

Detection and Identification of Tomato Ring Spot Virus in Ornamental Plants in North Khorasan Province

Javad Mahmoodi Safa ^{1*}, Saeed Nasrolahnejad ² and Farveh Sadat Mostafavi Neishaburi ³

^{1,2} Department of Plant Protection, Faculty of Plant Production, Gorgan University of Agriculture Sciences and Natural Resources, Gorgan, Iran

³ Department of Plant Protection, Faculty of Agriculture, University of Zabol, Iran

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*Corresponding author's email: javad.simsafa@yahoo.com

In recent years, the symptoms of viral diseases such as dwarfism, mosaic, discoloration, necrosis, and circular spots have been prevalent in ornamental plants in parks, gardens, and streets in North Khorasan Province. Tomato ring spot virus (ToRSV) belongs to the Nepovirus genus of Secoviridae family. This virus is a major cause of the infection of ornamental plants in the world. In this study, to evaluate the percentage of infection to ToRSV, in the early autumn of 2014, 400 samples of ornamental plants were collected based on the suspicious viral symptoms from parks, gardens, and streets of North Khorasan province (Bojnord and Shirvan Cities) and were transported to the laboratory in cold conditions. In the laboratory, DAS-ELISA serological test was performed to evaluate the presence of virus in the suspected samples. Then, the infected samples identified by ELISA test were inoculated to the test plants of *Cucumis sativus* L. (Cucumber), *Vigna unguiculata* L. (Cowpea) and *Nicotiana glutinosa* L. (Tobacco). After the appearance of symptoms, in order to verify the infection, they were tested again using DAS-ELISA. The molecular identification of the infected samples was done by Qiagene RNA extraction kit. Using specific primers in RT-PCR reaction, a fragment was amplified in the band of 449bp. The results of DAS-ELISA and RT-PCR tests proved the presence of the virus on ornamental plants in North Khorasan Province. In the mechanical inoculation of the virus, three above-mentioned plants showed the symptoms of the disease.

Abstract

Keywords: DAS-ELISA, Mechanical inoculation, Ornamental plants, RT-PCR, Viral diseases.

INTRODUCTION

The area under the cultivation of ornamental plants in the world is approximately 560,000 ha and the area of greenhouses for their cultivation is 120,000 ha (AIPH and Union Fleurs, 2011). Iran is a country that has four seasons with climate variability. Therefore, there are good potentials for the production of flowers and ornamental plants in Iran (Mostashar Nezami *et al.*, 2013). A large part of Iranian territory in the north, center, and south-west of the country is suitable for developing a variety of ornamental plants and flowers (NIOP, 2015). The total area under the cultivation of ornamental plants in Iran including greenhouses and outdoor areas is 5,734 ha, which is of economic importance (Anonymous, 2014). In recent years, flowers and ornamental plants industry in Iran has developed extensively (Ghotbi and Shahraeen, 2012). Ornamental plants are grown in all provinces of Iran and can be classified into four groups of cut flowers, potted flowers, trees and shrubs, and seasonal and transplanted plants. The ornamental plants are cultivated performed in both outdoor areas and greenhouses (Mahmoodi Safa *et al.*, 2015).

Viruses are the most common pathogenic factors in many ornamental flowers, but they are often overlooked because their symptoms are mild or there are no symptoms at all. The viral diseases are often asymptomatic and do not pose a problem, but the problem occurs when the virus had been transmitted to a susceptible host and has caused severe damages and even death. In fact, this asymptomatic factor acts as a source of the pathogen (Mathews, 2010). At least 125 different viruses that infect and cause disease in ornamental plants have been identified (Hsu and Maroon-Lango, 2003).

