCR, sensitivities of protocol by CHRISTENSEN *et al*. (2004) and NIKOLIĆ *et al*. (2010) were 10 and 100 times, respectively, higher and performed 5 and 45 times, respectively, lower $LOD_{95\%}$ than the detection limit of nested PCR.

Analysis of protocol sensitivities at detection of '*Ca*. P. pyri' (Fig. 2) showed that, both, protocols by CHRISTENSEN *et al*. (2004) and NIKOLIĆ *et al*. (2010) are capable to detect order of 10^1 target copies (Tab. 3). Computed $LOD_{95\%}$ (Tab. 2) of protocol by CHRISTENSEN *et al*. (2004) laid in the same order of target copies (10^1) as its sensitivity, however the $LOD_{95\%}$ of the protocol by NIKOLIĆ *et al*. (2010) was 100 times higher than its sensitivity. When comparing real-time PCR protocols to nested PCR, real-time PCR protocols showed 100 times higher sensitivities, but performed same (NIKOLIĆ *et al*. 2010) and approximately 42 times lower (CHRISTENSEN *et al*., 2004) $LOD_{95\%}$ than the detection limit of nested PCR.

Tab. 2 $LOD_{95\%}$ calculation of real-time PCR protocols

Pathogen	Protocol	$LOD_{95\%}$ (copies)	Residual std. error	Degrees of freedom	Function [a] (drc package, R program)	Nested PCR detection limit[b] (copies)
'Ca. P. prunorum'	CHRISTENSEN *et al*. 2004	$7.08x10^2$	$4.69x10^{-2}$	6	W2.2	$6.93x10^2$
	NIKOLIĆ *et al*. 2010[c]	$7.08x10^2$	$4.69x10^{-2}$	6	W2.2	
	YVON *et al*. 2009	$7.07x10^3$	$5.14x10^{-2}$	5	W2.2	
	JARAUSCH *et al*. 2010	$3.83x10^3$	$2.14x10^{-2}$	5	W2.2	
'Ca. P. mali'	CHRISTENSEN *et al*. 2004	$5.87x10^1$	$9.05x10^{-5}$	5	W1.2	$2.67x10^3$
	NIKOLIĆ *et al*. 2010[d]	$5.28x10^2$	$7.31x10^{-5}$	3	LL.3u	
'Ca. P. pyri'	CHRISTENSEN *et al*. 2004	$7.05x10^1$	$7.69x10^{-5}$	5	W1.2	$2.96x10^3$
	NIKOLIĆ *et al*. 2010 [e]	$3.02x10^3$	$5.14x10^{-2}$	5	W2.2	

[a] function showing the lowest residual std. error (regarding to MEHLE 2014)

[b] the lowest detected concentration (calculated from Fig. 2)

[c] ESFY probe was used, [d] AP probe was used, [e] PD probe was used

Dynamic range (Tab. 3) was at detection of '*Ca*. P. prunorum' and '*Ca*. P. mali' the same for all protocols, 10^3-10^6 copies/µl. At detection of '*Ca*. P. pyri' the dynamic range of the protocols was by 1 dilution point lower, 10^4-10^6 copies/µl.

Analysis of linear regression of real-time PCR protocols (Tab. 3) at detection of '*Ca*. P. prunorum' showed that protocols based on hydrolysis probes (CHRISTENSEN *et al*., 2004 and NIKOLIĆ *et al*., 2010) had lower slope (k), -3.59 and -3.45, respectively, *i.e.* closer to the optimal -3.32, than protocols based on intercalating dye (YVON *et al*., 2009; JARAUSCH *et al*., 2010), -3.74 and -3.67, respectively. Furthermore, the efficiency of amplification of protocols based on hydrolysis probes (CHRISTENSEN *et al*., 2004; NIKOLIĆ *et al*., 2010) was higher, 90.01% and 94.87%, respectively, than of the protocols based on intercalating dye (YVON *et al*., 2009; JARAUSCH *et al*., 2010), 84.99% and 87.27%, respectively.

The values of slope (k) and amplification efficiencies (E) of protocols by CHRISTENSEN *et al*., (2004) and NIKOLIĆ *et al*. (2010) at detection of '*Ca*. P. mali' were very similar. The same similarity was observed with these protocols at detection of '*Ca*. P. pyri'. However, at detection of '*Ca*. P. pyri' protocols showed worse values of slope (k) and amplification efficiencies (E) as compared to the performance of the same protocols at detection of '*Ca*. P. prunorum' and '*Ca*. P. mali' (Tab. 3).

