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REGULAR ARTICLE

Molecular detection of *Grapevine fanleaf Virus* by the isolation of ssRNA and dsRNA from *Xiphinema index*

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Abstract

Xiphinema index is an important grapevine pathogen nematode which also vectors *Grapevine fanleaf virus*. The viral genes involved in transmission by the vector nematode are mapped to the C-terminal residues of RNA2-encoded polyprotein. To recognize viruliferous nematodes, there are some serological and molecular methods. In this study, we extract RNA and dsRNA of the virus, then Reverse transcription-polymerase Chain Reaction was done with virus specific primers to detect virus in its vector. The virus was detected by visualizing the desired 350 and 750 bp gene fragments in electrophoresis. This study reduces the virus detection time to only couple of hours with least imposed charges, and could be employed in transmission experiments as well.

Key words: *Xiphinema index*, grapevine fanleaf virus (GFLV), dsRNA, Reverse transcription-polymerase chain reaction

Introduction

Grapevine fanleaf virus (GFLV) is a member of the genus *nepovirus* in the family *Secoviridae* (ICTV 2015) which is transmitted by Xiphinema species (*X. index*, *X. americanum*) specifically by *Xiphinema index* (Hewitt et al., 1958). The vector nematode acquires GFLV by feeding on the roots of infected vine (Leavitt, 2000). The coat protein of GFLV is determinant for the virus transmission by the vector (Andret-link et al., 2004). According to Taylor and Rasky (1964) during nematode molting, GFLV is lost. Therefore, could not be passed thorough eggs (McFarlane et al., 2002). Virus transmission is characterized by the specificity between GFLV and *X. index*. Xiphinema is known as a dagger nematode and is also considered among the major pests of woody plants (Brown et al., 1995). There are several methods for isolation of the nematode from plants or soil such as Cobb's sieving and gravity, Baermann funnel, Mist extraction and centrifugal flotation methods (Viglierchio and Schmitt, 1983; Evans et al., 1993; Shurtleff and Averre III, 2000). Soil samples are taken close to the vines up to a depth of 60 cm (Quader et al., 2003). Nematode species and soil type are the common factors affect the method of extraction (Brown and Boag, 1988). Nematode infected soil samples should be handled carefully, due to the susceptibility of Xiphinema species. Missing nematodes influences the extracted viral RNA concentration. Staudt (1997) used sieving technique to extract nematodes from soil (Flegg, 1967; Bleyer and Kassemeyer, 1992). He also applied ELISA (Bioreba Basel, Switzerland) test for detection of

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GFLV in *X. index* in the infected roots (Staudt, 1997).

The present study was conducted with an aim to detect GFLV in its vector nematode by molecular method. Soil sample for this purpose was prepared from up to 50 cm depth close to the GFLV-infected vine.

Material and methods

Sampling and nematode isolation from the soil.

Sampling was conducted from the rhizosphere of GFLV-positive vine which was previously tested for. Sampling was carried out from different depth (5, 10, 15, 20, 30, 40, 50 cm) of the soil. Isolation of the *Xiphinema index* was done following the Cobb's sieving method (Chawla and Prasad, 1974) and thereafter was morphologically identified.

Total RNA extraction from nematodes

Total RNA was extracted from 10-15 isolated nematodes according to Rouhani et al (1993). Isolated nematodes were slashed with sterile scalpel in 300 µl of RNA extraction buffer (21.7 g/l $K_2HPO_4.3H_2O$, 4.1 g/l KH_2PO_4, 100 g/l Sucrose, 1.5 g/l BSA, 20 g/l PVP, 5.3 g/l Ascorbic acid, pH.7.6) and then centrifuged at 11800 rpm for 15 min. The pellet was suspended in 200 µl of s buffer containing 10mM EDTA, 50mM Tris (pH. 8) and 0.1% Mercaptoethanol. Thereafter 25 µl of 10% SDS was added and incubated at 60°C in a water bath for 10 min. Then 80 µl 5M potassium acetate was added in and kept in ice for 30 min; subsequently centrifuged. Upper layer was transferred into a new microtube and 0.1× volume of the solution, 3 M sodium acetate (pH 5.2) and at the same volume isopropanol was added and placed at -20°C for 1 hour. After a 10 min centrifugation at 11800 rpm (4°C), upper layer was removed and the pellet washed in 80% ethanol. Extraction was terminated by a 4 min centrifugation at 11800 rpm. The pellet was dissolved in 50 µl ddH_2O and maintained at -20°C.

