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Seasonal colonisation of apple trees by '*Candidatus* Phytoplasma mali' revealed by a new quantitative TaqMan real-time PCR approach

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Abstract Apple proliferation (AP), caused by '*Candidatus* Phytoplasma mali', is an economically important disease affecting many apple-growing areas in Europe. A new TaqMan real-time PCR assay was established for absolute quantification of '*Ca.* P. mali' by using a single-copy gene of the host plant as a reference, which is amplified with the pathogen DNA in a single-tube reaction. Normalised estimates of phytoplasma concentration are ultimately expressed as the number of phytoplasma cells per host plant cell. The assay was used to monitor the '*Ca.* P. mali' titre over the course of two growing seasons in roots and branches of symptomatic and asymptomatic but AP-positive apple trees. All 252 root samples from symptomatic and asymptomatic trees tested positive, with an average number of 59.8±5.68 (standard error) and 55.1±9.83 '*Ca.* P. mali' per host cell, respectively. From the 378 shoot samples analysed, 81% of the symptomatic and only 20% of the asymptomatic samples were AP-positive with an average number of 9.4±1.04 and 0.7±0.13 '*Ca.* P. mali' per host cell, respectively. This strengthens evidence that not the pathogen occurrence alone but the presence of a certain quantity of '*Ca.* P. mali' in the aerial tree sections is involved in symptom expression. In addition, pronounced seasonality of the phytoplasma concentration was found, not only in branches, but also for the first time in roots of symptomatic and asymptomatic apple trees. Highest phytoplasma levels in roots were detected from December to May.

Keywords *ACO* gene · Apple proliferation phytoplasma · Internal normalisation standard · *Malus domestica* · Phytoplasma quantification

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Introduction

Apple proliferation (AP) is a disease of the apple tree (*Malus*×*domestica* Borkh.) causing increasing damage and substantial economic loss in apple orchards in Central and Southern Europe. The causative agent, '*Candidatus* Phytoplasma mali' or AP phytoplasma (Seemüller and Schneider 2004), is a quarantine organism belonging to the bacterial class Mollicutes. It resides in the phloem of infected trees and is transmitted through grafting of infected propagation material (Kartte and Seemüller 1988), via natural root grafts (Baric et al. 2008a; Ciccotti et al. 2008) and by sap-sucking insects (Frisinghelli et al. 2000; Tedeschi and Alma 2004, 2006).

'*Ca.* P. mali' causes a number of symptoms such as stunted growth, proliferation of axillary buds (witches' brooms), leaf yellowing or reddening, reduced yield, small and tasteless fruit, general decline and sometimes death of the infected plant

(Bertaccini 2007; Lee et al. 2000). However, the severity of AP symptoms can differ substantially between individual plants, and also between different years for the same tree. Once infected, the apple tree remains phytoplasma-positive throughout its life, even though symptom remission can occur, either transiently or permanently (Carraro et al. 2004; Schmid 1975; Seemüller et al. 1984a). Besides this so-called recovery, latent infections can occur, where the disease does not manifest itself despite the presence of the pathogen (Baric et al. 2007; Carraro et al. 2004). The first evidence that the expression of AP symptoms in infected plants correlates with the presence of '*Ca.* P. mali' in above-ground parts of the tree came from a study using DAPI staining for microscopic phytoplasma detection (Seemüller et al. 1984a). Using a combination of immunofluorescence microscopy, PCR, and ELISA, Carraro et al. (2004) studied the presence of '*Ca.* P. mali' in recovered, symptomatic and latent infected trees in more detail. They demonstrated that only the roots but not the aerial parts of recovered as well as latently infected trees were colonised by the pathogen.

Only a few studies have analysed the colonisation of an infected apple tree by '*Ca.* P. mali' in different organs over the course of a year. So far, all of them have used DAPI staining or immunofluorescence microscopy, techniques which are characterised by limited sensitivity and quantifiability (Loi et al. 2002; Pedrazzoli et al. 2008; Schaper and Seemüller 1984; Seemüller et al. 1984b). These studies revealed that the colonisation of the apple tree canopy follows a seasonal pattern: the phytoplasma is eliminated in aerial parts of the tree during winter but persists in the root system from where it recolonises the stem in spring. The disappearance of the phytoplasma in the above-ground parts was explained by the almost complete degeneration of the phloem in the trunk and branches in winter (Schaper and Seemüller 1982).

For this study, we refined a previously described TaqMan assay for the detection of '*Ca.* P. mali' (Baric and Dalla Via 2004) to enable absolute quantification of the pathogen. The first question addressed using this quantitative approach was whether the occurrence of AP symptoms depends on the presence and/or the phytoplasma titre in the affected tree. Furthermore, the phytoplasma concentration in two different plant organs, roots and branches, sampled at different time points over the course of two growing seasons, was analysed.

Materials and methods

Sample collection and DNA isolation

All samples were taken in a commercial orchard in Unterinn/Auna di Sotto at Ritten/Renon (910 m a. s. l.) in South Tyrol (Northern Italy). The orchard consisted of 1,013 apple trees belonging to the cultivar 'Golden Delicious' clone B on rootstock M9, and was planted in 1996. It was monitored for symptoms of AP over a five-year period from 2001 to 2005. In summer 2003, eight adjacent rows containing 345 trees were sampled and analysed using a high-sensitive qualitative TaqMan real-time PCR test in order to assess the exact infection rate, including also asymptomatic infections (Baric et al. 2007). For the present study, seven of the trees with similar growth characteristics that had tested positive for '*Ca.* P. mali' were selected for further analysis with the quantitative assay. Three of the trees did not display any AP symptoms over the five-year monitoring period. The remaining four trees showed specific AP symptoms such as enlarged stipules and in one case also witches' brooms as well as unspecific symptoms like premature bud break and leaf reddening. All trees were sampled monthly from March 2004 until October 2005, with the exception of November 2004 and January 2005, where no samples were taken due to frost. At each time point, two root samples from different parts of the root system as well as branch samples cut at three different heights (approximately 1.0 m, 1.5 m and 2.0 m) were taken from each tree. Samples were taken from the same branches at each time point. Sampling had to be terminated in October 2005 because the orchard was uprooted by the owner.

