COMPARISON OF DIFFERENT METHODS OF RNA ISOLATION FOR PLUM POX VIRUS DETECTION BY REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION

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Summary. – The diagnosis of plum pox virus (PPV) is still considered one of the most important aspects of the "sharka" problem. In fact, different studies demonstrated an uneven distribution of the virus in infected trees due to a high variability in virus concentration. These aspects complicate the PPV diagnosis. To date, biological, serological and molecular assays have been successively developed in order to obtain sensitive and efficient PPV detection techniques. In particular, the polymerase chain reaction (PCR) technique seems to be promising and can be considered the most sensitive and reliable one. Preparation of viral RNA is still a fundamental step in reverse transcription-PCR (RT-PCR) technique, especially when applied to large scale testing, i.e., for certification purposes. In order to find the most rapid and efficient procedure, we have compared three different procedures of extraction of viral RNA to be processed RT-PCR. Their common characteristics is their capacity to extract the RNA from a small amount of plant tissue without organic solvents in the extraction fluid. The procedures were as follows: an immuno-capture (IC) method using a specific antiserum, a silica-capture (SC) method using a non-specific matrix, and a simple and rapid RNA extraction (RE) method. They all were followed by one-tube RT-PCR. The obtained results show that all the three techniques allowed a successful amplification and detection of PPV in tested samples except the SC-PCR method which proved less effective. In fact, the IC-PCR and RE-PCR methods amplified and detected PPV in all isolates tested, while the SC-PCR method was able to reveal the presence of the virus in apricot and infected control samples only.

Key words: plum pox virus; diagnosis; reverse transcription-polymerase chain reaction

Introduction

PPV, the causal agent of the sharka disease, is considered the most dangerous viral pathogen of stone fruit crops in Europe and Mediterranean Basin (Roy and Smith, 1994).

As for other viral pathogens, the only way to control the sharka is a prevention. In particular, sanitary selection, eradication of affected trees and quarantine procedures are the only methods able to control the disease in countries where the virus is not in endemic status.

For these reasons, the diagnosis of PPV is still considered one of the most important aspects of the sharka problem. In fact, different studies have demonstrated an uneven distribution of the virus in infected trees due to a high variability in virus concentration. These factors complicate the PPV diagnosis.

Biological (Sutic, 1961; Németh, 1963), serological (Dunez, 1977) and molecular (Varveri *et al.*, 1987; Wetzel *et al.*, 1991) assays have been successively developed in order to obtain sensitive and efficient PPV detection techniques. In particular, the PCR technique seems to be promising and can be considered the most sensitive and reliable laboratory assay (Wetzel *et al.*, 1992; Kölber *et al.*, 1997).

Preparation of viral RNA is still a fundamental step in RT-PCR technique, especially when applied to large scale testing, i.e., for certification purposes.

In order to find the most rapid and efficient procedure, we compared different protocols of extraction of RNA to be subjected to RT-PCR.

The procedures have, as common characteristics, the possibility to extract the RNA from a small amount of plant tissue, and the lack of organic solvents in the extraction fluid.

Table 1. Efficacy of the three different PCR procedures of detection of PPV in different stone fruit species

Isolates	Strain	IC-PCR	SC-PCR	RE-PCR
Peach sel XIII C12	D	+	–	+
Peach 188 P4	M	+	–	+
Peach Calipso P1	D	+	–	+
Apricot Perla	D	+	+	+
Apricot 37 P5	M	+	+	+
Apricot 75 P1	D	+	+	+
Plum Morettini	D	+	–	+
Plum Favorita del Sultano	M	+	–	+
Plum Simka	D	+	–	+
GF 305 ispave 17	D	+	+	+
GF 305 ispave 11	M	+	+	+
GF 305 healthy	–	–	–	–

D = Dideron strain; M = Marcus strain; (+) = positive sample; (–) = negative sample.

Three RNA preparation methods were applied: an immuno-capture using a specific antiserum, a silica-capture using a non-specific matrix, and a simple and rapid extraction, all followed by one-tube RT-PCR.

Materials and Methods

Source of materials. Leaves naturally infected with PPV were taken from the following stone fruit isolates: 3 apricots (37 P5, 75 P1, and Perla), 3 peaches (sel XIII C12, 188 P4, and Calipso) and 3 plums (Morettini, Simka, and Favorita del Sultano). The isolates belonged either to Dideron (D) or Marcus (M) strain (Table 1). Moreover, leaves of infected (ispave 17, ispave 11) and healthy GF 305 were used as positive and negative controls.