Tomato ring spot virus (ToRSV) is a Nepovirus of Secoviridae family (Walker *et al.*, 2015). It is round isometric with the diameter of about 30 nm. When it is ultra-centrifuged, it can produce three components of sedimentation at sucrose density gradient (Stace-Smith, 1996). This Nepovirus is prevalent in America, has economic importance, and may have harmful effects in other locations outside the continent. The virus has a wide natural host domain and infects 285 species from 189 genera and 55 families of monocots and dicotyledonous plants. The most apparent leaf symptom caused by this virus is ring spots (EPPO, 2005). The virus is transmitted by mechanical ways, seeds, transplant, pollen, vegetative organs, and different species of *Xiphinema nematode* (Pinkerton *et al.*, 2008). Tomato ring spot virus is widely spread in Iran. The virus has been reported in soybeans farms in Golestan, Mazandaran, Khuzistan, Ardebil and Lorestan provinces (Golnaraqi *et al.*, 2004), the vineyards of the northern Iran (Rakhshandehroo *et al.*, 2005), ornamental plants of Tehran and Markazi provinces (Ghotbi and Shahraeen, 2009), tomato farms in the southern provinces of the country (Massumi *et al.*, 2009), and apple gardens of Khorasan province (Moini, 2010). ToRSV is among the major Nepovirus infections of ornamental flowers (Card *et al.*, 2007) and was first reported on *Gladiolus* (*Gladiolus communis* L.) in Iran (Kamran and Izadpanah, 1981).

It has been reported from different regions of Iran on the variety of plants like *Althaea officinalis* L., *Chrysanthemum indicum* L., *Rosa canina* L., *Pelargonium × hortorum*. and *Schefflera arboricola* (Hayata) M. (Ayazpour, 2014). Therefore, it is necessary to know the contamination level of the ornamental plants to the virus in Iran and to determine its prevalence in different areas in order to properly manage and control it on a large scale. Given the history of tomato ring spot virus in various agricultural products, gardens, and ornamental plants in Iran, easy transmission, wide host range of the virus, and viral-like symptoms on leaves of ornamental plants in some areas located in North Khorasan Province, attempts were made in this research to identify and detect the virus contamination in various ornamental plants in parks, gardens, and streets in some cities of North Khorasan province and finally, the percentage of tomato ring spot virus infection was investigated.

MATERIALS AND METHODS

Sampling

In the early autumn of 2014, 400 samples of ornamental plants (200 samples from each city) were collected from parks, gardens, and streets in Bojnord and Shirvan cities in North Khorasan province. The samples included different plant species based on the suspicious viral

symptoms collected at the time of sampling. They were from 9 families and 16 species of *Viola* sp. L., *Matthiola incana* L., *Gaillardia pulchella* Foug., *Calendula officinalis* L., *Chrysanthemum indicum* L., *Zinnia elegans* L., *Leucanthemum* × *superbum.*, *Ipomoea tricolor* Cav., *Ipomoea batatas* (L.) Lam., *Rosa hybrida* L., *Pelargonium* × *hortorum.*, *Amaranthus caudatus* L., *Lepidium draba* L., *Berberis Thunbergii* L., *Bellis perennis* L., and *Galium odorata* L. They included leaves and young stems of ornamental plants with different types of viral-like symptoms such as wane-ness, necrosis, chlorosis, mosaic, and ring stains or without obvious symptoms and were transported to the laboratory in cool conditions.

Detection and identification of ToRSV

Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA,) was used for ToRSV detection according to Clark and Adams (1977)'s method. In the test, the commercial kits of polyclonal antibodies (Bioreba, Switzerland), (dilution of 1000) were used to detect the virus. ELISA tests for the detection of the virus were done according to the manufacturer's instructions as follows: immunoglobulin (Virus-specific IgG) with coating buffer to the ratio of 1: 1000 was mixed and 100 ml was poured into each well. Then, the collected leaf samples were extracted at the ratio of 1:5 (1 g of leaf tissue in 5 ml of the extraction buffer) in the cold porcelain mortar. After adding the extract to the ELISA plate, IgG -AP was again diluted with the same ratio (1: 1000) by the conjugate buffer and added to the wells. To change the color of the wells, para-nitro-phenyl phosphate substrate (PNPP) was used. The plates were washed three times with PBS-Tween buffer. In this test, the color-forming reaction of the positive samples and the presence of yellow color were evaluated using ELISA reader (ELX 800-Biotek model) at 405 nm. The calculations were done and the positive samples were determined using the formula $R = x + 3 SD$, where x = attraction mean of negative samples, SD = standard deviation of wells, and R = threshold level of pollution.

