JOURNAL OF APPLIED Plant Biology 2023, Vol:1,No:1, pp29-35

# Isolation of Double-stranded (ds) RNA from Virus-symptomatic Soybeans and Molecular Detection of Soybean mosaic virus (SMV) in North of Iran

**Javad Ramezani Avval Reiabi**[1]

**Jihad Agricultural organization of South Khorasan province, Birjand, IRAN.**

*Corresponding author: jramezan@utk.edu

Original Article Received: 10 November. 2023, Revised: 15 November 2023 . Accepted:17 November. 2023, ePublished: 18 November . 2023

## Abstract

**Background:** There are a large number of pathogens which infect soybean crop. Viruses are one of these destructive agents. DsRNA extraction could be a reliable method to confirm the presence of a virus in a plant. To date, several attempts were conducted to extract dsRNA from different plants, but not from soybean.As the symptoms of nutritional deficiency and viral diseases are very similar, intending to assess the presence of the virus (es), and particularly SMV, in soybean farms in the north of Iran, the dsRNA from soybean fresh leaf tissues were extracted.

**Materials and Methods:** In this study, the soybean samples were collected from the north of Iran followed by extraction of dsRNA using CF-11 column chromatography. DsRNA was run on 1% agarose gel and SMV was recognized by RT-PCR. The PCR product was bidirectionally sequenced.**Results:** By using changes in other methods, we successfully extracted viral dsRNA from soybean. To confirm the dsRNA structure of the extracted molecules they were subjected to DNase I treatment; according to the results, dsRNA bands still existed after DNase I treatment which confirmed the RNA structure of the isolated molecules. According to the RT-PCR results, dsRNA extracted from SMV infected-soybean were confirmed through virus Helper Component Proteinase (HC-Pro) sequence amplification which verified the integrity of the extraction method.

**Conclusions:** In this study, it was demonstrated that dsRNA is a favorite tool for constructing of cDNA library, as it is more stable and resistant to RNase degradation in comparison with ssRNA, when the total RNA extraction is not feasible due to the low titer, instability of particles or the presence of inhibitors.

**Keywords:** Double-stranded RNA; Polymerase Chain Reaction; Soybean; soybean mosaic virus

## 1.Introduction

Soybean [Glycine max (L.) Merr., 2n = 40], an annual and autogamy plant species belonging to the Fabaceae, which provides both protein and oil for human nutrition is the most important legume crop worldwide. Different biotic and abiotic agents can cause significant losses in crops most particularly in soybean.

Viruses are including the destructive agents that make an impact on the quality and quantity of soybean. Plant viruses are known to cause considerable losses in crop yield, quality of plants, and plant products around the world. They pose a particular risk as they are difficult to detect and identify (11). Unlike other plant pathogens, there are no direct methods available yet to control viruses and, consequently, the current measures rely on indirect tactics to manage the diseases. Hence, methods for the detection and identification of viruses play a critical role in the management of the disease.

Accurate diagnosis of diseases is the first important step for any crop management system. With virus diseases, plant treatment after infection often does not lead to effective control. Accordingly, the diseases are managed more effectively if control measures are applied before infection occurs. The use of healthy plant propagation material is among the most effective approaches to adopt by farmers. One of the elements essential for successful certification programs to produce such propagation material is the availability of sensitive diagnostic methods (16). Due to the increased worldwide movement of germplasm through seed and other propagative material in global trade and agriculture, diagnosis of viruses in these materials assumes greater importance for national quarantine services to ensure the safe movement of germplasm across the borders.

More than 111 viruses/strains, belonging to different virus genera and families can infect soybean in field conditions. Soybean mosaic virus (SMV), one of the most economically damaging viruses and a member of the genus Potyvirus in the family Potyviridae, is a devastating soybean pathogen found in all soybean-growing regions and reduces soybean yields and seed quality worldwide (13).

