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Detection of '*Candidatus* Phytoplasma pyri' (pear decline agent) in pear trees by different diagnostic procedures in the seasonal course

Nachweis von *Candidatus* Phytoplasma pyri (Birnenverfallserreger) an Birne mit unterschiedlichen Diagnoseverfahren im Jahresverlauf

Abstract

The influence of three nucleic acid-based detection procedures and two sample sizes on the detection rate of '*Candidatus* Phytoplasma pyri' (pear decline agent) was studied in a factorial trial with three pear cultivar/rootstock combinations in the course of the year. Shoot samples were taken approximately every month over a period of 17 months from naturally infected trees. Nested PCR (nPCR), real-time LAMP (rtLAMP) and to a lesser extent real-time PCR (rtPCR) were used as detection methods. Sample sizes comprised two subsample sets consisting of three and two shoot pieces, respectively, (five in total) per tree. The detection level was highly dependent on the tested cultivar. The level was high for breeding code '48–40–95' (81.4%) but low for cvs. 'Conference' (26.2%) and 'Xenia' (19.2%), whose replicate trees partly failed to be tested positive for longer periods resulting in overall low detection rates. Nested PCR and rtLAMP showed no significant detection rate differences in the sum of all cultivars, but rtPCR resulted in significantly higher detection rates compared to the other detection procedures. By analysing both subsample sets instead of only one per tree, the detection rates were improved in most cases with an overall increase of 29.4% considering the methods and cultivars. Detection rates varied depending on the method and the cultivar in the course of the year, but most of the trees were tested positive in the period from late winter to early spring, except for '48–40–95', where the pathogen could be detected almost all year round. Comparing the impact for the factors investigated on the detection rate, the cultivar/rootstock combination was the greatest, followed by the method and sample size, both showing a similar effect.

Key words: nested PCR, real-time PCR, real-time LAMP, sample size, sampling date, pear cultivar

Zusammenfassung

In einem faktoriellen Versuch wurde der Einfluss von drei nukleinsäure-basierten Nachweisverfahren und zwei Probenumfängen auf die Nachweisrate von '*Candidatus* Phytoplasma pyri' (Birnenverfallserreger) bei drei Birnensorten/Unterlagenkombinationen im Jahresverlauf untersucht. Triebproben wurden in etwa monatlichem Abstand über einen Zeitraum von 17 Monaten von natürlich infizierten Bäumen entnommen. Als Nachweismethoden wurden die nested PCR (nPCR), real-time LAMP (rtLAMP) und in geringerem Umfang real-time PCR (rtPCR) verwendet. Die Probenumfänge umfassten zwei Teilprobenmengen, bestehend aus drei bzw. zwei Triebstücken (insgesamt fünf) je Baum. Das Nachweisniveau war stark von der getesteten Sorte abhängig. Es war hoch bei der Kreuzungsnummer '48–40–95' (81,4%), aber niedrig bei 'Conference' (26,2%) und 'Xenia' (19,2%), in deren Wiederholungsbäumen teilweise für längere Zeitabschnitte keine positiven Nachweise erhalten wurden, was zu insgesamt geringen Nachweisraten führte. In der Summe aller Sorten gab es keine signifikanten Nachweisunterschiede zwischen der nPCR und rtLAMP, dagegen wur-

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den mit der rtPCR signifikant höhere Nachweisraten im Vergleich mit den anderen Nachweisverfahren erzielt. Durch die Untersuchung von beiden Teilproben anstatt nur einer je Baum konnte die Nachweisrate in den meisten Fällen verbessert werden und ergab in der Summe aller Untersuchungen für die Methoden und Sorten eine Steigerung um 29.4%. Die Nachweisrate variierte im Jahresverlauf in Abhängigkeit von der Methode und der Sorte, die meisten Bäume konnten jedoch im Zeitraum Spätwinter bis Anfang Frühjahr positiv getestet werden, außer bei '48–40–95', in welcher der Erreger fast ganzjährig nachgewiesen werden konnte. Ein Vergleich des Einflusses der untersuchten Faktoren auf die Nachweisrate ergab für die Sorte/Unterlage den größten Effekt, gefolgt von der Methode und dem Probenumfang mit etwa gleich großen Auswirkungen.

