EvaGreen Real-time PCR protocol for specific 'Candidatus Phytoplasma mali' detection and quantification in insects

EvaGreen Real-time PCR protocol for specific '*Candidatus* Phytoplasma mali' detection and quantification in insects

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ABSTRACT

In this paper the validation and implementation of a Real-time PCR protocol based on ribosomal protein genes has been carried out for sensitive and specific quantification of '*Candidatus* (*Ca.*) Phytoplasma mali' (apple proliferation phytoplasma, APP) in insects. The method combines the use of EvaGreen® dye as chemistry detection system and the specific primer pair rpAP15f-mod/rpAP15r3, which amplifies a fragment of 238 bp of the ribosomal protein *rplV* (*rpl22*) gene of APP. Primers specificity was demonstrated by running in the same Real-time PCR '*Ca*. Phytoplasma mali' samples with phytoplasmas belonging to the same group (16SrX) as '*Ca*. Phytoplasma pyri' and '*Ca*. Phytoplasma prunorum', and also phytoplasmas from different groups, as '*Ca*. Phytoplasma phoenicium' (16SrIX) and Flavescence dorée phytoplasma (16SrV). '*Ca*. Phytoplasma mali' titre in insects was quantified using a specific approach, which relates the concentration of the phytoplasma to insect 18S rDNA. Absolute quantification of APP and insect 18S rDNA were calculated using standard curves prepared from serial dilutions of plasmids containing *rplV-rpsC* and a portion of 18S rDNA genes, respectively. APP titre in insects was expressed as genome units (GU) of phytoplasma per picogram (pg) of individual insect 18S rDNA. '*Ca*. Phytoplasma mali' concentration in examined samples (*Cacopsylla melanoneura* overwintered adults) ranged from $5.94*10^2$ to $2.51*10^4$ GU/pg of insect 18S rDNA. Repeatability and reproducibility of the method were also evaluated by calculation of the coefficient of variation (CV%) of GU of phytoplasma and pg of 18S rDNA fragment for both assays. CV less than 14% and 9% (for reproducibility test) and less than 10 and 11% (for repeatability test) were obtained for phytoplasma and insect qPCR assays, respectively. Sensitivity of the method was also evaluated, in comparison with conventional 16S rDNA-based nested-PCR procedure. The method described has been demonstrated reliable, sensitive and specific for the quantification of '*Ca*. Phytoplasma mali' in insects. The possibility to study the trend of phytoplasma titre in the vectors will allow a deepen investigation on the epidemiology of the disease.

Keywords: '*Candidatus* Phytoplasma mali', Real-time PCR, EvaGreen® dye, ribosomal protein gene, insects.

1. INTRODUCTION

Phytoplasma diseases are a group of severe plant disorders caused by obligate, cell wall-less bacteria, which are responsible of important yield losses in many crops worldwide [1,2], including ornamental plants and fruit crops.

Before the application of molecular biology as a tool in phytoplasma detection, the first approaches were mainly based on the observation of symptoms caused by different strains and the observation of the phytoplasma presence in sections of phloem tissues by electron microscopy [3]. However, many issues in phytoplasma detection can occur making difficult a correct phytoplasma diagnosis. They can include an unbalanced distribution of the phytoplasma in plant organs as well as the recovery phenomenon and a low concentration (especially in field-collected samples where the amount of phytoplasma DNA is less than 1% of the total amount of plant DNA) [4,1].
Therefore, the application of PCR assays in the early 1990s, in particular, the use of a nested-PCR approach, allowed the establishment of a more sensitive, specific and faster approach for the detection of different species and strains of phytoplasmas in both plants and insects using generic and specific primers based on conserved sequences (e.g. 16S rRNA, ribosomal protein (rp), *tuf*, 16S-23S rRNA intergenic spacer region sequence) [3].

Although the use of nested-PCR remains a sensitive tool for phytoplasma detection, possibilities of cross-contamination between the two amplifications, as well as time-consuming post-amplification steps, were overcome by the introduction of a Real-time PCR approach. These assays increased the accuracy and sensitivity of pathogen detection. However, the choice of the appropriate chemistry detection system is a critical point during the establishment of a Real-time PCR experiment. This assay, which permits the detection and quantification of specific targets of DNA, is based on the use of labeling systems for the DNA amplicons. Most of these systems use probes labeled with a fluorescent dye (such as TaqMan®probes) which bind specifically a target sequence of the amplicons, while others, called DNA dyes, bind the minor groove of all double strand DNA molecules. The first technology results very sensitive but also expensive. Furthermore, for those template sequences that tend to vary, this system might generate false negatives due to the base pair mismatches between the probe and the specific region [5,6]. This problem does not occur

when DNA dyes are used. This chemistry results less expensive and easier to use (because it does not require the design of a probe). However the detection of false positives might occur because of the non-specific binding capacity. To overcome this problem, melting curve analyses of each amplicon are necessary, in particular for those DNA samples extracted from woody plants collected from the field, and also from insect vectors [7] where the phytoplasma titre can be very low [1]. In those cases, the low concentration of the sample DNA results in very high threshold cycles (C_T), which correspond also to the C_{Ts} of non-specific DNA binding that can occur, resulting in the detection of false negatives [8,9]. Between the wide variety in commerce, most of the Real-time PCR approaches are based on the use of SYBR® Green I as DNA dye. SYBR® Green I is a high specific dye but its use has been shown to have some disadvantages, such as the inhibition of PCR reactions at high concentration [10,11], negative effect on DNA melting temperature [10,12], and preferential binding to GC-rich DNA sequences [12]. An alternative DNA dye is the EvaGreen®, which has been demonstrated having a higher reaction efficiency at different concentration as well as better melting curves with sharper peaks compared with the SYBR Green chemistry [13].