Inoculation to test plants

In order to see the symptoms and pathogenicity of the virus, the infected samples that were identified in ELISA were inoculated to the test plants. Profile and age of the inoculated plants are given in Table 1. The plants were planted directly. First, the test plant seeds were planted in medium-sized plastic pots composed of two layers of sand, two layers of peat, and one layer of manure. Then, they were kept in the greenhouse under the appropriate conditions of temperature, humidity, and light. To inoculate the test plants, the inoculation buffer containing potassium phosphate buffer (including K_2HPO_4 and KH_2PO_4), (0.01M with pH=7), which contained 2-mercaptoethanol (0.15 %), was prepared. The infected samples placed in the Chinese mortars were extracted with 2 ml of cold incubation buffer; then, carborundum was sprayed on the leaves to enhance the penetration of the virus and extract. Then, separately using the index finger, the leaves were inoculated (parallel to the veins, especially the central vein.) and, 3 min after inoculation, the leaves were washed to remove extra carborundum. Inoculation was done during the cool hours of the day (in the morning or at evening). The inoculated plants were kept at 25-30°C and indirect lighting in the greenhouse and, in order to prevent the insects carrying the virus, lacing was used around the pots. These pots were irrigated regularly and were visited to record the progress of work each day. After the symptoms appeared, all the plants were tested by DAS-ELISA to confirm the contamination.

Table 1. Test plants inoculated with virus and their inoculation age

Test plants	Scientific name	Inoculation age
Cucumber	<i>Cucumis sativus</i> L.	2 to 4 leaves
Cowpea	<i>Vigna unguiculata</i> L.	2 to 4 leaves
Tobacco	<i>Nicotiana glutinosa</i> L.	2 to 4 leaves

Molecular detection of ToRSV

For verification and closer examinations, the infected samples were evaluated in ELISA by RT-PCR test.

1. RNA extraction and cDNA synthesis

Since the genome of the virus is of RNA kind, by reverse transcription, RNA was changed to DNA and, then, PCR amplification was done on the obtained cDNA. RNA extraction was performed using Qiagen kit (Qiagen, United Kingdom). The absorbed RNA was used for reverse transcription reaction using Accupower™ RT Premix kit (Bioneer, South Korea). The process was as follows:

A. First, 6 µl of the extracted RNA and 1 µl of the reverse transcriptase enzyme were poured into 0.5 ml microtube.

B. Then, the micro-tubes were placed inside the heater block for 5 min at 70°C.

C. All the contents of the micro-tubes were transferred by the pipette into 0.5 ml micro-tubes of the kit. Then, 13 ml of the distilled water was added to have 20 ml of reaction volume.

D. Finally, the micro-tubes were centrifuged to be a little monotonous and, then, were placed in the thermocycler at 42°C for 1h for cDNA synthesis and 94°C for 5 min to deactivate RNase.

E. The micro-tubes were removed from the device and stored in the freezer at 20°C.

2. Polymerase chain reaction (PCR)

For this purpose, two pairs of specific primers designed from the ToRSV coat protein gene were used (Table 2).

These primers reached the appropriate concentration by autoclaved double sterilized water and stored in the freezer at -20°C. After the RT reaction and cDNA synthesis, the PCR reaction was done for cDNA amplification using Accupower™ RT Premix kit in accordance with the following instructions:

A. PCR micro-tubes contained some of the materials needed, such as PCR buffer, MgCl₂, dNTPS, and Taq polymerase enzyme for polymerase chain reaction. These micro-tubes were selected and named for testing; then, 5 µl of the cDNA was poured into the kits.

B. 1 ml of each of the primers was added to the kit.

C. At last, 13 ml of the distilled water was added to the micro-tubes and to make the final reaction volume of 25 µl (Table 3).

Table 2. Profile of the primers used in polymerase chain reaction to amplify coat protein gene

Virus	Name of primers	Directions	Sequence of primers	References
ToRSV	Oligo1n	Forward	5'-GAC GAA GTT ATC AAT GGC AGC-3'	John (1995)
ToRSV	Oligo 2n	Reverse	5'-TCC GTC CAA TCA CGC GAA TA-3'	John (1995)

Table 3. Ingredients and their dosage to prepare a PCR solution

Materials	The dosage used in each 25 µL reaction volume (µl)
Buffer PCR (10X)	2.5
Mgcl2 (50 Mm)	1
dNTPS (10 Mm)	0.5
Cdna	5
Taq DNA polymerase (5 U/µ)	1
Forward-primer (10 µM)	1
Reverse-primer (10 µM)	1
Deionized water	13

Table 4. Step of PCR test for ToRSV detection

Thermal cycle	Number of cycles	Temperature (°C)	Duration (min)
The initial denaturation	1	94	4
Denaturing		94	1
Gradual connection	35	56	2
Extension		72	2
A final extension	1	72	7

D. The micro-tubes were centrifuged at low speed until the ingredients were well mixed and did not stick to the walls of the micro-tubes.