Stichwörter: nested PCR, real-time PCR, real-time LAMP, Probenumfang, Probenahmezeitpunkt, Birnensorte

Introduction

Pear decline (PD), caused by the bacterium '*Candidatus* Phytoplasma pyri' ('*Ca.* P. pyri'), is a common disease in pear cultivation and in production of planting material. Its importance is emphasized by the fact that the disease is enclosed in the European Guideline EC 2009/29, which regulates the production and distribution of propagation material and plants for planting with respect to quarantine pests and diseases. A reliable detection of '*Ca.* P. pyri' is therefore of great importance to prevent or limit losses in commercial fruit production and planting material. However, plant phytoplasma titres can be affected by climatic conditions, host plant species, phytoplasma strain or species, time of infection and are often very low in woody hosts (Marzachi, 2004, IPPC, 2016). An inhomogeneous distribution and a low phytoplasma titre have been reported for phytoplasmas of the apple proliferation (AP) group, which includes '*Ca.* P. pyri' (Seemüller et al., 1984b, Berges et al., 2000). Therefore, the identification or diagnosis of phytoplasma diseases is not trivial, as stated by Firrao et al. (2007) and Galetto and Marzachi (2010).

For diagnosis, various detection procedures for laboratory use have been developed, outlined in the review by Seemüller et al. (2011). Different nucleic acid based amplification techniques are routinely used (Bertaccini and Duduk, 2009, Delić, 2012) and the International Plant Protection Convention, an institution of the FAO, recommends PCR techniques as the methods of choice for the detection of phytoplasmas listed in Annex 12 of the ISPM (International Standard for Phytosanitary Measures No. 27; IPPC, 2016). Amongst many PCR methods real-time PCR (rtPCR) is reported to be less prone to contamination and less work intense compared to nested PCR (nPCR) (Christensen et al., 2013). Recently protocols based on loop-mediated isothermal amplification (LAMP) have been propagated as a possible alternative to established PCR-techniques (reviewed by Le and Vu, 2017) and several fruit phytoplasmas have been detected by this method (Bekele et al., 2011, Kogovšek et al., 2015, De Jonghe et al., 2017). The LAMP method is described as being highly specific, efficient, rapid and labour saving (Ravindran et al., 2012), but only limited data are available for the detection of '*Ca.* P. pyri' (Neumüller et al., 2014).

Sampling is also an important factor for phytoplasma diagnosis because the titre and location in the plant may be influenced by seasonal changes (Seemüller et al., 1984a, Jarausch et al., 1999, Garcia-Chapa et al., 2003, Christensen et al., 2004). When investigating pear or apple trees for phytoplasma infection, roots are recommended for best test results, as it was shown that roots are colonised by relatively high numbers of phytoplasmas throughout the year in contrast to the aerial parts (Seemüller et al., 1984a, b). However, there are some situations, in which the testing of leaves or shoots instead of root material is indicated, like testing asymptomatic *Pyrus* species grafted on quince (*Cydonia oblonga*) (EPPO, 2017) or for the post-entry testing on quarantine organisms of imported scion material for propagation. Moreover, root sampling is hardly feasible when large plantations have to be tested within certification programs. Regarding the best sampling time, different information for optimal detection can be found in the literature. While late summer or fall is recommended most frequently (Seemüller et al., 2011, IPPC, 2016, EPPO, 2017), winter to spring was shown to be the best sampling period for above-ground parts of the plants by other authors (Garcia-Chapa et al., 2003, Kučerová et al., 2007). The erratic distribution of phytoplasmas in trees raises the question of sample size. Various recommendations and/or procedures on the sample size have been published for the diagnosis of fruit tree phytoplasmas. Several authors recommend to collect more than one sample per tree (Berges et al., 2000, Tomlinson et al., 2010, Olivier et al., 2014) and the European Plant Protection Organisation (EPPO) recommends the examination of shoots from at least three different locations of symptomatic trees for diagnosis of the three fruit tree phytoplasmas causing European stone fruit yellows, apple proliferation and pear decline. For testing asymptomatic plants limited experience is noted and root testing is recommended (EPPO, 2017). Furthermore, the influence of the cultivar (cv.) and/or rootstock on the detectability of the pathogen can play an important role, as shown by investigations of Poggi Pollini et al. (1995), Garcia-Chapa et al. (2003) and Torres et al. (2010). Therefore, with regard to the influencing factors mentioned above, many uncertainties still exist on how to improve the examination procedure for best detection results. In many studies the influence of just a single factor on the pathogen detection has been investigated but little research was done comparing several factors simultaneously in a factorial trial. To investigate situations closer to practical relevance, three pear cultivars, different sampling times, two sample sizes and three nucleic acid-based detection methods were com-

binedly analysed to determine the impact of the investigated factors on the detection rate and to adapt analysis conditions for improved detection results.