RNA preparation. The following three methods were compared. (1) *The IC method.* A plant extract was prepared by grinding (1/10 w/v) a sample (0.5 g) in sterile TBS buffer (10 mmol/l Tris-HCl pH 7.4, 140 mmol/l NaCl) supplemented with 2% polyvinylpyrrolidone K25 (PVP). After centrifugation at 10,000 x g for 5 mins, 50 µl of the supernatant was pipetted into a thin-wall tube (0.5 ml) precoated with a specific polyclonal PPV-antiserum (1 µg/ml in 0.1 mol/l borate buffer pH 8.5). The tube was then shaken for 2 hrs on ice. After washing with TBS-T (TBS with 0.05% Tween 20), the tube was well dried and 50 µl of RT-PCR mixture was added. (2) *The SC method.* A sample (0.5 g) was ground (1/10 w/v) in PBS-T buffer supplemented with 2% PVP and 20 mmol/l sodium diethyl dithiocarbamate. After centrifugation at 10,000 x g for 3 mins, 200 µl of the supernatant was transferred into a new tube and processed as described by Malinowski (1997). Finally, 5 µl of the obtained extract was added to 45 µl of RT-PCR mixture. (3) *The RE method.* Three – four disks of tissue cut with the cap of 1.5 ml microcentrifuge tube were ground in 800 µl of PBS-T supplemented with 2% PVP, 20 mmol/l sodium diethyl dithiocarbamate and 0.1% carborundum powder, using a micropestle. After centrifugation at 5,000 x g for 3 mins, 5 µl of the supernatant was added to 45 µl of RT-PCR mixture.

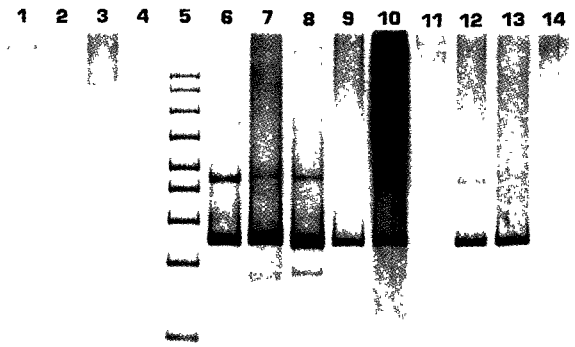

Fig. 1
Polyacrylamide gel electrophoresis of PPV cDNA amplified by IC-PCR, SC-PCR and RE-PCR from RNA of different stone fruit species
Water control (lane 1); healthy control preparations processed by IC-PCR (lane 2), RE-PCR (lane 3), and SC-PCR (lane 4); DNA size marker, 50 – 2,000 bp ladder (BioRad) (lane 5); apricot preparations processed by IC-PCR (lane 6), RE-PCR (lane 7), and SC-PCR (lane 8); plum preparations processed by IC-PCR (lane 9), RE-PCR (lane 10), and SC-PCR (lane 11); peach preparations processed by IC-PCR (lane 12), RE-PCR (lane 13), and SC-PCR (lane 14).

RT-PCR. The RT-PCR single step protocol (Krizbai *et al.*, 1998) was used for amplification purposes. Briefly, 50 µl of RT-PCR mixture containing 67 mmol/l Tris-HCl pH 8.8, 17 mmol/l $(NH_4)_2SO_4$, 0.2 mg/ml gelatine, 6 µmol/l EDTA, 16 mmol/l mercaptoethanol, 1 µmole of each primer, 250 µmol/l dNTPs, 2.5 U of avian myeloblastosis virus reverse transcriptase (Amersham), 2 U of Taq DNA polymerase (Promega), and 200 U of RNase (Gibco) was added to a washed capture tube for IC-PCR. In the case of the other two protocols, 5 µl of SC or RE preparation was added to 45 µl of the same mixture. PPV primers were chosen according to Candresse *et al.* (1995). The cDNA synthesis and amplification were carried out in a Perkin Elmer thermal cycler at 46°C for 30 mins, followed by denaturation at 95°C for 3 mins and 35 cycles of amplification (92°C for 30 secs, 55°C for 30 secs, and 72°C for 45 secs). After amplification, 10 µl of the PCR product was analysed by electrophoresis on a 6% polyacrylamide gel in TBE buffer and stained with silver nitrate.

Results and Discussion

The comparison of the IC-PCR, SC-PCR and RE-PCR methods indicated that they all allowed a successful amplification of PPV. However, the SC-PCR method proved less efficient than the two others. In fact, the obtained results (Table 1) show that the IC-PCR and RE-PCR methods

amplified and detected PPV in all isolates regardless of the original species, while the SC-PCR method was able to reveal the presence of the virus only in apricot and infected control samples. The efficacy of the SC-PCR method may depend on the virus concentration, which was probably higher in apricot and GF 305 than in plum and peach tissues tested.

No difference was observed between the D and M strains. No amplification was observed in the negative controls (Fig. 1).

Both the IC-PCR and RE-PCR methods avoid the use of organic solvents in the extraction fluid, are simple, rapid and very reliable; anyway, the RE-PCR method could be preferred as it avoids the capture phase and consequently the use of a specific antiserum.

The high dilution in the sample preparation (about 1/200 w/v) and the small volume of RNA (5 µl) used in the RE-PCR method allowed to overcome the problem of plant inhibitors usually present in nucleic acid extracts from woody plants. Moreover, the rapidity (6 – 7 hrs to analyse about 100 samples) and reliability of the PCR technique, obviously more sensitive than ELISA (Wetzel *et al.*, 1992; Krizbai *et al.*, 1996; Kölber *et al.*, 1997; Pasquini *et al.*, 1998), indicate that the RE-PCR method could substitute the serological assay also in large scale testing.

On the basis of these data, we suggest the use of the RE-PCR method in routine indexing programs.

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