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Short Communication

An efficient procedure to extract RNA from a single *Aphis gossypii* and *Aphis spiraecola* for detecting *Citrus tristeza virus* by nested RT-PCR

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ABSTRACT

A simple protocol to extract RNA from a single wingless aphid, *Aphis gossypii* and *Aphis spiraecola*, using liquid nitrogen and Tri-Reagent is described. A nested reverse-transcription polymerase chain reaction with degenerate primers enabled *Citrus tristeza virus* (CTV) detection in these aphid species collected from an infected tangor 'Ortanique' (*Citrus* × *sinensis* (L.) Osbeck × *Citrus reticulata* Blanco) in the field. The procedure enabled a CTV detection rate of 75% in the *A. gossypii* and *A. spiraecola* evaluated.

KEYWORDS: aphid, CTV, detection, diagnostic, RNA extraction.

INTRODUCTION

Citrus tristeza virus (CTV) is the causal agent of various diseases in citrus crops. CTV has a long flexuous virion (length 2000 nm) with an ssRNA(+) genome of approximately 19.3 kb, encapsidated by two coat proteins, p25 and p27, and is mainly restricted to phloem cells, although the virus has also been found in tracheid cells [1]. The virus has been introduced in many countries through the transport of infected plants and propagation material and has been spread by aphids [2]. The most efficient vector of CTV is the brown aphid *Toxoptera citricida* Kirkaldy [3]. *Aphis gossypii* Glover or cotton aphid is the second most efficient vector, followed by *A. spiraecola* Patch, both present in all countries of the Mediterranean basin [4-7]. Additional vectors are aphids *Toxoptera aurantii* Boyer de Fonscolombe, *A. craccivora* Koch, and *Dactynotus jacae* L. All these vectors transmit the CTV in a semi-persistent manner [8-10].

Field detection of CTV can be performed on the plant material or on aphid species that infest citrus and spread the virus. CTV detection in aphids has several advantages. Knowing the ratio of viruliferous aphids in the field or in an experimental area under study is useful to estimate the pressure of infection as a high incidence of aphids carrying CTV leads to a high CTV prevalence in field trees [6-7]. In addition, a CTV population can be segregated by single aphid transmission, according to studies performed either with *Toxoptera citricida* Kirkaldy [9, 11-14]

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or *A. gossypii* [15-16]. Thus, the occurrence of genetic bottlenecks during virus acquisition by aphids has been used to assess the complexity of a mixture of CTV isolates present in a host plant [17-18].

The detection of RNA viruses in an individual aphid has been based on PCR methodologies that make use of reverse transcription-PCR (RT-PCR) and other PCR-based techniques such as nested-PCR, heminested-PCR, ELISA-PCR, immunocapture-PCR (IC/RT-PCR), or real-time RT-PCR [6, 19-25]. More recently, the reverse transcription loop-mediated isothermal amplification (RT-LAMP) technique has been used due to the possibility of detecting a virus in the absence of an exhausting nucleic acid extraction procedure [26-28]. Common protocols for the RNA extraction from aphids immobilize the insect on a 3MM Whatman filter paper or nylon membrane and use a solution with Triton-X 100, a technique initially developed for *Plum pox virus* (PPV) detection [20, 22]. Another protocol uses a single micro centrifuged tube to perform both the RT-PCR and subsequent nested-PCR protocol in which the main disadvantage, according to the authors, is the need to accurately establish the ratio between primers in two liquid mixtures contained into a pipette tip [23]. The extraction of total RNA (totRNA) from aphids by crushing the aphid in Whatman filter paper or in nitrocellulose membrane is usually performed at room temperature and thus some RNA degradation may occur, contributing to the detection of a low percentage of viruliferous aphids, never above 45%, according to the literature [29].

In this work, we describe a protocol in which liquid nitrogen and Tri-Reagent were used to efficiently crush and extract totRNA from one young adult wingless aphid, followed by a nested RT-PCR for reverse transcription and PCR amplification. In this protocol degenerate primers were used in the RT-PCR amplification process and nested PCR and a CTV detection rate of 75% was attained in one of the aphid species analysed.