The first step to control viral diseases is to identify the virus. Double-stranded RNA (dsRNA), is an indicator of the presence of viruses in plants (14). Considering the stable structure of dsRNA in comparison to RNA molecules, dsRNA extraction is a liable tool and quick method to demonstrate the viral genome sequence (9-7) before processing a serological and molecular procedure that may be time-consuming and costly. The majority of the RNA, some of ambisense and DNA viruses are producing dsRNA molecules which are the copies of the virus genome. As an advantage, the thermodynamic stability of the dsRNA molecule and its resistance to RNase degradation makes it an excellent molecule for virus characterization when virus purification is not feasible due to low titer, instability of particles, or the presence of inhibitors. A disadvantage of this procedure is that would generally not be used to process a large number of samples at a time. Also, since dsRNA is related to replication of the virus, the titer of the dsRNA molecule (s) may increase or decrease in the plant at certain times of the year, thereby influencing detection. Summer is generally an unfavorable time of the year for plant growth and virus replication due to the heat. It's also important to note that this technique doesn't work for all positive ssRNA virus groups, especially the ilarviruses. It also doesn't work for the ambisense tospoviruses (Tomato Spotted Wilt and Impatiens Necrotic Spot viruses) but works well for the cucumoviruses, alfamoviruses, tobamoviruses, potyviruses, potexviruses, and closteroviruses. It is important to note that although most uninfected plants do not produce dsRNAs, there are some, like the cucurbits, that produce indigenous dsRNA molecules.

Several efforts have been done to extract dsRNA from many different plants such as barley (18), grapevine (2-15, 21), sour cherry (25), rice (19), potato (6), strawberry (12), European mountain ash (5), however, the procedure is either time consuming or relatively costly; Which sometimes is not affordable for some laboratories or student works without financial support.

Several methods have been reported to extract dsRNA (4-8, 17, 20, 23), however, the extraction time, tissue amount, infected plant species, viral group, and extraction costs have been important within the time. In most of the established methods, dsRNA is extracted through a cellulose column using buffers containing different percentages of ethanol (3-24) and phenol from infected plant tissues (18-24).

As sometimes the symptoms of a nutrient deficiency are very similar to viral symptoms that can confuse explorer, extracting dsRNA from viral-suspected plants can be very helpful. Hence, this study was carried out to extract dsRNA from soybean samples with viral symptoms and to prove the presence of the virus (es) in soybean.

## 2. Materials and Methods

### 2.1. Sample Collection

The samples of viral-symptomatic soybean leaves were collected from soybean growing farms in the north of Iran, Golestan, and Mazandaran provinces and stored in plastic bags at 4 ºC until use. Inoculations were done on different varieties of soybean including Williams 82, Essex, Amir, Sahar, Saman, and Gorgan 3, as well as on plants belong to several families.

### 2.2. DsRNA extraction

Extraction of dsRNA was conducted, in-room temperature (RT) conditions and 2ml tubes, from healthy and viral-symptomatic soybean leaves by homogenization of 0.2 g fresh leaf tissue in 1000 µl STE lysis buffer [500 µl of STE buffer pH 6.8 (200 mM Tris, 500 mM NaCl, 1mM EDTA), 500 µl of Saturated phenol, 100 µl of SDS 10% and 100 µl of Bentonit 2%]. After a one min vigorous vortex, centrifugation was done at 5,100 × g for 3 min. 400 µl of the supernatant was transferred into a new tube.

Thereafter, 440 µl of STE buffer, 160 µl of absolute ethanol, and 10 mg of CF 11 cellulose (Sigma-Aldrich Corp., Schnelldorf, Germany) was added and vigorously mixed by vortex for 10 s. After a 3 min centrifuge at 5,100 × g, the upper phase was removed and 1000 µl washing buffer (STE /16% ethanol) was added and vigorously mixed. After centrifugation at 5,100 × g for 3 min, the pellet and transparent aqueous phases were produced. The aqueous phase was removed and the washing step repeated. 500 µl of eluting buffer (STE) was added to the pellet and mixed vigorously by vortex for 5 s, followed by centrifugation at 5,100 × g for 3 min 400 µl of supernatant was transferred into a new tube, then 30 µl of Sodium Acetate *(3 M, pH 5.5) and 1200* µl of chilled absolute ethanol was added. The solution was gently vortexed and to precipitate the expected dsRNA (s), the tube was centrifuged at 8,490 × g for ten min. Afterward, the solution was removed carefully and the pellet was incubated at room temperature for 1 min. Eventually, dsRNA was dissolved in 35 µl of Nuclease-*Free Water* and maintained in -20 ºC (Fig. 1).To confirm the isolated dsRNA, agarose gel electrophoresis and molecular detection were carried out.