E. The micro-tubes were placed in the thermocycler device, the program of which is already given (Table 4).

To maintain PCR product, the end cycle of the reaction after the final expansion stage was set at 4 °C for 1h in all the programs.

3. Agarose gel electrophoresis

Agarose gel electrophoresis 1% in TBE 1X buffer was used to check the results of PCR. For buffer TBE 10X, 10.8 g Tris, 5.5 g boric acid, and 0.37 g EDTA were dissolved in 100 ml of distilled water and its acidity and pH were adjusted to 8.3 and (1x) diluted when being used. To prepare the gel, 0.5g agarose was dissolved in 50 ml of TBE 1X buffer. Agarose must be completely dissolved in the buffer to have a clear solution. After the solution was cooled slightly at room temperature, 5 ml of pre-prepared SYBR Green solution (EURX, Poland (prepared was added and stirred well to completely solve SYBR Green in the agarose solution. Then, the solution was poured into the tray for molding the gel and it was placed inside the electrophoresis so that the wells were placed next to the negative electrode. After 20 min, the comb was removed from the gel and the reservoir tank was filled with TBE 1X buffer so that the gel was completely covered and the wells were ready to be poured into the samples. Then, 5 ml of the extracted RNA was mixed with 2 ml of the dye solution and poured into the wells. The electrophoresis voltage was set to 90V and 60 min and the size was determined using the standard marker of 100 bp ladder to ensure the expected size and accuracy of the amplification reaction. After decolorization with distilled water, the gel was examined on a UV Transilluminator and, then, was photographed using gel documentation.

RESULTS

ELISA serological test results

Results of 400 samples of the collected ornamental plants showed high prevalence of viral-like symptoms in the plants in North Khorasan province (Fig. 1). In this study, 400 samples of 16 species of ornamental plants were tested for Tomato ring spot virus (ToRSV), which is an important and economic viral disease with global prevalence. Most of the tested plants were ornamental or used in outdoor gardens.

Results of ELISA serological test showed that among 400 ornamental tested plant samples, 29 samples were infected with the virus (7.25%) and 371 samples (92.75%) had no virus infection (Fig. 2). Results also demonstrated that the virus existed in both cities of Bojnord and Shirvan. ToRSV infections in the city of Shirvan were 11.5% and it was 2.5% in Bojnord city. Out of 16 species of the ornamental plants, two species were infected and 14 species were without infection to ToRSV. ToRSV was identified in 16 samples of *Ipomoea batatas* (L.) Lam. and 13 samples of *Galium odorata* L.

In the mechanical inoculation of ToRSV, the entire mentioned test plants showed the symptoms of the disease (Fig. 3). In addition, it was observed that, in most cases, the symptoms of the plants were worse in warmer conditions. ELISA confirmed the contamination of all the test plants inoculated with the virus.

