International Journal of Agriculture Innovations and Research
Volume 4, Issue 4, ISSN (Online) 2319-1473
Manuscript Processing Details (dd/mm/yyyy) :
Received : 16/02/2016 | Accepted on : 29/02/2016 | Published : 04/03/2016

Studies Of Real-Time Rt-PCR Detection and Regular PCR for Phenotyping the Peach Old Varieties

ION Ligia
University of Agronomic Sciences and Veterinary Medicine Bucharest, Marasti Street, No. 59 E-mail: ligiadion@yahoo.com

MOREAU Fabienne
Société ADNid Cap Alpha, France

BADULESCU Liliana
University of Agronomic Sciences and Veterinary Medicine Bucharest, Marasti Street, No. 59

***Abstract* – The TaqMan real time reversetranscriptaze (RT)- Polymerase chain reaction (PCR) using plant extract immobilized on a membrane (Whatman 3MM) to achieve targets for PPV is a procedure that has been developed to detect and quantify the virus Plum pox (PPV), in different varieties of peach . The sensitivity of the conventional real-time RT-PCR was 1,000 times higher than immunocapture (IC) -RT-PCR and 106-fold higher than the enzyme-linked immunosorbent assay (ELISA). The objective quantification of samples of PPV present in infected material which was then immobilized on the membrane Whatman 3MM can be used directly as a template without the need for a purification of RNA. In this study, different approaches were developed for sample preparation before PCR based Real Time Kit for detection TaqMan for a simple, rapidly, sensitive and universal quantification for PPV virus by real time RT PCR .Also the regular PCR was used to detect the PPV after artificial infection on the old Romanian varieties of peach („Novîi", „Socini"," Învingătorul", „Oriol", „De Voineşti"," Zalotnaia Oşeni", „Mioriţa", „Turist", „Veteran"," Cluj112").**

***Keywords* – Varieties, Peach, Detection, Strains, Resistance, Plum Pox Virus.**

I. Introduction

Plum pox (Sharka) is a serious disease caused by virus *Plum* pox virus (PPV) which attacks the genus *Prunus*, and is considered the most important viral disease of fruit trees in Europe and the Mediterranean region [8], [1]. How far can spread PPV-infective vectors and how much retains the ability of the infected are issues that remain unknown. In Europe, the primary vectors that transmit PPV aphids were identified in Romania [5], Spain [6], Hungary [4], and France [7]. Many questions remain to be elucidated about the range of host plants. Now we know that in the case of woody plants and herbaceous sensitivity of the host is dependent of the PPV strain used in the study [2].

One of the objectives of this study was to identify genotypes of peach able to support the long-term systemic infections with PPV. It has been launched the hypothesis that these genotypes could be more effective in maintaining the long-term infections with PPV being an important reservoir that could be an alternative to eradication program if the plants remain asymptomatic, at least the part of the year. Different PCR techniques have been described above, with or without immunocapture [9]. In all cases, plant extracts are needed, even if the plant mashed operation is time consuming and involves a risk of contamination and release of PCR inhibitors. Several reports have demonstrated the potential to use different methods of immobilization by immunocapture PCR viral particle.

II. Material and Methods

A. Plant Material

A total of 88 different genotypes of peach, coming from the National Collection, (Mioriţa, Turist, Partizan, Miur, Gloria, Frumos de Baneasa, Roşie marmorată, Cluj, Ideea, De Voineşti, Superbă de Toamnă, De Cândeşti, Veteran, etc.) including controls and the positive control, they were tested for their ability to support the PPV infection under natural conditions of infection in the field.

B. Method of Work

The analysis of real-time quantitative RT-PCR was performed on RNA in advance captured on the membrane (paper) Wathman 3MM by directly placing the plant extract, and then to use the specific primers designed to detect the PPV particles. For printing fresh peach leaf tissue infected or healthy were mashed and pipetted on the Wathman 3MM paper. Thus prepared material can be worked immediately or stored at room temperature for up to 1 month, without negative effects on the amplification process. Plant extracts were prepared with PBS buffer + 2% PVP, + 0.2% DIECA. The 5 to 10 ml were taken and placed on 3MM Wathman membrane spots. The spots Wathman paper were cut with scissors and put in sterile 1.5 ml Eppendorf tubes. In order to release the PPV particles from Wathman spot paper add 0.5% Triton X 100. You can use this form as evidence for RT -PCR without prior purification the ARN. The primers used are: P241 primer: 5", -CGT TTA TTT GAT GGA GGC TTG A- 3", the 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 samples: 5 - : 5' ˉ FAM CGT CGG AAC ACA AGA AGA GGA CAC AGA 3'; 1 x TaqMan Universal PCR Master Mix (Applied Biosystems); 1 x MultiScribe and RNase Inhibitor Mix (Applied Biosystems). For preparation of RT - PCR were used acquis 1 µM P241 primer, 0,5 µM P316D primer, 0,5 µM P316M primer, 200 nM TaqMan probe and 5 µl sample to a final volume of 25 ml.