Materials and methods

Plant material and nucleic acid extraction

From November 2012 to January 2013 about four years old pear trees growing in a fruit experimental station at Karlsruhe, Germany, were examined by nPCR to identify PD-infected plants for subsequent regular testing. Trees of the cultivars 'Xenia'® ('Nojabrskaja') on quince A, 'Conference' on quince Sydo and one cultivar with the breeding code '48–40–95', a crossbreed of 'Williams Christ' (syn. 'Bartlett') (*Pyrus communis* L.) and 'Nijisseiki' (*Pyrus pyrifolia* Nakai) from the University of Geisenheim, most likely grafted on 'Pyrodwarf'®, were found to be PD-infected. Three trees of each cultivar were selected for analysis. All fruits were removed some weeks after flowering to promote shoot growth. Sampling started in January 2014 and was carried out in about monthly intervals until May 2015. Five shoot cuttings with a length of 5 – 10 cm were collected randomly from different areas of each tree. Annual shoots were preferred, however sometimes two years old shoots were taken if the material was scarce. The plant material was stored at + 8°C and DNA extraction was performed within three weeks. Before extraction, the plant material from one tree was divided randomly into two different subsamples, the first consisted of three (set 1) and the second consisted of two shoot cuttings (set 2). Phloem tissue was collected after the bark was carefully scraped off with a scalpel blade. Approximately equal proportions were taken from each shoot piece and pooled for each subsample set, which were subsequently analysed separately. Extraction of total nucleic acids was performed by the silica capture method as described by Menzel et al. (2002), whereas 300 mg of tissue was used in a ratio of 1:10 (w/v) with grinding buffer instead of 100 mg. The tissue was homogenized in extraction bags with a HOMEX 6 apparatus (both from Bioreba Company, Reinach, Switzerland). In the final step, the pellets were resuspended in 150 µl TE-buffer buffer (10mM Tris-HCl, 1mM EDTA; pH 8.0).

Detection of 'Ca. P. pyri'

Three different detection procedures were performed and are briefly outlined below. For each protocol, the same DNA-extracts were used. A nested PCR was performed with the universal phytoplasma detection primers P1/P7 (Deng and Hiruki, 1991, Schneider et al., 1995) followed by a PCR with primer pair fU5/rU3 (Lorenz et al., 1995). The PCR was performed with the following conditions: The first round PCR was carried out in a total volume of 25 µl, consisting of 1 µl nucleic acid extract, 1.25 µl of each primer (10 µM), 0.5 µl dNTPs (40 µM), 2.5 µl PCR buffer (10-fold), 0.125 µl Qiagen HotStar Taq DNA Polymerase (5U/µl), adjusted with DEPC treated deionized water. The second round PCR contained the same reaction components in the same volume, except for primers and template, which were 1.25 µl (10 µM) of each primer (fU5 and rU3) and 0.5 µl of P1/P7 amplicons, respectively. The first round PCR products were neither purified nor diluted before added to the second round PCR. The cycling parameters for the first run included an initial denaturation step at 95°C for 18 min, followed by 31 cycles with 1 min at 95°C, 1 min at 52°C, 1 min at 72°C and a final extension step at 72°C for 7 min. For the second run similar conditions were chosen, however the annealing temperature was 55°C and 34 cycles were performed. To verify the identity of '*Ca.* P. pyri' in the samples, the fU5/rU3 fragment was purified and sequenced with an ABI Prism 310 sequencer. Data evaluation was performed with DNASTAR Lasergene 12.1 software and the identification was performed by BLAST comparison to sequences deposited in the NCBI database.

A real-time loop-mediated isothermal amplification (rtLAMP) protocol with primers outlined in Table 1 (Nußbaum, pers. communication, for affiliation see acknowledgement) was conducted based on the method described by Notomi et al. (2000). The LAMP procedure specifically detects phytoplasmas of the AP-group (apple proliferation, pear decline and European stone fruit yellows). Briefly, two µl of nucleic acid extract were added

Table 1. LAMP primers for detection of '*Ca.* P. pyri' [a]

Primer Name	Sequence (5'⟶3')
F3 Phyto n2	GAACGGGTGAGTAACACG
B3 Phyto n2	TGGTAAGCCGTTACCTTAC
FIP Phyto n2	GCCTTAAAACTTCCTATCCAGTCTTGTAACCTGCCTCTTAGACG
BIP Phyto n2	ATCTTGAAACTTTTAAAAGACCCGCCAACTAACTAATGTGCCGC
LF Phyto n2	AGCAGTCGTTTCCAACTGTTATC
LB Phyto n2	AAGGGTATGCTAAGAGATGGGC

[a] The LAMP primers were designed by Nußbaum using the Primer Explorer software (Eiken Chemical Co., Ltd.) based on a sequence of the 16S ribosomal RNA gene for *Candidatus* Phytoplasma mali isolate 1162010, acc. no. JN555596.

