

Quantitative Real-Time PCR Analysis of ‘*Candidatus Phytoplasma mali*’ Without External Standard Curves

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Abstract Apple proliferation caused by ‘*Candidatus Phytoplasma mali*’ is an economically important disease of apple (*Malus × domestica*). The availability of a simple quantitative approach to assess pathogen load in infected host plants would certainly contribute to a better understanding of pathogenesis and epidemiology. This study proposes a quantification approach not requiring the analysis of external standard curves. It is based on the simultaneous detection of the 16S rRNA gene of the pathogen and the 1-aminocyclopropane-1-carboxylate oxidase gene of the host plant in a single-tube reaction using TaqMan chemistry. The quantity of the phytoplasma relative to its host plant is determined as the difference between C_T values of the two target genes (ΔC_T). To assess the agreement of the relative quantification approach with a standard curve-based method, a dataset of 450 DNA samples from infected apple trees was reanalysed. Comparison of the ΔC_T -based relative quantities with the corresponding absolute values revealed high degrees of agreement between the relative and absolute quantification methods. The ΔC_T procedure can thus be considered adequate for quantification of the phytoplasma in infected host plant tissue. In addition, this approach ensures improved methodological standardisation and increased analysis throughput.

Keywords Apple proliferation · *Malus × domestica* · Phytoplasma · Relative quantification

Quantitative real-time PCR Analyse von ‘*Candidatus Phytoplasma mali*’ ohne externe Standardkurven

Zusammenfassung Die Apfeltriebsucht, verursacht durch ‘*Candidatus Phytoplasma mali*’, ist eine wirtschaftlich bedeutende Krankheit des Apfelbaumes (*Malus × domestica*). Die Verfügbarkeit einer einfachen und schnellen Analyse-methode zur Ermittlung der Erregermenge in infizierten Wirtspflanzen könnte zu einem besseren Verständnis der Pathogenese und der Epidemiologie der Krankheit beitragen. In dieser Arbeit wird ein Quantifizierungsverfahren vorgestellt, das ohne die Analyse externer Standardkurven auskommt. Es beruht auf der Verwendung der TaqMan Technologie, wobei in einer Reaktion gleichzeitig das 16S rRNA-Gen des Erregers und das 1-Aminocyclopropan-1-Carboxylatoxidase-Gen (ACO) der Wirtspflanze nachgewiesen werden. Die Erregermenge, relativ zur Anzahl der Genomeinheiten der Wirtspflanze, wird als Differenz der C_T Werte (ΔC_T) berechnet, die für die beiden Genabschnitte ermittelt werden. Zur Ermittlung der Übereinstimmung des relativen Quantifizierungsverfahrens mit der konventionellen Standardkurven-Methode wurde ein Datensatz mit 450 DNA-Proben von Apfeltriebsucht-infizierten Apfelbäumen reanalysiert. Der Vergleich der relativen ΔC_T -Werte mit den absoluten Erregermengen zeigte ein hohes Maß an Übereinstimmung. Das ΔC_T -Verfahren kann somit als geeignet für die Quantifizierung des Apfeltriebsucht-Phytoplasmas in infiziertem Wirtspflanzen-Gewebe angesehen werden. Zudem trägt die neue Quantifizierungsmethode zur besseren Standardisierung und durch den geringeren Arbeitsaufwand zur Erhöhung des Analysedurchsatzes bei.

Schlüsselwörter Apfeltriebsucht · *Malus × domestica* · Phytoplasma · Relative Quantifizierung

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Introduction

Phytoplasmas are Gram-positive bacteria of the class Mollicutes that cause diseases in hundreds of plant species worldwide and can induce serious yield losses of economically important crops (Hogenhout et al. 2008; Lee et al. 2000). These bacteria are limited to the phloem tissue of plants and their transmission occurs mainly through grafting of infected propagation material or by sap-sucking insect species of the order Hemiptera (Weintraub and Beanland 2006). So far, no attempt to cultivate phytoplasmas in vitro has been successful, making the investigation of this group of phytopathogens a particularly challenging task (Strauss 2009).

'*Candidatus* Phytoplasma mali' causes the quarantine disease apple proliferation (AP) and has been progressively spreading in European apple growing regions (Seemüller and Schneider 2004). The only possibilities currently available to confine the disease are vector control and uprooting of infected apple trees (*Malus × domestica* Borkh), as they remain positive throughout their lifespan and generally deliver fruit of inferior quality (Seemüller et al. 1984).

