QUANTITATIVE DETECTION OF PLUM POX VIRUS (PPV) IN SEVERAL VARIETIES OF PEACH BY REAL-TIME RT-PCR

DETECTIA CANTITATIVA A VIRUSULUI *PLUM POX* (PPV) LA UNELE SOIURI DE PIERSIC FOLOSIND TEHNICA REAL TIME RT- PCR

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Rezumat: *TaqMan real-time reverse transcriptase (RT)-polymerase chain reaction (PCR) using plant extract spotted onto a membrane (3MM Whatman) to realise PPV targets procedures was developed to detect and quantify Plum pox virus (PPV) in sevaral varieties of peach. The sensitivity of conventional real time RT-PCR was 1,000 times higher than immunocapture (IC)-RT-nested PCR and 106 times higher than enzyme linked immunosorbent assay (ELISA). Reliable quantitation of PPV targets present in infected plant material immobilized on paper can be directly used as template without the need of RNA purification. In the present study, different approaches for sample preparation prior to real-time RT-PCR based on the TaqMan chemistry for a simple, rapid, sensitive and universal detection and quantification of PPV, have been developed. This paper presents data from the Ph. degree thesis in the frame POSDRU/107/1.5/S/76888, project financed from the European Social Fund through the Sectorial Operational Programme for Human Resources Development 2007-2013.*
Key words: *varieties, peach, detection, strains, resistance.*

Abstract: *TaqMan real time reverstranscriptazei (RT)-polimerazei reacție în lanț (PCR) folosind extract de plante imobilizat pe o membrană (Whatman 3MM) pentru a realiza țintele de PPV este o procedură ce a fost dezvoltată pentru a detecta şi cuantifica virusul Plum pox virus (PPV), la diferite soiuri de piersic. Sensibilitatea în timp real convențional RT-PCR a fost de 1.000 de ori mai mare decât immunocapture (IC)-RT-PCR şi 106 de ori mai mare decât enzima legate immunosorbent assay (ELISA). Cuantificarea percisă şi obiectivă a probelor de PPV prezent în materialul infectat ce apoi a fost imobilizat pe membrană 3MM Whatman pot fi utilizate direct ca şablon fară să fie nevoie de o purificare prealabilă a ARN-ului. În studiul de față, au fost dezvoltate abordări diferite pentru pregătirea probei înainte de Real Time -PCR bazat pe Kitul TaqMan pentru o detectare simplă, rapidă, sensibilă şi universală cât şi cuantificarea virusului PPV, prin real time RT PCR. Lucrareade față prezintă date din teza de doctorat in cadrul POSDRU/107/1.5/S/76888, proiect finanțat din Fondul Social European prin Programul Operațional Sectorial pentru Dezvoltarea Resurselor Umane 2007-2013.*
Cuvinte cheie: *soiuri, piersic, detecție, suşe, rezistență*

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INTRODUCTION

Plum pox (Sharka) is a serious disease caused by virus Plum pox virus (PPV) attacking species of the genus Prunus, and is considered the most important viral disease of fruit trees in Europe and the Mediterranean region (Royand Smith, 1994). How far can travel PPV-infection vectors and how much infectaþie retain the capacity are issues which remain unknown. In Europe, the aphids primary vectors that transmit PPV's were identified in Romania (Isaac et al., 1998), Spain (Llacer and Cambra, 1998), Hungary (Gaborjanyi and Basky, 1995), and France (Labonne et al. 1995). Many questions remain to be elucidated about the range of host plants. We now know that both in the case of the woody plants and herbaceous host susceptibility is dependent PPV strain used in studies (Schneider et al., 2004). One objectives of this study was to identify genotypes of peach able to sustain long-term systemic infection with PPV. He hypothesized that these genotypes could be effective in maintaining long-term infection with PPV being an important reservoir that could be a danger to any attempt eradication program, especially if the infected plants remain asymptomatic, the least part of the year. Different PCR techniques have been described above, with or without imunocapture (Wetzel et al., 1991). In all cases plant extracts are necessary, even if grinding operation is time consuming and involves the risk of contamination and release of PCR inhibitors. Several reports have demonstrated the potential of using different methods to immobilize the PCR viral particle by imunocapture.