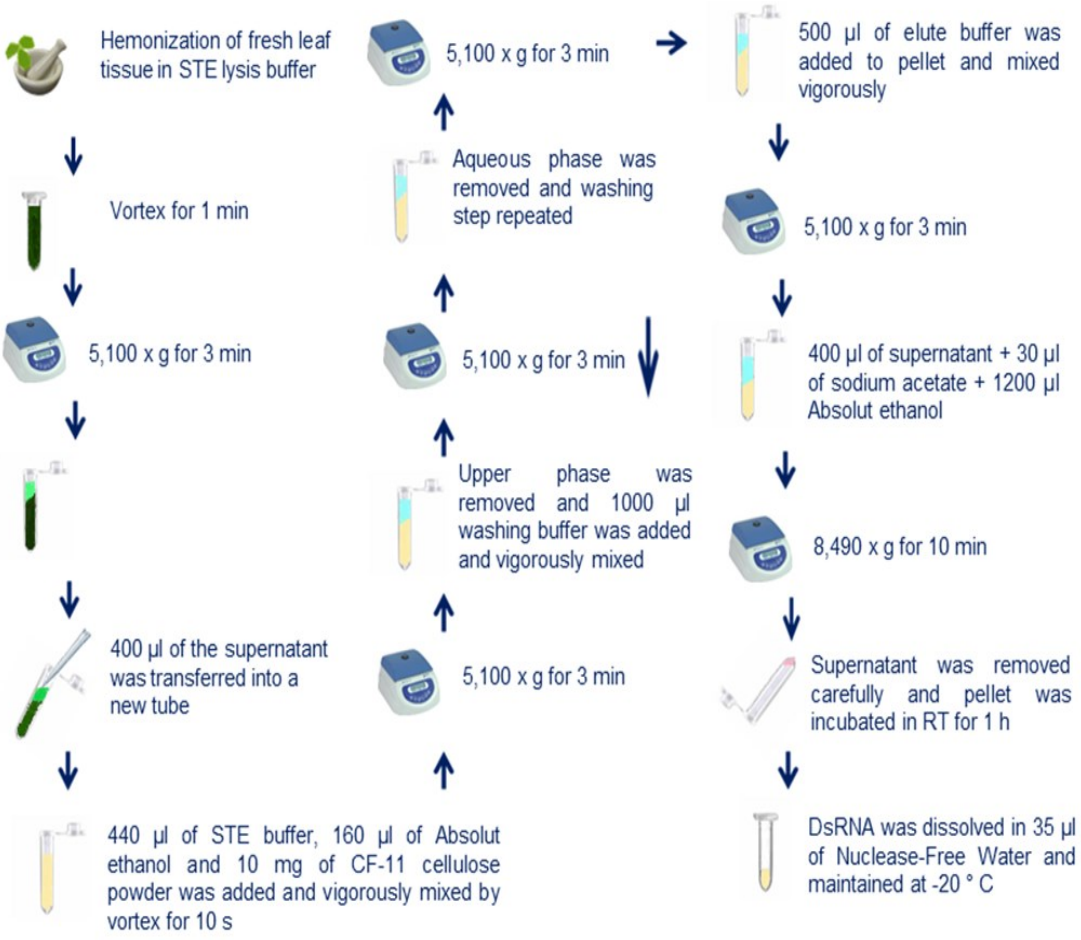


**Fig. 1** The schematic diagram of the viral dsRNA extraction method from infected leaves of soybean plants

## 2.3. DNase I treatment

To eliminate the possible host DNA contamination, extracted dsRNA were subjected to DNase I kit (DNAbiotech, Life Science, KalaZist, Cat #: DB9717-K, Iran). To perform the reaction, 8 µl of dsRNA, 1 µl of 10X reaction buffer (200 mM Tris HCL, pH 8.3, 20 mM MgCl2), and 1 µl of DNase I were added to an RNase free PCR tube. The reaction was mixed gently, and incubated for 5 min at room temperature. Then, 1 µl of stop solution (50 mM EDTA) was added to the mixture to bind calcium and magnesium ions and to inactivate the DNase I. 5 µl of the enzyme-treated dsRNA was electrophoresed on 1 % agarose gel (1X Tetraborate buffer) stained with safe nucleic acid stain (DNA green viewer, pars tous) and visualized using integrated electrophoresis system (All in One Electrophoresis System, SabzBiomedicals™, SB-14001) by safe blue light. 1 kb DNA ladder RTU GeneDirex® (Life Science Technology Inc., Seoul, Korea) was used as a size marker to identify the desired band.