MATERIALS AND METHODS

Aphid total RNA extraction

Young adult wingless aphids fed on the tangor 'Ortanique', previously confirmed to be infected with CTV, were collected from the leaves of three young shoots randomly selected from different parts of the canopy, in April 2020. Aphid individuals were identified using a stereomicroscope and divided into 2 groups: (i) aphids belonging to *A. gossypii* species and (ii) aphids belonging to *A. spiraecola* species. Each insect was placed separately in a 2 mL micro centrifuge tube with round bottom and maintained at -80 ºC. Twenty-four aphids were analysed from each species. TRI-Reagent (Sigma-Aldrich, USA) was used to extract totRNA from each aphid following the manufacturer's instructions and protocol, with some modifications, described below. Wingless *A. gossypii* and *A. spiraecola* reared in young *Citrus* × *sinensis* CTV-free plants maintained in environmental growth chambers under controlled conditions [22 ºC/18 °C (day/ night) and a photoperiod of 16/8 h (light/dark)], were used as controls and similarly managed for RNA extraction. A drop of liquid nitrogen was placed inside the micro centrifuge tube and the aphid was powdered with the aid of a syringe plunger. Aphid tissues were ground in 10 µL of Tri-Reagent, with the final volume adjusted to 150 µL, and then transferred to a 500 µL micro centrifuge tube (see Figure 1). Chloroform (30 µL) at 4 ºC was added to the tube, mixed well and incubated at room temperature for five minutes, then centrifuged for 15 minutes at 12000 g and 4 ºC. The supernatant was transferred into a new 500 µL micro centrifuge tube, 75 µL of cold isopropanol (4 ºC) was added and the tube was centrifuged at 13000 rpm for 15 minutes at 4 ºC. The supernatant was discarded and 500 µL of 75% ethanol at 4 ºC was added to the tube. A centrifugation at 13000 rpm for 10 minutes at 4 ºC was performed and the pelleted RNA was dissolved in 12 µL of RNase free water (Figure 1). TotRNA was stored at -80 ºC until use.

Amplification by nested RT-PCR of the CTV p25 gene

The integrity of aphid RNA samples was assessed by agarose gel electrophoresis and the RNA concentration was determined using a NanoDrop 1000 UV-Vis Spectrophotometer (Thermo Scientific, USA). RNA was analysed for CTV detection by nested RT-PCR. RT-PCR mix, prepared in a final volume of 25 µL, contained

