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Research Article

Application of Real Time PCR for the Prevalence Determination of Citrus Exocortis Viroid (CEVd) in Algeria

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Abstract
Citrus exocortis is a grafting disease caused by Citrus Exocortis Viroid (CEVd). The knowledge of the viroid's incidence and distribution are necessary to further apply control measures. The objective of this work was to apply the real time PCR assay for the detection of CEVd in samples collected from symptomatic CEVd-infected plants in Mitidja (North Algeria). The assay showed an excellent diagnostic specificity where 38 out of 50 samples showed a positive reaction for CEVd, which revealed the presence of CEVd in citrus orchards with a prevalence of 76%. Consequently, this work offers a quick alternative to conventional methods for the early diagnosis and the prevalence assessment of CEVd.

Keywords: Citrus Exocortis Viroid (CEVd), Real Time PCR, Diagnosis.

Introduction

Algerian citrus production represents, at present, one of the major concerns for decision-makers at the level of the Ministry of Agriculture. Indeed, the Algerian orange, like other varieties of citrus fruits, because of the very favorable environmental conditions (soil and climates) in which they are grown and their taste qualities occupy an important place on European retail shelves.

The anarchic uprooting caused by town planning, the aging of the orchards where 53.4% are over 30 years old, and the decrease in their sanitary

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condition, have significantly influenced yield, which led to a regression in exports affecting self-sufficiency of the country [1]. Besides, the new citrus plantations have been carried out using non-controlled plants where sometimes, it may pose serious risk in regards to the transmission of Citrus Exocortis. The latter is a viroid disease reported in the Mediterranean basin caused by Citrus Exocortis Viroid (CEVd) [2].

Citrus Exocortis is transmitted by grafting, and is considered a rootstock disease that may affect different parts of the tree including the rootstock (at bark and wood levels), scion, leaves, and fruits [3]. It can cause various types of damages such as bark scaling and cracking, bumps, severe stunting, low fruit-bearing and dwarfism of trees [4].

CEVd belongs to the *Pospiviroidae* family and *Pospiviroid* genus, represented by a 50 µm long ribonucleic acid (RNA) molecule comprising between 370 and 375 nucleotides depending on the strain [5]. Viroids are naked RNA-based infectious molecules that are single-stranded and covalently closed circular [6]. Cottilli *et al.* [7] reported that viroids do not code for any known protein and yet are able to infect plants resulting in serious diseases [7]. They are known as the smallest plant pathogens and can cause very severe diseases in many plants resulting in a range of symptoms very similar to those caused by viral infections [2–5].

During surveys carried out throughout the citrus growing seasons of 2009-2010, 2010-2011, 2011-2012 and 2012-2013 in the Mitidja region (North Algeria), we noticed the presence of typical symptoms of CEVd in plants, mainly peeling bark **(Figure 1A)**, leaf curl **(Figure 1B)** and tree dwarfing. The surveys covered citrus orchards having an area of 92.03 hectare and planted with different varieties of citrus plants, mainly Citrus limon, Citrus clementina, Citrus volkameriana, Thomson navel, Citrange Carrizo, Citrange troyer, Washington navel, Valencia, Valencia late, Mandarina, Double fine, Bitter orange and Poncirus trifoliata.The objective of this work was to characterize the Exocortis disease in those citrus plant samples using real-time PCR assay, which is considered to be rapid and sensitive tool for the diagnosis of plant pathogens.