## 2.4. SMV detection through RT-PCR

RT-PCR was performed by using Potyviruses degenerate primers, [helper component-proteinase (HC-Pro) and cylindrical inclusion (CI)] and SMV coat protein (CP) specific primer pair to confirm the identity of extracting dsRNA (Table 1). To synthesis, the cDNA, 3 µl dsRNA, and 1 µl random Hexamer primer (Yekta Tajhiz Azma Com., YT4550, Iran) was mixed and adjusted with ribonuclease-free water to the total volume of 12 µl and preheated at 95 ºC for 5 min and then chilled on ice and briefly centrifuged. It was added in 8 µl the prepared solution including 2 µl Thermo-resistant H Minus Moloney Murine Leukemia Virus (M-MuLV) Reverse Transcriptase (pars tous biotechnology, Mashhad, Iran), 4 µl 5 X RT buffer accompanied with the enzyme and 2 µl 10 mM dNTP mix and mixed by pipetting gently up and down. The mixture was incubated for 10 min at 25 ºC and then 60 min at 47 ºC. Eventually, the reaction was stopped by heating at 70 ºC for 10 min.

PCR amplification of the first-strand cDNA was performed in a total reaction mixture of 25 µl containing 12.5 µl Taq DNA Polymerase 2x Master Mix RED (Amplicon, Denmark), 0.5 µl of each forward and reverse primers (10 pmol/ µl), 2 µl cDNA and 7.5 µl PCR-grade H2O. PCR with Potyviruses degenerate primers was performed in 3-step cycling as follows: initial denaturation at 94 ºC for 3 min, followed by 40 cycles of denaturation at 94 ºC for 30 s, annealing at 40 ºC for 30 s, and extension at 72 ºC for 1 min and a final elongation step at 72 ºC for 10 min. Amplification of cDNA with SMV specific primers (CP) also carried out in 3-step: initial denaturation at 94 ºC for 2 min, 35 cycles of denaturation at 94 ºC for 40 s, annealing at 50 ºC for 40 s, and extension at 72 ºC for 90 seconds and a final elongation step at 72 ºC for 10 min.

| Primer name | Sequence* (5′-3′) | Annealing temperature (°C) | PCR product size (bp) | References |
|---|---|---|---|---|
| CIfor | GGIVVIGTIG-GIWSIGGIAAR TCIC | 40.0 | 700 | (10) |
| CIrev | ACICCRTTY-TCDATDATRTT IGTIGC | | | |
| HPfor | TGYGA-YAAYCARYTI GAYIIIAAYG | 40.0 | 700 | (10) |
| HPrev | GAIC-CRWAIGARTCI AIIACRTG | | | |
| CPf | TAGAGTGG-GAYAGRAGCA AAG | 50.0 | 1272 | (1) |
| CPr | TTCTCCCTGCY ATTCATAAAC | | | |

## 2.5. Sanger sequencing

To confirm the RT-PCR result, the PCR product was bidirectional sequenced (Macrogen, South Korea). The sequence was subjected to nucleotide BLAST program using NCBI BLAST tools.

## 2.6. Sequence Accessions and Phylogenetic Analysis.

Database accessions for sequences used here were from Iran and other several countries. The Plum Pox Virus [PPV; (GeneBank: M92280.1)] was used as an outgroup. The percentage of nucleotide substitution was obtained by the Maximum likelihood statistical method and Tamura-nei model (22). A phylogenetic tree was constructed using the Neighbor-Joining function of Clustal W using the Mega-X software and bootstrapped with 1000 replicates.

## 3. Results

The loading of the extracted dsRNA on 1% agarose gel showed several bands where an upper band (~ 9.5 kb) was presumably related to SMV (Fig. 2A) and dsRNA was unaffected by DNase I. The virus dsRNA from soybean fresh leaves was identified by RT-PCR. Agarose gel electrophoresis of the DNA products generated from RT-PCR using HC-Pro and CI degenerate primer and a CP specific primer