III. Results and Discussion

The PCR amplification by directly pipetting the vegetable juice on paper spots and used the Real Time-PCR are obtained the specific amplification results in

International Journal of Agriculture Innovations and Research
Volume 4, Issue 4, ISSN (Online) 2319-1473

some varieties of peach. Attempts to amplify fragments of the PPV captured on the Wathman spots paper the researches are indicating the need to use Triton x 100, in order to release viral particles, before the amplification. The RT - PCR technique is sensitive, fast, and safe, with low risk of contamination. A remark made during this works is that anti-PPV immunoglobulin specific primers are required for a successful capture RT-PCR.

The results demonstrated that among the 90 samples (Figure 1) only sample 88 the variety "Michelin" and of course the control sample revealed an upward curve (Figure 2, figure 3, figure 4 and figure 5).

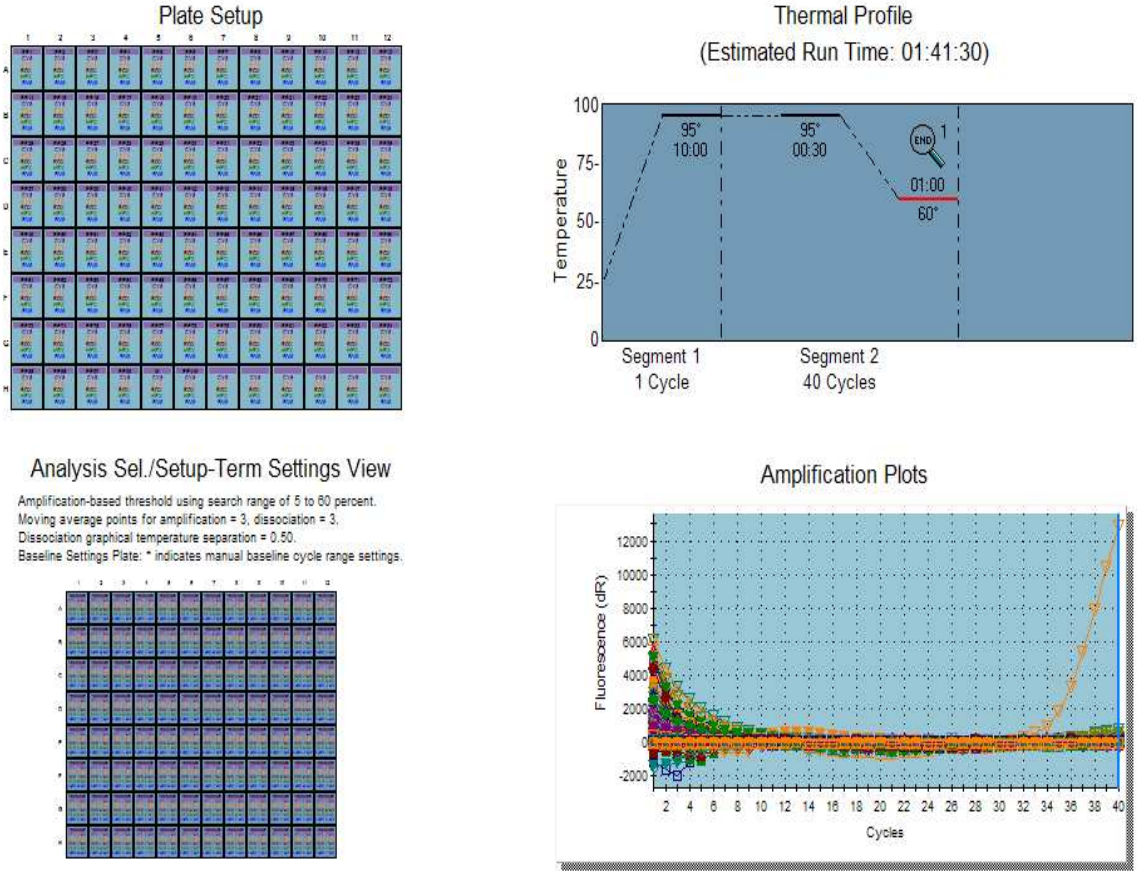

Fig. 1. Plates of Real Time RT- PCR, the program and the peach samples response.