Within two days of sampling, total genomic DNA was extracted from 100 mg dissected root or branch phloem using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), as described previously (Baric et al. 2006). The quality of the DNA was controlled by agarose gel electrophoresis. The Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA) was used to determine the DNA concentration of each isolate. The purity of DNA was assessed by measuring absorbance at 260 and 280 nm using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

Real-time PCR

The presence of '*Ca.* P. mali' in the apple tree samples was determined using a qualitative real-time PCR test described previously (Baric and Dalla Via 2004). For quantitative analysis, the same primer/probe set of the duplex TaqMan real-time PCR assay was used to amplify the 16S rRNA gene of '*Ca.* P. mali' (Baric and Dalla Via 2004). However, the chloroplast DNA gene for tRNA leucine (*trnL*) was substituted by the *M. domestica* single-copy gene for 1-aminocyclopropane-1-carboxylate oxidase (*ACO*) (GenBank accession number: Y14005) as internal positive reference.

The primers and probe for the *ACO* gene, designed using Primer Express software (Applied Biosystems, Foster City, CA, USA), were as follows: forward primer qMd-ACO-F (5′-CCA GAA TGT CGA TAG CCT CGT T-3′), reverse primer qMd-ACO-R (5′-GGT GCT GGG CTG ATG AAT G-3′), and the TaqMan probe qMd-ACO (5′-TAC AAC CCA GGC AAC G-3′). The 5′ end of the '*Ca.* P. mali' probe qAP-16S was labelled with the reporter dye FAM, whereas the probe amplifying the apple gene *ACO* was 5′-labelled with VIC, thus allowing distinction of the two amplification products. Both probes were conjugated with a Minor Groove Binder (MGB) and a non-fluorescent quencher dye (NFQ) at their 3′-ends (Applied Biosystems).

Qualitative real-time PCR reactions and the performance evaluation of the duplex real-time PCR assay were performed in an automated ABI PRISM 7000 Sequence Detection System (Applied Biosystems), while the quantitative PCR analyses, the testing of a possible inhibition effect and the assessment of the detection and quantification limits were performed in a 7500 Fast Real-Time PCR System of the same manufacturer. Quantitative real-time PCR analysis was performed in duplicates in 20 µl-reactions, containing 10 µl TaqMan Universal PCR Master Mix (Applied Biosystems), 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 probe and 2 µl template DNA, normalised to 10 ng/µl. Cycling conditions of the real-time PCR were as follows: 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. To correct for inter-plate variation, a calibrator sample was run on every plate, allowing manual adjustment of the threshold level so that the threshold cycle (C_T) values of the calibrator sample remained constant. Threshold cycles and standard curves for each PCR reaction were calculated using the 7500 Software version 2.0.1 (Applied Biosystems).

Standard curve preparation for quantitative analysis

For quantification of '*Ca.* P. mali' in its host plant, two standard curves were constructed by diluting plasmids containing a cloned '*Ca.* P. mali' 16S rRNA gene fragment (1784 bp) and the apple *ACO* gene fragment (587 bp). PCR amplification of the phytoplasma-specific fragment using the primer pair P1/P7 (Schneider et al. 1995) was performed in a reaction volume of 20 µl containing 200 µM of each dNTP, 1 µM of each primer, 2 mM $MgCl_2$, 2 µl 10× PCR buffer, 1 U AmpliTaq Gold polymerase (Applied Biosystems) and 40 ng total DNA isolated from a '*Ca.* P. mali'-infected apple tree. Cycling conditions were as follows: 94°C for 10 min, 40 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 1 min 20 s, followed by a final extension at 72°C for 5 min. The *M. domestica ACO* gene was PCR-amplified in 20 µl-reactions with the primers Md-ACO1F/Md-ACO1R (Costa et al. 2005). The reaction contained 250 µM of each dNTP, 200 nM of each primer, 1 mM $MgCl_2$, 2 µl 10× PCR Buffer, 1 U AmpliTaq Gold polymerase and 50 ng DNA of *M. domestica*, and was carried out under the following conditions: 94°C for 10 min, 35 cycles of 94°C for 45 s, 65°C for 45 s, 72°C for 2 min, followed by a final extension at 72°C for 8 min. The resulting PCR products were purified using Montage PCR Centrifugal Filter Devices (Millipore, Bedford, MA, USA) and cloned into the pGEM-T Easy plasmid vector (Promega, Madison, WI, USA), followed by purification with the Perfectprep Plasmid Mini Kit (Eppendorf, Hamburg, Germany).

Standard curves were prepared with 10-fold serial dilutions of the cloned '*Ca.* P. mali' 16S rRNA gene fragment (diluted in *ACO* plasmid solution containing 1000 copies / µl) and the *M. domestica ACO* gene fragment (diluted in 10 mM Tris-HCl, pH 8), ranging from a copy number of 10^2 to 10^7. The initial concentration of the purified plasmids was measured in triplicate using optical density spectrophotometry (NanoDrop 1000 Spectrophotometer, Thermo Fisher Scientific). Subsequently, the following equation was used to calculate the corresponding copy number per

microlitre: [plasmid DNA concentration (g/μl)] / [molecular weight of the plasmid + insert (u/copy) × atomic mass unit (g/u)], where the molecular weight of plasmid + insert was calculated as the sum of molecular weights of the total number of specific nucleotides and the atomic mass unit was 1.6605×10^{-24} g/u.

Standard curves were constructed by plotting the C_T values against the $\log_{10}$ of the copy number. On each PCR plate, in addition to the test samples, duplicates of serial 10-fold dilutions for the phytoplasma as well as for the apple DNA standard curves were analysed. The stability and linearity of standard curves over time was monitored by comparing C_T values of standards from different experiments.

