Bulletin of Insectology ***60*** *(2): 255-256, 2007*
ISSN 1721-8861

Establishing a real-time PCR detection procedure of "flavescence dorée" and "bois noir" phytoplasmas for mass screening

Massimo GORI[1], Roberto MONNANNI[1], Marcello BUIATTI[1], Emanuele GOTI[1], Silvia CARNEVALE[2], Luigi DA PRATO[3], Assunta BERTACCINI[4], Stefano BIRICOLTI[2]

[1]*Centro Interdipartimentale di servizi per le Biotecnologie di Interesse Agrario Chimico e Industriale (CIBIACI), University of Florence, Firenze, Italy*
[2]*Dipartimento di Ortoflorofrutticoltura, University of Florence, Firenze, Italy*
[3]*Applera Italia, Monza, Italy*
[4]*Dipartimento di Scienze e Tecnologie Agroambientali, Patologia vegetale, University of Bologna, Bologna, Italy*

Abstract

"Flavescence dorée" (FD) and "bois noir" (BN) are two grapevine diseases threatening many wine producing countries. Routine detection of FD and BN phytoplasmas mainly rely on nested PCR/RFLP analyses which are time and labour consuming. Real-time PCR can be employed to procedure speed up by coupling specificity of primers and probes and sensitivity of detection system. In this paper a real-time PCR based procedure to detect FD and BN phytoplasmas, suitable for large number of sample testing is described.

Key words: "Flavescence dorée", "bois noir", real-time PCR, phytoplasma detection, mass screening.

Introduction

"Flavescence dorée" (FD) is a well-known grapevine disease which cause severe losses in a number of grapevine growing countries. To prevent or, more realistically, to limit the spread of such disease, rapid and reliable methods to detect FD are useful to possibly eradicate sources of infection. "Bois noir" (BN) is the most wide-spread grapevine yellows disease in Europe and in the Mediterranean basin and is associated with stolbur phytoplasmas (ribosomal subgroup 16SrXII-A). Control to prevent FD and BN spread rely on very different strategies being the first an epidemic disease transmitted by a polyphagous insect vector and the second a quarantine disease transmitted by a specific insect vector. Routine surveys of BN and FD presence in Tuscany is based on detection of phytoplasma ribosomal DNA with nested-PCR of samples from symptomatic plants and on assessment of the FD-vector presence. Nested PCR technique is time consuming, requests much labour, and risks of cross contamination during manipulation occur. Real-time PCR is a promising alternative method because it couples specificity of primers and probes and sensitivity of detection system. The aim of the present study is to establish a detection procedure for FD and BN suitable for large numbers of sample testing.

Materials and methods

During the 2005 regional survey, leaves with petioles were taken from 130 symptomatic grapevine in vineyards located in various areas of Tuscany.

Total nucleic acid was extracted according to Angelini *et al*. (2001) or using the Invitek extraction kit according to the manifacturer's instructions using 100 mg from a pool of 1 g of leaf midribs grinded in liquid nitrogen. PCR was performed with the universal primer pair P1/P7 followed by nested amplification with a second primer set R16F2/R2; primers specific for 16SrI, II and XII (Lee *et al.*, 1994) and for 16SrV ribosomal groups (Lee *et al.*, 1995) were then employed. RFLP analyses with selected restriction enzymes allowed final phytoplasma identification when necessary. PCR products were subjected to electrophoresis in 1% agarose gel, stained with ethidium bromide, and then visualized with a UV transilluminator.

Primers and probes for real-time PCR were designed using the "Primer Express" software (Version 2.0; Applied Biosystems, Foster City, CA, USA). The design of the primer/probe set for detecting FD and BN phytoplasmas was based on an alignment of the 16S rRNA gene including all major phytoplasma ribosomal groups. Both probes were then conjugated with the reporter dye FAM at its 5'end. The probes were then attached with a non-fluorescent quencher dye (NFQ) at their 3' ends (Applied Biosystems).

To evaluate the performance of real-time PCR, grapevine FD and BN-positive samples were used to prepare serial dilutions ranging from an average of 100 ng to 10 pg total DNA. To test specificity of the real-time PCR assay samples infected with FD and BN were tested in separate tubes by running a matrix of primer and probe set combinations in order to assess the occurring of cross-detection of the two phytoplasmas. All positive samples tested with the real-time PCR assay were also tested with nested PCR/RFLP analyses. FD positive samples were further tested using *rpS3* gene (Botti and Bertaccini, 2007) to identify FD strain.

Results

Real-time PCR test showed that quality of DNA employed is of critical importance because amplification inhibitions can occur. While reliable results were obtained with total DNA concentration ranging from 0.1 to 1 ng, extracted according to the method described by Angelini *et al*. (2001), higher DNA concentrations did not anticipate the upturn of the amplification curve, indicating that the amplification was impaired.

When DNA was extracted with the kit, amplifications of the target DNA at higher concentrations (10 ng) occurred at the expected threshold cycle (figure 1).

The two probes revealed a high specificity and no cross detection occurred (figure 2).