Molecular genetic methods have become indispensable tools for phytoplasma detection, identification and/or classification. In recent years, qualitative and quantitative real-time PCR approaches have been increasingly used to gain more insight into the epidemiology and pathogenesis of AP (e.g. Baric et al. 2008; Bisognin et al. 2008; Mayer et al. 2009; Musetti et al. 2010, 2011; Rekab et al. 2010; Seemüller and Schneider 2007). The protocols for quantitative analysis of '*Ca. P. mali*' described so far are exclusively based on the application of external standard curves (Baric et al. 2011; Bisognin et al. 2008; Musetti et al. 2011; Rekab et al. 2010; Seemüller and Schneider 2007; Torres et al. 2005). However, the quality of standard curves can be impaired by errors during measurement of initial amounts of standard DNA and/or during the preparation of serial dilutions (Rutledge and Côté 2003). Moreover, the analysis of serially diluted standards in each real-time PCR experiment reduces the available number of target sample wells on a microtiter plate, and consequently leads to increased

analysis time and reagent costs. The main aim of this work was to assess the reproducibility and suitability of a simpler quantification procedure for '*Ca. P. mali*', based on the comparative threshold cycle (C_T) method and not requiring external standard curves.

Material and Methods

DNA samples used in the present study were previously described by Baric et al. (2011). The 450 DNA isolates investigated herein were obtained from phloem tissue of branches or roots of apple trees infected with '*Ca. P. mali*'. All isolates were tested positive using a qualitative real-time PCR approach (Baric and Dalla Via 2004; Baric et al. 2006) and quantified applying an absolute standard curve method (Baric et al. 2011).

In a first set of analyses it was aimed to assess the most appropriate procedure for real-time PCR data analysis in order to minimise inter-assay variation. For this purpose, a representative subset of 18 DNA isolates was analysed in six independent experiments. In each experiment, duplicate samples were amplified in a duplex TaqMan real-time PCR, targeting the 16S rRNA gene of '*Ca. P. mali*' (AP-16S) and the *M. domestica* gene for 1-aminocyclopropane-1-carboxylate oxidase (Md-ACO1) (Baric et al. 2011; Table 1). PCR analysis was performed in 20 µl reactions, containing 10 µl TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 900 nM of primers qAP-16S-F and qAP-16S-R, 200 nM of primers qMd-ACO-F and qMd-ACO-R, 200 nM of each MGB-probe qAP-16S and qMd-ACO, and 2 µl template DNA, normalised to 10 ng/µl (Baric et al. 2011). The following cycling conditions were applied on a 7500 Fast Real-Time PCR System (Applied Biosystems): 2 min at 50 °C, 10 min at 95 °C and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. After termination of amplification reactions, data were analysed using the automatic baseline setting of the 7500 Software Version 2.0.1 (Applied Biosystems), while three different approaches were employed to set the threshold: (i) automatic threshold setting as implemented in the 7500 Fast Real-Time PCR System analysis software; (ii) threshold fixed

Table 1 Primers and probes employed for relative quantification of '*Candidatus* Phytoplasma mali' in its host plant

Target gene	Primer/probe name	Primer/probe sequence	Reference
16S rRNA (' <i>Ca. P. mali</i> ')	qAP-16S-F	5'-CGAACGGGTGAGTAACACGTAA-3'	Baric and Dalla Via (2004)
	qAP-16S-R	5'-CCAGTCTTAGCAGTCGTTTCCA-3'	Baric and Dalla Via (2004)
	qAP-16S ^a	5'- FAM -TAACCTGCCTCTTAGACG-3'	Baric and Dalla Via (2004)
ACO1 (<i>M. domestica</i>)	qMd-ACO-F	5'-CCAGAATGTCGATAGCCTCGTT-3'	Baric et al. (2011)
	qMd-ACO-R	5'-GGTGCTGGGCTGATGAATG-3'	Baric et al. (2011)
	qMd-ACO ^a	5'- VIC -TACAACCCAGGCAACG-3'	Baric et al. (2011)

^aTaqMan-Probes were conjugated with a Minor Groove Binder (MGB) and a non-fluorescent quencher dye (NFQ) at the 3'-ends (Applied Biosystems)

manually at 0.05 for all amplification runs and both targets; and (iii) manual adjustment of the threshold level based on a so-called calibrator sample which was run on each plate. More precisely, the threshold level was set such that the C_T values for both target genes of the calibrator sample remained constant over all runs.

