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On the apple proliferation symptom display and the canopy colonization pattern of "*Candidatus* Phytoplasma mali" in apple trees

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Abstract Notwithstanding the availability of several different real time PCR protocols for "*Candidatus* Phytoplasma mali", it is still unclear how informative is the estimation of the concentration of phytoplasma cells in the leaves of apple proliferation infected trees, and how the reliability of the estimations may be affected by an erratic and uneven distribution of the pathogen in the host. Here we investigated these issues systematically and showed that phytoplasma concentration varies significantly among seasons, but not between two cultivars that appeared to have different degree of susceptibility on the basis of the symptoms displayed. In fully symptomatic trees sampled at the end of the season the phytoplasmas were detectable in most leaves, but in more than half of the leaves at low concentrations. Both the pattern of colonization of the canopy and the amount of phytoplasmas varied greatly in trees that show symptom remission, although a direct relation between symptom severity and colonization could not be established. The sampling of the apple canopy for the purpose of evaluation of concentration of "*Candidatus* Phytoplasma mali" should take into consideration the complex pattern of colonization and seasonal variation.

Keywords Real time PCR · Quantification · Phytoplasma · Apple · Proliferation

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The phytoplasmas are bacterial plant pathogens classified within the genus "*Candidatus* Phytoplasma" (IRPCM 2004; Firrao et al. 2005) that cause several hundred plant diseases. Among them, the agent of apple proliferation (AP), "*Candidatus* Phytoplasma mali", is regarded as a major threat for the apple industry, particularly in the light of the recent AP outbreaks in Europe (Loi et al. 1995; Bliefernicht and Krczal 1995; Springhetti et al. 2002). Due to the inability to grow in vitro phytoplasmas, DNA based techniques provide indispensable tools for the study and the control of the diseases they cause. With the continuous development of ever more powerful techniques, the recent research on apple proliferation has been characterized by the proposal of several new methods based on real time PCR (Baric and Dalla-Via 2004; Jarausch et al. 2004; Torres et al. 2005; Seemüller and Schneider 2007; Aldaghi et al. 2008; Ermacora et al. 2008), that may provide a previously unreachable ability to obtain precise quantitative information on the amount of pathogen DNA in

infected tissues. Indeed, quantification of "*Ca*. P. mali" in infected trees might be regarded as relevant information since previous studies highlighted changes in sugar content in the phloem of plants infected by phytopathogenic mollicutes (Lepka et al. 1999) and hence supported a hypothesis that symptoms may be connected with the depletion of plant nutrients. Experimental support for the hypothesis that the concentration of pathogenic mollicutes cells in the phloem may be critical in determining disease severity has been obtained for the distantly related *Spiroplasma citri* (Gaurivaud et al. 2000). It has been reported by Bisognin et al. (2008b) that *M. sieboldii* rootstocks contain, on average, a much lower titre of "*Ca*. P. mali" cells as compared with susceptible M9 rootstocks when experimentally infected. The same team (Bisognin et al. 2008a) evaluated quantitative differences of the pathogen titre in the in vitro screening for resistance. Notwithstanding the influence of the rootstock on the symptoms and pathogen titre, the physiology of the complex syndrome development in phytoplasma diseases of trees remains poorly understood. How the quantitative aspects are indeed related to symptoms during the natural infection of "*Ca*. P. mali" on susceptible trees remains to be determined. In the few surveys of naturally infected or graft inoculated apple trees that we are aware of, some variability has been detected in the amount of phytoplasma cells, but its significance appears not easy to interprete (Seemüller and Schneider 2007; Jarausch et al. 2004), and reliable results appear to be limited to the investigations restricted to the root system. Here, we present a statistically supported evaluation of quantitative aspects of "Ca. P. mali" colonization of apple tree canopy, that unveils some aspects that may not have not been investigated in sufficient detail.