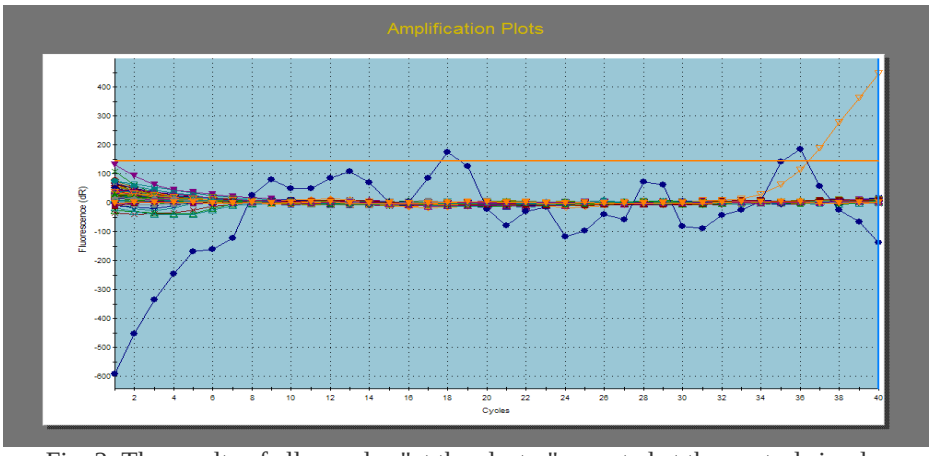

Fig. 2. The results of all samples "at the cluster" reported at the control simple.

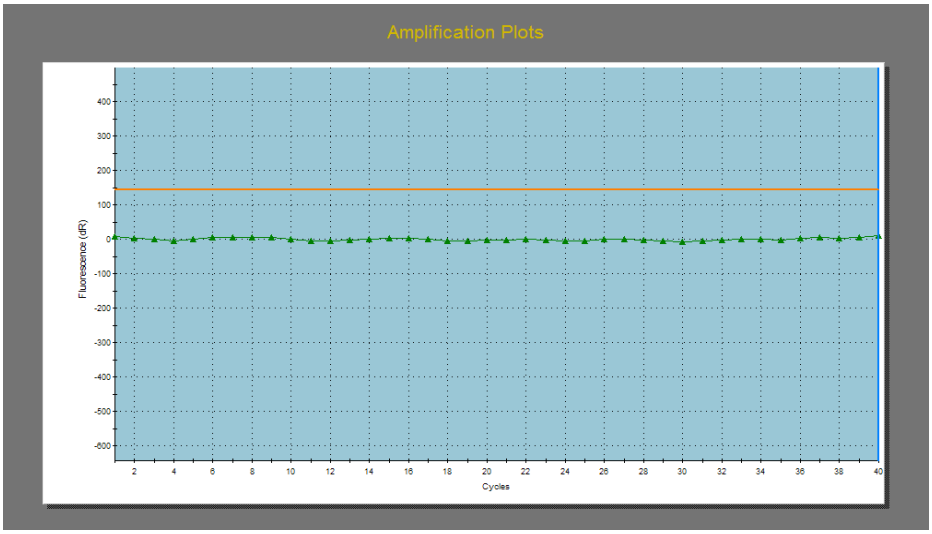

Fig. 3. The negative reaction to infection with PPV for peach cultivar "The Cândeşti"