to 20 µl of mastermix, containing 2.0 µl 10 × amplification buffer, 0.8 µl Bst 2.0 WarmStart DNA Polymerase (8,000 U/ml, both from New England BioLabs), 0.8 µl dNTPs (10 mM each), 1.5 µl MgSO4 (100 mM), 0.8 µl EvaGreen® (100 µM), 0.32 µl primers FIP Phyto n2 and BIP Phyto n2 (100 µM each), 0.32 µl primers LF Phyto n2 and LB Phyto n2 (50 µM each), 0.08 µl primers F3 Phyto n2 and B3 Phyto n2 (50 µM each), adjusted to 20 µl with nuclease-free water. The mixture was run on a CFX96 Real-Time PCR detection system with the CFX Manager 3.1 software (Bio-Rad Laboratories Inc.) for 30 min at 63°C. Relative fluorescence units were monitored in intervals of one minute using the detection settings for SYBR® Green. The baseline and threshold values were automatically set by the software and quantification cycle value (Ct) was determined by the system when the fluorescence signal crosses the threshold.

A real-time PCR assay for specific detection of '*Ca.* P. pyri' as described by NICOLIĆ et al. (2010) was performed, except 25 µl of final reaction volume was used instead of 10 µl, including 0.5 µl DNA extract and cycled on a Bio-Rad CFX96 Real-Time PCR Cycler. The running parameters were as described by the authors.

Statistical analysis

The raw data from the factorial trail were evaluated for the effects of the different factors on the detection rate. Statistical analysis of the data was performed using a generalized linear mixed model (GLMM), the Glimmix procedure of SAS 9.4, using a binomial distribution. The repeated sampling from the same tree (month* year* tree) was modelled by AR(1) structure. The analysis model was:

$y_{ijklmn} = \mu + \text{cultivar}_i + \text{detection method}_j + \text{number of investigated samples}_k + \text{year}_l: (\text{cultivar* tree})_{in} + (\text{month* year})_{lm} + (\text{month* year* tree})_{lmn} + e_{ijklmn}$. Where y_{ijklmn} is the value of the ijklmn observation of the i-th cultivar, j-th detection method, k-th number of investigated samples, l-th year, m-th month and n-th tree. All terms mentioned after the colon are random effect. A significance level of 0.05 was used.

Results

Detection methods and cultivar effects

The primer pairs P1/P7 and fU5/rU3 are not '*Ca.* P. pyri'-specific and were designed for universal phytoplasma detection. To confirm the identity of the phytoplasma, fU5/rU3 PCR fragments from six of the nine trees were sequenced one to several times in the course of the study and compared to sequences deposited in the NCBI-database. All sequences revealed homology rates between 99 and 100% to '*Ca.* P. pyri' accessions.

The results of the different diagnostic procedures for each cultivar on the detection rates for '*Ca.* P. pyri' for the sum of all taken samples are listed in Table 2. DNA-extracts from samples taken in 2015 were also analysed by rtPCR. The detection rates, regardless of the diagnostic procedure, were low for 'Xenia' and 'Conference' and ranged, depending on the method, between 14.3% to 36.7% and 20.9% to 33.3%, respectively. This was due to PCR results, which were negative for samples of some of the replicates taken over longer time periods. The values for cv. '48–40–95' were about three to five times higher. This

Table 2. Cumulative detection rates of '*Ca.* P. pyri' by nPCR, rtLAMP and rtPCR with samples of the different cultivars from the years 2014 and 2015

Cultivar	Detection method	Number of analyses[a]	Number of positive detections [b]	Positive detections in %	Positive detections (%) in the sum of all methods
'Xenia'	nPCR	84	12	14.3	
	rtLAMP	84	15	17.9	19.2
	rtPCR [c]	30	11	36.7	
'Conference'	nPCR	86	18	20.9	
	rtLAMP	86	25	29.1	26.2
	rtPCR [c]	30	10	33.3	
'48–40–95'	nPCR	90	71	78.9	
	rtLAMP	90	72	80.0	81.4
	rtPCR [c]	30	28	93.3	
Sum of all cultivars	nPCR	260	101	38.8	
	rtLAMP	260	112	43.1	
	rtPCR [c]	90	49	54.4	

[a] Sample sets of three and two stem cuttings included. [b] A sample was rated positive, if one or both of the sets tested positive.
[c] Method applied only for samples collected in 2015.

is also reflected in the sum of all detection rates, which was 19.2% for 'Xenia', 26.2% for 'Conference' and 81.4% for '48–40–95'. The differences between these values were statistically significant for cv. '48–40–95' compared to cvs. 'Xenia' and 'Conference', but not between the latter two. The statistical evaluation results are presented further below and summarized in Table 6. The nPCR and rtLAMP detection rates were similar for cv. '48–40–95', but were higher for rtLAMP in 'Xenia' and 'Conference'. However, the detection rates were highest with rtPCR for all cultivars. Comparing the number of positive detections as a total of all cultivars for the different methods, rtPCR with a rate of 54.4% was statistically significant in relation to rtLAMP (43.1%) and nPCR (38.8%), but there was no significance between the last two. It must be mentioned that in all cultivars some of the sample sets were only positively tested by one of the methods (data not shown).