The most important fruit tree phytoplasmas in Europe belong to the 16SrX-group, also called apple proliferation group, which includes the apple proliferation phytoplasma (APP) '*Candidatus* (*Ca.*) Phytoplasma mali' (16SrX-A, the causal agent of apple proliferation, AP), '*Ca.* Phytoplasma prunorum' (16SrX-B, the causal agent of European Stone Fruit Yellows, ESFY) and '*Ca.* Phytoplasma pyri' (16SrX-C, the causal agent of Pear Decline, PD) [14-17].

In the specific, AP is an endemic disease, which affects apple trees in central Europe, with important economical losses in particular in Germany and Italy [18]. The infected plants show typical symptoms that are consequences of the presence, multiplication, and spread of the phytoplasma in the phloematic cells. Most of the symptoms involve both plants and fruits, like witches' brooms, early leaves reddening, and poor taste and small size fruits, which cause important qualitative and quantitative reduction of the production. The transmission of '*Ca.* Phytoplasma mali' by insect vectors in Italy and Germany is associated with two different *Cacopsylla* species: *Cacopsylla picta* (Förster), the main vector in Germany and Northeast Italy [19- 21] and *Cacopsylla melanoneura* (Förster), which is the main vector in Northwest Italy [22-24].

In this article the validation of a Real-time PCR protocol was established using the species-specific primer pair rpAP15f-mod/rpAP15r3 previously designed in the variable region of the ribosomal protein (rp) gene *rpIV* (*rpl22*) of '*Ca*. Phytoplasma mali' [25], especially for the detection and quantification of this phytoplasma in insects. The choice of this gene for '*Ca*. Phytoplasma mali' detection is due to its major variability than the 16S rDNA gene [26], which has been already used

as a target for the specific detection and quantification of '*Ca*. Phytoplasma prunorum' in its natural hosts [27]. The method presented in this paper is implemented compared to the method published in the abstract [25] due to the use of EvaGreen® dye, whose advantages have been stated above in comparison to SYBR® Green I, and the utilization of a ready-to-use 2x supermix which makes the experiments faster and less prone to errors. Finally, the present work provided all the results needed to demonstrate that this Real-time PCR protocol can be used for a specific, sensitive and reliable quantification of '*Ca.* Phytoplasma mali' in insect.

2.MATERIALS AND METHODS

2.1. Phytoplasma sources and DNA extraction

Field collected '*Ca.* Phytoplasma mali'-infected *C. melanoneura* were used in this study. DNAs from overwintered adults were tested. To validate the Real-time PCR protocol, other field collected insects were tested: '*Ca*. Phytoplasma prunorum'-infected *Cacopsylla pruni* (Scopoli), '*Ca*. Phytoplasma pyri'-infected *Cacopsylla pyri* L., Flavescence Dorée (FD) phytoplasma (16SrV-C)-infected *Scaphoideus titanus* Ball and '*Ca*. Phytoplasma phoenicium' (16SrIX-D, the causal agent of almond witches' brooms, AlmWB)-infected insects [28].

Total DNA was extracted from single insects using a CTAB-based protocol developed for leafhoppers [29] and already applied to psyllids [22]. The DNA extracted from each insect was resuspended in 1X TE Buffer, quantified by using NanoDrop Spectrofotometer (Thermo Fisher Scientific, Wilmington, DE, USA), and stored at -20°C until use.

2.2. Phytoplasma detection by nested-PCR and RFLP analyses

The presence of phytoplasma in the insects was previously assayed by conventional nested-PCR using 16S rDNA universal and group specific primers followed by RFLP analyses.

In particular for the characterization of the 16SrX-group phytoplasmas, a direct PCR using the phytoplasma universal primer pair P1/P7 [30,31] was first carried out. Then, a 1:40 dilution of each amplicon was used as template in the following nested-PCR, by using the group-specific primer pair fO1/rO1 [15]. Direct PCR was performed in 20 µl reaction volume containing 1 µl of insect DNA, while nested PCR was performed in 40 µl reaction volume containing 2 µl of diluted P1/P7 amplicon. In order to discriminate '*Ca.* Phytoplasma mali', '*Ca*. Phytoplasma prunorum' and '*Ca*. Phytoplasma pyri' a RFLP analysis of fO1/rO1 PCR products was performed with the endonucleases *Ssp*I and *Rsa*I. Three microliters of the amplicons were digested with 3 U of *Ssp*I for 4.5 h at 37°C and with 5 U of *Rsa*I at 37°C overnight, respectively.

2.3. Establishment of a Real-time PCR procedure for the specific detection of **'Ca. *Phytoplasma mali' in* C. melanoneura**

A Real-time PCR protocol was set up using samples infected with '*Ca*. Phytoplasma mali' as well as with other phytoplasmas of different groups and sub-groups (PD, ESFY, FD and AlmWB phytoplasmas) as controls. For the detection and quantification of '*Ca.* Phytoplasma mali' two Real-time PCRs were used. In the first one rpAP15f-mod/rpAP15r3 primer pair (5'-TGCTGAAGCTAATTTGGC-3' / 5' -CCCATGAATATTAACCTCCT-3') [25] which amplify a specific fragment of 238 bp located in the variable region of the ribosomal protein (rp) *rplV* gene (*rpl22*), was chosen. To be able to normalize the data and quantify the phytoplasma titre, a second Real-time PCR was set up using a primer pair which has a portion of the 18S rDNA gene of the insect DNA as target. The primers MqFw/MqRv (5'-AACGGCTACCACATCCAAGG-3'/ 5'-GCCTCGGATGAGTCCCG-3') [32], which amplify a 98 bp region of 18Sr DNA, were used. They were previously designed on the consensus sequence obtained from the alignment of *Euscelidius variegatus* (Kirschbaum) (EVU15148), *Philaenus spumarius* L. (PSU06480), and *Trioza eugeniae* Froggatt (TEU06482) 18S rDNA sequences [32]. At the beginning of the assay, the efficiency of MqFw/MqRv primer pair for the amplification of 18S rDNA in *C. melanoneura* was evaluated. The Real-time PCR reactions were carried out in a 25 µl volume, which was composed by the following components: 12.5 µl of SsoFast™ EvaGreen® Supermix 2x (Bio-Rad, CA) (containing dNTPs, Sso7d fusion polymerase, $MgCl_2$, EvaGreen® dye, and stabilizers), 2.5 µl of each primer (3µM), 6.5µl of Milli-Q water and 1 µl of DNA templates at different concentrations (range from 19.91 to 392.31 ng/µl) (Table 2).