The fact that two copies of the *ACO* gene are present in the diploid genome of the *M. domestica* cultivar 'Golden Delicious' (Baric et al. 2008b; Costa et al. 2005) and that the 16S rRNA gene also exists in two copies in the '*Ca.* P. mali' genome (Schneider and Seemüller 1994), allows calculation of the absolute number of phytoplasma genomic units or cells per diploid host plant genome or cell.

The quantitative data were subjected to statistical analysis by SPSS Software version 12.0 (SPSS Inc., Chicago, IL, USA) and by SigmaPlot for Windows version 11.0 (Systat Software, Inc., Chicago, IL, USA). Amplification efficiency (AE) was calculated from the slope of the standard curve using the equation $AE=10^{(1/-slope)}-1$. Coefficients of variation (CV) were calculated by dividing the standard deviation of a value by its mean, multiplied by 100.

Results

Performance evaluation of the duplex real-time PCR assay

Performance characteristics of the new duplex real-time PCR assay were evaluated in comparison to singleplex reactions by amplifying four serial 10-fold dilutions of DNA isolates from ten phytoplasma-infected apple trees. C_T values obtained for each of the ten samples amplified as singleplex and as duplex reactions were plotted against the $\log_{10}$ of the DNA concentration. Amplification of the serial dilutions resulted in linear regression lines with a high correlation coefficient ($R^2\geq0.992$), confirming high linearity of the PCR reactions. Amplification of the '*Ca.* P. mali' gene resulted in linear regression lines with a slope between −3.563 to −3.380 (mean −3.471± 0.048 standard deviation [SD]) for singleplex amplification, and slopes between −3.550 and −3.315 (mean −3.385±0.071 SD) for the duplex reactions, indicating a mean amplification efficiency of 94.2% and 97.5%, respectively. For the apple gene, slopes between −3.525 and −3.360 (mean −3.428±0.064 SD) were obtained for singleplex amplifications, whereas they ranged from −3.548 and −3.280 (mean −3.401±0.071 SD) for duplex reactions, which corresponds to a mean amplification efficiency of 95.8% and 96.9%, respectively. The comparison of the amplification efficiencies of the '*Ca.* P. mali' gene and the host gene in the duplex real-time PCR assay revealed no significant difference (Wilcoxon Signed-Ranks Test; $n=10$, $p=0.445$), permitting direct calculation of the absolute number of phytoplasma genomic units per host genomic unit.

In order to assess the inter-assay reproducibility of the quantitative real-time PCR, ten randomly selected AP-positive apple DNA extracts were run on three different plates. The average coefficient of variation (CV) for the C_T values was 0.62% for the phytoplasma-specific PCR and 0.82% for apple-specific PCR, indicating that the real-time PCR results were highly reproducible. The average CV for the number of '*Ca.* P. mali' copies per host cell amounted to 7.88% (data not shown).

To check for a possible inhibitory effect between different types of samples, all DNA isolates were divided into 16 different groups, according to the type of plant organ, presence/absence of symptoms and sampling period. Out of each of the 16 groups, one DNA isolate was randomly selected and 10-fold serial dilutions, ranging from undiluted to 1,000-fold diluted, were prepared. Each of the four serial dilutions was analysed with the duplex real-time PCR assay, and the C_T values were plotted against the $\log_{10}$ of the initial DNA amount. The correlation coefficients of the linear regression lines ranged from 0.9905 to 0.9998 (mean 0.9973±0.0025 SD) and from 0.9882 to 0.9995 (mean 0.9965±0.0031 SD) for the '*Ca.* P. mali' gene and the apple gene, respectively. The slopes of the linear regression lines for the two genes ranged from −3.513 to −3.183 (mean −3.350±0.093 SD) and −3.533 to −3.162 (mean −3.300±0.086 SD),

resulting in a minimum amplification efficiency of 92%. Based on these results, no inhibitory effect in the real-time PCR assay, driven by tissue type or seasonal physiological changes in the apple tree, could be observed.

Evaluation of the experimental variability of the quantitative real-time PCR assay

Experimental variability of the quantitative real-time PCR procedure was assessed using samples from four apple trees, which were previously artificially inoculated with '*Ca.* P. mali' and maintained in the experimental orchard of the Research Centre Laimburg. From each tree, three samples from different parts of the rootstock as well as three independent DNA isolates from each of the root samples were obtained, using the same procedure as described before. All 36 DNA isolates were analysed in duplicate by means of the quantitative real-time PCR.

The average coefficient of variation for the number of '*Ca.* P. mali' per plant cell among the three DNA isolation replicates amounted to 11.02%. The variability between the quantitative results was generally lower for samples with less than 100 phytoplasma cells (CV from 2.10 to 14.25%; mean 6.79%; n=8) and higher in samples with more than 100 phytoplasma cells per plant cell (CV from 11.64 to 26.04%; mean 19.47%; n=4) (see also Fig. 1). The '*Ca.* P. mali' quantity varied considerably among the different root samples of the same tree (CV up to 65.0%) (Fig. 1), most probably due to an uneven distribution of the pathogen within the host plant rootstock. For this reason, quantitative data from different trees were grouped for statistical analyses (i.e. roots from symptomatic trees; roots from asymptomatic trees; shoots from symptomatic trees; shoots from asymptomatic trees), to account for experimental variability and irregular pathogen distribution, and no conclusions were drawn for single samples or single trees.

Stability and linearity of quantification

Standard curves showed high stability over the entire period in which the experiments were performed. The linear correlations of the standard curves from single experiments were almost perfect over six orders of magnitude, with $R^2 \geq 0.994$ for the '*Ca.* P. mali' gene and $R^2 \geq 0.999$ for the apple gene. The mean amplification efficiencies of the standard curves from all experiments reached 97.62%±1.239 SD for the '*Ca.* P. mali' gene and 92.91%±1.154 SD for the apple gene.