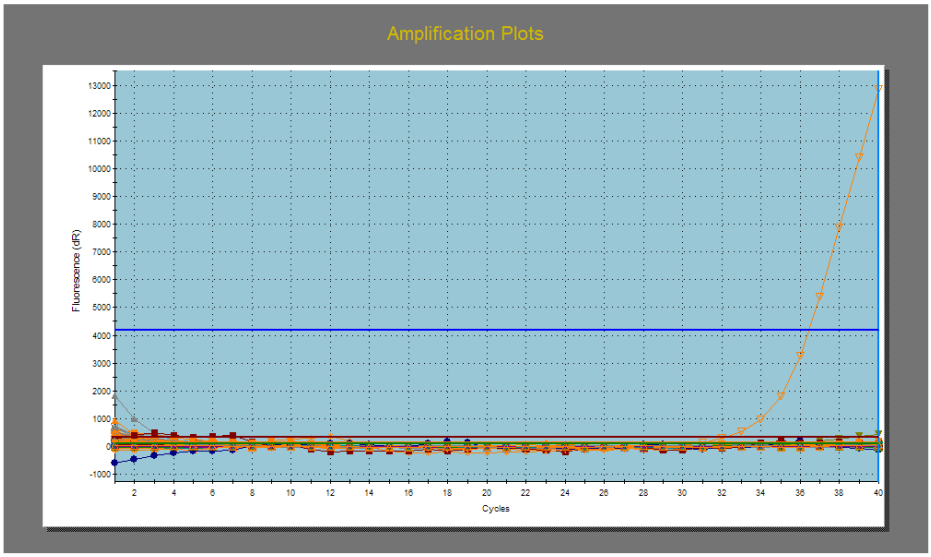

Fig. 4. The positive reaction in Real Time RT-PCR for the peach variety "Michelini"

Below is the latest evidence of peach values which were revealed to be infected with PPV based on threshold values in relation to infection control. It is noted in the last column as evidence that the variety 88 "Michelin" has been positive for different fluorescent 36.48 to 35.07 in comparison of the control (so close in value).

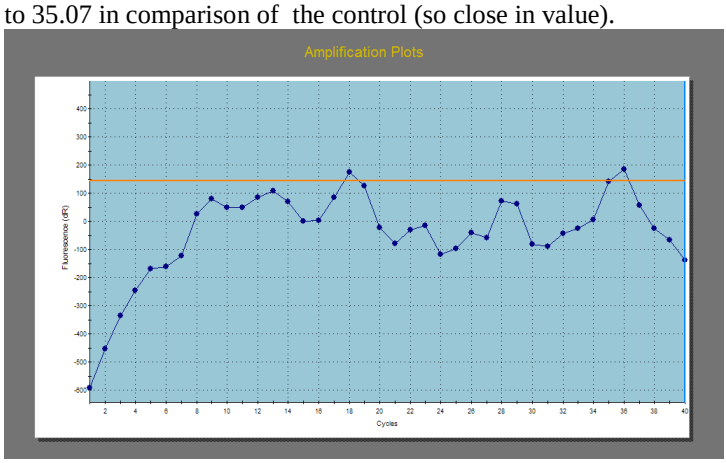

Fig. 5. Positive image on blank expression.

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

Fig. 6. The values of the peach varieties on RT PCR

In figure 6 were expressed in value all peach samples for all 5 fluorescence analysis, and the figure is presented only the final tests with the positive variety Michelini, along with the positive control and some negative samples for comparison.

All these varieties were tested under field conditions by, Real Time PCR technique, but for the greater part eloquence the most important of these varieties with the Romanian origin were indexed on GF 305 (PPV indicator for sensitivity) in artificially infected conditions with PPV strain M infection. Samples were taken from both the varieties and rootstocks and the samples were analysed by serological and molecular tools. (Table1.)

Table 1: Serological and molecular tests of peach genotypes artificially infected with PPV.

No. crt.	Genotypes	2014 Das Elisa	2014- RT -PCR	2015 DAS Elisa	2015 RT-PCR
1.	Novîi	-	-	-	-
2.	Socini	-	-	-	-
3.	Ribet	+	+	+	+
4.	Uneeda	+	+	+	+
5.	Invingatorul	-	-	-	-
6.	Ranger	+	+	+	+
7.	Oriol	-	-	-	-
8.	Superbă de toamnă	-	+	+	+
9.	De Voinești	-	-	-	-
10.	Zalotnaia Oseni	-	-	-	-
11.	Mioriț̦a	-	-	-	-
12.	Frumos de Baneasa	-	-	-	+
13.	Turist	-	-	-	-
14.	Veteran	-	-	-	-
15.	Cluj 1112	-	-	-	-
16.	SEO (martor)	-	-	-	-

Some peach varieties developed clear symptoms, while others have no symptoms and PPV is detectable only through highly sensitive analysis, RT-PCR. Some varieties of germplasm collection appear to be highly resistant to PPV based on the percentage of infection and RT-PCR.
(Figure 7)

International Journal of Agriculture Innovations and Research
Volume 4, Issue 4, ISSN (Online) 2319-1473