For each reaction, 96-wells plates were used and the standards and samples were run in triplicates. An additional sample, containing water instead of DNA, was added to each plate in triplicate as DNA-free negative control. The reaction was conducted in a DNA Engine Opticon ™ System (Bio-Rad, CA). For the amplification of '*Ca*. Phytoplasma mali' the following thermal conditions were used: incubation step at 95°C for 2 min, 40 cycles of amplification at 94°C for 15 s, 56°C for 15 s, 72°C for 20 s, and a final extension step at 72°C for 8 min. On the contrary, for the quantification of 18S rDNA, the following thermal protocol was used: incubation step at 94°C for 3 min, 37 cycles of amplification at 94°C for 45 s, 65°C for 1min. Two melting curve profiles, the first ramp from 65°C to 95°C at 0.2°C/sec and the second one from 65° to 94°C at 0.5°C/s were run. Both absolute quantifications, expressed as genome units (GU) for '*Ca.* Phytoplasma mali' and pg for insect 18S rDNA, were achieved plotting the samples C_T against the standard curves prepared from serial dilutions of plasmids containing *rplV-rpsC* and a portion of 18S rDNA gene, respectively.

Analyses of melting curves generated from each assay were also carried out for the detection of possible non-specific products. Furthermore, in order to validate the method, accuracy, repeatability and reproducibility of the assay were assessed.

2.4. Establishment of calibration curves for the quantification of phytoplasma and insect DNAFor quantification of '*Ca*. Phytoplasma mali' in *C. melanoneura* a standard curve was established by diluting plasmid pGEM® Easy Vector (Promega, WI, USA) containing rp genes (*rplV-rpsC*, EF193366) of AP15 phytoplasma (here called AP-15 clone) [25]. Furthermore, for quantification of insect 18S rDNA another standard curve using the plasmid pGEM® Easy Vector (Promega, WI, USA) containing the 98 bp fragment of the insect 18S rDNA sequence (here called Mq clone) [32] was established. Both plasmids were first quantified by using NanoDrop Spectrofotometer and then used to prepare 1:10 serial dilutions in Milli-Q water.

For quantification of '*Ca.* Phytoplasma mali' the concentration ng/µl of AP-15 clone was converted to copy number of the insert, rp genes, (equal to GU of phytoplasmas), therefore the dilutions ranged from $1.15*10^9$ to 115 GU/µl.

On the other hand, the quantification of the whole *C. melanoneura* 18S rDNA was estimated starting from the quantity of 18S rDNA inserted fragment. This estimation was carried out considering that 18S rDNA of Sternorrhyncha (which includes psyllids, whiteflies, aphids and scales) has a long length (~ 2200 to ~ 2500 bp) due to the presence of internal expansions [33] and that the only complete 18S rDNA sequence for a psyllid is the one from *T. eugeniae* (GenBank accession n° U06482; 2199 bp), which was also one of the species used for the MqFw/MqRv primer pair design [32]. Since the inserted fragment amplified by the MqF/MqR primer pair (98bp) is about 1/20 of the whole 18S rDNA of *T. eugeniae*, we estimated the amount of the whole 18S rDNA of *C.melanoneura* multiplying by 20 the quantity assessed for the fragment; therefore the Mq clone dilutions ranged from $4.47*10^3$ to $4.47*10^{-4}$ pg of 18S rDNA/µl.

The standard curves were constructed by the interpolation of the log10 of the copy number of each standard plotted along the x-axis with their respective Ct values along the y-axis.

'*Ca.* Phytoplasma mali' titre in insects was expressed as genome units (GU) of phytoplasma per picogram (pg) of individual insect 18S rDNA.

2.5 Sensitivity evaluation of the Real-time PCR approach for the detection of* 'Ca. *Phytoplasma mali'

In order to test the sensitivity of the Real-time PCR method, a comparison with the conventional PCR approach for the detection of '*Ca*. Phytoplasma mali' was carried out. In the specific, 10-fold serial dilutions (from 10^9 to 1) of total DNA extracted from a '*Ca*. Phytoplasma mali'-infected insect were prepared in Milli-Q water. Dilutions were tested by direct PCR with the universal primer pair P1/P7 followed by nested-PCR using the AP-group specific primer pair fO1/rO1. Five µl of each fO1/rO1 amplicon were run on a 1% electrophoresis gel, stained in Etidium Bromide and visualized under UV light. Real time quantitative PCR with the primer pair rpAP15f-mod/rpAP15r3 was also carried out for the simultaneous detection and quantification of '*Ca*. Phytoplasma mali'. Standards and samples were also run in triplicates.

3. RESULTS

3.1. Specificity of the Real-time PCR protocol

On a total of 61 samples included in the assay only sample DNA of '*Ca.* Phytoplasma mali' infected insects and the AP-15 clone were successfully amplified using the primer pair rpAP15Fmod/rpAP15r3 (Tab.1). No amplification curves were observed with *C. pruni* and *C. pyri* samples infected with '*Ca*. Phytoplasma prunorum' and '*Ca*. Phytoplasma pyri', respectively. On the contrary, some of the examined *S. titanus* specimens infected with FD phytoplasma showed amplification curve at C_T 13.72 (±0.5), while all the other FD phytoplasma samples did not show any amplification curves. The analysis of the dissociation (melting) curve revealed a single peak at 75.5°C (±0.26) for all the '*Ca.* Phytoplasma mali'-infected samples and for the AP-15 clone, while no peaks were observed for the other phytoplasmas (Fig. 1), including FD.