Tab. 3 Range of detection, dynamic range and linear regression of real-time PCR protocols

	Protocol	Range of detection (copies/µl)	Dynamic range (copies/µl)	Linear regression			Melting temp. (Tm)
				Slope (k)	R^2	E (%)	
'*Ca*. P. prunorum'	CHRISTENSEN *et al*. 2004	$6.93x10^6 – 6.93$	$6.93x10^6 – 6.93x10^3$	-3.59	0.999	90.01	
	NIKOLIĆ *et al*. 2010[a]	$6.18x10^6 – 6.93$	$6.93x10^6 – 6.93x10^3$	-3.45	0.998	94.87	
	YVON *et al*. 2009	$6.93x10^6 – 6.93x10^1$	$6.93x10^6 – 6.93x10^3$	-3.74	0.996	84.99	83.6 °C
	JARAUSCH *et al*. 2010	$6.93x10^6 – 6.93x10^1$	$6.93x10^6 – 6.93x10^3$	-3.67	0.996	87.27	72.2 °C
'*Ca*. P. mali'	CHRISTENSEN *et al*. 2004	$2.67x10^6 – 2.67x10^1$	$2.67x10^6 – 2.67x10^4$	-3.46	0.998	94.41	
	NIKOLIĆ *et al*. 2010[b]	$2.67x10^6 – 2.67x10^2$	$2.67x10^6 – 2.67x10^4$	-3.46	0.994	94.69	
'*Ca*. P. pyri'	CHRISTENSEN *et al*. 2004	$2.96x10^6 – 2.96x10^1$	$2.96x10^6 – 2.96x10^3$	-3.67	0.997	87.32	
	NIKOLIĆ *et al*. 2010 [c]	$2.96x10^6 – 2.96x10^1$	$2.96x10^6 – 2.96x10^3$	-3.74	0.992	85.00	

[a] ESFY probe was used

[b] AP probe was used,[c] PD probe was used,R^2: regression coefficient,E: efficiency of amplification

Tab. 4 Results of phytoplasma detection by nested PCR and real-time PCR protocols