In a second step, the real-time PCR raw data of 450 DNA samples obtained by Baric et al. (2011) were reanalysed using a fixed threshold of 0.05 for all experiments and both targets. Subsequently, the average C_T values of the two target genes were subtracted for each sample ($\Delta C_T = C_T \text{ AP-16S} - C_T \text{ Md-ACO1}$) (Gachon et al. 2009). ΔC_T values were also calculated using the original dataset with baseline setting relying on a calibrator sample (Baric et al. 2011).

The limits of agreement method described by Bland and Altman (1999) was used to assess the average differences between each of the relative quantification approaches and the standard curve-based technique. This statistical procedure is commonly used in medical studies to examine the degree by which two measurement methods disagree (Bland and Altman 1999). Initially, a non-linear regression was used to calculate the model parameters "constant" and "beta". Subsequently, ΔC_T s were converted to absolute values (AV) using the formula: $AV = \exp(\text{constant} + \text{beta} * \Delta C_T)$. These values were then used for the Bland-Altman analysis (Bland and Altman 1999) with the logarithmically (ln) transformed differences between quantities obtained by the relative and absolute quantification plotted against their logarithmically transformed average. The variance of the measurement error, bias and 95% limits of agreement were calculated as described in Bland and Altman (1999). In addition, intraclass correlation (ICC) analysis using SPSS Statistics (IBM Corporation, Armonk, NY, USA) was performed to assess the degree of accordance among each of the relative quantification approaches with the previously published standard curve-based method (Baric et al. 2011).

Results

The analysis of a subset of 18 DNA isolates in six independent experiments and the subsequent application of different approaches for threshold setting showed that the default threshold option of the 7500 Fast Real-Time PCR System analysis software resulted in higher degrees of variation between different runs compared to the two manual threshold setting procedures (Table 2). In addition, the automatic threshold setting delivered consistently lower ΔC_T values than the two manual approaches (Table 2).

Due to the higher inter-assay variation by the application of the automatic threshold algorithm, which was more than twice as high (Table 2), the computation of ΔC_T values for the dataset of 450 samples was based exclusively on the two

manual threshold setting procedures. The ΔC_T values ranged from -8.829 to 8.054 using the fixed threshold setting and from -9.003 to 7.592 using the reference sample-based threshold setting, where negative values stand for higher phytoplasma loads, while the positive values indicate lower phytoplasma concentration per host cell. These values correspond to a range of pathogen concentration from 0.01 to 726 'Ca. P. mali' cells per host plant cell as obtained by Baric et al. (2011).

The model parameters obtained in non-linear regression analysis and used for conversion of ΔC_T values into absolute quantities ($AV = \exp(\text{constant} + \text{beta} * \Delta C_T)$) were as follows: constant = 0.681 and 0.836, and beta = -0.668 and -0.645 for the fixed and the calibrator sample-adjusted threshold settings. The Bland-Altman plots displayed a good agreement among the relative quantification methods and the standard curve-based method (Fig. 1). The standard deviations (s) of the average differences were 0.17 for the fixed threshold setting and 0.11 for the calibrator sample-adjusted threshold setting that, back-transformed (antilog), correspond to 1.2 and 1.1 pathogen cells per plant cell. However, the relative quantification based on the fixed threshold setting displayed a bias compared to the standard curve procedure, leading to underestimates of the phytoplasma load particularly in the low concentration range (Fig. 1a). For the relative quantification using calibrator sample-adjusted threshold setting the bias was only minimal (Fig. 1b).

Intraclass correlation (ICC) analysis demonstrated high degrees of accordance among the standard curve-based quantification approach and the two relative quantification procedures, with ICC coefficients of 0.996 (95 % confidence intervals: 0.995–0.996) for the fixed threshold and 0.999 (95 % confidence intervals: 0.999–1.000) for the calibrator sample-adjusted threshold. The two relative quantification approaches showed an ICC coefficient of 0.993 (95 % confidence intervals: 0.992–0.994).

