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# A Simple, Rapid and Efficient Method of *Pepino mosaic* Virus RNA Isolation from Tomato Fruit

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## Abstract

The main concern in molecular detection of RNA viral pathogens in plants is the achievement of good quality of the extracted RNA. Various methods of isolating RNAs from both polysaccharide-rich and poor tissues and other recalcitrant plants are available. However, the use of time and reagent consuming methods and those involving hazardous chemicals is somewhat cumbersome and problematic, especially when it is not necessary for specific purposes like isolating viral RNA from tomato fruit, hence the objective of this paper. We describe an alternative, simple and rapid method for preparing viral RNA from tomato fruit without RNA extraction and purification steps, case of *Pepino mosaic* virus (*PepMV*).

The method employs mechanical treatment and suspension in water. The quality of RNA obtained was judged by spectrometric readings and validated in RT-PCR assays. The used protocol was compared with the usual TRIzol method. The results showed that the yield and the quality of RNA obtained using the proposed method are efficient and highly yielded in comparison with TRIzol method. Moreover, the developed method successfully allowed a sensitive and reproducible detection of *PepMV* predicted bands in RT-PCR. Thus, molecular detection of *PepMV* from tomato fruit can be performed routinely without fastidious RNA isolation. As well, this will make the diagnosis of other RNA viruses infecting tomato crops easier and less time-consuming, in comparison with the other methods performed with expensive commercial kits and those involving toxic chemicals. Finally, the described established method will contribute effectively in strategies of phytosanitary and certification programs of tomato crops worldwide.

**Keywords**: Tomato; RNA isolation; *Pepino mosaic* virus; Molecular diagnosis

## Introduction

Tomato (*Solanum lycopersicum*) is one of the most widely grown vegetable crops. In many worldwide regions where tomato is cultivated, viral diseases have become one of the main limiting factors in tomato production. Over the past few years, *Pepino mosaic* virus (*PepMV*), genus Potexvirus, Family Flexiviridae, has caused significant economic losses in tomato production areas in Europe [1-4], North America [5], South America [6], and Asia [7]. Detection and prevention are the main control measures. Diagnosis based on disease symptoms is not reliable, because symptoms can vary according to the *PepMV* isolates [8].

Several methods have been developed for the detection of *PepMV* in plants. Serological techniques like ELISA have been developed and used successfully for a number of years for the detection of plant viruses. Recently, the trend toward molecular biology techniques such as RT-PCR and nucleotide sequencing has risen in last years. The use of RT-PCR in diagnosing *PepMV* is described in Hasiów et al. [9]. An immunocapture-retrotranscription-PCR (IC-RT-PCR) approach is detailed in Mansilla et al. [10].

Prior to RT-PCR, RNA virus isolation is a critical step. For the majority of plant material containing high levels of polysaccharides and polyphenols, pigments and *RNase* is a challenging starting material for high-quality RNA isolation in reasonable amounts because of different amounts of those substances in diverse tissues.

A large number of methods have been developed or widely modified [11,12] for RNA isolation and purification from plant tissues and adopted by researchers and laboratories. The RNA isolation method developed by Chomcynski and Sacchi [11] employ a single extraction with acid guanidinium thiocyanate-phenol-chloroform mixture [11]. This method incorporated TRIzol, a ready-to-use reagent. Since its introduction, this method has become widely used for isolating total RNA from biological samples of different sources, during last few decades [13]. Thompson et al. [14] used the Plant RNeasy Kit produced by QIAGEN to quickly extract high-quality total RNA from strawberry leaves, but the kit is expensive [14]. In addition, protocols designed for plant are time consuming, require hazardous products and are tissue specific [12,15,16].

In the case of tomato, the level of polysaccharides and other interfering components varies in dependence of fruit ripening stages and nature of tissue (leaf, stem, and root, flower, fruit). Consequently, the yield of total RNA extracted differs from a tomato tissue to another [15]. The method described by Wang et al. [15] for isolation of total RNA from tomato fruit was used in gene expression studies at the microarray-based level [15].

However, isolation of viral RNA from infected tomato fruit is not as difficult and requiring as isolation of functional RNA and small RNAs [17]. Optimizing protocols of RNA virus isolation step, is important in order to meet sensitivity, specificity and rapid means of detecting RNA plant viruses, including *PepMV*, in tomato tissues.