In a first experiment, we have identified, in a single plot located in the region Trentino Alto Adige (Northern Italy), four 13-year-old trees that were infected by "*Ca*. P. mali", as detected by PCR. Two trees were from a selection named PSERT11 (corresponding to Co-op 28, Korban et al. 1990) and showed severe symptoms of the disease, while the other two trees were of cv. Brina (Bergamini and Giongo 2002) and showed very mild symptoms. Both PSERT11 and Brina were grown on MM106 rootstock, but the selection PSERT11 is highly sensitive to apple proliferation, while cv. Brina is tolerant (De Salvador, unpublished observation) and our interest was to determine whether this difference in the severity of symptom display was related to differences in the amount or pattern of colonization of the pathogen. The trees were sampled three times, at the beginning of June (spring), end of July (summer) and beginning of October (fall). Each tree was subdivided into three or four homogeneous sampling zones, depending on the tree size, and from each zone three samples were taken. Each sample was composed of 8 leaf disks, about 1.4 cm of diameter (0.35 mg total on average), including the middle of leaf midribs. This sampling method guaranteed the collection of a standard amount of phloem per mg of sample, that otherwise would be variable if a phloem enrichment procedure had been used. Total nucleic acids were extracted according to Doyle and Doyle (1990) and resuspended in 100 µl TE. This simple extraction procedure does not include any phytoplasma enrichment step that may introduce additional variability due to the different efficiency from preparation to preparation.

As a quantitative system, we used the primers and method of the real time PCR procedure developed by Baric and Dalla-Via (2004). Amplification reactions, from one µl of the extraction as a template, were carried out with the primer pair qAP16SF/qAP16SR and the FAM labeled probe qAP-16S for the detection of "*Ca.* P. mali", and the primer pair qMd-CPleuf/qMd-CPleur with the JOE labeled probe qMd-CPleu for quantification of apple chloroplast DNA. The gene fragments of phytoplasma and of plant chloroplast were purified and cloned in *E. coli*. Recombinant plasmids were used as standards in real time PCR, and the system proved to work with good linearity ($r^2>0.97$) within the range of 3 to $3*10^7$ copies per reaction (not shown), allowing quantification of phytoplasma concentrations ranging from 500 to $5*10^9$ per gram of tissue in the experimental conditions adopted. All reactions were carried out in parallel to determine the amounts of phytoplasma cells and plant chloroplasts. Each real time PCR assay was repeated twice from the same DNA extract with similar results. The total chloroplast number in the samples varied between $6*10^6$ and $2.7*10^8$, with season as a most significant source of variance; nine samples for which figures lower than $2*10^6$ chloroplasts per sample were estimated were excluded from statistical analysis, that was performed on the

results of qPCR quantitation of 117 samples. The distribution of the log transformation of chloroplast number estimated was normal.

The analysis was carried out for both: (1) phytoplasmas, as absolute amount of gene copies per sample; and (2) phytoplasmas per million chloroplasts. The evaluation of phytoplasmas per chloroplast compensated for repeatability in DNA extraction, although it could be sensitive to chlorosis and variations during season; there were however only minor differences in the results of the two statistical analyses, and therefore we present them below only as phytoplasmas per million chloroplasts. Due to the occurrence of negative samples, the data set comprising individual sample estimates did not pass the check for normal distribution carried out with the Kolmogorov-Smirnov test, nor the Levene's test for homogeneity of variance in most comparisons. Hence the factors were analyzed with Wilcoxon rank sum test. When the sample estimate values were pooled, the resulting dataset was shown to have a normal distribution with homogeneity of variance in all comparisons, and was therefore analyzed with one way ANOVA. All statistical analyses were carried out with R (R Development Core Team 2008).