Influence of the number of examined stem cuttings per tree

The detection rates of '*Ca.* P. pyri' obtained by analysing one sample set (set 1) per tree and the subsequent improvement achieved by the inclusion of the second sample set (set 2) are presented in Table 3. From 305 PCR assays, positive amplification results were obtained for 163 samples. Of those, 100 samples showed positive results in both sets, 26 only in those with set 1and 37 only in those with set 2. The effects were different for the detection methods and cultivars. The detection rates with nPCR and rtLAMP increased in all cultivars by analysing both sets per tree. For 'Conference' the detection rates were almost doubled from 16.3% to 30.2% by nPCR and from 33.3% to 60.0% by rtPCR. For '48–40–95' an increase from 71.1% to 93.3% and from 77.8% to 91.1% was observed by nPCR and rtLAMP, respectively. For 'Xenia' the larger sample size had minor effects on the detection rates. The detection rate of rtPCR remained the same for 'Xenia' (46.7%) and '48–40–95' (93.3%) regardless of the sample size. Comparing the detection rates for set 1 and set 1 and 2 by the sum of all cultivars and methods (41.3% and 53.4%, respectively), the difference (relative increase of 29.4%) was statistically significant.

Date of sampling

The influence of the sampling date on the detection of '*Ca.* P. pyri' in the individual tree replicates during the observation period is presented in Table 4. A tree was considered positive, when at least one sample of both sets gave a positive PCR result. The monthly detection varied considerably depending on the individual tree. In '48–40–95', PD was detected in all replicates most of the time, except for January 2014 where two trees were tested negative. For 'Conference' a remarkable lack of PD-positive samples was apparent in summer to autumn, except for positive reactions in one tree in October and November. In the trees 'Conference' 1 and 'Conference' 3, the pathogen could not be detected for a period of about one year. For the cultivar 'Xenia' the pathogen detection was even more difficult. While the first replicate, 'Xenia' 1, tested positive by at least one of the methods with some exceptions throughout the year, the second and third replicate gave only few positive results. Positive detections for this cultivar were only achieved from January to July 2014 with rtLAMP. For 'Xenia' and 'Conference' a pattern regarding the monthly detection percentages became evident and the periods from January to April or May in 2014 and 2015 were most suited for PD-detection. During this period in 2015 the number of positive test results was highest and was achieved by rtPCR.

Table 3. Detection rates of '*Ca.* P. pyri' in the three cultivars by nPCR, rtLAMP and rtPCR analysing one and two sample sets.

Cultivar	Detection method	Samples analysed [a]	Positive detections (% in brackets) with	
			set 1	set 1 + 2 [b]
'Xenia'	nPCR	42	7 (16.7)	9 (21.4)
	rtLAMP	42	8 (19.0)	12 (28.6)
	rtPCR	15	7 (46.7)	7 (46.7)
'Conference'	nPCR	43	7 (16.3)	13 (30.2)
	rtLAMP	43	11 (25.6)	16 (37.2)
	rtPCR	15	5 (33.3)	9 (60.0)
'48–40–95'	nPCR	45	32 (71.1)	42 (93.3)
	rtLAMP	45	35 (77.8)	41 (91.1)
	rtPCR	15	14 (93.3)	14 (93.3)
Sum of samples		305	126 (41.3)	163 (53.4)

[a] Cumulated number of samples from January 2014 to May 2015 (nPCR, rtLAMP) and from January to May 2015 (rtPCR). [b] A sample was rated positive, if one or both of the sets tested positive

Table 4. Detection of '*Ca.* P. pyri' in trees of different cultivars by nPCR, rtLAMP and rtPCR in the course of the year.