Discussion

The present study demonstrates the suitability of the comparative C_T procedure for quantification of 'Ca. P. mali' relative to the host plant genome. The approach is simple to apply as the pathogen and the plant target genes are detected simultaneously in a single-tube duplex reaction using Taq-Man chemistry. Finally, the relative quantity is determined as the difference between the C_T of the pathogen AP-16S rRNA gene and the plant Md-ACO1 gene (ΔC_T). This assay represents an advancement of a recently described quantification method for 'Ca. P. mali' based on the application of two sets of external standard curves, which are to be analysed on each real-time PCR plate to determine the number of phytoplasma cells per host plant cell in the test

Table 2 Mean C_T and ΔC_T values and the standard deviations (SD) for the ‘*Candidatus Phytoplasma mali*’ 16S rRNA (AP-16S) gene and the *Malus domestica* ACO1 gene (Md-ACO1) analysed in 18 DNA samples in six independent real-time PCR experiments. Calculation of threshold values (C_T) was based on the automatic threshold setting as implemented in the 7500 Fast Real-Time PCR System analysis software (Auto THR), on the manual threshold setting at 0.05 for all experiments and both targets (Fixed THR), and on the manual adjustment of the threshold level using a calibrator sample (Cal THR)

Sample	N ‘Ca. P. mali’/host plant cell ^a	C_T -AP-16S			C_T -Md-ACO1			ΔC_T (AP-16S–Md-ACO1)											
		Auto THR		SD	Auto THR		SD	Auto THR		SD	Fixed THR		SD	Cal THR		SD			
		Mean	SD		Mean	SD		Mean	SD		Mean	SD		Mean	SD				
01	10.1	20.70	0.44	22.17	0.30	22.19	0.22	23.82	0.53	24.50	0.31	24.46	0.41	-3.12	0.61	-2.32	0.18	-2.28	0.21
02	20.5	20.43	0.43	21.94	0.28	21.95	0.22	24.73	0.57	25.52	0.37	25.49	0.43	-4.30	0.45	-3.58	0.14	-3.53	0.29
03	21.8	20.13	0.37	21.68	0.26	21.68	0.25	24.53	0.54	25.34	0.28	25.31	0.42	-4.40	0.52	-3.66	0.16	-3.63	0.24
04	47.2	19.12	0.45	20.62	0.32	20.64	0.26	24.97	0.61	25.88	0.30	25.84	0.55	-5.84	0.72	-5.26	0.21	-5.21	0.33
05	14.4	21.12	0.48	22.58	0.41	22.58	0.20	24.65	0.55	25.37	0.41	25.33	0.31	-3.53	0.53	-2.80	0.15	-2.75	0.18
06	2.4	23.21	0.45	24.82	0.40	24.81	0.13	23.85	0.34	24.42	0.18	24.39	0.21	-0.63	0.43	0.40	0.25	0.43	0.24
07	9.3	20.89	0.47	22.52	0.31	22.52	0.19	23.78	0.35	24.46	0.23	24.42	0.29	-2.88	0.41	-1.93	0.11	-1.90	0.13
08	34.7	18.96	0.51	20.54	0.34	20.55	0.16	24.02	0.45	24.85	0.24	24.81	0.41	-5.06	0.56	-4.31	0.18	-4.26	0.31
09	40.8	18.49	0.45	19.98	0.30	19.97	0.14	24.44	0.71	25.37	0.45	25.32	0.38	-5.94	0.74	-5.39	0.26	-5.35	0.26
10	11.0	20.54	0.50	22.13	0.32	22.13	0.13	23.70	0.37	24.40	0.20	24.37	0.29	-3.16	0.56	-2.27	0.14	-2.24	0.20
11	85.9	18.87	0.44	20.45	0.36	20.46	0.23	25.27	0.53	26.27	0.26	26.23	0.36	-6.41	0.72	-5.82	0.16	-5.76	0.29
12	5.6	21.25	0.39	22.76	0.32	22.77	0.05	23.60	0.36	24.24	0.23	24.21	0.17	-2.35	0.40	-1.48	0.13	-1.45	0.15
13	11.6	20.23	0.40	21.80	0.29	21.80	0.11	23.73	0.36	24.45	0.19	24.42	0.26	-3.51	0.50	-2.65	0.11	-2.61	0.19
14	58.5	18.76	0.44	20.28	0.32	20.28	0.06	24.80	0.47	25.78	0.39	25.73	0.29	-6.05	0.61	-5.50	0.22	-5.44	0.24
15	25.5	19.74	0.50	21.33	0.34	21.34	0.11	24.57	0.37	25.39	0.29	25.36	0.32	-4.83	0.49	-4.06	0.21	-4.02	0.30
16	0.8	24.17	0.50	25.92	0.40	25.93	0.07	23.52	0.32	24.07	0.24	24.04	0.15	0.66	0.43	1.85	0.18	1.89	0.16
17	3.0	22.23	0.41	23.82	0.35	23.83	0.11	23.41	0.39	24.00	0.20	23.96	0.09	-1.18	0.49	-0.18	0.21	-0.14	0.17
18	1.2	23.80	0.47	25.51	0.38	25.51	0.11	23.37	0.31	23.94	0.24	23.91	0.22	0.43	0.41	1.57	0.18	1.60	0.21
Average			0.45		0.33		0.15		0.45		0.28		0.31		0.53		0.18		0.23
Min	0.8		0.37		0.26		0.05		0.31		0.18		0.09		0.40		0.11		0.13
Max	85.9		0.51		0.41		0.26		0.71		0.45		0.55		0.74		0.26		0.33