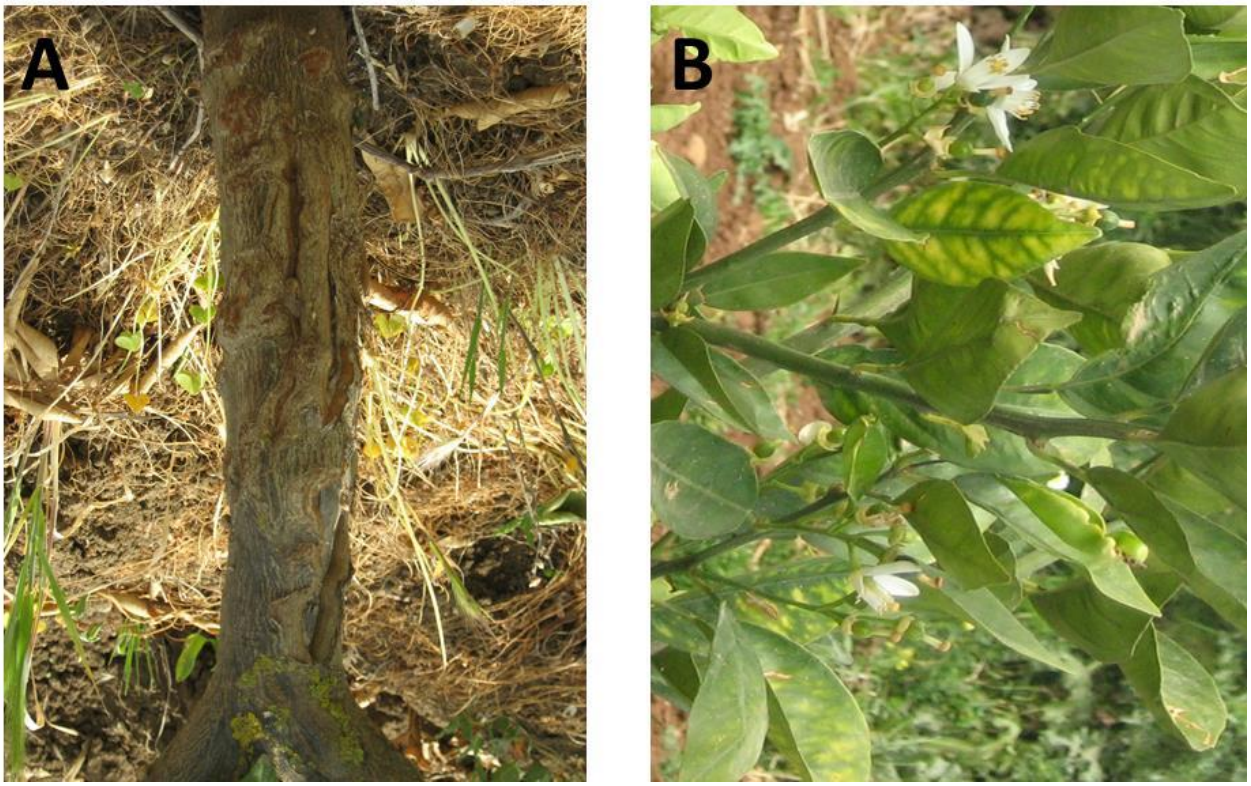


Figure 1. Peeling bark (A) and leaf curl (B) typical symptoms related to Citrus exocortis viroid (CEVd) during surveys carried out in citrus orchards in the Mitidja region (North Algeria).

Materials and Methods

Source of CEVd

The plants suspected of being infected by CEVd came from surveys carried out on different citrus species in three citrus-growing areas of Mitidja (Hadjout, Chebli and Boufarik) during the citrus-growing campaigns of 2009-2010, 2010-2011, 2011-2012 and 2012-2013. The aim of this survey was to identify plants showing symptoms observable with the naked eye suspected of being caused by Exocortis. Sampling parts of the tree were pieces of bark, aspen eyes and leaves with leaf epinasty, curling, cracks on the underside of the midribs and petioles, browning of the first shoots and the peeling of the bark of the tree. The samples collected are preserved on indexed plants of Citrus volkameriana, Poncirus trifoliata and bitter orange.

Choice of samples to be tested for molecular detection

Among the 128 collected plant samples, there were 111 displaying the characteristic CEVd symptoms. The molecular detection study was carried out on only 50 positive samples. Sample selection was based according to the quantity (yield ≥ 50 µg) and quality (A 260/280 ratio of ~2) of their representative RNA obtained after extraction.

RNA extraction method

Total RNA was extracted using the TRIzol reagent according to Rio *et al.* [8]. Briefly, after grinding 30 mg of fresh tissue in liquid nitrogen, the obtained powder was homogenized in 1 mL of TRIzol and incubated for 5 min at room temperature. Then 0.2 mL of chloroform was added and the homogenate was shaken, incubated for 3 min at room temperature before a centrifugation step for 15 min at 12000 *g* at 4°C. Afterwards, 0.5 mL of isopropanol was added to the obtained supernatant and the mixture was centrifuged for 10 min at 12000 g at 4°C. The supernatant was discarded carefully, and RNA pellet was washed with 1 mL of 75% ethanol by centrifugation at 7500 g at 4°C. The washed pellet was air dried for 5–10 min, then dissolved in 100 µL of RNase Free water [8] and stored at -20°C until needed for PRC reactions.

