Improvement of phytoplasma diagnostics in grapevine and fruit trees: from grinding to real time PCR analysis

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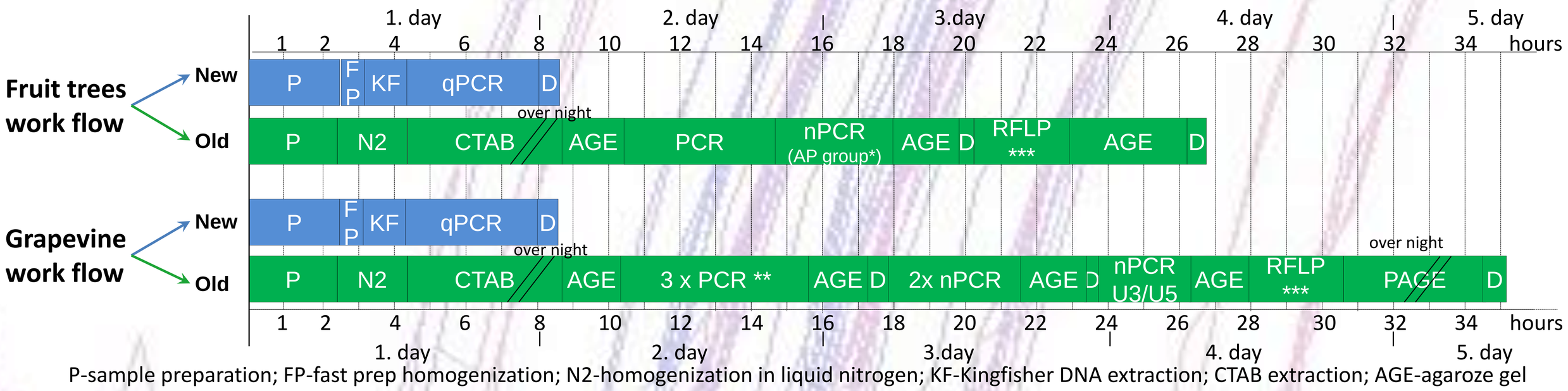
Introduction

Phytoplasmas are plant pathogenic bacteria without cell wall and may cause severe damage to their plant hosts. Examples of such pathogens are *Flavescence doreé* (FD) and *Bois noir* (BN) phytoplasma causing grapevine yellows and '*Candidatus* Phytoplasma mali' (AP), '*Ca.* P. prunorum' (ESFY) and '*Ca.* P. pyri' (PD) from apple proliferation group phytoplasmas on fruit trees. All of them can cause great crop losses and economical damage if not detected soon enough and properly managed. Because phytoplasmas cannot be grown in vitro, their diagnostics depends only on molecular and serological methods. The most commonly applied assays involve laborious and time consuming steps of DNA extraction followed by the conventional PCR and several PCR- RFLP analyses with agarose or polyacril amid gel electrophoresis. Through recent years our laboratory developed and improved methods for fast, Real time PCR based diagnostics of phytoplasma in grapevine and fruit trees with higher sensitivity, specificity and fewer possibilities for contaminations

Methods

We compared the homogenization of plant tissues using mortar and pistil in liquid nitrogen with a Fast Prep® homogenizer and additionally compared both methods using real-time PCR described below. For a DNA extraction CTAB method and KingFisher® automatic extraction procedure based on magnetic beads (Pirc et al., 2009) were compared with pipetting of a large set of samples using an automatic pipetting workstation. The PCR-PFLP analyses were compared with a real-time PCR assay, using different primers and probes. We use primers and probes for 18S rRNA gene (Applied Biosystems, USA) as an internal control of DNA isolation, primers and probes for universal phytoplasma (Hren et al., 2007) detection phytoplasma and from apple proliferation group (AP, PD, ESFY) (Nikolić et al., 2010)

Results



P-sample preparation; FP-fast prep homogenization; N2-homogenization in liquid nitrogen; KF-Kingfisher DNA extraction; CTAB extraction; AGE-agaroze gel electrophoresis; PCR- polimeraze chain reaction; nPCR-nested PCR; qPCR-real time polimeraze chain reaction; RFLP-restriction fragment lenght polimorphism.

Work flow time scales are approximately determined for 10 samples.

*The time scale of fruit trees work flow is for positive samples and in case of negative results of nPCR for AP group phytoplasma, another nPCR with universal U3/U5 primers for phytoplasma detection is required which is not included in this time scale.

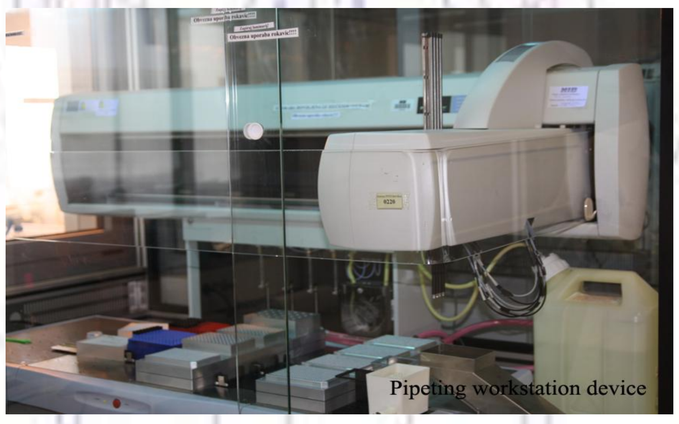
** Time of the old workflow depends on the number of PCR machines on disposal and on the infection of samples (only negative samples or also positive ones). This time scale is for 3 simultainious PCR reactions (using phytoplasma universal, BN and FD specific primers) and in case of positive and negative samples.

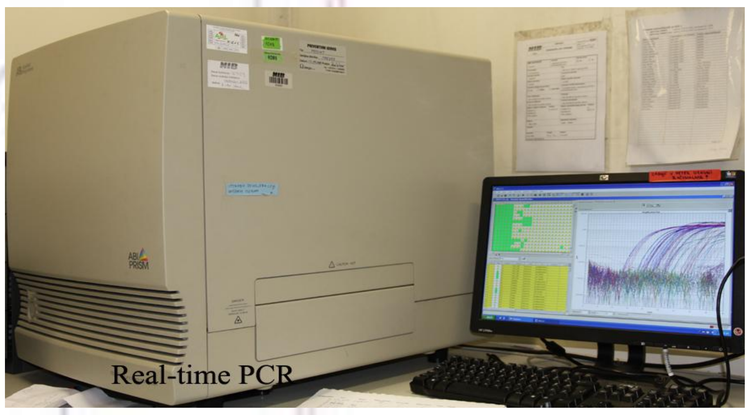
***RFLP only for positive samples.

New work flow

Homogenization

DNA extraction


Pipeting workstation device


Real-time PCR

Conculsions

With a new procedure, which combines a homogenization with a Fast Prep® homogenizer, KingFisher® automatic extraction procedure based on magnetic beads, pipetting of a large set of samples by an automatic pipetting workstation and real-time PCR assays we increased the number of processed samples in time, diminished the possibilities of contaminations, improved the sensitivity and specificity of detection and shortened the time for the diagnosis for approximately three times. **Therefore, new protocols combined enable high throughput routine phytoplasma diagnostics.**

Literature

-Hren M., Boben J., Rotter A., Kralj P., Gruden K., Ravnikar M. (2007). Plant Pathology 56 (5), 785-796.

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-Pirc M., Ravnikar M., Tomlinson J., Dreo T. (2009). Plant Pathology, 58 (5): 872-881.