MATERIAL AND METODS

A total of 88 different peach genotypes from the national collection (Miorița, Turist, Partizan, Miur, Gloria, Frumos de Baneasa, Roşie marmorată, Cluj, Ideea, De Voineşti, Superbă de Toamnă, De Cândeşti, Veteran, etc.) including witnesses and positive control, were tested for their ability to support infection with PPV infection in natural conditions in the field. Quantitative analysis of real-time RT-PCR was performed on RNA previously captured on the membrane (paper) Wathman 3MM putting it directly extracts and then to use specific primers designed to detect PPV's presence. Print fresh peach leaf tissue infected or healthy previously mortar and pipettes on to Whatman 3MM paper. This prepared material can be worked immediately or stored at room temperature for up to 1 month without negative effects on the process of amplification.

Plant extract is prepared with PBS buffer +2% PVP, 0.2% DIECA. Take 5 to 10 ml and placed on 3MM Wathman membrane spots. Spots Wathman cut paper with scissors and placed in sterile 1.5 ml eppendorf tubes to free PPV on paper Wathman spot plus 0.5% Triton X-100. You can use this form as a sample for real time RT-PCR without prior purification ARN. The Primers used are:

P241 primer: 5'-CGT TTA TTT GGC TTG GAT GGA A- 3', P316D primer : 5'-GAT TAA CAT CAC CAG CGG TGT G- 3', P316M primer : 5'-GAT TCA CGT CAC CAG CGG TGT G- 3', PPV-DM probe: 5'- FAM CGT CGG AAC ACA AGA AGA GGA CAC AGA – TAMBRA 3', 1 x TaqMan Universal PCR Master Mix (Applied

Biosistems);1 x MultiScribe şi RNase Inhibitor Mix (Applied Biosistems).For real time RT – PCR preparation were used: 1 µM P241 primer, 0,5 µM P316D primer, 0,5 µM P316M primer, 200 nM TaqMan probe şi 5 µl plants probe at the final volume 25 µl.

RESULTS AND DISCUSSIONS

The PCR amplification attempts by pipetting the vegetable juice directly onto Wathman paper spots, the Real Time-PCR resulted in specific amplification of some peach varieties. Attempts to amplify fragments that highlights PPV's presence in extracts released on to membrane Wathman or commercials without Triton X-100 proved to be unsuccessful, indicating the need to use Triton in order to release viral particles that must be amplified. Real time RT - PCR sensitivity is fast, secure, with low risk of contamination. An observation made during this study is that anti-PPV immunoglobulins specific primers are required for a successful capture RT-PCR. The results showed that among the 90 samples presented in figure 1, only the probe number 88, the variety "Michelin", prove that is positive and certainly the control probe materialised by un upward curve like in figure 2 or figure 4.

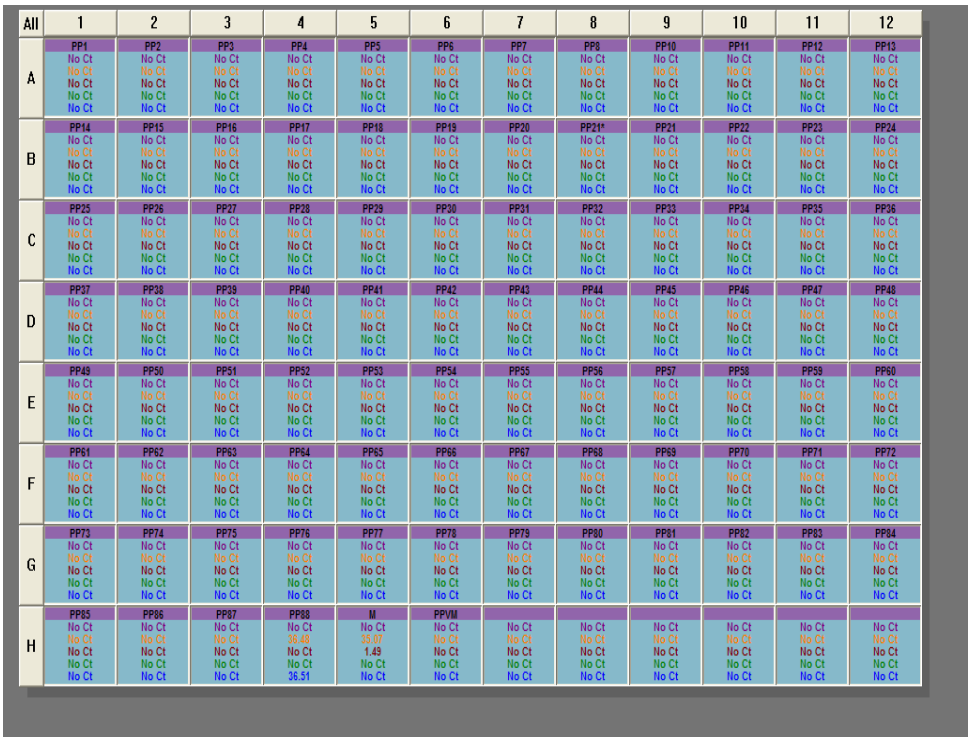

Fig.1 - Placing the peach samples in Real Time RT-PCR plate.