Real-Time PCR:

Real time PCR reactions were carried out in an iCycler iQ5™ Real Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using the SYBR Citrus Exocortis viroid kit (Qualiplante, CLAPIERS, France), supplied with CEVd qPCR Master Mix, reverse transcriptase enzyme, one positive control and one negative control. RT-qPCR reactions were carried out in a total volume of 20 µL consisting of 2 µL of template RNA and 18 µL of CEVd one Step master mix. The thermal cycling conditions were as follows: 40°C for 15 min for reverse transcription, followed by 95°C for 10 min for enzyme activation, and then 45 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. Melting curve analysis was performed using the following conditions: 1 min denaturation at 95°C, 1 min annealing at 60°C, followed by 180 cycles with annealing temperatures increasing in 0.2°C increments (10 sec each) beginning at 55°C with continuous reading of fluorescence. According to the supplier kit, the specific product of CEVd generates an amplicon that melts between 84.5 and 85°C and the threshold cycle value (Ct) of the amplification curve is lower than 35. Conversely, a sample is considered negative when melting temperature differ more than ±1°C of positive control and the Ct value of the amplification curve is greater than 35.

Results and discussion

Viroids are known as the smallest plant infectious agents and consist of a naked, covalently closed, circular RNA molecule. To date, 29 viroid species have been identified and classified into two major families, namely *Pospiviroidae* and *Avsunviroidae*, according to their structural characteristics and sequence similarity [9]. CEVd is classified within the *Pospiviridae* family and has been known to infect citrus trees. It has been listed as regulated pathogens in many countries [10] for its phytosanitary problem, especially for the production of disease-free propagating material of fruit tree crops.

Up to now, various laboratory methods have been developed and applied for CEVd diagnosis. In general, molecular-based detection approaches such as real- time PCR have significantly revolutionized the detection of various plant pathogens [11].

The aim of this study was to evaluate a real-time PCR assay for the CEVd detection in plant samples collected from the citrus orchards in the Mitidja region (North Algeria). We analyzed 50 samples suspected to have CEVd disease by observation with the naked eye. The assay showed an excellent diagnostic and analytical specificity where 38 out of 50 samples showed a positive reaction for CEVd, which revealed an incidence of 76%. The threshold cycles obtained were less than 35 (**Figure 2**), which confirms the presence of CEVd in our tested samples.

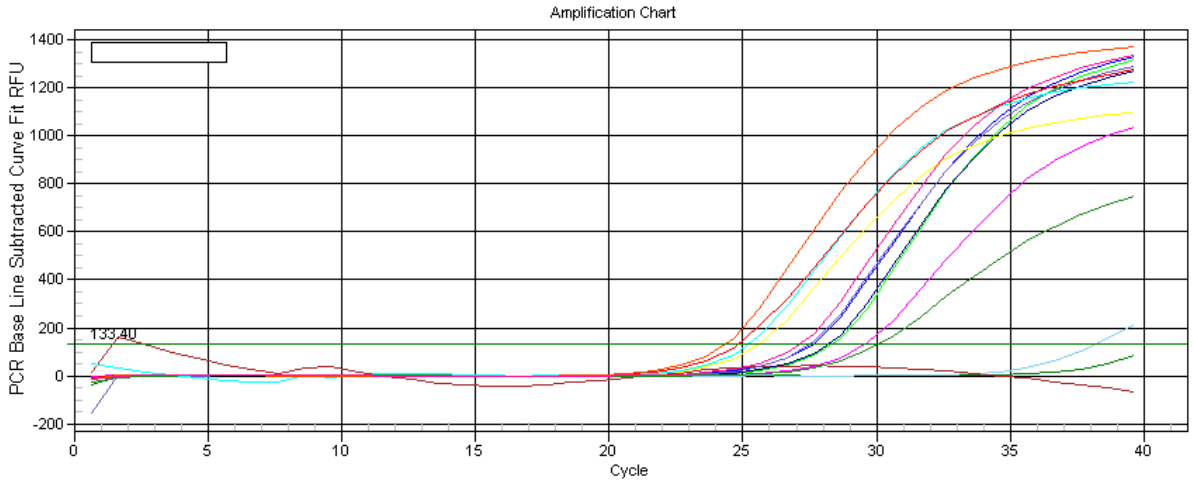


Figure 2. Amplification curves of the analyzed samples showing the fluorescence signal plotted against PCR cycle number