The amount of phytoplasmas detected spanned five orders of magnitude, varying from 2,250 to $1.5*10^8$ per gram of tissue and from 3 to $2*10^6$ per million chloroplasts. The differences among sampling dates resulted significant, and in particular the samples collected in mid summer had fewer phytoplasmas than those collected in spring and fall ($p<10^{-5}$). Table 1 (left) shows the values of phytoplasmas per million of chloroplasts averaged for each sampling date. The number of negative samples was smaller in October than in any other collection date. None of the other variables examined resulted in significant differences at $p<0.05$. In particular, there was no significant difference in the mean phytoplasma cell concentrations in cv. Brina or selection PSERT11, despite the obvious difference in the symptoms displayed. The results also showed a high dispersion of the data, although the samples collected in fall showed a standard deviation smaller than those collected in other dates, as shown by the boxplot in Fig. 1a. Aggregated samples, made by pooling three samples, (corresponding to 24 leaf disks) allowed the analysis of three aggregated samples for each of the four trees for each sampling date. Pools were made choosing randomly one sample per zone, but similar results were obtained by aggregating the three samples of each collecting zone. Pooling reduced the number of samples that resulted negative or with a low copy number and their effect on mean and dispersion (Table 1, right), thus the resulting dataset fitted a normal distribution. The boxplot of aggregated samples, shown in Fig. 1b, and the table of mean and standard deviation (Table. 1, right) provide graphic and numeric representation of the dispersion of the samples of 24 leaf disks collected in the fall for estimation of the average number of phytoplasmas in the tree. The analysis of the variance of pooled samples gave results similar to the analysis of individual samples, i.e. the sole significant effect detected was that of the sampling date.

The data collected were used to estimate the number of samples necessary for the comparison to be significant. In particular we could determine, using power analysis, the number of samples that was necessary to assess that the phytoplasma concentrations were significantly different in the different sampling dates. In the case of pooled samples (=24 leaf disks), power analysis showed that a minimum of 2.9 samples would be necessary to achieve a power of 0.8 with a significance $p=0.05$. Thus, collecting four samples of 24 leaf disk, i.e. one sample for each tree, would be sufficient to show a significant difference among the sampling dates (power = 0.95, significance $p=0.05$).

The results of this first experiment showed that the amount of AP phytoplasmas in the same trees varies significantly with the season. Therefore, the results of studies aimed at comparing the amount of "*Ca*. P. mali" phytoplasmas in samples collected in different dates or in different geographical regions could be misleading. This first experiment also showed that the differences in symptom display between trees having a different genetic background may not correspond to differences in phytoplasma concentrations and colonization pattern. Therefore accessions visually selected as tolerant on the basis of symptoms may support phytoplasma growth as well as highly symptomatic trees. The results of this experiment did not show a direct relation between the amount of pathogen cells in the phloem and disease severity. Moreover the first experiment showed that a sample of 8 leaf disks is too small, but a sample of 24 leaf disks, particularly if collected in the fall, may provide a reliable means to

Table 1 Mean and standard deviation of $\log_{10}$ transforms of the phytoplasma cell number estimated from the analysis of samples collected four plants of cv. Brina (B1 and B2) and PSERT11 (P1 and P2) in three different sampling dates. Left: real samples made of 8 leaf disks; right: aggregate values of three samples of 8 disks each

	Samples of 8 leaf disks				Samples of 24 leaf disks			
	B1	B2	P1	P2	B1	B2	P1	P2
Spring	3.98±1.18	3.99±1.84	3.28±2.17	3.47±1.96	4.58±.47	4.97±.69	4.62±1.32	4.51±1.85
Summer	2.18±1.11	2.30±1.3	1.13±.53	2.74±1.02	2.78±.64	2.67±1.38	1.00±.83	2.81±.5
Fall	4.00±.62	3.07±1.13	3.03±1.23	3.65±1.12	4.06±.6	3.44±.61	3.47±.46	3.93±.29

estimate differences in phytoplasma concentration of about two orders of magnitude, as observed in this work among the sampling dates. When less pronounced differences are to be compared, the sample size required would have to be much larger. Finally, the results of the first experiment showed that the samples collected in fall were less variable than those collected in other sampling dates, and a lower number of samples resulted negative. Conversely most samples collected in summer were negative or contained a relatively low number of phytoplasma cells, much lower than those counted in the spring and fall samples. This result counter-indicates a continuous progression of the infection with time, and may be due either to the reduced growth of the pathogens at the higher temperatures, or to host physiological conditions that are less conductive for phytoplasma invasion. It may be worth noting that host stress is a known cause of recovery from phytoplasma infection (Osler et al. 1993).