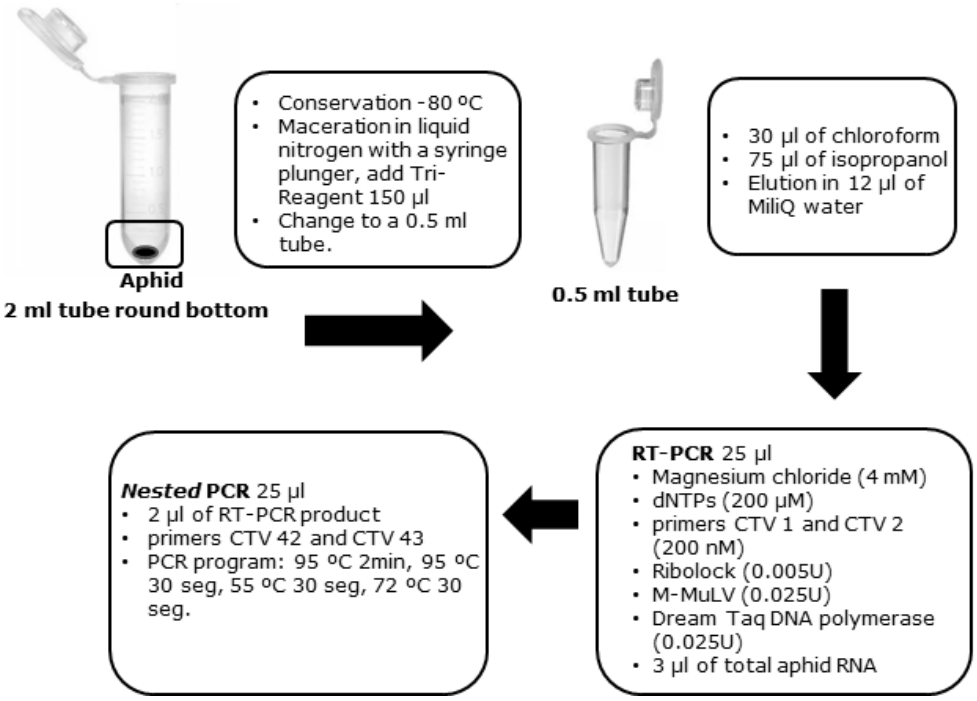

Figure 1. Schematic representation of the experimental protocol performed to extract RNA from a single aphid. Grinding with liquid nitrogen was carried out in a 2 mL round-bottom tube. After adding 150 µL of Tri-Reagent, the entire volume was transferred to a 0.5 mL tube, for an easy handling of the subsequent RNA extraction steps. 3 µL of the extracted total RNA was used for the RT-PCR reaction. 2 µL of the RT-PCR product was used for the nested PCR reaction.

4 mM of $MgCl_2$, 200 µM of dNTPS (Invitrogen, USA), 200 nM of each primer, 8 U of Ribolock (Thermo Scientific, USA), 10 U of M-MuLV Reverse Transcriptase (Roche, Sigma-Aldrich), 0,625 U of Dream Taq DNA polymerase (Thermo Scientific, USA), Dream Taq buffer 1x and 3 µL of totRNA. Primer pairs specific for CTV used were CTV1 Fw (5′ ATGGACGACGARACAA AG 3′, with R = A/G) and CTV2 Rv (5′ TCAACGTGTGTTYAATTTCC 3′, with Y = C/T), to amplify the complete sequence (672 bp) of the p25 gene [30]. A T100 Thermal Cycler (BioRad, USA) was used for RT-PCR with the following parameters: one step at 37 ºC for 60 min, one cycle at 94 ºC for 2 min followed by 35 cycles at 94 ºC for 30 s, 52 ºC for 40 s and 72 ºC for 40 s, with an extension time of 72 ºC for 5 min. For nested PCR, 2 µL of the generated RT-PCR product was used as template, prepared in a final volume of 20 µL. Nested PCR mix contained also Dream Taq Buffer 1x, 1 mM of $MgCl_2$, 200 µM of dNTPS (Invitrogen, USA), 200 nM of each primer and 0.5 U of Dream Taq DNA polymerase (Thermo Scientific, USA). Primer pairs used were CTV43 Fw (5′ ATGTTGTTGCNGCNGAGTC, with N = A/G/C/T) and CTV42 Rv (5′ CTCAAATTGCGRTTCTGTCT 3′, with R = A/G [18], which amplify an internal sequence of the p25 gene with 415 bp. Negative control was the reaction mix without the RT-PCR product and positive control was a miniprep of the p25 gene from T318A isolate, previously cloned into pGEM T-Easy. The nested PCR products were analysed by agarose gel electrophoresis, purified using the DNA Clean & Concentrator-5 Kit (Zymo research Corp., USA) following the manufacturer's instructions and TA cloned into the pGEM-T Easy vector (Promega, USA). The recombinant plasmid was used to transform competent DH5α *Escherichia coli* cells. The cloned amplicon was sequenced in both directions and their identity confirmed by searching the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the blastx algorithm.

Table 1. Amplification procedures used in the detection of the *Citrus tristeza virus* in single aphids.