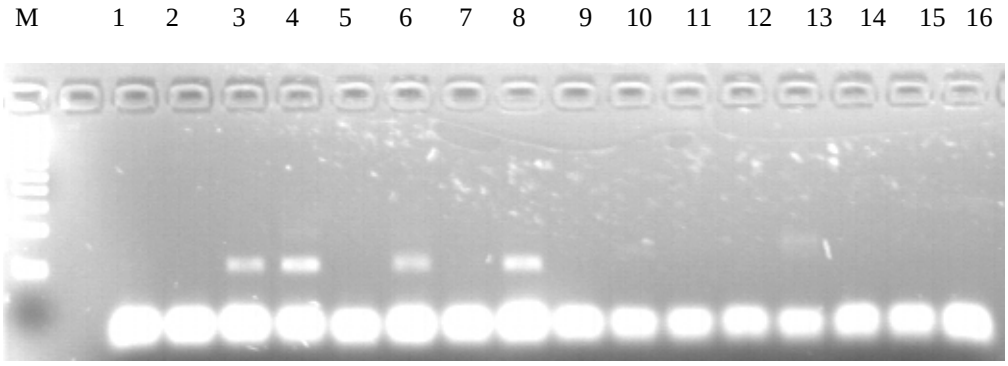

Fig. 7. Image of the agarose gel electrophoresis to detect infection with PPV on peach genotypes.

Among the 12 varieties of peach analysed the results showed in electrophoresis gel, only 5 varieties were found to be infected with PPV and so sensitive, the varieties „Ribet", „Uneeda", „Ranger", „Superbă de toamnă", „Frumos de Băneasa". The remaining varieties are resistant to PPV analysed and studied so interesting in terms of the interaction plant / parasite.

It is important to note that there is considerable variability among isolates of PPV-D and among varieties.

The resistant varieties „Novîi", „Socini"," Învingătorul", „Oriol", „De Voineşti"," Zalotnaia Oşeni", „Mioriţa", „Turist", „Veteran"," Cluj112" and of course PPV control resistant, the variety 'SEO' (Stark Early Orange) will be further analysed by serological and molecular tools during at least 3 cycles of vegetation. In the reason to focus better on the most appropriate detection techniques and rapid analysis we limited the PPV infection on the 12 varieties of peach.

IV. Conclusions

Preparation of prints is simple and much faster than DNA extraction or kits isolation and can be used for the quarantine viruses without risk. Another advantage is that, plant extracts printed on membranes or vegetable juice spots can be stored for a long time before being used or can be sent by mail, thus allowing they direct training field, if necessary. Method Real Time RT-PCR is simple, fast, inexpensive and sensitive, and is therefore very well suited for use in routine indexing programs. In addition, this technique should be easily adapted to detect plant viruses and other pathogens. RT-PCR amplified fragments were of the expected size 420 pb, thus confirming the reliability of the method.

Among the 12 local peach varieties that were tested under artificial infection the varieties „Novîi", „Socini"," Învingătorul", „Oriol", „De Voineşti"," Zalotnaia Oşeni", „Mioriţa", „Turist", „Veteran"," Cluj112" they have been shown to be resistant to PPV.

References

[1] Dicenta, F., Pe´rez-Campoy, P.J., Martı´nez-Go´mez, P., Garcı´a-Brunton, J., Abad, E., 1999. Plum pox virus situation in Murcia (Spain). *Acta Hort*. 488, 769– 774.

[2] Dicenta, F., Audergon, J.M., 1998. Inheritance of resistance to plum pox potyvirus (PPV) in 'Stella' apricot seedlings. *Plant Breed*. 117, 579–581

[3] Frederick, R. D. 2004. Specific detection and quantification of Plum pox virus by real-time fluorescent reverse transcription-PCR. *J. Virol. Methods* 120:97-105.

[4] Gaborjanyi, R., and S. Basky. 1995. Correlation between migration of aphid vector and natural spread of plum pox virus. *Acta Hortic*. 386: 201-206.

[5] Isaac, M., S. Preda, and M. Marcu. 1998. Aphid species vectors of plum pox virus. *Acta Virol*. 42: 233-234

[6] Llacer, G., and M. Cambra. 1998. Thirteen years of sharka disease in Valencia, Spain. *Acta Hortic*. 472: 379-384.

[7] Labonne, G., M. Yvon, J. B. Quiot, L. Avinent, and G. Llacer. 1995- Aphids as potential vectors of plum pox virus: comparison of methods of testing and epidemiological consequences. *Acta Hortic*. 386: 207-218

[8] Roy, A. S., and Smith, I. M. 1994. Plum pox situation in Europe. *EPPO Bull*. 24:515-523.

[9] Wetzel, T., Candresse, T., Ravelonandro, M. and Dunez, J. (1991) – *The PCR methods.J. Virol. Methods* 33, 355–365