Cultivar/ Tree No.	Jan 14	Feb 14	Mar 14	Apr 14	May 14	Jun 14	Jul 14	Aug 14	Sep 14	Oct 14	Nov 14	Dec 14	Jan 15	Feb 15	Mar 15	Apr 15	May 15
'Xenia' 1		●	●	/		/	●		○●	●	○●	/	○●Δ	○●Δ	○●Δ	○ Δ	○ Δ
'Xenia' 2				●		/		○			/	/	Δ				
'Xenia' 3	●					/					/	/				○●Δ	
'Conference' 1	○●	○●	●			/					/	/				Δ	○●Δ
'Conference' 2	○●	○●	○●	○●	○●	/				●	●	/	Δ	Δ	○●Δ	●Δ	○ Δ
'Conference' 3		○●	○●	○●		/					/	/			Δ		Δ
'48–40–95' 1	○●	○●	○●	○●	●	/	○●	○●	○●	○●	○●	/	○●Δ	○●Δ	○●Δ	○●Δ	○●Δ
'48–40–95' 2		○●	○●	○●	○	/	○●	○●	○●	○●	○●	/	○●Δ	○●Δ	○●Δ	○●Δ	○●Δ
'48–40–95' 3		○●	○●	○●	○	/	○●	○●	○●	○●	○●	/	○●Δ	○●Δ	○●Δ	○●	○●Δ
Positive trees (%) [a]																	
nPCR	33	67	56	62	33		33	44	44	33	[b]		44	44	56	56	67
rtLAMP	44	78	78	75	22		44	33	44	56	[b]		44	44	56	56	44
rtPCR	n.t.	n.t.	n.t.	n.t.	n.t.		n.t.	n.t.	n.t.	n.t.	n.t.		67	56	67	78	78

Positive by ○ = nPCR, ● = rtLAMP, Δ = rtPCR (performed only with samples taken in 2015), negative results are indicated by missing symbols. [a] A tree was considered positive, when at least one of the two sample sets gave a positive testing result. [b] Not calculated due to small amount of tested trees, n.t. = not tested, / = no samples taken

Table 5. Statistical parameters used with the generalized linear model and their probabilities (p).

Factor	$DF_{Num.}$	$DF_{Den.}$	p-value
Cultivar	2	6	<u>0.0185</u>
Detection method	2	556	<u>0.0030</u>
Sample set	1	556	<u>< 0.0001</u>
Year	1	13	0.1974

DF = degrees of freedom, Num. = numerator, Den. = denumerator
Underlined data show significant differences (level of statistical significance: $p < 0.05$)

Statistical analysis

The statistical parameters used by GLMM and the calculated p-values for the different factors are given in Table 5. Significant differences in detection were obtained for the cultivar, detection method and number of sample sets ($p < 0.05$), but not for the year (raw data not shown). Regarding the individual factor levels (Table 6), significant differences of mean values were observed between cultivar '48–40–95' and the other cultivars, between rtPCR and the other detection methods and between the number of sample sets analysed.

Discussion

'*Ca.* P. pyri', the causal agent of pear decline, is notoriously difficult to detect in infected pear trees. Important factors therefore are the uneven distribution of the pathogen in plants, a seasonal variation in pathogen titre and host characteristics of cultivar and rootstock. To investigate these variables for an improved molecular detection, three cultivar/rootstock combinations were examined with two different sample sets, monthly sampling and three diagnostic procedures. DNA amplification methods have been proven to be the most sensitive diagnostic tools for the detection of fruit tree phytoplasmas of the AP-group, including '*Ca.* P. pyri'. In this study '*Ca.* P. pyri' was detected in the same DNA extracts most frequently by rtPCR, followed by rtLAMP and nPCR. A higher detection rate with rtPCR compared to nPCR was also reported for PD-infected pear samples (Nicolić et al., 2010), for phytoplasma-infected grapevine (Hren et al., 2007) and ESFY-infected apricot trees (Johnston et al., 2014). However, Mehle et al. (2010) found rtPCR more sensitive than nPCR in root samples of pears taken in autumn but not during wintertime. Moreover, Torres et al. (2005, 2010) reported converse results for PD-infected pear trees. In one study nPCR was more sensitive than rtPCR and in

Table 6. Estimates for the factors cultivar, detection method and sample set numbers obtained from the GLMM statistics.

Factor	Factor level	Mean	Standard Error
Cultivar	'Xenia'	0.2174 [A]	0.1236
Cultivar	'Conference'	0.2897 [A]	0.1481
Cultivar	'48–40–95'	0.9098 [B]	0.0600
Detection method	nPCR	0.3809 [a]	0.1148
Detection method	rtLAMP	0.4738 [a]	0.1212
Detection method	rtPCR	0.6736 [b]	0.1199
Sample set	1	0.3980 [α]	0.1160
Sample set	1 + 2	0.6232 [β]	0.1137

Mean values were calculated as probabilities (0–1) and their standard errors. Factor levels followed by the same letter are not significantly different. Various letter types (capital, small, Greek) differentiate the factors analysed.

another study no discrepancy was noted for both methods. An interlaboratory ringtest conducted by the EUPHRESCO network to compare detection methods for phytoplasmas of the AP-group found both methods identically sensitive (Anonymous, 2011). The divergent results observed by the different authors may be due to deviations in laboratory protocols for the methods, sampling time, pear cultivar and extraction procedure all influencing the sensitivity.