3.2. Method validation, evaluation of the efficiency of the assay and quantification of **'Ca. *Phytoplasma mali' titre***

Before sample quantification, the efficiency of standard curve for 18S rDNA gene in *C. melanoneura* was evaluated. A mean slope of -3.474 (±0.05) and a R^2> 0.999 demonstrated an efficiency of 94% (Fig 2 b). For '*Ca*. Phytoplasma mali' quantification, standard curves resulted in a mean slope of -3.345 (±0.08) and a correlation coefficient (R^2) > 0.998, which demonstrated an efficiency of 99%. Phytoplasma titre was quantified in all *C. melanoneura* overwintered adults included in this study. Samples with C_T higher than the one of the last standard (standard 10^{-8}), which corresponds to C_T 30, were considered negative. Results of the quantification of phytoplasma titre are illustrated in Tab. 2. In the specific, infection level of *C. melanoneura* overwintered adults showed an interval of phytoplasma concentration from $5.94*10^2$ to $2.51*10^4$ GU/pg of insect 18S rDNA .

Accuracy of the assays were evaluated by running three replicates for each sample and standard dilution in each plate. Intra-variance (intra-assay repeatability) was calculated by considering three replicates of the same sample in the same run, while inter-variance (inter-assay reproducibility) was evaluated by running the same sample in two different runs in different days. For phytoplasma qPCR assay, the tests revealed an intra-variance with an average coefficient of variation (CV) equal to 5.26%, and an inter-assay variance of 6.79% (Tab.3a and 4a). Instead, for insect qPCR assay, an average CV equal to 5.89% for the reproducibility was revealed, while a mean CV of 5.03% was tested for the repeatability assay (Tab. 3b and 4b).

3.3. Sensitivity comparison between Real-time PCR and conventional approach for the detection of **'Ca.** *Phytoplasma mali'*

Figure 3 shows the results obtained with the two approaches. For conventional PCR approach both direct and nested PCR are shown in order to better visualize the differences in band intensity of the different sample dilutions (Fig. 3). For Real-time PCR method, C_T averages and quantification for each sample dilution are shown. Same level of sensitivity in APP detection was observed in both conventional and Real-time PCR. The last dilution detected by both methods was the 10^5-fold one corresponding to the C_T 31.62 and to the quantity of 38 GU/µl in Real-time PCR.

4. DISCUSSION

Several Real-time PCR approaches have been developed for the detection and quantification of 16SrX phytoplasma group and '*Ca*. Phytoplasma mali' using different labeling systems and different target sequences. These works are based on the use of SYBR Green technology for quantification of 16SrX phytoplasma group in plants [9] and specific '*Ca.* Phytoplasma mali' quantification in host plants and vectors [8, 20]. On the other hand, Baric & Dalla-Via [34] and Aldaghi [35] developed and used, respectively, a TaqMan Minor Groove Binding (MGB) probe system for the specific detection of '*Ca.* Phytoplasma mali' in plant material, similarly Nikolić [36] used the same technology for the specific detection and discrimination of '*Ca.* Phytoplasma mali', '*Ca.* Phytoplasma prunorum' and '*Ca.* Phytoplasma pyri'. In this work a Real-time PCR protocol, based on the use of EvaGreen® intercalating dye and a primer pair previously designed on the ribosomal protein *rplV* gene, has been developed for the specific detection and quantification of '*Ca.* Phytoplasma mali' in insects. Since its commercialization, the use of EvaGreen® dye has been greatly increasing. In fact, its application varies from quantitative PCR, DNA conformation detection, double-stranded DNA tracing and quantification in capillary electrophoresis to melting

analysis on a Lab-on-Chip, and Real-time isothermal DNA amplifications. Even though this chemistry detection system is not as specific as a TaqMan®probes or Molecular Beacons (based on the highly specific bind to target sequence), it results less expensive and easy to use because it only requires the design and synthesis of two PCR primers, which decreases assay setup and costs. Furthermore, EvaGreen technology also shows many advantages comparing with the most used intercalating dye SYBR® Green I. In 2007 Mao et al. [37], who developed EvaGreen® dye, compared its physicochemical properties to SYBR® Green I characteristics. In this study they demonstrated for EvaGreen® a higher reproducibility, a less PCR inhibition effect, weak binding for short dsDNA fragments (which reflects the low tendency to promote not specific amplification), as well as, higher and narrowed melting curves compared with SYBR® Green I, also demonstrated by Eischeid [13]. So, findings from this work and results from Mao [37] and Eischeid [13] demonstrated the efficiency of EvaGreen chemistry and its application in qPCR and post-PCR DNA melting curve analysis, which is required when nonspecific binding dye is used in a qPCR assay. In fact here, from the specificity test, a clear dissociation peak at 75°C for all '*Ca.* Phytoplasma mali' samples was observed, while the phytoplasmas used as controls showed no peaks, indicating that the fluorescence signals were caused by amplification artifacts. In fact, an example of the importance of melting curve analysis was demonstrated from some of the FD phytoplasmas DNA, which showed amplification curves at C_T 13.72 (±0.5). However, from an accurate melting curve analysis, no peaks were observed for all of them, which indicate fluorescence artifacts.

Furthermore, the results also validated the specificity of the rpAP15f-mod/rpAP15r3 primer pair. The gene *rplV* (*rpl22*) was chosen as amplification target [26,25] for the higher variability of ribosomal protein genes compared to 16S rDNA, which substantially increase the resolving power of differentiating and classifying distinct phytoplasma strains within a given 16Sr group [26,38,39].