Pathogen	Host plant	Variety	Origin	Nested PCR (P1/P7 + fO1/rO1)	CHRISTENSEN *et al.* 2004		NIKOLIĆ *et al.* 2010[a]	YVON *et al.* 2009[b]	JARAUSCH *et al.* 2010[c]
					copies/µl[d]	ct ± SD	ct ± SD	ct ± SD	ct ± SD
'Ca. P. prunorum'	*P.armeniaca*	Saldcot	Lednice	+	$4.85x10^4$	27.84 ± 0.11	28.02 ± 0.33	26.64 ± 0.09	27.29 ± 0.06
		Gvardejskij	Lednice	+	$1.30x10^5$	26.24 ± 0.11	27.12 ± 0.09	25.31 ± 0.07	25.60 ± 0.12
		Gvardejskij	Lednice	+	$1.99x10^5$	25.55 ± 0.10	26.02 ± 0.01	24.68 ± 0.10	24.94 ± 0.17
		Churmai	Lednice	+	$1.42x10^4$	29.83 ± 0.15	30.48 ± 0.48	29.44 ± 0.11	30.21 ± 0.12
		NJA35	Lednice	+	$3.00x10^5$	24.89 ± 0.19	25.59 ± 0.66	23.09 ± 0.08	23.94 ± 0.10
		NJA35	Lednice	+	$9.69x10^4$	26.74 ± 0.30	26.75 ± 0.62	26.01 ± 0.10	26.00 ± 0.02
		Veselka	Lednice	+	$1.49x10^5$	26.02 ± 0.07	26.20 ± 0.17	24.44 ± 0.14	25.54 ± 0.09
		Hativ Colmer	Lednice	+	$6.93x10^5$	23.52 ± 0.03	24.35 ± 0.21	22.44 ± 0.06	22.75 ± 0.07
		Hativ Colmer	Lednice	+	$6.18x10^5$	23.71 ± 0.09	25.02 ± 0.05	23.46 ± 0.05	23.96 ± 0.14
		Hargrand	Lednice	+	$3.86x10^4$	28.22 ± 0.11	28.75 ± 0.10	27.19 ± 0.02	27.41 ± 0.06
		Hargrand	Lednice	+	$1.37x10^5$	26.15 ± 0.07	26.71 ± 0.24	25.40 ± 0.10	25.75 ± 0.08
		Olimp	Lednice	+	$2.55x10^5$	25.15 ± 0.15	26.06 ± 0.08	24.99 ± 0.06	24.37 ± 0.10
		Olimp	Lednice	+	$1.85x10^5$	25.68 ± 0.27	25.71 ± 0.00	23.90 ± 0.03	24.53 ± 0.16
		Poljus	Lednice	+	$3.15x10^5$	24.80 ± 0.00	25.15 ± 0.05	23.76 ± 0.08	23.88 ± 0.08
		Poljus	Lednice	+	$1.30x10^5$	26.24 ± 0.13	26.64 ± 0.43	25.34 ± 0.01	25.45 ± 0.11
		Arzami Aromat	Lednice	+	$1.86x10^5$	25.66 ± 0.07	26.49 ± 0.11	25.71 ± 0.17	25.46 ± 0.01
		Reale d´Imola	Lednice	+	$1.83x10^5$	25.69 ± 0.05	25.89 ± 0.09	24.14 ± 0.13	24.8 ± 0.04
		NJA1	Lednice	+	$1.63x10^5$	25.89 ± 0.27	26.40 ± 0.06	24.74 ± 0.16	24.82 ± 0.07
		Hargrand	Kobylí	+	$1.14x10^5$	26.45 ± 0.10	26.58 ± 0.18	25.37 ± 0.12	25.70 ± 0.30
		Hargrand	Kobylí	+	$1.57x10^5$	26.00 ± 0.53	26.36 ± 0.05	25.13 ± 0.08	25.40 ± 0.05
		Hargrand	Kobylí	+	$1.22x10^5$	26.35 ± 0.09	27.09 ± 0.04	25.7 ± 0.11	25.85 ± 0.16
		Hargrand	Kobylí	+	$7.81x10^4$	27.07 ± 0.16	27.53 ± 0.31	26.6 ± 0.07	26.43 ± 0.02
	P.persica	Envoy	Lednice	+	$3.48x10^4$	28.38 ± 0.07	28.66 ± 0.14	27.14 ± 0.14	27.56 ± 0.19
		Victory	Lednice	+	$5.12x10^4$	27.77 ± 0.24	28.23 ± 0.07	27.12 ± 0.08	27.23 ± 0.03
		Jantze	Lednice	+	$1.21x10^4$	30.09 ± 0.01	30.93 ± 0.05	30.56 ± 0.10	30.5 ± 0.16
		Velkobítešská	Lednice	+	$3.50x10^4$	28.37 ± 0.03	28.68 ± 0.09	27.62 ± 0.02	27.87 ± 0.04
		Veteran	Lednice	+	$4.14x10^4$	28.10 ± 0.08	28.86 ± 0.12	28.39 ± 0.13	28.31 ± 0.08
		Efekt	Lednice	-	ND	ND	ND	ND	ND
		seedling	Lednice	-	ND	ND	ND	ND	ND
	P.amygdalus	Filippo Ceo	Lednice	+	$1.00x10^5$	26.68 ± 0.29	26.8 ± 0.05	25.23 ± 0.07	25.77 ± 0.12
'Ca. P. mali'	*M.domestica*		Lednice	+	$2.09x10^4$	29.21 ± 0.06	29.10 ± 0.11	ND	ND
			Lednice	+	$2.67x10^5$	25.07 ± 0.10	24.88 ± 0.02	ND	ND
			Lednice	+	$7.71x10^4$	27.09 ± 0.06	27.05 ± 0.04	ND	ND
			Lednice	+	$1.43x10^5$	26.09 ± 0.04	26.20 ± 0.09	ND	ND
			Lednice	+	$1.31x10^5$	26.23 ± 0.16	26.20 ± 0.10	ND	ND
			Lednice	+	$1.47x10^5$	26.05 ± 0.14	25.91 ± 0.11	ND	ND

		RBIP						
	P.communis	Holovousy	+	$2.96x10^5$	24.91 ± 0.18	25.25 ± 0.14	ND	ND
		RBIP						
'Ca. P. pyri'		Holovousy	+	$1.23x10^5$	26.33 ± 0.13	26.89 ± 0.07	ND	ND
		RBIP						
		Holovousy	+	$4.56x10^4$	27.94 ± 0.04	28.27 ± 014	ND	ND
		RBIP	-	ND	ND	ND	ND	ND
		Holovousy						

ND: not detected

[a] For detection of '*Ca*. P. prunorum' ESFY probe was used, for '*Ca*. P. mali' AP probe was used, for '*Ca*. P. pyri' PD probe was used

[b] Melting temperature (Tm): 83.6 °C

[c] Melting temperature (Tm): 72.2 °C

[d] Efficiency (E): 85.12 %; slope (k): -3.74; R^2: 0.991

DISCUSSION

The work was focused on comparison of detection capabilities of real-time PCR protocols using DNA samples isolated from phloem of two-year-old fruit tree shoots. DNA isolated from plants is often linked with higher concentration of PCR inhibitors (GREEN *et al*., 1999). Therefore, as written by MASKOVA *et al*. (2009), focus should be kept on the method of DNA extraction as well. Afterall, PCR inhibition was not observed at any sample tested by any PCR protocol. Lately, new approaches to test the presence of inhibitors and especially the quality of DNA extraction were established. They are based on detection of plant matrerial DNA by real-time PCR (BARIC and DALLA VIA, 2004; CHRISTENSEN *et al*., 2004). Nonetheless, it is questionable what results are achieved at detection of plant DNA isolated from phloem, since phloem sieve tubes do not contain ribosomes or nuclei (VAN BEL, 2003).