Regarding the LAMP assay, a comparative trial with nPCR or rtPCR for the detection of '*Ca.* P. pyri' has not been conducted or has not been published. Although a tendency for slightly higher detection rates by rtLAMP than by nPCR was noted in the study presented, there was no evidence of a significant difference. This is in accordance with the results of Neumüller et al. (2014), who compared a Blue LAMP procedure with a direct PCR assay, both modifications of the methods used in this study. Like in the comparison of rtPCR and nPCR sensitivity, divergent sensitivities of the LAMP assay compared to rtPCR or nPCR were reported for the detection of bacterial and viral plant pathogens in other plant species (Harper et al., 2010, Tomlinson et al., 2010, Kogovšek et al., 2015, 2017).The results published in the above mentioned reports seem not to allow to favour one method over the other. Differences in the sensitivity of a method or between methods can be caused by a number of factors. Beside execution, hardware and reaction components like primers and polymerases, the single highest impact on the sensitivity might be the quality and quantity of input DNA. In our hands rtPCR was the most sensitive detection method for '*Ca.* P. pyri'. Although the results for this method were based only on the study of samples from winter to spring, it can be assumed that this would also apply for samples taken at other seasons. The choice of detection methods may not be crucial in case of a high-titre plant but important when low pathogen titres are present and this might also be one factor for the contrarian sensitivity results published in the literature.

For reliable diagnostic results samples should be a pool of subsamples taken from different levels or parts of the individual plants (Errea et al., 2002, Firrao et al., 2007). Serial sampling along the stem axis of '*Ca.* P. pyri'-infected ex vitro pear rootstocks identified phytoplasma-free regions up to 20 cm in length (B. Schneider, pers. communication). Such an uneven distribution was also found when studying branches of several pear cultivars at different altitude levels of the stem by Errea et al. (2002). However, little attention was paid to quantitative effects of different sample sizes on the pathogen detection. In this study, it was shown that the detection rate could partly almost be doubled by analysing two instead of only one sample set (total of five shoot pieces) per tree. From a statistical point of view, increased sampling would be expected to result in a higher detection rate due to an erratic distribution of the phytoplasma in the tree. However, this did not apply to cvs. 'Xenia' and '48–40–95' for rtPCR. The reason for the missing increase for 'Xenia' is unclear but might indicate that the number of analyses by rtPCR was too small to finally assess the reason therefor. For '48–40–95', the Ct-values (data not shown) consistently ranked at a lower level, indicating higher phytoplasma titres. Moreover, the generally high detection rate for this cultivar suggests a more even distribution of the phytoplasma in the tree. Both conditions might be responsible that the detection rate could not be further increased by analysing the second sample set. The gain of detection by 29% in the sum of all investigations, however, underlines that the sample size is a significant factor for an improved detection of the phytoplasma in pear trees. Nonetheless it seems to be difficult defining an optimum sample size in general due to varying influencing factors. Garcia-Chapa et al. (2003) analysed 20 – 30 shoot pieces per pear tree for their investigations and Johnston et al. (2014) discussed the option to sample approximately 100 or more leaves per tree to maximize the probability of phytoplasma detection in fruit trees. However, such sample sizes would be difficult to implement in large scale testing in a defined time and would find their limits in personnel and monetary capacities. An alternative way to increase the sample size with a justifiable in-

put of resources would be an upscaling of the extracted tissue per sample. This might also expand the detection rate, especially when low phytoplasma concentrations are present.

Regarding the cultivar effect on the detection rates, low values were found for 'Conference' and 'Xenia' in contrast to '48–40–95'. A lasting stretch of negative results for individual trees and consequential effects on detection rates have been also reported for other pear cultivars by Garcia-Chapa et al. (2003). The authors reasoned the different detection levels to susceptibilities of the cultivar and/or phytoplasma concentration. In other studies the level of some fruit tree phytoplasmas was shown to correlate with the degree of resistance of a cultivar or rootstock (Seemüller et al., 1986, Bisognin et al., 2008, Jarausch et al., 2011). It should be considered, that on grafted trees the severity of the cultivar symptoms will also be influenced by the susceptibility of the rootstock type, as shown for some combinations of quinces and pears by Seemüller et al. (1986) and Poggi Pollini et al. (1995). The first authors could furthermore correlate the symptom severity of the grafted cultivar to the colonization frequency and density of '*Ca.* P. pyri' in different susceptible rootstocks. With respect to the cultivars in this study, 'Conference' has been described to be little susceptible to PD, regardless of the rootstock type (Giunchedi et al., 1995). For 'Xenia' and '48–40–95' no information on PD susceptibility is available. However, offspring of *Pyrus pyrifolia* genotypes are known to be predominantly susceptible (Westwood, 1976, cited by Seemüller and Harries, 2010) and also 'Williams Christ' (syn. 'Barlett'), being the second parent of '48–40–95', is reported to be susceptible (Giunchedi et al., 1995). With respect to the rootstocks, quince A and Sydo are classified as moderately susceptible (Weber, 2004), the former one being less susceptible than pear seedlings (Seemüller et al., 1986). In contrast, 'Pyrodwarf' was assessed to be susceptible (Monney and Egger, 2013). Thus, these different host properties might explain a higher phytoplasma titre and the significantly higher detection rate found in '48–40–95'/'Pyrodwarf' compared to the other cultivar/rootstock combinations. Phloem necrosis seems to play an important role in resistance, as concluded by Bisognin et al. (2008) from their investigations on AP-infected apomictic *Malus* rootstock types. However, detailed studies on the influence of this impairment on the phytoplasma distribution in pear trees are not available.