In our work rpAP15f-mod/rpAP15r3 primer pair, obtained from the multiple alignment of *rplV* gene of several phytoplasma strains [25], allows not only the discrimination of phytoplasmas belonging to different groups, but also of the subgroups within the 16SrX group (which also includes '*Ca.* Phytoplasma prunorum' and '*Ca.* Phytoplasma pyri').

As for phytoplasma detection, no difference in terms of sensitivity was detected comparing the Real-time PCR assay with classic APP detection approach, which includes a direct PCR with universal primers P1/P7 [31], a nested-PCR with the group-specific primer pair fO1/rO1 [15] followed by a RFLP analysis for the '*Ca.* Phytoplasma mali' discrimination within the 16SX group [15]. These data indicates that both the approaches are sensitive for APP detection. However, Real-time PCR assay has more advantages, such as the rapidity of the assay, the quantification of

phytoplasma titre as well as less chances of false-positive results (due to cross-contaminations between direct and nested-PCR). In fact, Real-time PCR approach is a faster detection system than nested-PCR since it avoids post-PCR amplification processes, as gel electrophoresis to verify sequences amplification, and RFLP analysis for the confirmation of '*Ca.* Phytoplasma mali' presence.

In order to quantify '*Ca.* Phytoplasma mali' titre in insects, two absolute Real-time PCR were carried out for each sample: one for the quantification of the phytoplasma cells and the other for the quantification of *C. melanoneura* 18S rDNA. In particular, this second assay is necessary to avoid the fluctuations in yields during DNA extractions [32], resulting in a relative quantification of the phytoplasma in relation to each insect 18S rDNA. MqFw/MqRv primer pair, designed on the consensus sequence obtained from the alignment of *E. variegatus*, *P. spumarius*, and *T. eugeniae* 18S rDNA sequences [32] was used, and the efficiency was evaluated at the beginning of the assay. A value of 94% (for 18S rDNA) and 99% (for *rplV* gene), achieved from the mean of each run for both assays, were obtained, so indicating a good amplification efficiency. Besides that, it has been also demonstrated its repeatability and reproducibility. Referring to Bustin [40] in our work the variance of phytoplasma GU and pg of insect 18S rDNA were taken into account, because C_T values generated from different runs are subjected to inherent inter-run variation [41]. Both tests evaluated the variance expressed as coefficient of variation (CV%) of GU and pg, which were calculated between replicates of the same sample within the same assay (intra-variance or repeatability) and between different runs for the same sample (inter-assay or reproducibility). In the specific, coefficient of variation of less than 9 and 10% (reproducibility test) and less than 13% and 5.88% (repeatability test) for phytoplasma and insect qPCR assay were obtained, respectively. Therefore, these tests demonstrated that the method is reliable and also robust in terms of repeatability and reproducibility.

For the quantifications, considering that the ribosomal protein gene *rplV* belongs to the *S10-spc-α* operon, which is present in a single-copy in the genome of phytoplasmas and other *Mollicutes* [42,43], the final phytoplasma concentrations were calculated as the ratio of APP GU per pg insect 18S rDNA.

Results from the quantification assay, showed a range of phytoplasma infection level from $5.94*10^2$ to $2.51*10^4$ GU/pg insect 18S rDNA. This variability in phytoplasma titre within the examined group (overwintered adults) could be explained by the fact that each insect can have different acquisition efficiency, and moreover, some of them can have acquired the APP during the previous

season and possibly recharged it after the overwintering period and others can have acquired the phytoplasma only after the overwintering period.

This implemented Real-time PCR protocol has been demonstrated being a valid and efficient approach for the quantification of '*Ca.* Phytoplasma mali' titre in insects. The possibility to study the trend of phytoplasma titre in the insect will allow a deepen investigation on the epidemiology of the disease. Furthermore, it can help to better understand the biology of the pathogen, as well as the interaction between phytoplasma and insect vector. Finally, it can also be useful for developing better vector control strategies.

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FIGURE CAPTIONS

Fig. 1 Melting curve analysis of a) AP-15 clone b) *'Ca*. Phytoplasma mali' c) '*Ca*. Phytoplasma phoenicium' d) FD phytoplasma e) '*Ca*. Phytoplasma prunorum' f) '*Ca*. Phytoplasma pyri' g) DNA-free control.

Fig. 2 Representative standard curves of 8-fold dilution series (10^{-8} to 10^{-1}) for quantification of a) '*Ca*. Phytoplasma mali' titre, E= 100% and b) *C. melanoneura* DNA; E= 96%.

Fig. 3 Sensitivity comparison between conventional PCR and Real time PCR approach for '*Ca*. Phytoplasma mali' detection. Dilutions, C_T and quantification (genome units, GU) for each sample dilution from Real-time PCR, direct and nested PCR results; C+ = positive control; C- = negative control; M= 1kb-molecular marker; ND= not detected.