A second experiment was carried out next year on the same two PSERT11 trees; one of them was still highly symptomatic while the other presented a reduction in symptoms that was thought to be associated with the initiation of the process of recovery. The phenomenon of recovery, still poorly understood, has been described previously in AP infected apple and in other phytoplasma infected trees and consists in a spontaneous remission of symptoms, associated with the disappearance of the phytoplasmas from the canopy while roots remain infected (Carraro et al. 2004).

For this experiment, 72 samples were collected for each tree in October, each sample consisting of a single leaf disk. The extracted DNA was evaluated first by nested PCR, using a generic, in our hands highly reliable, detection procedure for the phytoplasmas that uses primers P1/P5 (Smart et al. 1996) for the first round and primers U3/U5 (Lorenz et al. 1995) for the nested amplification. For the highly symptomatic tree, 64 samples (88.9%) resulted positive, as well as 17 samples (23.6%) from the tree showing mild symptoms. When evaluated by real time PCR using the described method, all samples of the tree with mild symptoms gave results below the quantification threshold, while for the highly symptomatic tree 19 samples (26%) could be quantified. Thus, 45 single leaf disk samples (70%) of the highly symptomatic tree and 17 samples (100%) of the tree with mild symptoms that were positive to the nested

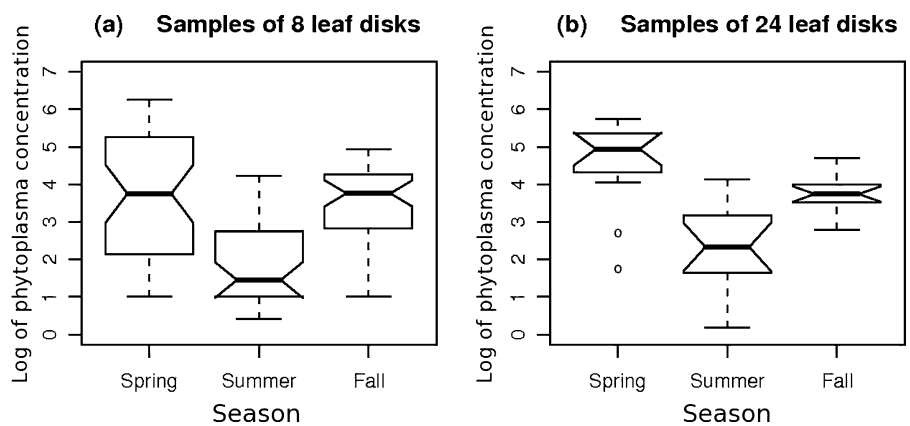

Fig. 1 Boxplot of the $\log_{10}$ estimates of "*Ca.* P. mali" concentrations in DNA extracts from samples made of **a** 8 leaf disks and **b** 24 leaf disks, according to their season of collection

PCR contained less than 500 phytoplasmas per gram, the lower limit of quantification with the method adopted. To compare the quantitative results obtained from individual leaf disks to those obtained from pools of 8 leaf disks in the previous year, we estimated virtual samples obtained by summing the figures of eight randomly chosen disks and compared them with the 24 similar samples collected in the same season of the previous year from selection PSERT11. As shown in Fig. 2, the distribution of phytoplasmas per samples were, although not identical, similar in the 2 years.

Furthermore, we collected 15 samples from each of nine cv. Golden Delicious trees showing different degrees of symptoms, each sample consisting of a single leaf disk. The extracted DNA was evaluated by nested PCR. As reported in Table 2, the trees with stronger symptoms revealed on average a higher number of positive samples, although a strict correlation between the severity of the symptoms and the percentage of sample positive to the nested PCR could not be established, as cases of symptom reduction with a high and very high percent of positive samples to nested PCR were found.