Methodology used	Aphid	Aphid conservation	RNA extraction methodology and solutions used	Acquisition time	Percentage of aphids with virus (total number of aphids assayed)	References
RT-PCR	AG; MP; TC	N/A	Tris-HCl and EDTA; Gene Releaser	48 h	N/A	[21]
RT-PCR IC/Nested PCR in a single tube	AG (WL)	N/A	N; Triton X-100	2 h	N/A	[23]
Nested RT-PCR	AG (WL)	N/A	N; water	48 h	5 (20)	[35]
Heminested-PCR; nested-PCR	AG; AN; TC; HP (WL)	4 ºC imprinted; room temperature	WhP; Triton X-100	48 h and Field plants	44.4 (9)*	[29]
Nested RT-PCR in a single tube	AG; TA	4 ºC; 70% ethanol	WhP; Triton X-100	Field plants	27 AG (76400); 23 AS (42830); 20 TA (22390)	[6]
Real-time RT-qPCR, TaqMan	AG (W)	N/A	WhP; N; Triton X-100	48 h	34.7 (129)	[25]
Nested RT- PCR	AG (W)	N/A	WhP; Triton X-100	Field plants	19.3 (280)*	[25]
Real-time RT-qPCR	AG; AS (W)	70% ethanol	WhP; N; Triton X-100	Field plants	35.4 (50), AG; 28.8 (207), AS	[7]
Nested RT-PCR	AG (WL)	N/A	WhP; Triton X-100;	48 h	0 (40)	[35]
Nested RT-PCR	AG (WL)	N/A	Homogeneization glass pestle; sodium sulfite	48 h	27 (48)	[35]
Nested RT-PCR	AG (WL)	N/A	Homogeneization glass pestle; PBS-Tween-PVP	48 h	11.1 (18)	[35]
RT-PCR	AG (N/A)	N/A	Trizol	48 h	50 (10)	[15]
Nested RT-PCR	AG (WL)	N/A	Trizol	48 h	42 (38)	[35]

Table 1 continued..

Methodology used	Aphid	Aphid conservation	RNA extraction methodology and solutions used	Acquisition time	Percentage of aphids with virus (total number of aphids assayed)	References
Real-time RT-qPCR	AG (WL)	N/A	Trizol	48 h	85 (20)	[35]
RT-PCR, nested PCR	TC (WL)	70% ethanol	Trizol	Field plants	60 (24)	[18]
Real time RT-qPCR	TC (WL)	N/A	Trizol	24 h	80.6 (196)	[13]

IC, immunocapture; AG, *Aphis gossypii*, AS, *A. spiraecula*; AN; MP, *Myzus persicae*; TA, *Toxoptera aurantii*; TC, *T. citricida*; W, winged; WL, wingless. Aphid RNA extraction: crushing into Whatman paper (WhP) or onto a membrane of either nylon or nitrocellullose (N). N/A, not available.

*When several field and greenhouse test results were reported, only the highest value was indicated.

RESULTS AND DISCUSSION

Detection of CTV in a single aphid by nested RT-PCR

The procedure of squash-capture to immobilize the aphid followed by the isolation of total RNA with a buffer containing Triton X-100 is a current protocol possibly with some limitations, since the rate of CTV detection, estimated by RT-PCR and real-time RT-qPCR, according to the literature, reached only 44.4% (Table 1). Some factors that can contribute to a lower detection of CTV beyond the RNA extraction procedure may be (i) the uneven distribution of CTV in different parts of the plant [31], which depends on the time elapsed after viral infection, (ii) the differential susceptibility of citrus species to distinct CTV isolates [1, 32] or (iii) the time of virus acquisition, which is high at 48 h in comparison with 24 h [25]. Regarding the time elapsed before RNA extraction from aphids, some published protocols refer to the maintenance of crushed samples at 4 ºC for several days [29] to up to 3 years [6] or even the maintenance of samples at room temperature which may also affect the detection rate of CTV in aphids.