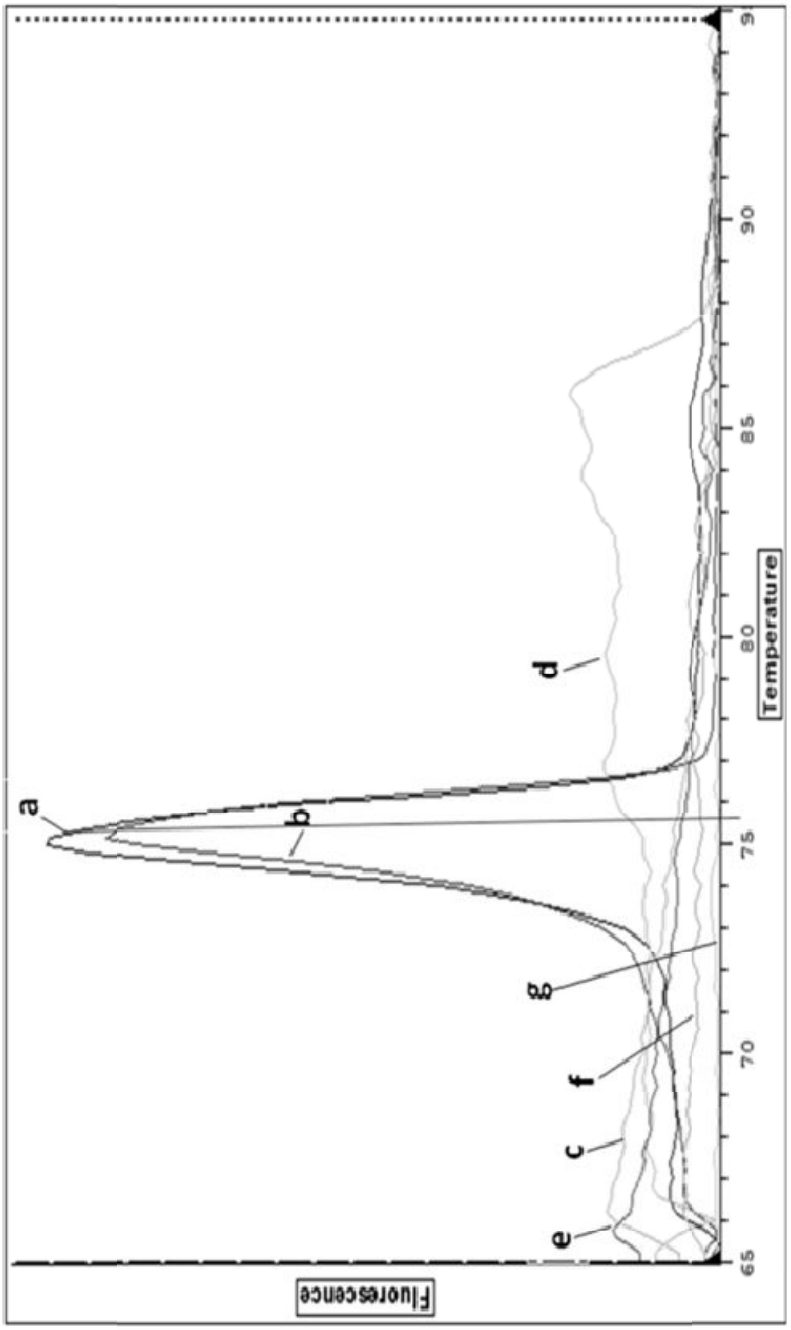


Figure 1

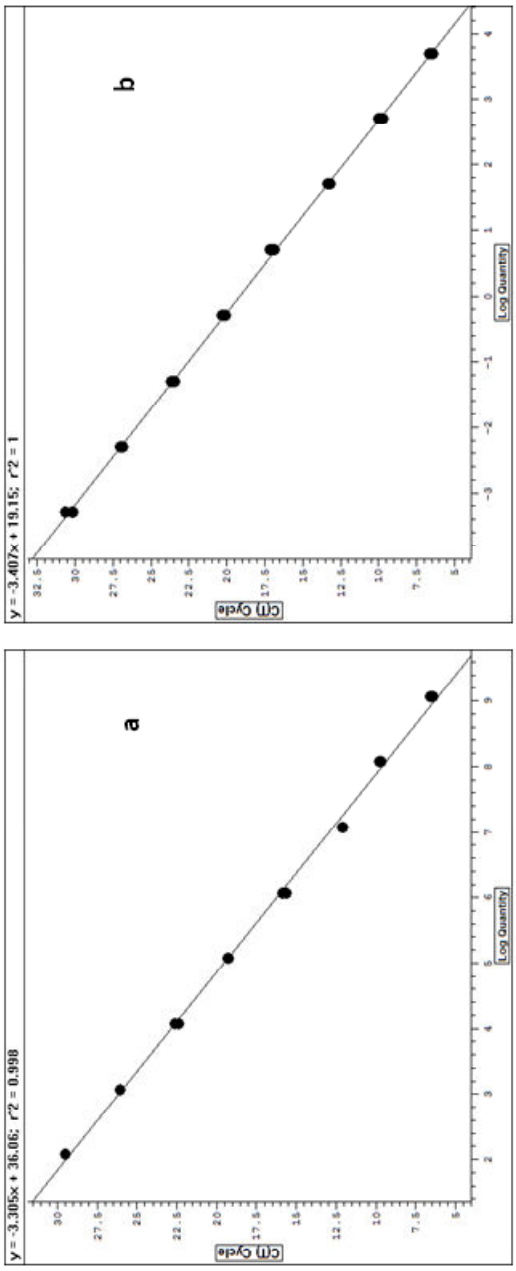
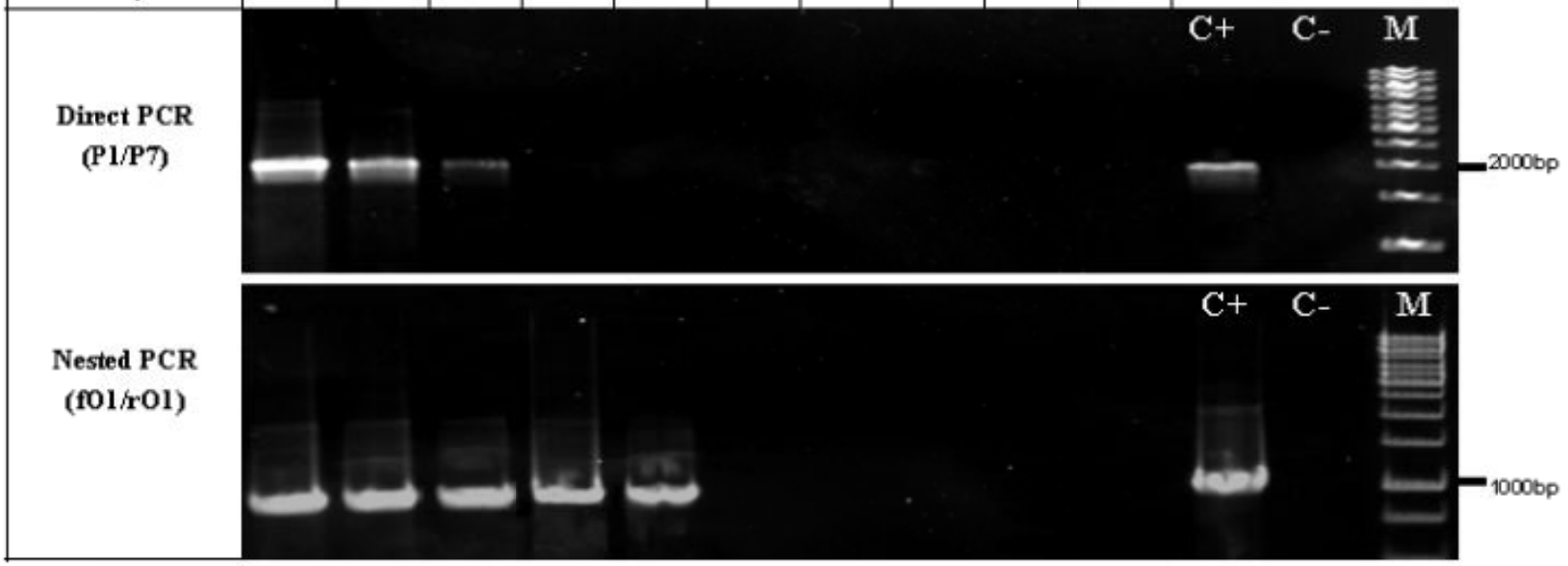