DsRNA extraction from nematode

To study the virus replication in the vector nematode, we tried to extract viral dsRNA. 10-15 nematodes were chopped up in 300 µl of dsRNA extraction buffer (200 mM Tris, 500 mM NaCl, 10 mM $MgCl_2$, 3% SDS, 10% Ethanol, 1% 2-Mercapto ethanol). After a 5 s vigorous vortex, the microtubes were incubated at 37°C water bath for 10 min. Subsequently, 1:1 chloroform was added and centrifugation was done at 11300 rpm for 20 min at 4°C. The upper layer transferred into a new microtube and chloroform was added and followed by a 7 min centrifugation at 11300 rpm (4°C) (this step could be repeated to remove leftover tissue particles). Upper layer was placed into a new tube; 0.2 ml absolute ethanol was added per 1 ml of the supernatant and followed by a 5 min centrifugation. In order to enmesh the nucleic acid, 15 mg CF-11 cellulose (Sigma-Aldrich Corp., Schnelldorf, Germany) was added in each tube, and kept at room temperature for 5 min. After a 7 min centrifugation at 11300 rpm (4°C), the upper phase was removed and 1 ml washing buffer (1X STE [10mM Tris-HCL, 1mM EDTA, 100mM NaCl]/16% ethanol) was added into the tubes. Centrifugation for 5 min separated the pellet and aqueous phases. Removing the transparent phase, washing step was repeated. To elute the pellet after removing upper phase, 150 µl 1X STE was added and incubated at room temperature for 5 min. After 5 min centrifugation at 11300 rpm, upper phase was transferred into new tubes. Absolute ethanol was added in 2× volume of the solution in each tube and kept at -20°C for an hour. To precipitate the expected dsRNA, the microtubes were centrifuged at 11300 rpm for 20 min. Pellet was dissolved in 30 µl ddH_2O and was treated by RNase A in 300 mM NaCl. Then it was incubated at 37°C for 30 min. The RNase A treatment was done to eliminate ssRNAs. Subsequently, it was maintained at -20°C.

RT-PCR

Reverse transcription was conducted to confirm the extracted viral RNA, by the use of specific primers (Table 1). cDNA synthesis was performed by utilizing RevertAid Reverse Transcriptase (Thermo Fisher Scientific Inc., Waltham, USA), accompanied with 1X RT buffer, 20 U RNase inhibitor, 1mM dNTPs and reverse specific primer were used. A 2.5 µl aliquot of each RNA sample was preheated at 80°C for 5 min, and followed by 60 min incubation at 42°C and then incubation at 70°C for 10 min. PCR amplification of first strand cDNA was performed by using one unit of *Taq* DNA Polymerase (SinaClon Co., Tehran, Iran) in a total reaction mixture of 20 µl containing 1X reaction buffer, 1.5 mM $MgCl_2$, 1 mM dNTPs and the 0.25 pmol of each primer. The optimized reaction conditions were as follows: initial denaturation at 94°C for 1 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s and a final elongation step at 72°C for 7 min.

Table 1. Specific viral primers used for RT-PCR.

Primer name	Primer Sequences	Expected fragment size(bp)
Coat protein [a]	F: 5′-GAACTGGCAAGCTGTCGTAGAA-3′	
Cp433	R: 5′- GCTCATGTCTCTCTGACTTTGACC-3′	350
Cp912		
	F: 5′-ACACAAACAGCAGTCTGATGGA-3′	
Satellite [b]	R: 5′-GCTGAGGAAAAACTGTTCGGA-3′	750
Gf750		
Gr 750		

a; Nucleotide positions correspond to the genomic RNA3 of GFLV (Izadpanah et al. 2003).
b; Our designed primers.

Results

Nematode isolation from soil

Sieving the soil was concluded in finding different nematodes such as *Longidorus* and *Xiphinema* species, specifically *Xiphinema index* (Fig. 1). To study GFLV vector, we selected *X. index* populations. Morphological studies of the extracted nematode revealed characteristics of the stylet (known as odontostylet), dorylaimid esophagus, Z-organs in female nematodes demonstrated the identity of nematode (Fig. 2). The mentioned characteristics were evident in the nematodes which were selected for GFLV extraction.

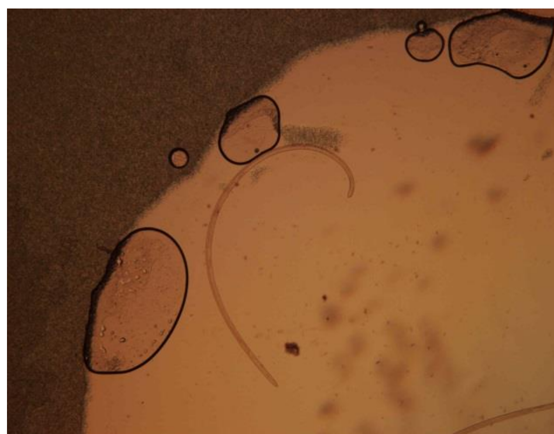

Fig. 1. *Xiphinema index* isolated from the other extracted nematodes from soil by Cobb′s sieving method.