Determination of the quantification and detection limits

In order to estimate the limit for accurate quantification, two-fold serial dilutions of plasmid DNA, containing the '*Ca.* P. mali' 16S rRNA or the *M. domestica ACO* gene fragment, were measured. The target gene copy numbers per assay ranged from 2000 to 62.5, each analysed in six replicates on the same plate (Table 1). The coefficient of variation of the estimated gene copy number was below 10% for 2000 and 1000 target copies for each of the two genes (Table 1). Furthermore, for 2000 and 1000 target copies the deviation of the estimated gene copy number from the expected was ≤10.9% for the '*Ca.* P. mali' 16S rRNA gene and ≤6.1% for the *M. domestica ACO* gene (Table 1). Since these results were associated with C_T values lower than 30, it was decided to exclude all samples with higher C_T values

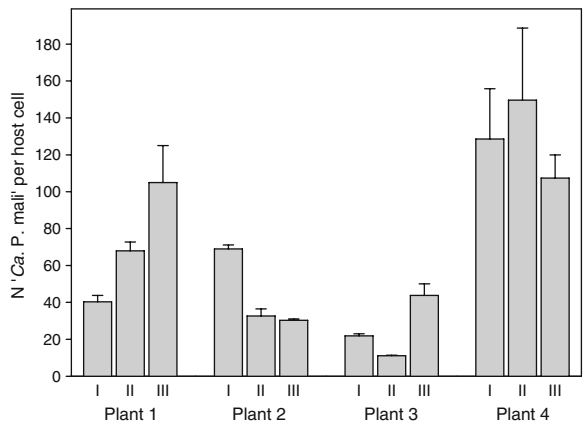


Fig. 1 Determination of the experimental variability of the real-time PCR assay for quantitative detection of '*Ca.* P. mali' in apple trees. Each bar represents the average number of '*Ca.* P. mali' per plant cell ± standard deviation of three DNA isolation replicates. The experiment included four artificially inoculated trees (Plant 1–4), of which three independent root samples were taken (denoted by roman numerals)

Table 1 Determination of the lower limit of quantification using two-fold serial dilutions of plasmid DNA containing the '*Ca.* P. mali' 16S rRNA gene and the *M. domestica ACO* gene fragments. Each sample, containing a defined gene copy number, was analysed in six replicates on the same plate

Gene	N gene copies expected	C_T			N gene copies estimated			% relative error[a]
		Mean	SD	CV (%)	Mean	SD	CV (%)	
AP-16S	2000	28.52	0.09	0.32	2151	136.2	6.33	7.6
	1000	29.45	0.10	0.34	1109	78.1	7.04	10.9
	500	30.24	0.18	0.58	632	81.2	12.84	26.4
	250	31.11	0.13	0.42	339	32.2	9.49	35.8
	125	32.07	0.36	1.12	175	43.3	24.74	40.1
	62.5	32.64	0.32	0.97	116	13.1	11.31	85.6
Md-ACO	2000	28.30	0.11	0.39	2122	151.5	7.14	6.1
	1000	29.48	0.14	0.47	975	86.1	8.83	−2.5
	500	30.66	0.25	0.80	455	75.4	16.59	−9.1
	250	31.91	0.25	0.78	200	31.3	15.62	−20.0
	125	32.95	0.31	0.93	102	21.9	21.44	−18.2
	62.5	34.19	0.22	0.64	45	6.4	14.29	−28.3

[a] The % relative error was calculated using the following equation: ([estimated value – expected value] / [expected value])*100

for any of the two genes from the quantitative approach.

To determine the limit of detection, two-fold serial dilutions of plasmid DNA ranging from 60 to 3.8 copies were prepared. For the '*Ca.* P. mali' 16S rRNA gene all eight replicates resulted positive down to 15 gene copies per assay, while for the *M. domestica ACO* gene this was the case down to 30 copies per assay (Table 2).

Absolute quantification of '*Ca*. P. mali' in symptomatic and asymptomatic apple tree samples

Prior to quantification, a qualitative real-time PCR assay was performed to test for the presence of '*Ca.* P. mali' in the seven apple trees included in this study. The results showed that the four trees with AP symptoms tested positive for '*Ca.* P. mali' in all root samples, but also in 175 out of 216 (81%) shoot

Table 2 Determination of the limit of detection using two-fold serial dilutions of plasmid DNA containing the '*Ca.* P. mali' 16S rRNA gene and the *M. domestica ACO* gene fragments. Each sample, containing a defined gene copy number, was analysed in eight replicates on the same plate

Gene	N gene copies expected	C_T			N positives / N experiments
		Mean	SD	Min - Max	
AP-16S	60	34.05	0.27	33.75–34.48	8 / 8
	30	35.32	0.47	34.81–36.14	8 / 8
	15	35.90	0.49	35.28–36.63	8 / 8
	7.5	37.03	0.63	36.32–38.24	7 / 8
	3.8	37.72	0.59	37.28–38.39	3 / 8
Md-ACO	60	33.48	0.53	32.64–34.44	8 / 8
	30	35.40	1.26	34.40–37.70	8 / 8
	15	35.74	1.01	34.46–36.92	5 / 8
	7.5	37.15	0.95	36.13–38.44	5 / 8
	3.8	35.95	0.70	35.46–36.44	2 / 8

samples. In the trees that never showed any symptoms over the monitoring period, root samples were AP-positive in all cases, whereas only 32 out of 162 (20%) shoot samples tested positive (Fig. 2). Notably, '*Ca.* P. mali' could be detected in above-ground parts of the three asymptomatic trees in at least a third of the 18 sampling time points.