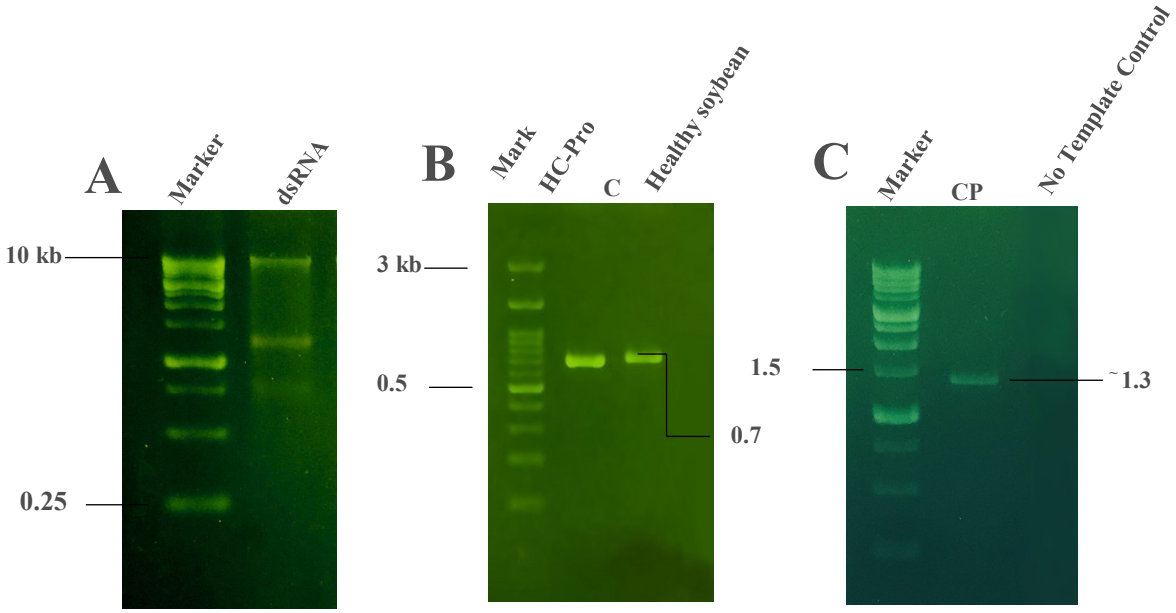


**Fig. 2** Agarose gel electrophoresis (1 %) pattern of viral dsRNA extracted from symptomatic soybean (**A**); RT-PCR with potyviruses degenerate primers. Marker: 100 bp ExcelBand™ DNA ladder, Smobio Technology Inc., Hsinchu, Taiwan (**B**) and RT-PCR with SMV coat protein-specific primer. Marker: 1 kb DNA ladder RTU GeneDirex®, Life Science Technology Inc., Seoul, Korea (**C**).

Of potyviruses revealed the presence of a ~ 700 bp (Fig. 2B) and ~ 1300 bp (Fig. 2C) fragment, respectively, while, no amplicon obtained for the non-template control (NTC) and healthy plants (Fig. 2B&C), indicating successful detection of SMV in virus-symptomatic soybean samples. This was similar to the results that came from total RNA as a template for RT-PCR.

The nucleotide sequence data of the HC-Pro coding region came from macrogen was compared to other viral sequences using the BLAST computer program in GenBank. The results showed that the SMV segment amplified by HPfor/HPrev primer pair was of 700 bp, and the sequence similarity with other sequences in GenBank ranged from 92 % to 94 % where the lowest similarity was found with SMV strain G5 (AY294044) and SMV strain G6 (FJ640980) from Korea, and the highest similarity of 94% was found with SMV isolate XFQ001 (KP710871) and SMV strain G2 (KP710874) from China. To determine the relationship of this isolate with other SMV isolates, a phylogenetic tree was constructed. The constructed tree was shown that this isolate has no relationship with others already reported SMV isolates from Iran (Fig. 3).


**Fig. 3** The constructed phylogenetic tree related to the **HC-Pro** gene of the **SMV** isolate under study here and 20 SMV isolates from different countries. Numbers on or near branches represent bootstrap values over 50 % as calculated by maximum likelihood. 0.2.

## 5. Discussion

There are some works of literature published on plant virus (es) isolated by dsRNA methods. To date, several studies were conducted in isolation of dsRNA from several plant species such as barley (18), grapevine (2), sour cherry (24), rice (19), potato (6); however, no reports were found in which Virus dsRNA could be extracted because organic reagent enables protein denaturalized, and dsRNA was directly extracted from soybean plants. dsRNA was combined with cellulose powder in 15% ethanol, and the purified dsRNA was obtained after DNA and ssRNA were completely washed off (18). So, in this study, we used Sodium dodecyl sulfate (SDS) 10%, where is a synthetic organic compound to denature the protein, and cellulose powder in 16% ethanol. There are many dsRNA extraction methods which majority of them use a relatively large amount of plant tissue and are time-consuming and costly (15).

