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Optimization of extraction procedure can improve phytoplasma diagnostics

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Abstract

Phytoplasma cause many important vector-borne and graft-transmissible plant diseases. They can affect different host plants. In this work we concentrated on the optimization of diagnostic procedures of pyhtoplasma on fruit trees. The extraction of DNA from the samples is necessary for the molecular analyses that follow. In our experiment we compared two extraction methods – CTAB and automatic extraction procedure on 61 different samples of fruit trees. After the analysis with real-time PCR we concluded that both extraction procedures give the same result, but the automated procedure is less time consuming and therefore has advantage over the CTAB method.

Key words: automated DNA extraction, CTAB, fruit tree phytoplasma, PCR, real-time PCR.

Introduction

Different phytoplasma strains, mostly in apple proliferation (AP) group (16SrX), were reported to be associated with fruit trees (Seemüller *et al*., 2004). More frequently reported phytoplasmas are: '*Candidatus* Phytoplasma mali' on apple trees (*Malus domestica*), '*Candidatus* Phytoplasma pyri' on pear trees (*Pyrus communis*), and '*Candidatus* Phytoplasma pruni' on stone fruits (peaches, apricots, prunes, cherries) (IRPCM, 2004). There were also some reports of other phytoplasma – host combinations like '*Ca.* P. mali' in stone fruits (Mehle *et al*., 2007).

Sensitive methods need to be implemented in order to monitor the presence and spread of phytoplasma infections. Recently, there were reports about the development of different real-time PCR tests for analysis of phytoplasma in general and also of the different types of fruit tree phytoplasma (Hren *et al*., 2007; Torres *et al*., 2005; Bianco *et al*., 2004). Quality of DNA is of key importance in molecular diagnostics, since it can affect the final result. Detection procedures have to be sensitive and also have to be quick.

In the year 2006 two different methods for DNA extraction were employed and compared in different types of fruit tree samples. Success of the extraction procedures and their impact on the whole detection protocol was determined.

Materials and methods

In 2006 22 apple trees (*M. domestica*), 9 pear trees (*P. communis*), and 30 stone frut trees (peach trees – *Prunus persica*, apricot trees – *Prunus armeniaca*, and plum trees – *Prunus salicina*) showing symptomsw related to phytoplasma presence were sampled and analysed. Either leaves or roots of the sampled trees were tested.

CTAB extraction procedure. For DNA extraction from the different species of fruit tree samples, a modified protocol by Ahrens and Seemüller (1992) was used. Final elution of DNA was performed in 50 µl of TE buffer (pH 8.0). The success of the DNA extraction procedure was checked by amplifying eukaryotic 18S rRNA in real-time PCR using 18S rRNA kit (Applied Biosystems, USA).

Automated DNA extraction procedure. DNA was isolated from 200 mg of homogenized material of fruit tress samples using QuickPick™ Plant DNA kit (Bio-Nobile, Finland) and KingFisher mL (Thermo Scientific, USA) machine. Final elution was performed in 200 µl of sterile double distilled water.

Direct PCR and nested PCR. Samples from the different fruit trees were anaylsed for the presence of phytolasma using PCR and nested PCR methods. Initial PCR was performed using 6F/7R – slightly modified primers by Schneider *et al*. (1995), nested PCR reactions that followed were done using f01/r01 (pair of primers specific for AP group) (Lorenz *et al.*, 1995) and using a pair of universal primers U3/U5 (Lorenz *et al.*, 1995). Products were visualized on 1 % agarose gels, stained using ethidium bromide.

Real-time PCR. A real-time PCR procedure using universal primers UniRNA as described by Hren *et al.* (2007) was employed to test the fruit trees samples for the presence of phytoplasma. An eukaryotic 18S rRNA TaqMan assay (Applied Biosystems, USA) was performed along with the universal testing for the presence of phytoplasmas to evaluate the efficacy of the extraction procedure.

All real-time PCR reactions were run in 10 µl reaction volumes under standard conditions on 7900 HT Sequence Detection System (Applied Biosystems, USA). The results of amplifications were analysed using SDS 2.2 software (Applied Biosystems, USA).

Results

Direct PCR and nested PCR. Based on the results of direct and nested PCR, efficacies of the two extraction procedures were similar or improved slightly when using the automated extraction procedure.

Despite the procedure of DNA extraction that was used, 8 samples tested positive for '*Ca.* P. pruni', 3 positive for '*Ca.* P. pyri', and one was positive for '*Ca.* P. mali'. The direct PCR reaction with 6F/7R universal primers using samples prepared by CTAB extraction often yielded many non-specific bands on the agarose gel. Using DNA samples obtained by the automated extraction procedure in first PCR, only 5 samples (of 16) of stone fruits, tested for the presence of '*Ca.* P. pruni', gave expected results. When the same samples were analysed after the CTAB extraction, unspecific bands were observed on agarose gel, whereas they were not observed (samples were negative) when automated procedure was used.

Real-time PCR. DNA was extracted from fruit tree samples by two different methods and analysed for the presence of phytoplasma using universal primers and MGB probe UniRNA. Extraction procedures were checked by amplifying 18S rRNA internal control. According to the results of 18S rRNA the CTAB extraction procedure gave lower Ct values in most of the analysed samples (usually 1 to 3 cycles). The detection of phytoplasma with UniRNA gave similar Ct values in the case of CTAB or automated extraction procedure.

Discussion

In real-time PCR reactions two different amplicons were analysed. One, UniRNA universally detects phytoplasma and the other 18S rRNA presents an internal control and is used to determine the quality of the extraction procedure. To determine the success of the extraction procedure one has to take into account the volume of either TE buffer or sterile double distilled water that was used to elute the DNA. When CTAB method was used, DNA was eluted in 50 µl of TE buffer, whereas it was eluted in 200 µl of sterile double distilled water in automated procedure. Since the dilution of DNA was higher when using automated procedure the lower Ct values in case of CTAB method correspond to the real dilution of DNA. Such difference in Ct values was not observed in the case of UniRNA amplicon.

When additional experiments were performed, elution was in both extraction procedures done in 200 µl of either TE buffer or sterile double distilled water; the difference in Ct values was not observed (data not shown). Since the automated procedure is much less time consuming, especially when combined with real-time PCR analysis, the use of it in routine detection of fruit trees phytoplasmas is suggested. The same comparison experiments in grapevine and in insect vector samples, leading to improved diagnostics of grapevine yellows phytoplasma are in progress.

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