^aObtained by Baric et al. (2011)

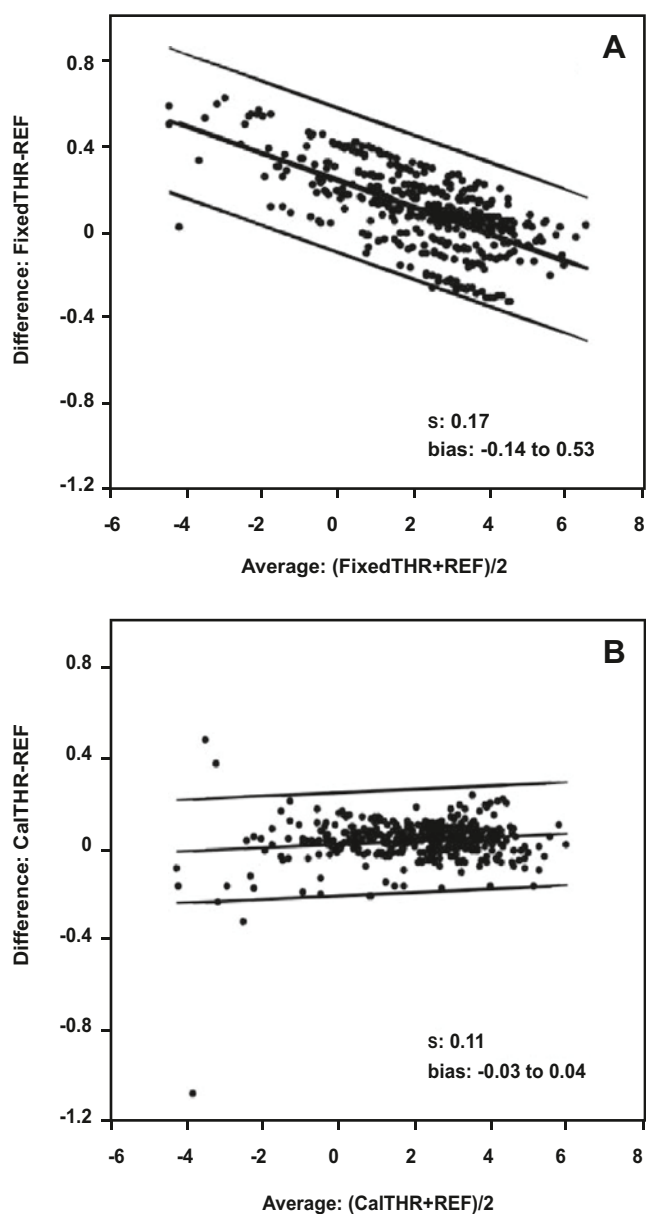


Fig. 1 Bland-Altman plots of differences in phytoplasma titre measured by relative and absolute quantification (REF) against their averages ($N=450$) (Bland and Altman 1999). In plot A threshold was set at 0.05 for all experiments and both targets (Fixed THR), while in plot B a calibrator sample was used to adjust the threshold level (Cal THR). All values were logarithmised prior to Bland-Altman analysis (Bland and Altman 1999). The middle line shows the bias, i.e. the systematic error of the measurement method. The outer lines define the 95 % limits of agreement, indicating the random measurement error. s standard deviation of the differences

samples (Baric et al. 2011). The novelty of that approach was the parallel analysis of the Md-ACO1 gene, mapped as a single-copy gene to chromosome 10 of *M. domestica* (Costa et al. 2005; Velasco et al. 2010), and used as a refer-

ence to relate the phytoplasma titre. Since phytoplasmas are obligate endoparasites, they cannot be separated from their host plant. Thus, nucleic acid isolates from infected tissue will always contain a mixture of both, pathogen and plant DNA. The relative proportion of the two kinds of DNA measured is therefore expected to reflect the pathogen load in plant tissue.