b
y = -3.407x + 19.15; r^2 = 1
CT(Cycle
Log Quantity
a
y = -3.305x + 36.06; r^2 = 0.998
CT(Cycle
Log Quantity

Figure 2

Figure 3

Dilutions	10^9	10^8	10^7	10^6	10^5	10^4	10^3	10^2	10	1
Real-time PCR C_T	17.4	21.5	24.7	28.2	31.6	ND	ND	ND	ND	ND
Real-time PCR *GU/µl*	$5.7*10^5$	$3.9*10^4$	$4.4*10^3$	365	38					



TABLES

Table 1. Origin and PCR results of samples included in the assay for the validation of method specificity; n.t.= not tested

Phytoplasmas	**Number of samples**	**Origin**	**PCR and RFLP analysis Results**	
			NESTED-PCR (fO1/rO1)	**Real-time PCR** (rpAP15fmod-rpAP15r3)
'*Ca*. Phytoplasma mali' (16SrX-A strain AT-1)	33	*Cacopsylla melanoneura*	+	+
'*Ca*. Phytoplasma prunorum' (16SrX-B)	7	*Cacopsylla pruni*	+	-
'*Ca*. Phytoplasma pyri' (16SrX-C)	7	*Cacopsylla pyri*	+	-
'*Ca.* Phytoplasma phoenicium' (16SrIX-D)	7	Insects	n.t.	-
Flavescence Dorée phytoplasma (16SrV-C)	7	*Scaphoideus titanus*	n.t.	-

Table 2. Real-time PCR quantification results for apple proliferation phytoplasma (APP) in *Cacopsylla melanoneura* overwintered adults.