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For this purpose, we attempted to change the time- and reagent-consuming RNA extraction procedures by a simple isolation method that yield a good quality of RNA from tomato fruit, when compared with TRIzol method, cost effective, and not require ultra-centrifugation or toxic chemicals. The developed method was validated in assays of repeatability and reproducibility performed in RT-PCR. In addition, we evaluate the integrity of the viral RNA obtained and the broad applicability for both high and low titers. The RT-PCR assays were conducted for *PepMV*, targeting various regions of the viral RNA genome; so, the obtained viral RNA from tomato samples would be appropriate in downstream RT-PCR detection of *PepMV*. This is the first study to report detection of plant virus infecting tomato crops without chemical reagents for RNA isolation.

## Materials and Methods

### Virus isolates and screening of positive samples

Tomato fruit showing characteristic symptoms of *PepMV* infection were collected from fresh vegetables markets in Rabat and other production fields in Morocco. This study was conducted on a total of 20 tomato samples. The experiments were carried out at Laboratory of Sanitary Control, Control Unit of Plants, Domaines Agricoles Maâmora, Sale, Morocco. Samples were analyzed for determination of *PepMV* presence using a double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) employing monoclonal antibodies, 1b11-G10 and 5A1-G5, which were produced in a previous study. The samples were rated positive if the OD exceeded the mean value of two negative control wells by three times [18].

### Design of experimental study

In order to develop and optimize the simple and rapid method of viral RNA extraction from tomato fruit, an experimental study was performed following four major phases: the first one was to extract samples (n=10) by phenol-guanidine isothiocyanate (TRIzol®) method, the second was to prepare suspension from the duplicate of each sample using Milli-Q water, and this is considered as an alternative to RNA extraction. The third phase was to perform RNA qualification and quantification and compare the yield between both methods. Finally, the last phase was to evaluate the RNA obtained from both methods by testing in one step RT-PCR the detection of RNA genomic targets of *PepMV*. Reproducibility and repeatability tests were performed in RT-PCR analysis to evaluate the reliability of the developed RNA preparation method. Also, to investigate the sensitivity of viral RNA detection, for both high and low titer of the virus, a serial dilution of RNA extracts was prepared and used in RT-PCR assays.

### TRIzol-based RNA extraction

The total RNA from each of the pulp and skin samples was extracted from the infected tomato (90 mg of homogenized fruit sample) by using the phenol-guanidine isothiocyanate procedure according to the manufacturer instructions (TRIzol® reagent; Invitrogen). RNAs thus obtained are stored at -80°C [11].

### RNA preparation method

A suspension was prepared by grinding 100 mg of pulp, skin and juice of tomato with 100 µl of Milli-Q water (RNase Free) in a 1.5 ml microtube using a pestle, then total volume was made up to 1 ml with Milli-Q water. After precipitation for 1 hr at 4°C, the supernatant was then recovered in a new microtube and stored at -80°C until use in downstream application.

### Quantity and quality control

RNA concentration and purity were assessed using Spectrophotometer (NanoDrop Technologies Inc.). The water was used as blank. Sample optical density was measured at wavelengths of 260 and 280 nm, and the 260/280 ratio was used to assess RNA purity. RNA purity was considered adequate when the 260/280 ratio was between 1.8 and 2, as a lower ratio could indicate the presence of proteins, phenol, or other contaminants that typically show strong absorbance at 280 nm [19].

### *PepMV* RNA detection by RT-PCR

RNA preparations were used to perform RT-PCR analysis to detect *PepMV*-RNA using and optimized one step protocol. For gene-specific amplification primers *Pep3* (5'-ATGAGGTTGTCTGGTGAA-3') and *Pep4* (5'-AATTCCGTGCACAACTAT-3') specific for a part of RNA-dependent RNA polymerase (*RdRp*), primers *PepRecB-D* (5'-GAACTAAATGCCAGGTCT-3') and *PepRec-R* (5'-GTGACTCCATCGAAGAAGT-3') specific for half of triple gene block (TGB) and half of capsid protein, primers *PepUSTGB-D1* (5'-TCACAAACTCCATCAAGG-3') and *PepUSTGB-R* (5'-TTAGAAGCTGTAGGTTGGTTTT-3') specific for TGB, and primers *PepCP-D* (5'-CACACCAGAAGTGCTTAAAGCA-3') and *PepCP-R* (5'-CTCTGATTAAGTTTCGAGTG-3') specific for CP were synthesized to amplify overlapping reading frames of *RdRp* (624 bp), TGB-CP (1028 bp), TGB (1317 bp) and CP (845 pb). RNAs were reverse transcribed using *M-MLV* reverse transcriptase (Promega) and polymerase chain reaction PCR amplified using Taq polymerase (Promega). Briefly, 25 µl of reaction was carried out in 0.2 ml tube containing 1 µl of prepared RNA and 5 µl of M-MLV buffer 5x, 2 µl of $MgCl_2$ (25 mM), 1 U M-MLV reverse transcriptase, 1000 U RNasin (RNasin® Ribonuclease Inhibitor, promega), 0.2 mM each dNTPs, 0.4 µM each primer and 0.05 U Taq polymerase. Ultra-pure water was used as negative control. The cycling parameter were reverse transcribed for 30 min at 50°C, denaturated for 2 min at 94°C, followed by 40 cycles of denaturation at 94°C for 15 sec, annealing at 45°C to 52°C depending on the specific primers, for 30 sec and extension cycle 60°C for 45 sec with a final extent cycle of 7 min at 68°C. An aliquot of RT-PCR preparation (6 µl) from each reaction was applied onto 1.5% agarose gel for electrophoresis and the RT-PCR product were visualized under UV.