This study shows that quantitative analysis of 'Ca. P. mali' in its host plant is possible without the use of standard curves but by direct subtraction of C_T values of the pathogen and host plant target genes. Such an approach requires comparable amplification efficiencies between the targets (Pfaffl 2004), which were previously confirmed for the AP-16S rRNA/Md-ACO1 system (Baric et al. 2011). The proposed relative quantification procedure contributes to a substantial increase in analysis throughput and decrease in reagent/consumable costs per sample compared to the standard curve-based method. In the latter, 25 % of the positions on a 96-well microtiter plate are occupied by serial dilutions of standards (two standard curves with six dilutions analysed in duplicates). Furthermore, generation of standard curves, on which the accuracy of quantitative results depends, is a critical step (Pfaffl 2004). First, preparation of recombinant plasmid DNA involves time-consuming cloning and plasmid purification steps. Second, determination of the initial plasmid DNA concentration is vulnerable to errors and the stability of standard curves over time can be compromised (Pfaffl 2004). One has to consider that only minor discrepancies in the initially measured copy number of plasmids used to construct the two standard curves can have a major impact on the outcome of absolute quantification results.

One of the disadvantages of the ΔC_T procedure for quantification of 'Ca. P. mali' is that only relative values are obtained. However, the scope of most studies applying quantitative real-time PCR analysis is not to determine absolute phytoplasma numbers but to assess whether there are differences in pathogen load relative to sampling season, plant organ, symptom expression, endophytic colonisation or genotype of the host plant or pathogen (Baric et al. 2011; Bisognin et al. 2008; Musetti et al. 2011; Rekab et al. 2010; Seemüller and Schneider 2007). For such applications, the relative quantification procedure is well suited. Since the accordance of ΔC_T values and the corresponding absolute quantities as determined by ICC was remarkably high, relative data could be easily converted using the model parameters described in this study in cases where absolute values were needed for comparison with previous data (see above for more details). However, when comparing converted values to data obtained by the standard curve procedure, one has to consider that the application of a fixed threshold consistently tended to result in lower pathogen quantities, in particular in the lower concentration range.

The assessment of the inter-assay reproducibility indicated a major effect of the mode of threshold setting which defines the point at which C_T values are recorded (also referred to as crossing point, C_p). In particular, the default automatic threshold option implemented in the 7500 Fast Real-Time PCR analysis software led to higher degrees of variability among replicate samples, while the two manual procedures delivered less variable results. This is in agreement with the findings of Liu et al. (2009) who developed an assay for mRNA quantification on the same instrument type and advised against the use of the automatic threshold setting option due to significantly higher levels of variation. Since manual threshold changes may be vulnerable to subjectivity, it is of the utmost importance to adjust the threshold line within the logarithmic phase of amplification. Furthermore, Nolan et al. (2006) already recommended keeping the threshold level constant whenever samples are analysed in different runs and/or different target genes of the same sample are compared. In the present study, the best performance was achieved using a calibrator sample to adjust the threshold level. Nevertheless, fixing the threshold at a defined level for both target genes and all runs can be seen as a valid alternative for relative quantification, since the amount of DNA of a calibrator sample is usually limited and different samples would be used across different laboratories. In any case, each real-time PCR run should involve the analysis of at least one common reference sample (e.g. positive control) as a means to control the inter-assay variation of quantitative data.

Quantitative analysis of phytoplasmas still carries technical difficulties. One of them is certainly the uneven distribution of the pathogen in the host plant (Rekab et al. 2010). However, the availability of a simple, robust and reasonably priced quantitative assay for ‘*Ca. P. mali*’ could facilitate addressing methodological issues such as the most appropriate sampling practice, the best tissue preparation technique or the optimisation of DNA isolation protocols. All these technical improvements could finally contribute to a series of studies analysing different aspects of pathogen biology, its interaction with the host plant or the effectiveness of potentially bacteriostatic substances and/or resistance inducers. The application of a relative quantification approach based on the analysis of original C_T values, and not on values derived from standard curves, would definitely ensure better methodological standardisation and generation of comparable data across different laboratories. By choosing appropriate amplification targets for the simultaneous analysis of pathogen and plant DNA, the principle of relative quantification could be applied to other phytoplasma species or uncultivable phytopathogens.

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