High CEVd prevalence was reported in other studies. For instance, Mohamed *et al.* [15] analyzed the presence of viroids in samples from 24 citrus species and cultivars collected in 2008 from several citrus-growing regions in Sudan and showed that CEVd was detected in 87% of samples using molecular methods. In Italy, results of survey conducted in Campania region showed that CEVd was found to be the most widespread viroids infecting 68% of the tested samples [16]. Rizzo *et al.* [17] found that CEVd was the most widespread viroid in ornamental nurseries in Tuscany (Central Italy), with an incidence reaching almost 50% in three years of monitoring. In Greece, CEVd was shown in high frequency as fourth most prevalent viroids where it was detected in 27.25% of samples collected from different geographical district of the country [18].

In this study, real time PCR assay was based on SYBR Green I as dsDNA intercalating molecule which binds non-specifically to the PCR amplicons. For this reason, a melting curve analysis is crucial to confirm the specificity of the assay. The 38 positive samples tested all presented melting curves between 84 and 84.5°C (**Figure 3**), which corresponds to the melting temperature of CEVd. According to Tse and Capeau [12], the melting curve makes it possible to verify that there is only one type of amplified DNA and therefore that the assay used is very specific.

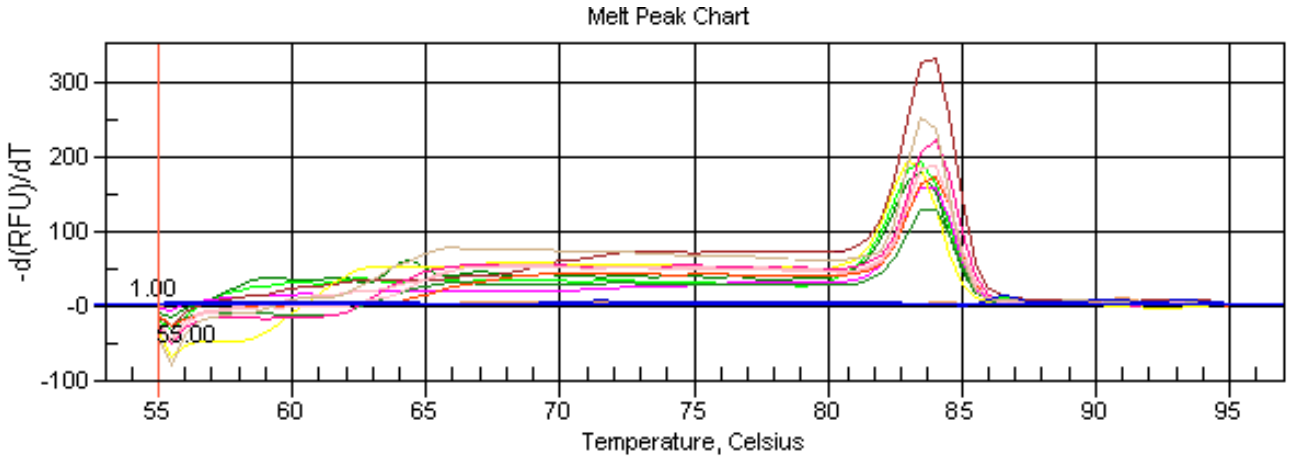


Figure 3. Melting-curve profile obtained by fluorescence measurement during the slow denaturation of PCR amplicons by increasing the temperature. The melting temperature of the target amplicon occurs at 84 and 84.5°C and no contaminating products are present in the reaction.

Quantitative real-time PCR is of great interest in quantifying the titer of viruses in the early stages of the disease [13]. The incidence rate obtained in our analysis (76%) can be explained by the sensitivity of Sour orange to CEVd on the one hand and to the age of the orchards on the other hand because according to Sagouti [14], the symptoms of the Exocortis disease are favored by the susceptibility of the attacked plant, the nature of the strain of the CEVd (strong or weak) and the ambient conditions for the replication of the viroid [14]. In conclusion, the successful CEVd detection in citrus samples by real- time PCR is one approach of the best disease management practices providing sensitive, reliable and affordable detection assay for pathogen surveys in orchards [15].

Conclusions

This study used a fast, sensitive, and quantitative detection tool of Citrus Exocortis viroid. This molecular tool using a real time PCR method was applied for the prevalence assessment of CEVd in the region of north Algeria suggesting its usefulness in the early diagnosis of CEVd compared to other conventional methods.

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