The specificity of real-time PCR is reliable to discriminate between samples infected by the two phytoplasmas. Of the 130 grapevine samples tested during 2005, 18 resulted positive to FD phytoplasmas after nested-PCR and the results were confirmed by real-time PCR. They were further subjected to RFLP and to analysis of ribosomal protein (rp) in order to further characterize the FD strains. All the samples were identified by RFLP analyses as belonging to ribosomal subgroup 16SrV-C; except the two that were not amplified in the rp gene 8 samples belonged to FD-type Piemonte/Liguria and 8 to the FD-type Treviso/Serbia (Botti and Bertaccini, 2007).

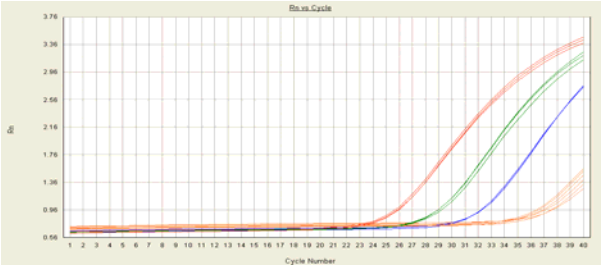

Figure 1. Amplification of the 3 replicas of DNA samples extracted with the kit, diluted at the concentration of 10 (red lines), 1 (green lines) and 0.1 ng (blue lines) and amplified with the FD probe.
(In colour at www.bulletinofinsectology.org).

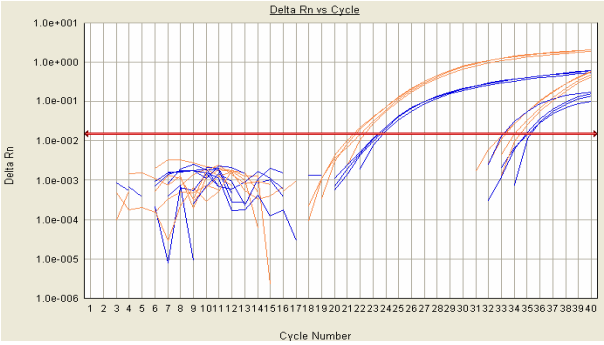

Figure 2. Amplification of 4 replicas of the FD infected samples with FD and BN probes (orange lines) and of the BN infected samples (blue lines). DNA was diluted at the concentration of 1 ng.
(In colour at www.bulletinofinsectology.org).

Discussion

Real-time PCR revealed to be a powerful tool for detection of FD and BN phytoplasmas in grapevines. Even if the system does not enable to discriminate among the different FD ribosomal subgroups or strains, a simple, quick and reliable method for mass diagnosis is greatly improving ability to carry out molecular mass screening in vineyards. The sensitivity of real-time PCR to detect phytoplasmas is comparable to the nested PCR assay routinely employed. Detection of FD phytoplasmas at very low total DNA concentrations (corresponding to 10 pg) was achieved confirming the high sensitivity of the system. However, high quality DNA template from grapevine was shown to be a key factor for the correct interpretation of results. Variability of Ct values can be observed if low quality undiluted DNA is used as template, and this is possibly due to the carry-over of contaminants during DNA extraction that could impair the PCR reaction. Even if a signal can be detected after proper dilutions of low quality DNA, this problem could be better solved by using extraction methods producing highly purified DNA. In fact DNA extracted with the silica column kits gave the best results and seems to be the best one to be reliably coupled with real-time PCR.

Acknowledgements

We thank the "Agenzia Regionale per la Protezione Ambientale della Toscana" (ARPAT) for providing grapevine material for testing and for supporting financially the research.

References

ANGELINI E., CLAIR D., BORGO M., BERTACCINI A., BOUDON-PADIEU E., 2001.- "Flavescence dorée" in France and Italy. Occurrence of closely related phytoplasmas isolates and near relationships to Palatinate grapevine yellows and alder yellows phytoplasmas.- *Vitis*, 40: 79-86.

BOTTI S., BERTACCINI A., 2007.- Grapevine yellows in Northern Italy: molecular identification of "flavescence dorée" phytoplasma strains and of "bois noir" phytoplasmas.- *Journal of applied microbiology* [online], doi:10.1111/j.1365-2672.2007.03467.x.

LEE I.-M., GUNDERSEN D. E., HAMMOND R. W., DAVIS R. E., 1994.- Use of mycoplasma like organism (MLO) group specific oligonucleotide primers for nested-PCR assays to detect mixed-MLO infections in a single host plant.- *Phytopathology,* 84: 559-566.

LEE I.-M., BERTACCINI A., VIBIO M., GUNDERSEN D. E., 1995.- Detection of multiple phytoplasmas in perennial fruit trees with decline symptoms in Italy.- *Phytopatology*, 85: 728-735.

Corresponding author: Stefano BIRICOLTI (e-mail: stefano.biricolti@unifi.it), Dip. Ortoflorofrutticoltura, viale delle Idee 30, 50019 Sesto Fiorentino (Firenze), Italy.