ESTABLISHMENT OF A NEW METHOD FOR RAPID AND PRECISE ESTIMATION OF APPLE PROLIFERATION PHYTOPLASMA CONCENTRATION IN PERIWINKLE

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SUMMARY

Quantification of a plant pathogen is essential to study its population dynamic in various conditions and to relate symptom expression with pathogen concentration. Up to now, very few methods have been published to quantify phytoplasmas. So, the objective of this work was to establish a method able to quantify the Apple Proliferation (AP) phytoplasma populations in periwinkles. The present work was based on a method previously published to detect AP phytoplasma. This method was optimized to transform it into a quantitative method. First, a new probe specific for AP detection was applied. This probe successfully detected only AP isolates (versus closely related ESFY and PD phytoplasmas). Secondly, the method was adapted to allow the quantification of phytoplasma in periwinkle leaves. For quantification, the calibration curve was built on serial dilutions of a plasmid containing the amplified fragment (phytoplasma 16Sr gene). The limit of detection of the method was one copy of cloned phytoplasma DNA in the reaction while the lower and upper limits of quantification were 10^2 and 10^8. Sample DNA extracts were diluted 100X before amplification and standards were prepared in 100x diluted DNA extract from healthy plant. Using the calibration curve, the concentrations in the tested samples were calculated at $2x10^5$ to 10^6 individuals per mg of fresh midrib. This work is a preliminary step to study the interaction of phytoplasmas with their hosts in relation to symptoms expression.

Key words: Apple proliferation, phytoplasma, real-time PCR, detection, quantification.

INTRODUCTION

The discovery of mycoplasma-like organisms (MLOs) as the probable cause of plant "yellows diseases" in 1967 was followed by a steady growth in knowledge of these unique microbes and their relatives (Davis, 1999). To date, these plant pathogens have been associated with diseases in several hundred plant species (McCoy *et al.*, 1989). MLOs (trivially phytoplasmas) are phloem-limited plant pathogens that are found primarily in the sieve elements of infected plants (Lee *et al.*, 2000). They are transmitted *via* propagation material and by specific phloem-feeding *Euhemiptera* (*Cicadellidae* and *Fulgoromorpha*) and *Psyllidae* (Tanne *et al.*, 2001). Unlike most human and animal mycoplasmas, phytoplasmas cannot be cultured *in vitro* in cell-free media.

Quantitative assays will be useful to evaluate the infection ability of insects and to monitor the phytoplasma movement in plants by time. The relatively low titre of phytoplasma, their uneven distribution in plant hosts and their inability to be cultured *in vitro* have complicated the development of an accurate procedure for their quantification. In this context, the Real-Time PCR

has a great potential as this technique has many advantages over the conventional PCR in terms of accuracy, dynamic range, high-throughput capacity, and absence of post-PCR manipulations. Torres *et al.* (2005) developed a real-time PCR for simultaneous detection and quantification of European fruit trees phytoplasmas (AP, PD, and ESFY) by the fluorescent SYBR Green I and primers targeting the 16Sr gene region. AP specific and quantitative detection was described by a quantitative PCR (qPCR) assay based on SYBR Green technology for quantification of AP phytoplasmas in plants and in insect vectors (Jarausch *et al.*, 2004). Baric and Dalla-Via (2004) established a new approach for specific diagnosis of AP phytoplasma in plant material by a multiplex real-time PCR assay using MGB probes.
The goal of this present study was to develop a sensitive real-time PCR technique able to quantify specifically the AP population in periwinkle plants.

MATERIAL AND METHODS

Phytoplasma isolates and plant material

Six isolates of AP, 3 isolates of ESFY and 2 isolates of PD obtained as periwinkle infected plants were maintained on periwinkle by grafting. Five additional isolates of AP were obtained from apple branches.

Phytoplasma DNA extraction

The method described by Zhang *et al.* (1998) was applied with some modifications for DNA extraction. Half gram of fresh leaf midribs of apple trees or periwinkle was grounded in 4 ml CTAB extraction. After the nucleic acid precipitation in 20 mM sodium acetate and ethanol, nucleic acids were pelleted at 16,100 g during 8 min at 4°C. The pellet was further rinsed, dried, and re-suspended in water.