Different PCR chemistry than in original articles was used in performance of real-time PCR. For protocols based on intercalating dyes GoTaq® qPCR Master Mix (Promega) where instead of SYBR Green, Bryt™ Green was used as intercalating dye. Bryt™ Green has no such inhibitory effect on performance of PCR and produces brighter fluorescence signal than SYBR Green (REECE *et al*., 2009). For protocols based on hydrolysis probes in house mixes using GoTaq® G2 Hot Start polymerase (Promega) were used. All protocols using these chemicals were functional and did not show any errors in detection. However, different performance compared to original PCR chemistry listed in original protocols can not be ruled out.

Results from the work do not confirm the statement of GALETTO *et al*. (2005), that non-ribosomal primers have lower detection sensitivity than primers designed in ribosomal region. Thus, both protocols by JARAUSCH *et al*. (2010), using non-ribosomal primers and protocol by YVON *et al*. (2009), using primers designed in ribosomal region showed the same PCR performances (Fig. 2, Tab. 2 and Tab. 3).

Lower mean Ct values of protocols based on intercalating dyes could be caused besides of formation of primer dimers by the fact that amount of fluorescence molecules per dsDNA amplicon is higher than with use of hydrolysis probes (JOSEFSEN, 2012), resulting in possible earlier amplification signal and thus increase of the sensitivity at lower concentrations of target DNA. However increased sensitivity of protocols based on intercalating dyes was not proved in

this work, which confirms the results of other authors, where protocols based on intercalating dyes showed same sensitivity as nested PCR (YVON *et al*., 2009) and less sensitivity than protocols based on hydrolysis probes (BARIC and DALLA VIA, 2004; CHRISTENSEN *et al*., 2004; NIKOLIĆ *et al*., 2010).

In conclusion, all tested real-time PCR protocols have confirmed their specificity/versatility of detection of 16SrX group phytoplasmas in samples isolated from phloem of fruit trees. High homogeneity of PCR performances was shown by inter plate control at detection of '*Ca*. P. prunorum'. When selecting an appropriate real-time PCR protocol for phytoplasma detection of samples isolated from phloem attention should be taken on presumed pathogen concentration in the sample. The reasons are low titers of phytoplasmas in fruit trees (JARAUSCH *et al*., 2004) and the fact that not all protocols are equaly sensitive.

All tested real-time PCR protocols showed 10 to 100 times higher detection sensitivities than nested PCR, whereas protocols based on hydrolysis probes (protocol by CHRISTENSEN *et al*., 2004; NIKOLIĆ *et al*., 2010) were 10 times more sensitive than protocols based on intercalating dyes (protocol by YVON *et al*., 2009; JARAUSCH *et al*., 2010).

When comparing both tested real-time PCR protocols based on hydrolysis probes, protocol by CHRISTENSEN *et al*. (2004) showed, in some cases, higher detection sensitivity ('*Ca*. P. mali') and lower $LOD_{95\%}$ values ('*Ca*. P. mali' and '*Ca*. P. pyri'). However, when distinguishing of 16SrX group phytoplasma species is necessary, better choice is the protocol by NIKOLIĆ *et al*. (2010) due its possibility to specifically detect separately '*Ca*. P. prunorum', '*Ca*. P. mali' and '*Ca*. P. pyri'.

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POREĐENJE REAL-TIME PCR METODA ZA OTKRIVANJE I KVANTIFIKACIJU FITOPLAZMI IZ GRUPE 16SRX KOD STABALA VOĆKI.

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Izvod

U ovom radu testirane su dve real-time PCR metode bazirane na interkalacionoj boji i dve bazirane na hidroliznim sondama kod prikupljenih uzoraka stabala voćki zaraženih fitoplazmom grupe 16SrX (proliferacija jabuke, propadanje kruške i evropsko žutilo koštičavih voćaka). Glavni parametri testiranja bili su specifičnosti i osetljivost metoda i efikasnost amplifikacije. Rezultati real-time PCR metoda upoređeni su sa nested PCR. Sve real-time PCR metode potvrdile su svoje specifičnosti otkrivanja fitoplazmi. Sve real-time PCR metode bile su 10-100 puta osetljivije od nested PCR metode. Sve PCR metode bazirane na hidroliznim sondama bile su 10 puta osetljivije od metoda baziranih na interkalacionoj boji. Među metodama baziranim na hidrolizujućim sondama, nešto bolje karakteristike otkrivanja fitoplazmi pokazala je metoda prema CHRISTENSEN et al. (2004).

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