The sampling in monthly intervals revealed quite different results on the detection rate for the cultivars. While sampling time was crucial for cvs. 'Conference' and 'Xenia', this was hardly the case for '48–40–95'. Individual trees of the cvs. 'Conference' and 'Xenia' were characterized by periods of failing detection regardless of the method employed. Although seasonal changes in PD detectability due to the annual process of phytoplasma recolonization of the aerial parts were described (Schaper and Seemüller, 1984, Seemüller et al., 1984a, b), another phenomenon was likely to be involved in the longer-lasting lack of detection observed in this study. This might have been the erratic recolonization of the aerial parts in pear trees with a longer disease history (Schaper and Seemüller, 1984, Seemüller et al., 1984b, Marcone, 2010, Seemüller et al., 2011). The reason for this is unknown but a possible explanation might be the phytoplasma concentration in less susceptible cultivars, which decline to a level near to or below the detection limit and this temporary fluctuation then causes irregular detection results irrespective of the chosen detection method.

In addition, locations of phytoplasma presence might be reduced in plants of little susceptibility, thus making it even harder to detect '*Ca.* P. pyri' by routine sampling procedures. As a consequence repeated testing and/or raising the sample size could increase the probability of detection. Apart from that, testing of aerial parts is generally indicated in seasons, where the phytoplasma titre is highest, which is according to available data late summer or fall (Seemüller et al., 2011, IPPC, 2016, EPPO, 2017). An extended testing period for PD was suggested by Errea et al. (2002), who found that the diagnosis with stem tissue is not only possible in summer and early autumn, but most of the year. The successful detection of PD almost around the year might therefore be primarily related to more suitable host properties of the cultivar or rootstock, which can be assumed for '48–40–95' grafted on 'Pyrodwarf' but not for 'Conference' grafted on quince Sydo and 'Xenia' on quince A. This is mostly supported by the findings of Garcia-Chapa et al. (2003) and Kučerová et al. (2007), who received the best results from December to April or March/April. The results obtained in this study and others are in contrast to recommended sampling periods and might indicate that some flexibility in the sampling period has to be considered depending on cultivar, rootstock and geographic region.

Comparing the influencing value of the single factors (method, sample size, cultivar), demonstrated by the spread of the detection rate under the conditions of this study, a strong domination of the cultivar or cultivar/rootstock combination was apparent. The impact of the detection method and sample size was smaller and almost similar. However, sampling time is assessed to be generally more relevant for the detection rate than the used detection method and sample size due to a possible temporary undetectability of '*Ca.* P. pyri' in individual trees. Because the detection rate is likely related to the phytoplasma titre in the plant, the choice of the detection method as well as the sample size and sampling time is relevant for improved detection results, when low titres are expected. Therefore, in the future, emphasis should be given on information on the susceptibility of cultivars and rootstocks to PD in order to better estimate the effects of the individual factors on the detection rate in a given situation.

In conclusion this study revealed that the pathogen cannot be reliably detected at any time in any cultivar regardless of the detection procedure and sample size.

Nevertheless, the presented results can help to better understand possible reasons for this deficiency, but further efforts are needed to improve the reliability of detection for a better control of this important plant disease.

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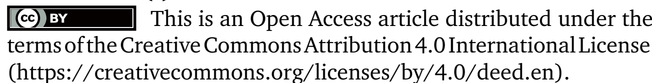
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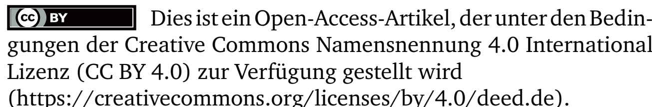
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