Next, shoot and root samples that had tested positive with the qualitative assay were analysed using quantitative real-time PCR, to determine the absolute concentration of '*Ca.* P. mali'. Seven positive samples were excluded from the quantitative analyses due to C_T values above 30 (see Fig. 2; NQ samples). The quantitative approach was performed for a total of 452 samples and produced C_T values ranging from 15.81 to 29.54, with a mean of 19.74 (±2.39 SD) for the phytoplasma 16S rRNA gene. The reproducibility of duplicates analysed in the same run was very high with standard deviations of C_T values being ≤0.47 (mean SD: 0.09). The total number of phytoplasma copies per reaction ranged from 612 to 7.1×10^6 (or 1224 to 1.4×10^7 AP-16S gene copies). For the *ACO* gene of *M. domestica*, C_T values from 19.65 to 28.80 (mean 22.59±1.42 SD) were obtained. Reproducibility of the technical replicates was high also for this gene, with standard deviations of the C_T values being ≤0.50 (mean SD: 0.12). The total number of diploid plant cells per assay was estimated to range from 732 to 2.5×10^5, corresponding to 1464 to 5.0×10^5 *ACO* gene copies.

The phytoplasma titre determined in the positive shoot samples from the three asymptomatic trees was low, with an average of only 0.7 phytoplasma cells per host cell (Table 3, Fig. 2). In contrast, quantitative analysis of the AP-positive shoot samples taken from symptomatic apple trees yielded an average concentration of 9.4 phytoplasma cells per host cell (Table 3). Repeated-measures ANOVA showed that the difference in the '*Ca.* P. mali' concentration between symptomatic and asymptomatic shoots was highly significant ($p<0.001$).

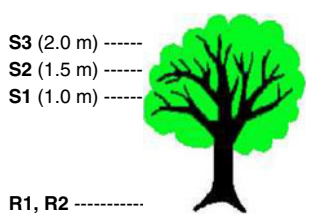
Analysis of the root samples revealed a more homogenous picture: all 144 root samples from symptomatic trees and 108 root samples from asymptomatic trees tested positive for the presence of '*Ca.* P. mali' with an average of 59.8 and 55.1 phytoplasma cells per host cell, respectively (Table 3). This difference was not statistically significant ($p=0.076$; repeated-measures ANOVA). The '*Ca.* P. mali' titre in root samples from symptomatic as well as from asymptomatic trees varied considerably, ranging from 1.4 to 727 phytoplasma cells per host cell (Fig. 2).

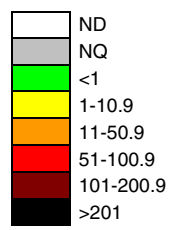
Seasonal variation of '*Ca.* P. mali' titres in root and shoot samples

The seasonal fluctuation of the '*Ca.* P. mali' titre was analysed in root and shoot samples over the course of two growing seasons. Figure 3 shows that '*Ca.* P. mali' could be detected in all root samples throughout the year, regardless of the time point of sampling, albeit at greatly varying concentrations. A visual inspection of the data indicated higher '*Ca.* P. mali' titres in the roots of both symptomatic and asymptomatic trees in the period from December to May. Thus, the results were grouped in the following way: (i) June to October 2004, (ii) December 2004 to May 2005 and (iii) June to October 2005, and Kruskal-Wallis one-way analyses of variance on ranks were performed. For both symptomatic and asymptomatic root samples a statistically significant difference between the median values of the three groups was found ($p<0.001$). A subsequent Dunn's test showed that in both cases (symptomatic and asymptomatic root samples) there was a difference at the 0.05 significance level in the phytoplasma concentration between the periods June–October 2004 and December 2004–May 2005, and June–October 2005 and December 2004–May 2005, while there was no difference between the periods June–October 2004 and June–October 2005.

'*Ca.* P. mali' could be detected at all sampling time points in at least one shoot sample from each symptomatic tree. The only exception was Tree 4, for which no shoot sample tested positive in June 2005 (Fig. 2). In the shoot samples from asymptomatic trees, '*Ca.* P. mali' was detected at 14 of the 18 sampling time points, however, the concentration was generally very low (Fig. 2). The highest number of 2.9 '*Ca.* P. mali' cells per host cell was found in a single shoot sample taken in December 2004. Also in symptomatic trees, the highest mean value of 13.7 '*Ca.* P. mali' cells per host cell was found at this time point (Fig. 3). A Kruskal-Wallis test ($p<0.001$) and the subsequent Dunn's test comparing the concentrations measured in the time periods (i) March to August 2004, (ii) September 2004