### Repeatability and reproducibility tests

To assess the repeatability of the obtained RNA extracts in downstream RT-PCR experiments, RNA extraction method was repeated 3 times for 10 samples from infected and non-infected tomato fruits, in the same conditions and same operator.

For the reproducibility assays, we work with the same triplicate of RNA extract samples (n=10) under the previous conditions but we varied the parameter of time by separating the triplicates by one day between each RT-PCR assay, also the experiment was tested by multiple users.

### Limit of detection

To study the limit of detection (LOD) of *PepMV* RNA in RT-PCR assays, total RNAs from tomato fruit was prepared in two-fold serial dilutions in water. This experiment was conducted for RNA obtained with the proposal method in comparison with Trizol method following the same conditions. The initial concentration of undiluted RNA was 140 ng/µl, and all amplifications were carried out with 1 µl in a final volume of 25 µl.

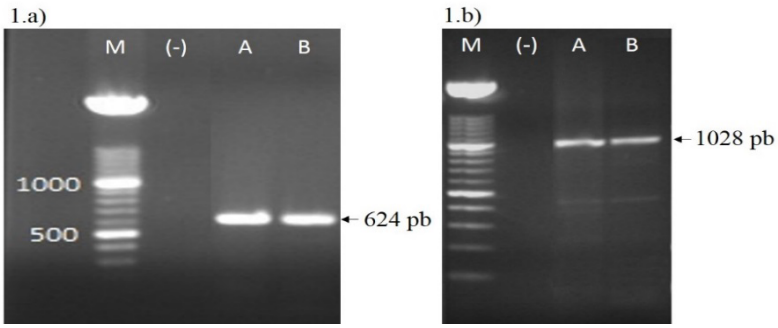


**Figure 1:** Agarose electrophoretic profile of RT-PCR product of PepMV. (1a) amplification of RdRp gene, (1b) amplification of TGB-CP gene, M: DNA marker 100 pb, (-): Negative control, A: Trizol-based RNA extraction, B: RNA preparation method.

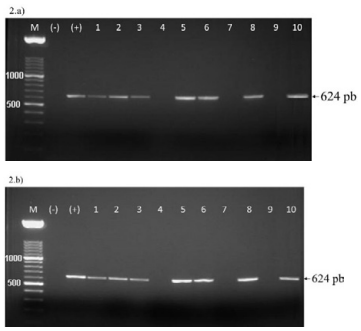


**Figure 2:** Representative results of repeatability and reproducibility tests for detection of PepMV viral RNA in tomato extract. (2a): Repeatability, (2b): Reproducibility, Target: *PepMV RdRp* gene (624 bp); (-): Negative control, (+): positive control, 1 to 10: RNA template from infected and non-infected tomato, M: DNA marker 100 pb.

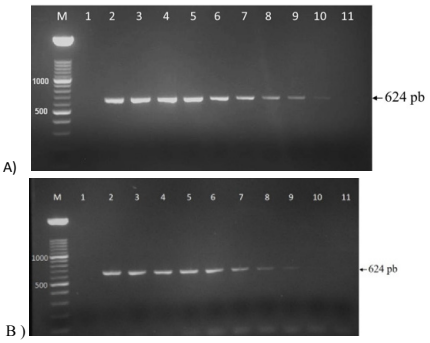


**Figure 3: Detection of PepMV viral RNA.** Two-fold serial dilution of extracted RNA were prepared in water and used as template in RT-PCR reactions. 3a) developed method, 3b) Trizol method, *Target: PepMV RdRp* gene (624 bp); Template quantity (25 µl of reaction): (1) no template, (2) 140 ng, (3) 70 ng, (4) 35 ng, (5) 17.5 ng, (6) 8.75 ng, (7) 4.37 ng, (8) 2.19 ng, (9) 1.09 ng, (10) 0.55 ng, (11) 0.27 ng.