DNA conc. (ng/µl)	APP DNA in insect DNA		Insect 18S rDNA fragment		APP GU/ pg of insect total 18S rDNA
	Ct (mean ± SD)	Quantity (GU; mean ± SD)	Ct (mean± SD)	Quantity (pg; mean ± SD)	
81.50	15.41 (±0.10)	$2.84*10^6$ ($±1.82*10^5$)	15.85 (±0.07)	8.91 (±0.37)	$1.59*10^4$
36.13	15.07 (±0.07)	$3.55*10^6$ ($±1.54*10^5$)	15.68 (±0.12)	10 (±0.81)	$1.78*10^4$
44.09	15.20 (±0.03)	$3.26*10^6$ ($±5.94*10^4$)	16.33 (±0.09)	6.48 (±0.38)	$2.51*10^4$
47.53	15.44 (±0.07)	$2.78*10^6$ ($± 1.38*10^5$)	16.09 (±0.05)	7.6 (±0.26)	$1.83*10^4$
68.04	15.25 (±0.06)	$3.15*10^6$ ($±1.29*10^5$)	16.29 (±0.05)	6.7 (±0.23)	$2.35*10^4$
42.79	15.33 (±0.09)	$2.98*10^6$ ($±1.85*10^5$)	15.79 (±0.30)	9.37 (±1.75)	$1.59*10^4$
81.92	15.26 (±0.14)	$3.15*10^6$ ($±2.87*10^5$)	15.78 (±0.06)	8.64 (±0.34)	$1.82*10^4$
52.49	17.31 (± 0.03)	$2.47*10^5$ ($±4.21*10^3$)	15.16 (±0.07)	5.54 (±0.26)	$2.23*10^3$
253.76	17.92 (± 0.23)	$1.62*10^5$ ($±2.52*10^4$)	13.97 (±0.10)	12.48 (±0.89)	$6.51*10^2$
66.27	16.54 (±0.52)	$4.43*10^5$ ($±1.50*10^5$)	14.07 (±0.06)	11.67 (±0.50)	$1.90*10^3$
71.31	17.95 (±0.08)	$1.58*10^5$ ($±9.15*10^3$)	14.35 (±0.05)	9.63 (±0.30)	$8.22*10^2$
392.31	20.66 (±0.24)	$7.82*10^4$ ($±1.23*10^4$)	15.86 (±0.07)	9.18 (±0.40)	$5.94*10^2$
31.13	15.12 (±0.12)	$1.69*10^6$ ($±1.39*10^5$)	16.94 (±0.05)	4.37 (±0.13)	$1.93*10^4$
37.87	17.41 (±0.06)	$3.36*10^5$ ($±1.44*10^4$)	15.22 (±0.19)	13.51 (±1.64)	$1.24*10^3$
105.69	16.74 (±0.04)	$5.35*10^5$ ($±1.54*10^4$)	15.6 (±0.11)	10.51 (±0.73)	$2.55*10^3$
74.87	15.41 (±0.08)	$1.37*10^6$ ($±7.01*10^4$)	14.14 (±0.08)	27.05 (±1.57)	$2.53*10^3$
28.99	15.65 (±0.15)	$1.16*10^6$ ($±1.18*10^5$)	17.33 (±0.07)	3.41 (±0.15)	$1.70*10^4$
34.41	15.86 (±0.10)	$1.00*10^6$ ($±6.43*10^4$)	16.44 (±0.12)	6.06 (±0.46)	$8.25*10^3$
28.31	16.81 (±0.14)	$5.13*10^5$ ($±4.76*10^4$)	15.7 (±0.06)	9.79 (±0.36)	$2.62*10^3$
38.14	16.20 (±0.04)	$7.84*10^5$ ($±2.01*10^4$)	16.23 (±0.08)	6.96 (±0.34)	$5.63*10^3$
40.85	15.86 (±0.06)	$1.00*10^6$ ($±4.25*10^4$)	15.04 (±0.08)	15.13 (±0.77)	$3.30*10^3$
32.58	15.51 (±0.16)	$1.28*10^6$ ($±1.40*10^5$)	15.98 (±0.06)	8.16 (±0.36)	$7.84*10^3$
33.29	16.20 (±0.06)	$7.87*10^5$ ($±3.61*10^4$)	13.89 (±0.06)	31.95 (±1.19)	$1.23*10^3$
34.82	14.88 (±0.35)	$2.02*10^6$ ($±5.24*10^5$)	15.62 (±0.05)	10.31 (±0.29)	$9.80*10^3$
55.12	17.64 (±0.03)	$2.87*10^5$ ($±6.40*10^3$)	14.9 (±0.01)	16.49 (±0.03)	$8.70*10^2$
31.53	18.32 (±0.07)	$1.78*10^5$ ($±8.22*10^3$)	15.52 (±0.02)	11 (±0.10)	$8.09*10^2$
44.22	15.68 (±0.16)	$1.13*10^6$ ($±1.24*10^5$)	15.66 (±0.04)	10.08 (±0.23)	$5.61*10^3$
25.76	16.11 (±0.17)	$8.43*10^5$ ($±9.61*10^4$)	15.6 (±0.06)	10.49 (±0.37)	$4.02*10^3$
29	17.53 (±0.08)	$3.09*10^5$ ($±1.67*10^4$)	16.26 (±0.03)	6.83 (±0.13)	$2.26*10^3$

30.65	15.22 (±0.06)	$2.02*10^6$ ($\pm 8.34*10^4$)	15.57 (±0.11)	9.95 (±0.76)	$1.02*10^4$
15.88	16.05 (±0.05)	$1.14*10^6$ ($\pm 3.96*10^4$)	15.81 (±0.08)	8.45 (±0.40)	$6.75*10^3$
57.1	17.05 (±0.02)	$5.65*10^5$ ($\pm 5.37*10^3$)	15.19 (±0.07)	12.71 (±0.60)	$2.22*10^3$
19.91	16.40 (±0.06)	$8.91*10^5$ ($\pm 3.89*10^4$)	15.90 (±0.10)	7.97 (±0.51)	$5.59*10^3$

Table 3. Reproducibility test for quantification of a) '*Ca*. Phytoplasma mali' GU and b) pg of insect 18S rDNA fragment.

a)

Samples	Mean GU	SD	CV%
P12	$2.63*10^6$	$1.28*10^4$	4.73
P5	$3.13*10^6$	$2.10*10^5$	7.11
P23	$2.88*10^6$	$7.16*10^4$	2.59
P15	$2.36*10^6$	$1.44*10^5$	6.31
P8	$2.84*10^6$	$1.90*10^5$	6.98
P20	$2.49*10^6$	$3.03*10^5$	13.63
P9	$3.07*10^6$	$1.90*10^5$	6.10

b)

Samples	Mean pg	SD	CV%
C2	14.24	0.82	5.80
C4	17.49	0.47	2.74
C20	12.51	1.02	8.2
C13	2.74	0.15	5.39
C11	22.76	1.35	5.88
C9	8.37	0.36	4.38
C13	14.83	0.43	2.85

Table 4. Repeatability test for quantification of a) '*Ca*. Phytoplasma mali' GU and b) pg of insect 18S rDNA fragment.

a)

Samples	Mean GU	SD	CV%
1	$2.84*10^6$	$1.82*10^5$	6.4
2	$3.55*10^6$	$1.54*10^5$	4.3
3	$3.26*10^6$	$5.94*10^4$	1.8
4	$2.78*10^6$	$1.38*10^5$	4.9
5	$3.15*10^6$	$1.29*10^5$	4.1
6	$2.98*10^6$	$1.85*10^5$	6.2
7	$3.15*10^6$	$2.87*10^5$	9.1

b)

Samples	Mean pg	SD	CV%
1	14.05	0.89	6.3
2	16.9	0.76	4.5
3	12.21	1.23	10.1
4	2.95	0.11	3.6
5	23.74	1.6	6.7
6	7.77	0.49	6.26
7	15.4	0.59	3.8