			30/03/2004	27/04/2004	25/05/2004	22/06/2004	21/07/2004	24/08/2004	21/09/2004	25/10/2004	06/12/2004	16/02/2005	22/03/2005	26/04/2005	26/05/2005	28/06/2005	26/07/2005	31/08/2005	26/09/2005	24/10/2005
SYMPTOMATIC	Tree 1	S3	8.0	6.9	0.846	ND	ND	1.2	7.7	17.2	15.4	15.5	18.8	11.6	3.1	2.2	2.0	19.7	1.8	0.740
		S2	28.0	4.8	0.750	1.3	1.4	ND	15.2	12.2	47.2	13.1	5.6	3.2	0.827	3.6	3.0	1.6	1.2	6.0
		S1	10.1	1.6	0.160	0.201	18.0	58.4	37.8	0.644	26.8	36.4	7.8	2.1	ND	ND	ND	ND	0.029	1.1
		R1	31.1	46.2	14.6	10.9	11.6	20.7	17.6	43.7	25.3	38.9	85.9	50.1	58.5	16.0	15.2	15.0	37.9	15.4
		R2	124.5	47.0	15.8	30.0	22.1	23.3	19.2	16.6	57.2	13.8	36.7	102.7	25.5	14.5	15.2	16.0	3.0	28.2
	Tree 2	S3	6.2	ND	2.9	5.6	ND	0.019	1.3	7.0	13.7	7.4	0.028	3.8	ND	ND	6.7	0.750	ND	ND
		S2	ND	ND	ND	ND	0.862	9.7	2.6	2.7	13.1	2.6	3.0	0.791	2.7	1.0	1.5	1.9	0.434	3.5
		S1	0.109	0.093	ND	ND	3.8	ND	16.7	6.9	0.251	16.6	7.9	3.5	NQ	ND	ND	ND	ND	2.0
		R1	81.1	109.1	59.4	39.4	60.2	26.4	50.9	33.1	18.0	104.3	130.8	92.5	42.9	30.6	41.8	87.2	29.0	19.1
		R2	67.0	59.6	20.9	20.5	47.2	35.8	9.3	41.2	49.2	25.8	83.1	257.6	56.1	12.7	42.1	20.1	65.1	27.1
	Tree 3	S3	20.3	ND	0.803	8.4	14.4	25.9	34.7	60.8	13.7	11.0	5.5	2.5	ND	3.0	4.0	8.4	4.7	4.7
		S2	14.6	7.5	1.2	5.3	13.5	6.1	36.8	40.8	5.6	24.1	8.6	3.2	6.0	2.4	4.6	32.9	9.6	17.7
		S1	2.5	0.479	0.666	7.4	20.9	34.9	46.7	71.0	91.6	15.7	4.3	2.6	ND	ND	7.6	3.9	23.5	3.3
		R1	37.9	224.1	176.7	4.3	25.7	17.5	18.1	44.8	19.2	48.3	92.0	1.6	21.8	78.7	41.6	46.0	26.6	73.0
		R2	35.4	100.2	119.0	24.9	14.7	152.5	27.7	12.7	19.5	39.1	24.1	238.5	166.1	12.6	13.0	90.8	2.7	14.8
	Tree 4	S3	4.2	7.8	0.634	ND	0.263	1.0	2.9	4.3	1.3	3.8	ND	ND	0.436	ND	ND	2.0	0.134	20.3
		S2	10.4	4.0	NQ	1.2	0.578	5.0	11.4	17.4	13.0	12.3	ND	ND	ND	ND	0.161	5.4	1.5	6.2
		S1	0.226	3.9	1.2	NQ	3.3	2.4	2.6	2.2	ND	ND	0.569	0.638	0.039	ND	ND	3.0	18.4	2.8
		R1	43.5	175.2	4.5	27.4	21.8	90.6	52.3	38.5	122.0	77.7	103.7	336.4	13.3	32.0	95.8	48.5	15.4	54.8
		R2	19.7	458.1	164.4	110.0	55.5	320.5	11.9	32.0	31.4	240.5	63.6	159.0	59.9	90.4	90.8	26.7	32.2	21.7
ASYMPTOMATIC	Tree 5	S3	ND	ND	ND	ND	ND	ND	ND	ND	0.160	ND	ND	ND	ND	ND	1.8	ND	ND	ND
		S2	ND	ND	ND	ND	ND	ND	ND	ND	2.9	ND	ND	ND	ND	NQ	ND	NQ	ND	0.118
		S1	ND	ND	ND	ND	ND	0.717	0.752	0.506	0.343	ND	ND	ND	ND	ND	0.097	0.394	0.084	ND
		R1	34.4	64.8	4.7	5.3	17.6	10.2	27.0	46.1	69.9	44.6	84.4	6.8	17.3	63.3	19.9	8.4	9.7	5.9
		R2	30.1	6.1	4.9	1.4	9.0	24.6	45.5	7.0	18.1	38.7	26.6	27.0	34.9	17.6	6.3	2.7	11.8	3.1
	Tree 6	S3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.255	1.746
		S2	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.724	0.388	ND	NQ	ND	ND	ND	ND	ND
		S1	ND	ND	ND	ND	ND	ND	NQ	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1.5
		R1	65.7	564.2	2.1	4.2	144.4	38.6	18.3	85.8	79.4	116.4	7.9	40.7	32.8	53.9	22.2	13.5	14.0	95.5
		R2	110.1	53.0	9.4	7.0	412.3	41.0	42.4	27.9	37.2	207.4	112.0	109.5	78.7	30.5	7.5	14.1	4.9	47.5
	Tree 7	S3	ND	ND	ND	ND	ND	ND	ND	0.211	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
		S2	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.011	0.554	0.920	0.885	1.0	ND	ND	ND	ND
		S1	ND	ND	0.012	ND	ND	0.268	1.2	0.945	ND	ND	ND	ND	ND	ND	ND	ND	0.048	0.079
		R1	202.0	32.8	3.6	20.5	15.9	31.7	9.9	56.6	57.8	362.6	59.3	29.3	11.6	9.8	88.3	8.7	4.2	16.9
		R2	68.7	28.0	8.5	30.3	42.1	11.4	26.5	23.2	17.7	726.5	67.4	82.6	26.5	12.1	29.3	77.1	42.3	2.6





◀ **Fig. 2** Presence of '*Ca.* P. mali' in four symptomatic and three asymptomatic but infected apple trees. Two root (R1, R2) and three shoot (S1–S3) samples were collected from each tree at 18 time points between March 2004 and October 2005. Samples testing positive for '*Ca.* P. mali' with both qualitative and quantitative real-time PCR are represented by coloured rectangles, each colour defining an exact range of phytoplasma cells per host cell. For each sample tested positive and quantified, the number of '*Ca.* P. mali' per host cell is given in the rectangles. Seven AP-positive samples (marked in light grey; NQ) could not be quantified as their C_T values were higher than the quantification limit. White squares indicate that the phytoplasma was not detected (ND)

to February 2005 and (iii) March to August 2005 showed that in the shoots of symptomatic trees there was a difference at the 0.05 significance level in the phytoplasma concentration between the periods March–August 2004 and September 2004–February 2005, and the periods March–August 2005 and September 2004–February 2005. No statistically significant difference was found between the periods March–August 2004 and March–August 2005.