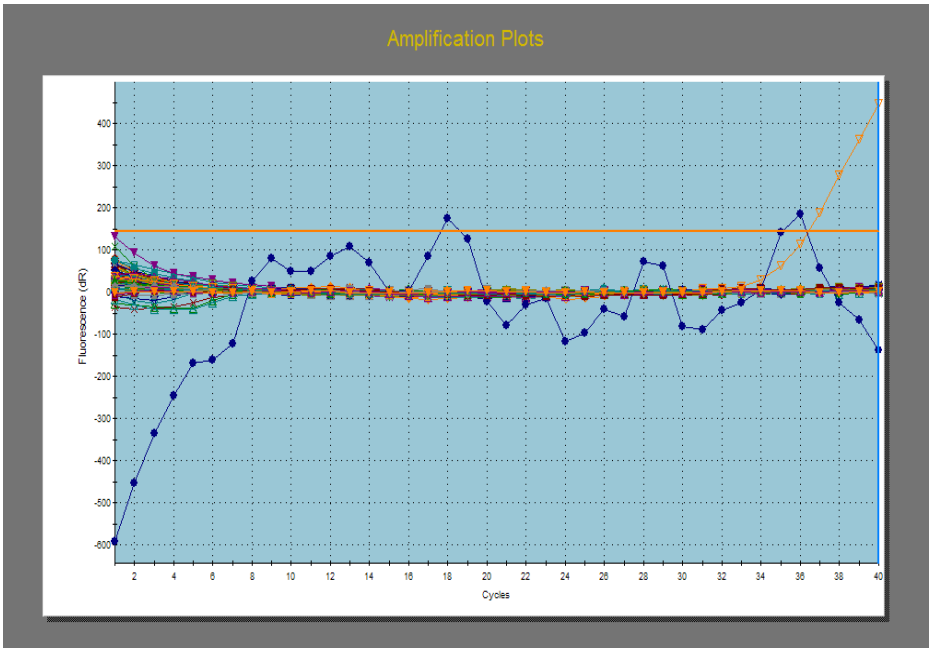

Fig.2 - The results of all tests "in cluster" reported to the control probs

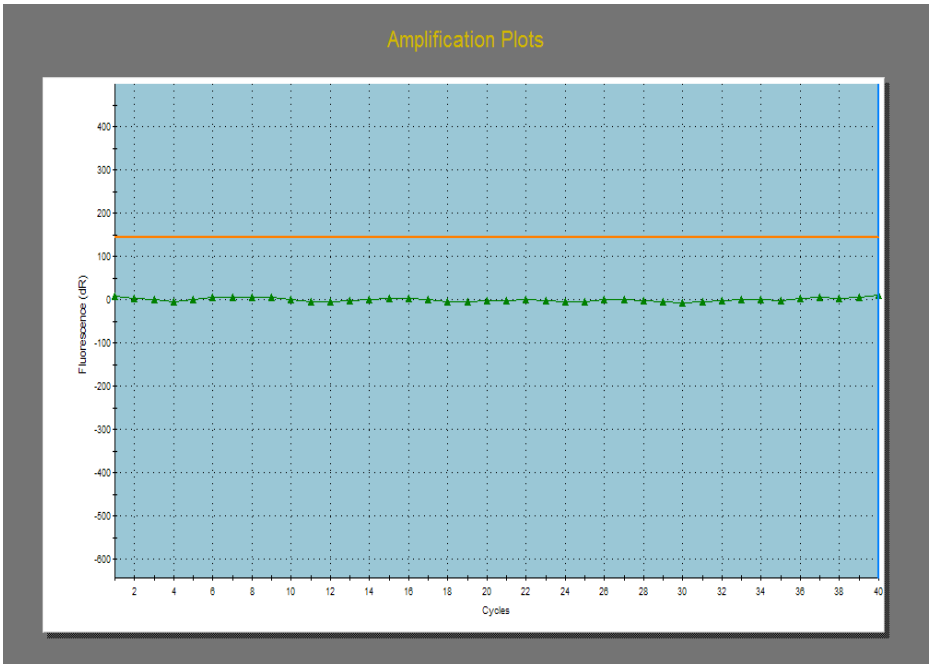

Fig.3 - Negative reaction to infection with PPV to peach variety "De Candesti"