In another study, self-prepared cellulose powder-based micro-spin columns were used to purify dsRNA molecules that effectively reduced the

expenses and extraction time, however, the extraction was carried out on some limited plants that could be fracturable, as extract compounds from different hosts enable impress the dsRNA extraction procedure (19). In this study, for the first time, we extracted dsRNA from 0.5 g fresh leaf tissue of soybean and squash (not published) in a short period (30 min). According to the earlier studies, plant host and virus are considered as the principal factors interfering with ribonucleic acid extraction (15).

Based on our experiences from this study, using dsRNA as a template compared with total RNA, to construct a cDNA library is an acceptable way to sequence the SMV genome that originated from different geographical regions.

## 6. Conclusions

In this study, the system of dsRNA extraction from soybean plants and its identification using agarose gel electrophoresis followed by RT-PCR was well revealed that type of plant and concentration of virus are two important factors for dsRNA isolation successfully. We could not be able to isolate dsRNA from soybean and cucurbits dried leaves, so only fresh leaves from the young leaves of in vitro plantlets or field-grown plants were appropriate for dsRNA extraction. Using dsRNA for cDNA library construction and sequence, compared with total RNA, obtained more reliable results, as sometimes non-specific PCR product from the total RNA cDNA library may interfere to achieve the desired result.

## References


1. Ahangaran A, Habibi MK, Mohammadi GH, Winter S, García-Arenal F. Analysis of Soybean mosaic virus genetic diversity in Iran allows the characterization of a new mutation resulting in overcoming Rsv4-resistance. *Journal of General Virology*. 2013;**94**(11):2557-68. DOI: 10.1099/vir.0.055434-0
2. Azzam OI, Gonsalves D, Golino D. Detection of dsRNA in grapevines showing symptoms of rupestris stem pitting disease and the variabilities encountered. *Plant Disease*: An International Journal of Applied Plant Pathology. 1991;**75**(9):960-964. DOI: 10.1094/PD-75-0960
3. Barber R. The chromatographic separation of ribonucleic acids. *Biochim. Biophys. Acta.* 1966;**114**(2):422-4. DOI: 10.1016/0005-2787(66)90326-1
4. Bar-Joseph M, Rosner A, Moscovitz M, Hull R. A simple procedure for the extraction of double-stranded RNA from virus-infected plants. *J. Virol. Methods*. 1983;**6**(1):1-8. DOI: 10.1007/978-1-4939-1743-3_3
5. Benthack W, Mielke N, Büttner C, Mühlbach HP. Double-stranded RNA pattern and partial sequence data indicate plant virus infection associated with the ringspot disease of European mountain ash (Sorbus aucuparia L.). *Arch. Virol.* 2005;**150**(1):37-52. DOI: 10.1007/s00705-004-0397-5
6. Blouin AG, Ross HA, Hobson-Peters J, O'Brien CA, Warren B, MacDiarmid R. A new virus discovered by immunocapture of double-stranded RNA, a rapid method for virus enrichment in metagenomic studies. *Mol. Ecol. Resour.* 2016;**16**(5):1255-63. DOI: 10.1111/1755-0998.12525
7. Decker CJ, Parker R. Analysis of double-stranded RNA from microbial communities, identifies double-stranded RNA virus-like elements. *Cell reports*. 2014;**7**(3):898-906. DOI: 10.1016/j.celrep.2014.03.049
8. DePaulo JJ, Powell CA. Extraction of double-stranded RNA from plant tissues without the use of organic solvents. *Plant disease*. 1995;**79**(3):246-248. DOI: 10.1094/pd-79-0246
9. Espach Y, Maree HJ, Burger JT. The complete genome of a novel endornavirus assembled from next-generation sequence data. *J. Virol.* 2012**; 86(23):13142. DOI:** 10.1128/JVI.02538-12
10. Ha C, Coombs S, Revill PA, Harding RM, Vu M, Dale JL. Design and application of two novel degenerate primer pairs for the detection and complete genomic characterization of potyviruses. *Arch. Virol*. 2008;**153**(1):25-36. DOI: 10.1007/s00705-007-1053-7

