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Detection of "flavescence dorée" in grapevines by RT-PCR

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Abstract

A rapid protocol for extracting crude sap from grapevine samples, and successive one-tube amplification by reverse transcriptase PCR (RT-PCR), was applied on symptomatic and symptomless plants to detect the presence of "flavescence dorée" phytoplasma. Results were compared with those obtained by utilizing nested-PCR on phytoplasma DNA-enriched preparations. Statistical analyses showed a good correlation between the two sets of results, with advantages for the RT-PCR as being rapid and reliable when used for large scale screening.

Key words: Phytoplasma, "flavescence dorée", molecular diagnosis, RT-PCR, grapevine.

Introduction

"Flavescence dorée" (FD) is an important grapevine disease subject to quarantine restrictions in Europe. It is caused by a phytoplasma of the 16SrV group and its natural vector is the leafhopper *Scaphoideus titanus* Ball (Homoptera Cicadellidae).

Rapid and reliable detection with constant monitoring of the infection are fundamental in limiting the spread of FD. The presence of compounds inhibitory for PCR enzymes and the low phytoplasma concentration in the plant (Berges *et al*., 2000) make detection of the disease more difficult. Many molecular diagnostic methods, relying on PCR and on a relatively pure DNA template, have been developed but they are often expensive and time consuming, making them unsuitable for large-scale use. We propose a simple, quick, and sensitive method for large scale screening of FD, based on a rapid extraction protocol followed by a one-tube RT-PCR for amplification of the 16S rRNA of the phytoplasma. The results obtained were compared with those from nested PCR on phytoplasma DNA-enriched template.

Materials and methods

A total of 232 grapevine samples were collected from June to October 2005 in several vineyards and nurseries of Piedmont region (north-west Italy) and tested in parallel by RT-PCR and nested PCR for the presence of FD phytoplasma.

For nested PCR assay an extract enriched in phytoplasma DNA (Ahrens and Seemüller, 1992) and two consecutive PCR amplifications, the first with universal phytoplasma primers, the second, nested, PCR using 16SrV group specific primers were employed.

For the RT-PCR, crude sap was extracted from 0.2 g of leaf tissue and/or 0.2 g of midribs of each sample following the protocol of Osmann and Rowhani (2006). To release the nucleic acid, 2 µl of the extract were boiled in 25 µl of sterile GES buffer (0.1 M glycine-NaOH pH 9.0, 50 mM NaCl, 1 mM EDTA, 0.5% Triton X-100). An aliquot of 2 µl was used directly as template for a single-tube RT-PCR. Specific primers were designed by aligning the transcribed region of the 16S rRNA from at least one phytoplasma for each of the 15 groups available in databases.

The level of agreement between RT-PCR and nested-PCR results, was estimated using Cohen's kappa coefficient (Cohen, 1960). Relevant parameters such as sensitivity, specificity and efficiency of RT-PCR versus nested-PCR were calculated as previously reported (Salomone *et al*., 2004). It was assumed that the correlation between the two techniques was good when parameter values were greater than 90%.

Results

Specificity of the primers designed for RT-PCR was evaluated on phytoplasmas of different groups maintained in periwinkle plants collection. Positive results were obtained only from plants infected by phytoplasmas of 16SrV ribosomal group.

Table 1 illustrates the results of detection obtained with both methods. Of the 135 samples that tested FD-positive with nested PCR, 115 were also positive with RT-PCR (85.2%) and of the 97 samples that were FD-negative by nested PCR, 90 were also negative by RT-PCR (92.8%).

Table 1. Results obtained in tests for FD with nested-PCR and RT-PCR.

Sample number	nested-PCR		RT-PCR	
	Negative	Positive	Negative	positive
	97	-	90	7
232	-	135	20	115
	97	135	110	122

Table 2. Parameters used for comparing RT-PCR and nested PCR and values obtained. TP: true positives, taken as samples positive in nested PCR; TN: true negatives, taken as samples negative with nested PCR; FP: false positives, taken samples positive only in RT-PCR; FN: false negatives, taken as samples negative only with RT-PCR.

	RT-PCR and nested PCR	%
Sensitivity	(TP/TP+FN)x100	87.1
Specificity	(TN/TN+FP)x100	93.3
Positive Predictive Value	(TP/TP+FP)x100	95.1
Efficiency	(TP+TN)/(TP+FP+TN+FN)x100	89.6

Table 3. Parameters comparing nested PCR and RT-PCR as used for phytoplasma detection in grapevine samples.

Parameter	Nested PCR	RT-PCR
Execution time	2 days	3-4 h
Number of samples processed/day	10	40
Difficulty	Medium	Very easy
Hazardous reagent	Chloroform	None
Costs	High-Medium	Medium
Sensitivity	Very High	High

A Cohen kappa index of 0, 0.0-0.2, 0.21-0.4, 0.41-0.60, 0.61-0.80, 0.81-1.00 indicates poor, slight, fair, moderate, substantial and almost perfect agreement, respectively. A Cohen kappa index of 0.76 was obtained, suggesting a substantial agreement between the results of the two methods.

Other diagnostic parameters to compare RT-PCR and nested-PCR, were calculated as reported in table 2, arbitrarily considering that the nested PCR results as true, and as false positive and false negative those RT-PCR results which did not correspond with them.

Discussion

The method proposed is based on rapid RNA extraction followed by a one-tube RT-PCR. The reverse transcription takes advantage of the high ribosomal RNA copy number present in the pathogen cell that increases the quantity of initial template, while only two copies of phytoplasma 16SrRNA gene (Schneider and Seemüller, 1994) are available for nested-PCR assay. In addition, the quick sample extraction protocol saves time and permits handling of more samples; it limits the risk of losing the DNA pellet or co-precipitating inhibitory plant compounds; it improves the operator's safety, avoiding toxic reagents (table 3).

The parameters used to compare RT-PCR with nested PCR (table 2), although sometimes less then 90%, indicated a satisfactory level of agreement. As a baseline, we assumed as true the results obtained by nested PCR, but this method introduces high risks of contamination, increasing the possibility of false positive results. Cohen kappa statistical analysis showed a substantial agreement between the two methods, although RT-PCR failed to detect a number of samples that were positive by nested PCR.

It should be remembered that we were comparing a direct RT-PCR with a nested PCR. For this reason, we are adapting the RT-PCR method for use in nested or real time PCR, in an attempt to increase sensitivity. Overall we suggest the employment of RT-PCR for large-scale screening of field infections, but we would be cautious in employing it for phytosanitary certification of reproductive material, because of the risk of false-negatives.

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