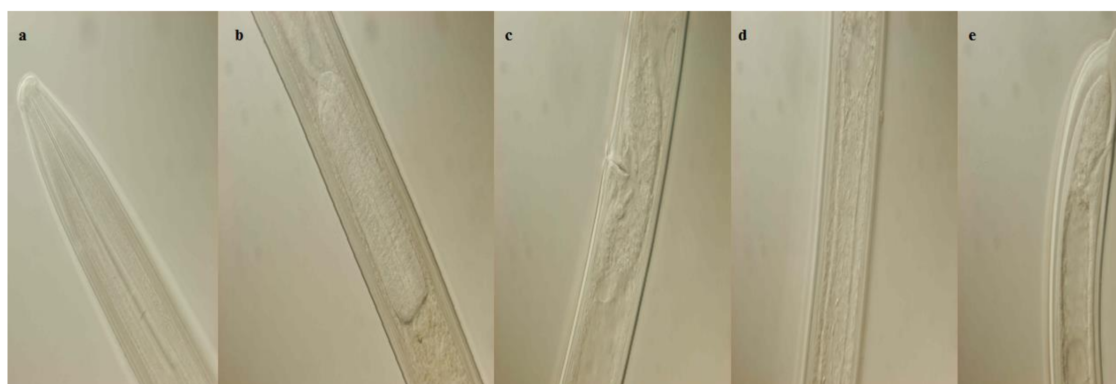

Fig. 2. Morphological analysis of *X. index*. a; Stylet (odontostyle), b; Dorylaimid esophagus, c; lateral view of vulva of a female, d; Z-organs of a female nematode, e; Tail region of an adult female nematode with an anus and small mucron at the end.

DsRNA electrophoresis and RT-PCR

As it was expected, because of low concentration of dsRNA in nematodes, we did not observe any sharp bands on 1.6% agarose gel. According to the previous studies, no virus replication was report in GFLV vector nematode. However, RT-PCR with *Grapevine fanleaf virus* coat protein and satellite primers on total RNA and dsRNA produced the expected 350bp fragment for coat protein primers and 750bp for satellite ones. Gel electrophoresis of RT-PCR products (on dsRNA template) showed GFLV replication in ector nematode (Fig. 3, 4). DsRNA is the intermediate replication form of the virus, so it was predictable not to get appropriate bands for dsRNA in vector nematode. But RT-PCR on dsRNA amplified the template and demonstrated virus replication in vector.

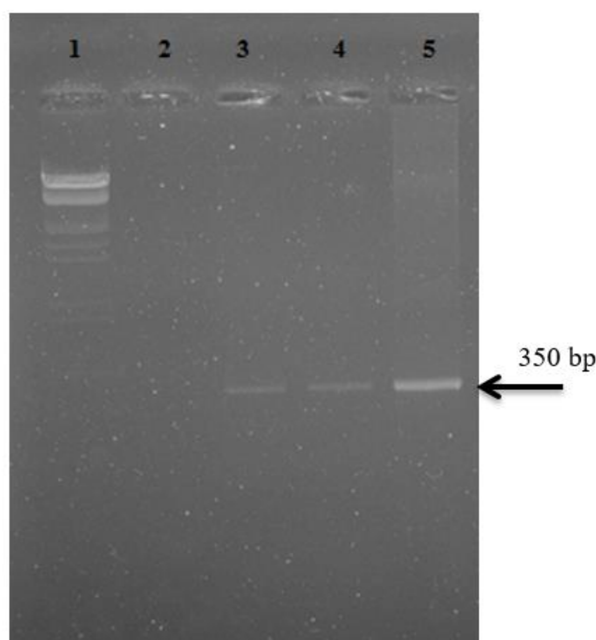

Fig. 3. 1% agarose gel electrophoresis of RT-PCR products performed on extracted total RNA. 1; Lambda DNA-*Eco*RI plus *Hind*III size marker, 2; Negative control: PCR master mix without any template DNA, 3; Amplified coat protein from total RNA extracted of *X. index*, 4; Amplified coat protein from total RNA extracted of *Chenopodium amaranticolor*, 5; Positive control: Amplified coat protein from a plasmid contains GFLV coat protein.