Discussion

Phytoplasmas are obligatory intracellular plant pathogens which cannot be grown in vitro in cell-free media. Therefore, pure phytoplasma DNA cannot be obtained but only in combination with nucleic acid of its host plant or insect vector. Previous quantitative real-time PCR approaches for '*Ca.* P. mali' related the phytoplasma copy number to the fresh weight of plant tissue used for DNA isolation (Bisognin et al. 2008; Jarausch et al. 2004; Torres et al. 2005; Seemüller and Schneider 2007). Such an approach, lacking an internal standard for parallel detection of a host plant gene, shows decreased accuracy, since it does not take into account the variability in the DNA yield between separate extractions (Brunner et al. 2009). The quantity of nucleic acid isolated from plant tissue largely depends on the efficiency and reproducibility of the disruption of the rigid plant cell walls using mechanical methods (Varma et al. 2007). Furthermore, woody plants often contain high amounts of secondary metabolites, such as polysaccharides and polyphenols, which can interfere with the DNA isolation procedure as well as with downstream processes, including real-time PCR. All these factors can impair the reproducibility of the plant genomic DNA isolation procedure and ultimately influence the DNA yield, making the fresh weight of plant tissue less suitable as a reference point for phytoplasma quantification. To overcome this problem, Christensen et al. (2004) suggested the parallel amplification of the 18S rRNA gene of the host plant to be used for normalisation. By dividing the amount of phytoplasma DNA by the amount of plant DNA for each sample, the authors obtained the number of phytoplasma cells per microgram of plant DNA. The reliability of ribosomal RNA genes as standards for data normalisation, however, needs to be questioned. In the plant genome, these genes are present in varying copy numbers, not only across species and within populations (Rogers and Bendich 1987), but also within individual organisms, depending on the tissue type, the developmental stage or the influence of environmental factors (Lloyd et al. 1994).

The advantage of the approach described here over the currently used quantitative real-time PCR procedures lies in the use of a single-copy gene of the host plant as a reference, which is amplified simultaneously and with comparable efficiency with the pathogen DNA in a single-tube reaction. Estimates of the phytoplasma infection level are ultimately expressed as the number of phytoplasma cells per host plant cell, rendering the quantitative results more reproducible and comparable. A limitation of this approach is that the phytoplasma load is averaged over the total number of host plant cells from which DNA was isolated. Since mature sieve elements, which are the main habitat of phytoplasmas, do not contain nuclei, these cells are not considered in the calculation. Moreover, it cannot be distinguished whether a small number of plant cells showed a high phytoplasma load or a larger number of plant cells was colonised by a lower number of pathogen cells. Although quantification of uncultivable microorganisms remains a challenging task, we suggest that reference gene-based approaches should become a future standard for more reliable quantitative phytoplasma determination, as is already the case for other plant pathogens (e.g. Brunner et al. 2009; Valsesia et al. 2005).

Table 3 Number of samples testing positive for '*Ca.* P. mali', and the average phytoplasma titre in quantifiable positive samples. Two root and three shoot samples were taken from each of the trees analysed at one-month intervals over a period of 20 months, except for November 2004 and January 2005, where no samples were taken due to frost. Seven AP-positive samples could not be quantified as their C_T values were higher than the quantification limit

	N positive / N tested samples (%)	Average No. of '*Ca.* P. mali' per host cell	Results of the repeated-measures ANOVA
Shoot samples			
Symptomatic	175/216 (81%)	9.4 (±1.04 SE)	$p<0.001$
Asymptomatic	32/162 (20%)	0.7 (±0.13 SE)	
Root samples			
Symptomatic	144/144 (100%)	59.8 (±5.68 SE)	$p=0.076$
Asymptomatic	108/108 (100%)	55.1 (±9.83 SE)	

The perennial analysis of seven full-grown apple trees from a commercial orchard in South Tyrol revealed that all root samples from symptomatic as well as asymptomatic trees tested positive for '*Ca.* P. mali'. In shoots, the phytoplasma could be detected in 81% of the samples from symptomatic and in 20% of the samples from asymptomatic trees. Previous studies showed that only roots but not aerial parts of recovered and latent infected apple trees were colonised by '*Ca.* P. mali' (Carraro et al. 2004; Musetti et al. 2004), suggesting that the phytoplasma presence in aerial sections of the tree strictly correlates with the occurrence of visual symptoms. This study demonstrates the presence of '*Ca.* P. mali' in the shoots of all three examined asymptomatic trees in at least one third of the sampling time points, albeit at very low concentrations. The average phytoplasma titre in shoot samples from symptomatic trees was shown to be 14 times higher compared to the one from asymptomatic trees. Therefore, not the pathogen occurrence alone but the presence of a high number of '*Ca.* P. mali' cells in the phloem of aerial sections of infected apple trees is involved in the development of severe AP symptoms, such as witches' brooms or undersized fruit, as shown in previous studies (Bisognin et al. 2008; Schaper and Seemüller 1984; Seemüller et al. 1984a, b). A high phytoplasma concentration could induce the symptoms by a number of mechanisms, such as by directly impairing phloem transport, or indirectly by provoking a disease response in the plant or by releasing virulence factors (Christensen et al. 2004; Hogenhout et al. 2008).

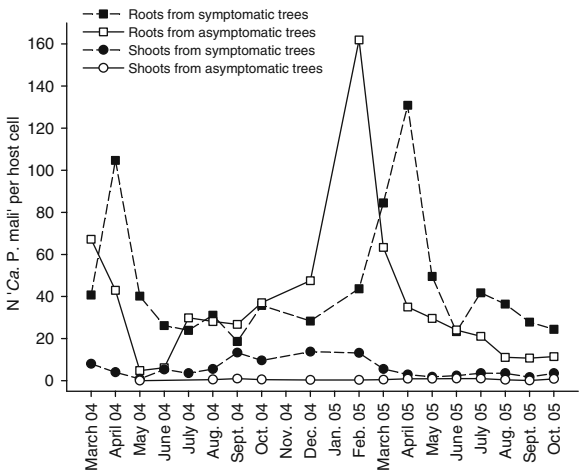


Fig. 3 Mean number of '*Ca.* P. mali' per host cell in root samples from four symptomatic (filled squares, dashed line) and three asymptomatic (empty squares, solid line) apple trees, as well as in AP-positive shoots from symptomatic (filled circles, dashed line) and asymptomatic trees (empty circles, solid line). Absence of data points indicates that '*Ca.* P. mali' was undetectable. Samples were taken at approximately 4-week intervals between March 2004 and October 2005. Due to frost, no samples were taken in November 2004 and January 2005