and complete genomic characterization of potyviruses. *Arch. Virol*. 2008;**153**(1):25-36. DOI: 10.1007/s00705-007-1053-7
11. Hadidi A, Czosnek H, Barba M. DNA microarrays and their potential applications for the detection of plant viruses, viroids, and phytoplasmas. *J. Plant Pathol.* 2004;**86**(2):97-104. DOI: jstor.org/stable/41998178
12. He LI, Dai HY, Zhang ZH, Gao XY, DU GD, Zhang XY. Isolation and identification of virus dsRNA from strawberry plants. ***Agric. Sci. China.*** 2007;**6**(1):86-93. DOI: 10.1016/S1671-2927(07)60020-0
13. Ishibashi K, Saruta M, Shimizu T, Shu M, Anai T, Komatsu K, Yamada N, Katayose Y, Ishikawa M, Ishimoto M, Kaga A. Soybean antiviral immunity conferred by dsRNase targets the viral replication complex. *Nat. Commun.* 2019;**10**(1):1-10. DOI: 10.1038/s41467-019-12052-5
14. Jordan R.L. Diagnosis of Plant Viruses Using Double-Stranded RNA. In: Zimmerman R.H., Griesbach R.J., Hammerschlag F.A., Lawson R.H. (eds) Tissue culture as a plant production system for horticultural crops. *Current Plant Science and Biotechnology in Agriculture*, Springer, Dordrecht. 1986;**2**: 125-134. DOI: 10.1007/978-94-009-4444-2_10
15. Khabbazi AD, Bashir NS, Khabbazi SD, Ighani H. Extraction and molecular detection of viral dsRNA from different infected plants. *J. Sci. Agric*. 2017;**1**:197-203. DOI: 10.25081/JSA.2017.v1.54
16. Makkouk KM, Kumari SG. Molecular diagnosis of plant viruses. *Arab J. Pl. Prot.* 2006;**24**(2):135-138.
17. Marais A, Faure C, Bergey B, Candresse T. Viral double-stranded RNAs (dsRNAs) from plants: alternative nucleic acid substrates for high-throughput sequencing. *Methods Mol Biol*. Humana Press, New York, NY. 2018. p. 45-53. DOI: 10.1007/978-1-4939-7683-6_4 PMID: 29492885
18. Morris TJ, Dodds JA. Isolation and analysis of double-stranded RNA from virus-infected plant and fungal tissue. *Phytopathology*. 1979;**69**(8):854-8. DOI: 10.1094/Phyto-69-854
19. Okada R, Kiyota E, Moriyama H, Fukuhara T, Natsuaki T. A simple and rapid method to purify viral dsRNA from plant and fungal tissue. Journal of General Plant Pathology. 2015;**81**(2):103-107. DOI: 10.1007/s10327-014-0575-6
20. Peyambari M, Habibi MK, Fotouhifar KB, Dizadji A, Roossinck MJ. Molecular characterization of a novel putative partitivirus infecting Cytospora sacchari, a plant pathogenic fungus. *Plant Pathol. J.* 2014;**30**(2):151. DOI: 10.5423/PPJ.OA.01.2014.0005
21. Rezaian MA, Krake LR, Cunying Q, Hazzalin CA. Detection of virus-associated dsRNA from leafroll infected grapevines. *J. Virol. Methods*. 1991;**31**(2-3):325-34. DOI: 10.1016/0166-0934(91)90170-5
22. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular biology and evolution. 2011;**28**(10):2731-9. DOI: 10.1093/molbev/msr121
23. Tzanetakis IE, Martin RR. A new method for extraction of double-stranded RNA from plants. *J. Virol. Methods*. 2008;**149**(1):167-70. DOI: 10.1016/j.jviromet.2008.01.014
24. Valverde RA, De La Torre-Almaraz R. Extraction and purification of large dsRNAs from virus-infected plants and fungi; applications in virus detection and identification. *Rev. Mex. Fitopatol*. 2017;**35**(1):80-105. DOI: 10.18781/R.MEX.FIT.1606-9
25. Zhang YP, Uyemoto JK, Kirkpatrick BC. A small-scale procedure for extracting nucleic acids from woody plants infected with various phytopathogens for PCR assay. *J. Virol. Methods.* 1998;**71**(1):45-50. DOI: 10.1016/S0166-0934(97)00190-0