## Sensitivity

In order to evaluate the viral RNA detection at low titer of virus infection, the neat extract from the infected tomato was diluted in extract from healthy tomato using the developed method. Thereby, the background level of inhibitor present in tomato remains the same, but the amount of RNA decreases.

# Results

## Yield comparison of two total RNA isolation method

In order to compare the yield of total RNA extracted with both methods, quantitative measurements were conducted to determine the nucleic acid concentration and the ratio OD260 nm/OD280 nm. The new developed method shows a high RNA concentration of 140 ng/µl and a good purity of 1.72. Both methods led to slightly identical results. However, the eluted volume is considered more important in the developed method (800 µl from 100 mg of tissue), whereas only 60 µl from 90 mg of tissue in case of TRIzol method.

## Detection of *PepMV* viral RNA

Results related to RNA *PepMV* detection from total RNA extracted by both described methods was checked using RT-PCR. 100 ng/µl of nucleic acid was used as template in this experiment. Through electrophoresis migration of RT-PCR amplified products, reliability of the proposal RNA isolation was evaluated by comparing to the TRIzol method. As shown in the Figure 1a and 1b, the correctly sized RT-PCR product (624 bp) and (1028 bp) were obtained for each of the RNA method after amplification with specific *PepMV* primers.

## Validation in RT-PCR assays

### Repeatability and reproducibility tests

The obtained results of both repeatability and reproducibility assays were satisfactory. Non-infected samples were negative and the positive samples remained positive in each triplicate tests. A full success of the evaluated criteria was rated at 100% for each sample. The representative results are shown in Figure 2 of one of each triplicate, a repeatability assay (Figure 2a) and a reproducibility assay (Figure 2b).

### Limit of detection

LOD was investigated including a comparison using RNA from the Trizol method compared to the new method. This approach aims to evaluate how much RNA is available for the PCR amplification in the new method compared with an established method. Two-fold dilutions were achieved for both cases with an equal amount of starting RNA templates.

The results of this part of study indicated that PCR products derived from viral RNA obtained with the proposed method were generated at lower template dilution in comparison with the established method TRIzol.

Indeed, the specific band (624 pb) was visible until the dilution corresponding to a quantity of 0.55 ng of total RNA (Figure 3A), as a low titer, while it became undetectable beyond 1.09 ng for TRIzol method (Figure 3B).

### Sensitivity

Sensitivity of viral RNA detection was assessed by simulating

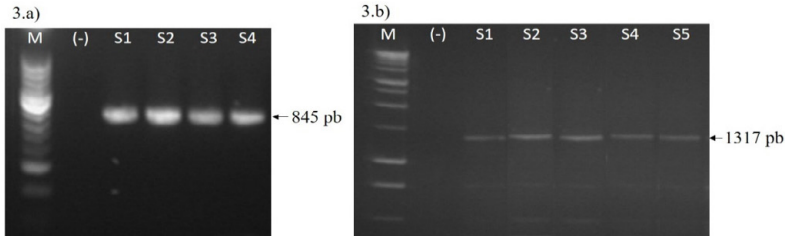


**Figure 4:** Agarose electrophoretic profile of RT-PCR product of PepMV. (3a) amplification of CP gene. M: DNA marker 100 pb, (-): Negative control, S1 to S4: Tomato RNA samples, (3b) amplification of *TGB* gene, M: DNA marker 1Kb, (-): Negative control, S1 to S5: Tomato RNA samples.

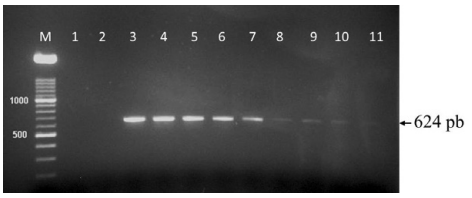


**Figure 5:** Detection of PepMV viral RNA in tomato extract. Two-fold serial dilution of extracted RNA were prepared in healthy plant extract and used as template in RT-PCR reactions. Target: PepMV RdRp gene (624 bp); Template quantity (25 µl of reaction): (1) No template, (2) Healthy plant extract, (3) 140 ng, (4) 70 ng, (5) 35 ng, (6) 17.5 ng, (7) 8.75 ng, (8) 4.37 ng, (9) 2.19 ng, (10) 1.09 ng, (11) 0.55 ng.

higher and lower virus titer by a serial dilution of RNA template in healthy tomato extracts (Figure 5).