This second experiment carried out on single leaf disks confirmed the reliability of the evaluation of phytoplasma concentration in the fall. Moreover, it showed that in the diseased trees the phytoplasmas can be consistently detected by nested PCR in a high fraction of the leaves (>80%), although most samples contained a low amount of phytoplasmas and only one third showed high titre. The standard method of pooling subsamples collected from several leaves or phloem masks the difference that exists among the various subsamples. Therefore, the presence of phytoplasma is not erratic, but it is uneven. Conversely, trees undergoing recovery showed a uniform, relevant reduction of both the number of leaf containing phytoplasmas and number of phytoplas-

Table 2 List of the fraction of 15 samples that reacted positively to a nested PCR diagnostic reaction for phytoplasmas in 9 apple trees showing various degree of symptoms of apple proliferation

Symptom severity	nPCR positives	Percent
Strong symptoms	15	100,00
Mild symptoms	14	93,33
Mild symptoms	10	66,67
Mild symptoms	9	60,00
Very mild symptoms	2	13,33
Very mild symptoms	5	33,33
Very mild symptoms	8	53,33
Very mild symptoms	6	40,00
Very mild symptoms	6	40,00

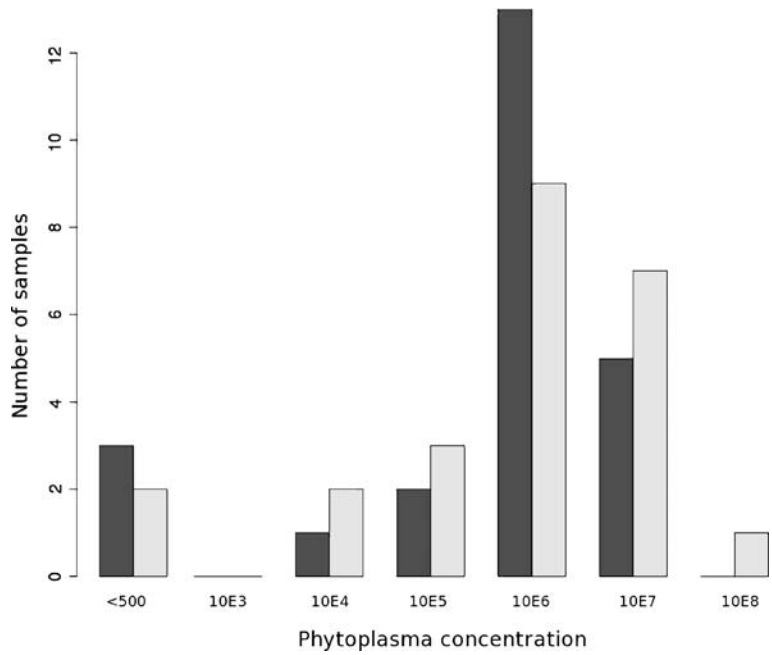

Fig. 2 Comparison of the distribution of "*Ca.* P. mali" concentration values in DNA extracts from samples of 8 leaf disks collected in the first year (dark gray) and random aggregates of eight samples of individual disks collected in the second year (light gray)

mas in infected leaves, but a synchrony between these two events could not been evidenced by our experimental work.

In conclusion, the final picture that we obtained is that the phytoplasmas are present in low concentration in most leaves of the highly infected tree at the end of the season, with some hot spots with high concentration. The sampling for the purpose of evaluation of concentration should take into consideration this complex pattern of colonization and seasonal variation. The amount of phytoplasma is not related to the apparent behaviour of cultivars as tolerant or susceptible; however both the pattern of colonization of the canopy and the amount of phytoplasmas may vary greatly in trees that show symptom remission, although a direct relation between symptom severity and colonization could not be established.

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