In the present protocol, liquid nitrogen was used to efficiently grind aphid tissues and Tri-Reagent solution that preserves totRNA extracted. The protocol was performed after a positive PCR amplification for CTV in the tangor 'Ortanique' plant. A procedure using liquid nitrogen has been previously reported in *Myzus persicae* RNA extraction that persistently transmit the *Potato leafroll virus* (PLRV) [33] with a virus detection rate in 90% of the analysed aphids [34]. Trizol, similarly to Tri-Reagent, is a mixture of guanidine thiocyanate and phenol and the former was described to be the most suitable template for CTV detection by nested RT-PCR and real-time PCR in aphids [35]. The combined use of liquid nitrogen and Trizol or Tri-Reagent was never assayed for RNA extraction from aphids that affect the citrus plants.

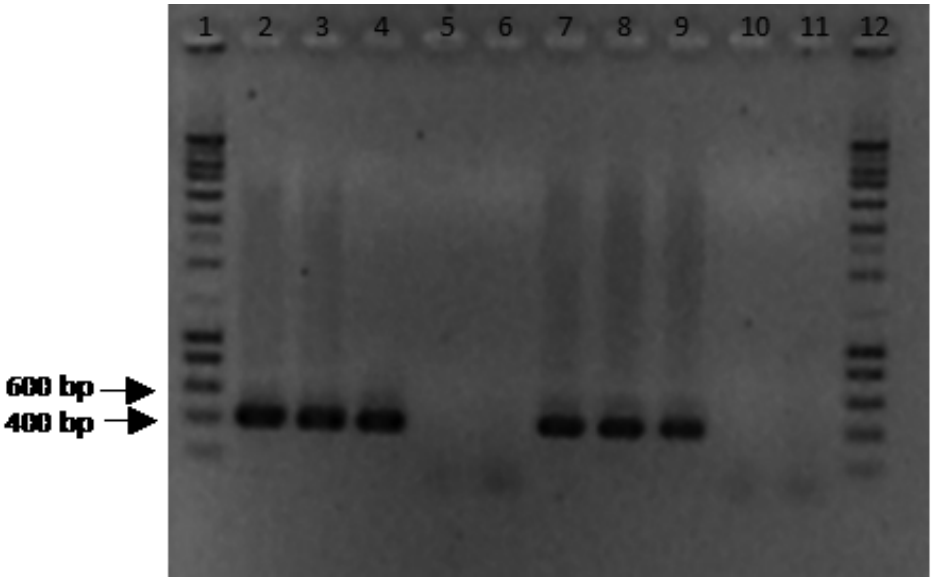

Figure 2. Agarose gel eletrophoresis showing the amplification of the CTV p25 gene partial sequence from single aphids by means of a nested RT-PCR (RT-PCR followed by nested-PCR). Amplification product of 415 bp was obtained using specific internal primers of the p25 gene. 5 µl of the amplified product was loaded. Lanes 1 and 12, NZYDNA ladder III (size marker 200 bp-10000 bp, Nzytech, Portugal); lanes 2 to 3 and 7 to 9, nested RT-PCR amplified product from single *A. gossypii* and *A. spiraecola* aphids, respectively; Lane 4, amplified DNA fragment from the plasmid pGEM T-Easy containing the CTV T318A p25 gene (nested PCR positive control); Lanes 5 and 10, negative control (aphids collected from the virus-free plants); Lane 6 and 11, nested PCR negative control, mix without RT-PCR product.

Degenerate primers were used for the amplification of the entire CTV p25 gene (coat protein) by RT-PCR as well as for the reamplification of an internal sequence of the p25 gene with 415 bp. Amplified fragments corresponding in size to the CTV-*p25* partial sequence were obtained from 70% and 75% of the evaluated single *A. gossypii* and *A. spiraecola* aphids, respectively, fed on a tangor 'Ortanique' (Figure 2). No amplification products were obtained from aphids reared on CTV-free plants (Figure 2).

CONCLUSION

The present protocol using liquid nitrogen and Tri-Reagent ensured an efficient extraction of total RNA from a single aphid. The rate of viruliferous aphids, estimated by nested RT-PCR using degenerate primers, revealed to be 70% and 75% for young adult wingless *A. gossypii* and *A. spiraecola*, respectively, captured on a tangor 'Ortanique' in the field infected with CTV.

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CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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