Gel migration reveals that the optimal quantity of total RNA that gives a good aspect of the specific band corresponds to 8.75 ng per 25 µl RT-PCR reaction. This amount of nucleic acid is greater than that required for the previous experiment, because the dilution was performed in tomato extract rather than in water, the amplification may be affected by the level of inhibitor present in tomato Figure 5.

**Amplification of other regions of the *PepMV* genome**

Further, to evaluate the integrity of viral RNA and the performance of the simplified method for RNA isolation developed in this study, total RNA from each of the tomato samples (n=10) was subjected to other RT-PCR reactions using the set of *PepMV* specific primers cited above. Examples of viral RNA detection in electrophoresis agarose gel 1.5% are shown in Figure 4. The results of these assays were successful in all RNA samples tested. All RT-PCR reactions yielded a DNA fragment at the molecular weight expected, ranged from 624 pb to 1317 pb, from *PepMV*-RNA for the chosen genomic regions.

## Discussion

In the present work, we attempted a novel method of viral RNA isolation from tomato fruit that is of good quality and suitable for downstream molecular analysis, namely RT-PCR. This is the first study to report reliable and efficient detection of plant virus infecting tomato crops without chemical and toxic reagents. A comparison with the reference TRIzol method, repeatability and reproducibility were performed to assess the validation process of this procedure using RT-PCR targeting viral RNA of *PepMV*.

The results of the comparison with the reference TRIzol method showed that, the purity of tomato fruit RNA prepared using mechanical treatment and suspended in water was comparable to that extracted with TRIzol. The ratio of $A_{260}/A_{280}$ was approximately 1.8 in all samples, this indicate high purity of RNA and the absence of protein, phenol or other contaminants that absorb strongly at or near 280 nm.

Besides, for RNA yield per gram tomato fresh weight, the results of the developed method were found higher than the obtained yields using TRIzol protocol, and those reported yields in other studies using protocol developed for tomato fruit [20]. This difference is due to the elution volume in the final step, which is important (800 µl) in our method that do not use ultra-centrifugation and purification steps, thus all the RNAs are recovered including DNA traces. Moreover, we noticed that standard deviation of yield from RNA preparations is little significant. This can be explained by the non-homogeneous fruit maturation stage between the tomato samples, which affect the yield of total RNA, as shown by Wang et al. [15], the RNA yield (µg/g) vary between tomato green fruit, orange fruit, red fruit [15]. Nevertheless, the concentration and purity remained comparable for both protocols. For another part of this comparison, all RNA samples achieved with both methods have been amplified successfully for the specific *PepMV* targets and exhibited good gel migration.

In addition, through the number of RT-PCR performed in this study, the results confirm the high repeatability and reproducibility of the viral RNA detection using the extracts obtained with our developed method, making it a reliable technique for RNA preparation from tomato fruit. The use of a set of primers targeting variable viral RNA regions and different length, between 624 pb and 1317 pb, have allowed to evaluate the integrity of the viral RNA, which was successful.

The only limit of the developed method, is the possible degradation of RNA due to the presence of *RNase* and other unpurified components in the RNA suspension after a long storage. In preference, tomato extracts should be used in the same day of their preparation or in the next few days in RT-PCR assays to ensure good quality of results and rapidity of the test. The *RNases* that may remain in an RNA sample are trapped by using protein *RNase* inhibitors (RNasin) in enzyme reactions of RT-PCR.

In the other hand, knowing that the concentration of *PepMV* particles in tomato fruit is high, the broad applicability for both high and low titer of the technique has been investigated. For this purpose, we determined the sensitivity of RNA detection using a serial dilution of total RNA extracted in healthy tomato extract. Also, we noted a very low interference by the constituents of tomato fruit with RT-PCR reactions.

Moreover, the limit of detection of viral RNA study, conducted for both the proposal method and the established TRIzol RNA extraction, give significantly satisfactory results. High dilutions lead to early disappearance of the bands when TRIzol is used due to its PCR inhibitors like ethanol and phenol components. These findings validate the use of relatively small amount of total RNA template for *PepMV* viral detection in tomato fruit using the described method in this paper.

## Conclusion

In summary, we developed a simple and reliable method based on mechanical extraction and ultrapure water as the only additive. This method has allowed the isolation of the viral RNA from tomato fruits which is correctly detectable by RT-PCR. This method provides a significant advantage to any laboratory, including those of limited-resource, interested in implementing procedures for viral RNA preparation from tomato fruits without the use of hazardous and noxious chemicals for the manipulator and the environment. Also, the potential of a one-step RT-PCR for rapid detection of *PepMV* would be helpful for both epidemiological studies and genetic characterization.

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