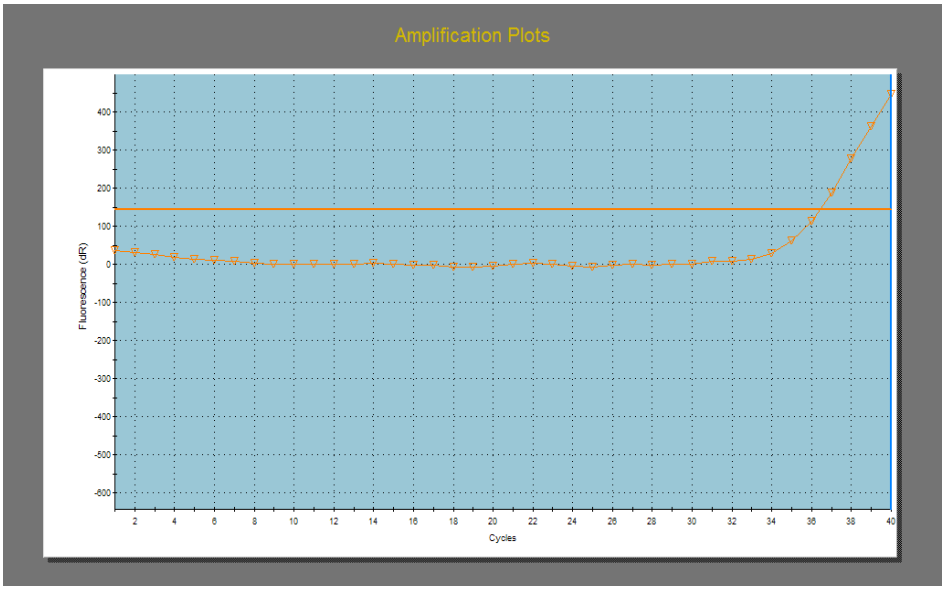

Fig.4 - Positive reaction in Real Time RT-PCR peach variety "Michelin"

Below are samples of peach last values that were revealed to be infected with PPV based on threshold values in relation to infection control. Shown in the last column as evidence that the variety 88 "Michelin" has been positive for different fluorescent 36.48 to 35.07 in rapport of the witness (so close in value).

H2	PP86	FAM	FAM	Unknown	"4,186,715"	No Ct
H3	PP87	CY5	CY5	Unknown	"3,367,025"	No Ct
H3	PP87	CY3	CY3	Unknown	"144,736"	No Ct
H3	PP87	ROX	ROX	Unknown	"317,820"	No Ct
H3	PP87	HEX	HEX	Unknown	"89,525"	No Ct
H3	PP87	FAM	FAM	Unknown	"4,186,715"	No Ct
H4	PP88	CY5	CY5	Unknown	"3,367,025"	No Ct
H4	PP88	CY3	CY3	Unknown	"144,736"	"36,48"
H4	PP88	ROX	ROX	Unknown	"317,820"	No Ct
H4	PP88	HEX	HEX	Unknown	"89,525"	No Ct
H4	PP88	FAM	FAM	Unknown	"4,186,715"	"36,51"
H5	M	CY5	CY5	Unknown	"3,367,025"	No Ct
H5	M	CY3	CY3	Unknown	"144,736"	"35,07"
H5	M	ROX	ROX	Unknown	"317,820"	"1,49"
H5	M	HEX	HEX	Unknown	"89,525"	No Ct
H5	M	FAM	FAM	Unknown	"4,186,715"	No Ct

CONCLUSIONS

Preparation of prints is simpler and faster than DNA extraction or isolation kits and can be used with quarantine viruses without risks. Another advantage is that, unlike plant extracts, membranes spots printed or vegetable juice can be stored at the room temperature for a long period of time before being used or can be sent by post, therefore allowing their training directly field, if necessary. Method of Real Time RT-PCR is simple, fast, inexpensive and very sensitive, and is thus very well suited for use in routine indexing programs. In addition, this technique should be easily adapted to detect plant viruses and other pathogens. Amplified by RT PCR fragments were expected size, confirming the reliability of this method.

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