The phytoplasma load in the rootstock, in contrast, does not seem to have a direct effect, neither on the colonisation intensity of the aerial parts of the apple tree nor on AP symptom expression. This conclusion is based on the observed lack of a statistically significant difference between the average phytoplasma titre in the roots of symptomatic and asymptomatic apple trees. Seemüller and Schneider (2007) reported that rootstocks of apple trees ('Golden Delicious' grafted on M11) inoculated with different

'*Ca.* P. mali' strains displayed similar phytoplasma concentrations despite differences in virulence. It can thus not be excluded that the asymptomatic apple trees tested in the present study were colonised by avirulent or mild strains of '*Ca.* P. mali', which could have similar multiplication rates in rootstocks as severe strains, but differ in their ability to colonise the canopy of apple trees and induce AP symptoms. Alternatively, microgeographic variation of abiotic factors, such as temperature, light, soil properties or water supply, could have modulated the physiological conditions of plants with the same genotype, leading to different responses to phytoplasma strains of similar virulence (Abdel-Farid et al. 2009). Furthermore, analyses of rootstocks derived from apomictic *Malus* species indicated that AP symptom development rather depends on qualitative than quantitative factors, as phytoplasma concentration in the rootstocks could not be linked with the severity of disease symptoms developed by the top grafted cultivar 'Golden Delicious' (Bisognin et al. 2008).

Although the infection level in the roots showed a great degree of variation within and between trees, the mean phytoplasma number was at least 5.8 times higher compared to the shoot samples. The finding of a greater density of the phytoplasma in the underground parts than in the aerial sections of the host plant is in agreement with the previously observed difference in '*Ca.* P. mali' detectability in shoots and roots using DAPI staining microscopy, immunofluorescence microscopy and quantitative real-time PCR (Bisognin et al. 2008; Loi et al. 2002; Seemüller et al. 1984b). Likewise, *Chrysanthemum carinatum* soon after experimental inoculation showed a significantly higher titre of chrysanthemum yellows phytoplasma in the roots than in the leaves (Saracco et al. 2006). The authors suggested that this was due to differences in the amount of phloem tissue between plant organs, due to more active translocation of the pathogen from above-ground organs to the roots and/or due to a more efficient multiplication of the phytoplasma in the roots. The latter assumption seems to be supported by another study showing that phytoplasmas tend to multiply more efficiently in non-photosynthetic tissue than in photosynthetically active plant components (Sears et al. 1997).

Regarding the seasonal variation of the phytoplasma infection level, previous studies using DAPI staining and immunofluorescence microscopy demonstrated that in late winter and spring '*Ca.* P. mali' is almost undetectable in aerial parts of the tree, but is still present in the roots (Loi et al. 2002; Pedrazzoli et al. 2008; Seemüller et al. 1984b). The highest phytoplasma detectability in aerial parts of infected apple trees was seen from August to December, whereas '*Ca.* P. mali' was difficult to detect from March to May (Loi et al. 2002; Pedrazzoli et al. 2008; Seemüller et al. 1984b). In the present study, the application of the quantitative real-time PCR approach revealed '*Ca.* P. mali' in the canopy of AP symptomatic trees throughout the year, with a detectability from 42 to 100%. Similar to previous studies, the highest percentage of AP-positive shoot samples were found in the period from September to February. The seasonal fluctuations of '*Ca.* P. mali' in the aerial parts of the apple tree were already explained by the degeneration of sieve tubes during winter (Evert 1963; Schaper and Seemüller 1982). The phloem of roots, in contrast, is regenerated continuously, being essential for the survival of the phytoplasma during the dormant season and the potential recolonisation of the stem during a new growing season (Schaper and Seemüller 1982). However, Schaper and Seemüller (1982) also reported the existence of apple trees with a pronounced and functional replacement phloem in the shoots, which was formed most probably as a consequence of the phytoplasma infection and possibly persisted throughout the winter. We speculate that this may have occurred in the trees investigated in the present study, explaining the presence of '*Ca.* P. mali' in the shoots throughout the year, though at different concentrations. Surprisingly, seasonal fluctuations of the phytoplasma titre were not only found in the canopy, but also in the roots of both symptomatic and asymptomatic trees, with the highest values from December to May and the lowest from June to October. This pattern coincides with the accumulation of carbohydrate reserves in the root system which peaks early in the winter (Tromp 1983) and, at least in the stem, remains constant until the time of bud break in spring (Sivaci 2006). As phytoplasmas utilise carbohydrates as their main source of energy (Oshima et al. 2007), it can be hypothesised that their higher availability during wintertime could promote the multiplication of the pathogen in the root system. In addition, lower temperatures seem to have a beneficial effect on phytoplasma multiplication, since these organisms

tended to reach higher titres in micropropagated plantlets when kept for 1–3 months at 4°C than at a constant temperature of 20°C (Kaminska et al. 2002).

In summary, a quantitative real-time PCR assay with a *M. domestica* single-copy gene as an internal standard was employed to study the seasonal colonisation pattern of '*Ca.* P. mali' in infected symptomatic and asymptomatic apple trees. This approach was shown to allow specific and quantitative detection of '*Ca.* P. mali' in sampling material from infected trees, even in the absence of symptoms. This finding could have implications for the production of propagation material: even if an apple tree does not manifest AP symptoms over a number of years, scions taken for propagation purposes could be colonised by the phytoplasma and hence represent a potential infection source and a risk for the spread of the disease. It would be necessary to confirm this assumption in further studies, involving inoculation experiments with a larger number of '*Ca.* P. mali'-positive scions taken from asymptomatic apple trees at different time points. In this context, it needs to be stressed that PCR-based techniques have the drawback that they do not allow distinction between DNA of viable and non-viable cells. Therefore, we cannot exclude the possibility that the low signals detected in some samples could be generated by persisting undegraded DNA from dead phytoplasmas. Nonetheless, the here presented method will be useful for studying the interaction between phytoplasma, host plant and insect vector in greater detail.

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