Detection and quantification of AP phytoplasma by real-time PCR

For detection and quantification of AP phytoplasma, the qAP-16S-F/R primers (Baric & Dalla-Via, 2004) and the AP-MGB probe (Aldaghi *et al.*, in press) were used in a newly-optimized real-time PCR. The PCR reactions were performed in a total volume of 25 µl containing 5 µl of known template DNA or known standard, 1x qPCR Master Mix (Eurogentec) containing dUTP, 400 nM of each primer and 200 nM of the probe. Amplification and detection were performed using the GeneAmp 5700 Sequence Detection System (Applied Biosystems). Other conditions of amplification were adjusted as mentioned by Aldaghi *et al.* (in press). Nonsymptomatic periwinkles were included in all experiments as a control.
Plasmid pCR® 2.1 containing the fragment amplified by qAP-16S primers was used as a standard. The plasmid was purified and the copy number of plasmid was estimated by measuring the optical density and by calculating its molecular mass. Ten-fold serial dilutions of the construct, ranging from 1 to 10^8 target copies per reaction, were prepared. Each sample and each standard were amplified in triplicate.

The concentration of phytoplasma was calculated using the equation of the calibration curve.

RESULTS

Specific amplification by real-time PCR and its limit of detection

In our experiments, a specific detection of all subtypes AP phytoplasma, AT (including AT1 and AT2) and AP (Jarausch *et al.*, 2000), was achieved. The threshold line was set by 10x standard deviation of the fluorescence values during the initial cycles. No amplification products were obtained from other closely related phytoplasmas (SFY and PD) or healthy plants. The calculated PCR efficiency (by PCR efficiency = $(10^{(1/-S)})$-1 formula, Ginzinger, 2002) in real-time PCR was 98%. Finally, the real-time PCR methodology successfully detected a single copy of a cloned DNA fragment per reaction.

Quantification of AP phytoplasma concentration

The PCR amplification is very sensitive to an excess of DNA extract in the sample. Preliminary results showed that a 100x dilution of the DNA extract before amplification minimized the inhibitory effects. So, the samples were always diluted 100x before amplification and standards were prepared in 100x diluted DNA extract from healthy plants.
A calibration curve was constructed from the cloned fragment of phytoplasma DNA into plasmid. A linear relationship for calibration curve was observed on serial dilution ranging 10^2-10^8 (Figure 1).

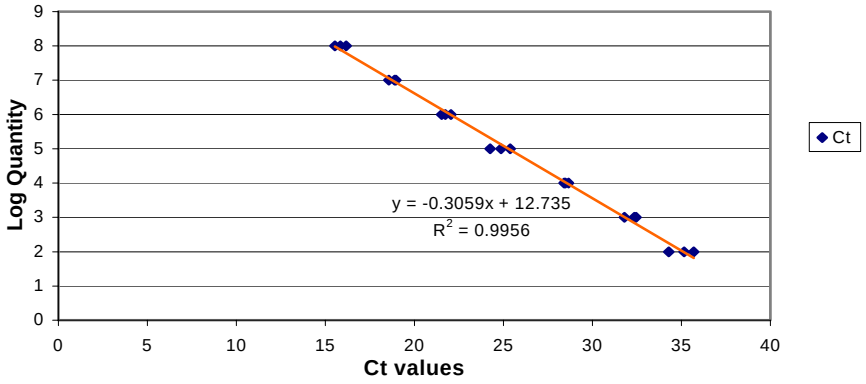

Figure 1. Calibration curve by plotting the log concentration (10^2-10^8copies) of standard against their C_t values

Using calibration curve, the concentrations of phytoplasma in periwinkle midrib was calculated from $2x10^5$ to 10^6 individuals per mg fresh midrib, considering that two copies of the targeted sequence (corresponding to the fragment of 16S ribosomal DNA) are present in each phytoplasma cell (Schneider & Seemuller, 1994). The concentrations of phytoplasma esti-

mated by different equations (obtained from separate runs) were very similar (statistical analyses done by LSD test using a general linear model (GLM) available as SAS software and judged at the $P < 0.05$ level).

DISCUSSION

In this study, we specifically detected and quantified by real-time PCR the AP phytoplasma from periwinkle leaves infected by different isolates of the phytoplasma. Our new MGB probe successfully detected a single copy of cloned DNA target per reaction. This corresponds to the lowest limit of detection among the published methods. In addition, this method allowed the quantification of a phytoplasma concentration ranging from 10^2 to 10^8 in plants (expressed as individuals per mg of fresh midrib).

The major limiting factors that affect the sensitivity or the reliability of a PCR assay are the presence of reaction inhibitors, the presence of insufficient target DNA and the uneven distribution of the pathogen. Our real-time PCR assay minimizes the effect of inhibitors by using 100x diluted DNA extract. Furthermore, it has a limit of detection of one cloned copy of target DNA. To circumvent the effect of uneven distribution of the phytoplasma, this real-time PCR methodology should be associated with an appropriate sampling procedure.

So, this method will be transferred on apple trees to evaluate its potential as a reliable procedure for phytoplasma certification and quantification. Additionally, this method may also be used to monitor the biological cycle of phytoplasma on apple tree.

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