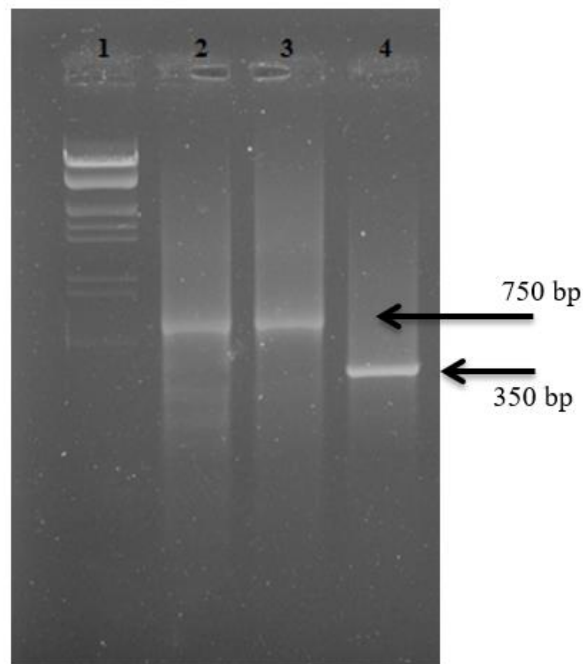

Fig. 4. 1% agarose gel electrophoresis of RT-PCR products performed on extracted dsRNA from *X. index* . 1; Lambda DNA-*Eco*RI plus *Hind*III size marker, 2; Satellite fragment amplified from the extracted dsRNA of *X. index*, 3; Satellite fragment amplified from the extracted dsRNA of *Chenopodium amaranticolor*, 4; Coat protein fragment amplified from the extracted dsRNA of *X. index*.

Discussion

Molecular approaches in comparison with serological assays are more accurate and rapid for detection of viruliferous nematodes. An efficient transmission of GFLV isolates by different populations of *X. index* has previously been reported (Catalano et al., 1989; 1991; 1992). *X. index* can retain GFLV for at least 4 years (Demangeat et al., 2005). Based on Demangeat et al. (2010) findings with seven *X. index* lines from diverse geographical locations, it is reasonable to expect that most, if not all, *X. index* populations are able to transmit natural GFLV isolates. Therefore, in a season that plant material is not accessible, RNA extraction from its vector could be useful in viral infection recognition. We extracted viral RNA from vector nematode by the method which was applied for extraction of the virus from plants. By using this, we could reduce the study cost. It is a time-saving method compared to the serological methods like ELISA (Staudt, 1997). Also, for performing ELISA, roots were needed

but in our method we isolated nematodes from soil.

Demangeat et al. (2004), extracted total RNA based on use of glass beads in a Mixer Mill MM 200 shaker (Retsch) to disrupt extracted nematodes and total RNA extraction with the RNeasy plant mini kit (Qiagen). But in our method no kit or phenol, isoamilalchol treatment was used. So, the cost was reduced. It is true that applying kits could be time-saving, but would be cost-consuming especially for under developed countries.

To identify the RNA identity, RT-PCR was carried out by GFLV coat protein and satellite specific primers which is not time consuming as the serological and electron microscopy approaches (Roberts and Brown, 1980; Wang and Gergerich, 1998). RT-PCR and RFLP was applied to characterize specific isolates of virus (Demangeat et al., 2004; Boutsika et al., 2004). We also used dsRNA extraction for the first time in order to test virus replication in its vector nematode. This would be a rapid method in virus recognition and it was tested on virus-infected. Also, dsRNA would be stable for long time at laboratory conditions. As mentioned in results, no sharp bands of dsRNA were observed; however, RT-PCR performed by RNase A treated dsRNA produced the expected gene fragment. No production of sharp bands on agarose gel could be because of the low population of isolated *X. index* used in dsRNA extraction or low concentration of dsRNA in vector nematode. The amount of dsRNA drastically depends on the extraction method and plant species, therefore, developing more effective and practical extraction method is necessary to enmesh the low quantity dsRNA molecules (Li et al., 2007). This study could be applied for virus detection in vineyards and virus-nematode interaction studies (Demangeat et al., 2004). Due to the RT-PCR sensitivity, this technique can be used in epidemiological studies, too (Esmenjaud et al., 1992; Taylor and Brown, 1997; Vuittenez and Legin, 1964).

Conclusions

In this research, we extracted *Grapevine fanleaf Virus* RNA by optimized protocol for viral infected plants. We detected virus without using any commercial kits. We detected GFLV by total RNA extraction from vector nematode for the first time in Iran and also demonstrated that the virus was replicated in *X. index* by dsRNA isolation which was thought not to have replication in its vector nematode.

Authors' contributions

A. D. Khabbazi conducted the study and prepared the manuscript. N. S. Bashir supervised all the research activities and critically evaluated the manuscript. E. Z. Asl contributed to nematode isolation and identification. S. D. Khabbazi contributed to molecular assays and manuscript preparation.

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