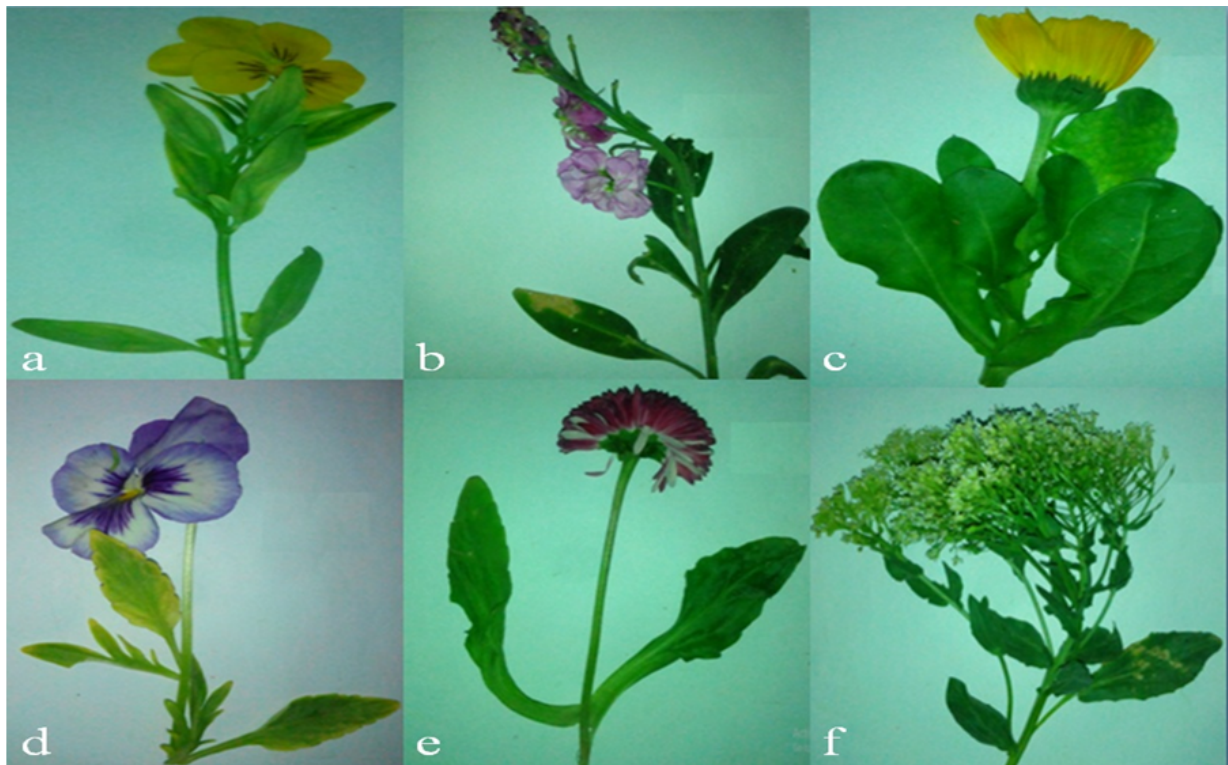


Fig. 1. Viral-like symptoms in ornamental plants in North Khorasan province. (a and d) Uniform chlorosis symptoms on *Viola* sp. L. (b) Necrotic spots symptoms on *Matthiola incana* L. (c) Mottling and malformation symptoms on *Calendula officinalis* L. (e) Shrinkage and deformities symptoms on *Bellis perennis* L. (f) Necrotic spots symptoms on *Lepidium draba* L.

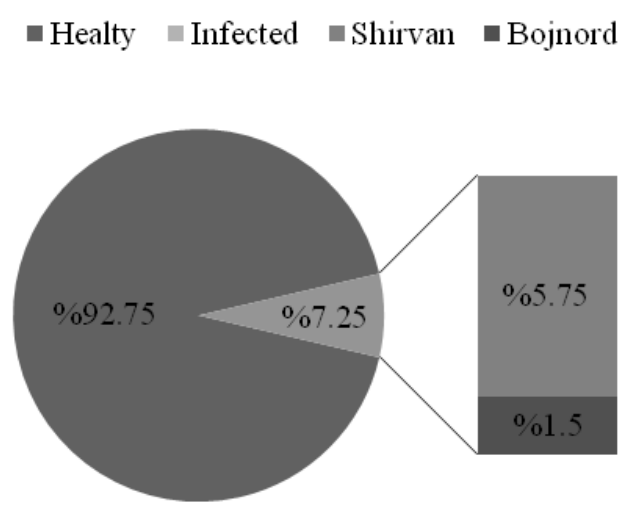


Fig. 2. Infection percentage of ornamental plants to ToRSV in North Khorasan Province

RT-PCR test results

The results of RT-PCR using specific primers to detect ToRSV are presented in Fig. 4. In this way, a region of the virus coat protein gene was amplified and a fragment of 449 bp of the infected *Ipomoea batatas* (L.) Lam. sample was obtained. These tests conclusively proved the presence of ToRSV in the province.



Fig. 3. Symptoms of infection to ToRSV.

(a) White veins and necrosis spots sporadically in *Cucumis sativus* L. (b) Systemic mosaic and malformation of leaves in *Vigna unguiculata* L. (c and d) Mottling and vein clearing in *Nicotiana glutinosa* L. (e, f and g) control plants *Cucumis sativus* L., *Vigna unguiculata* L., and *Nicotiana glutinosa* L., respectively

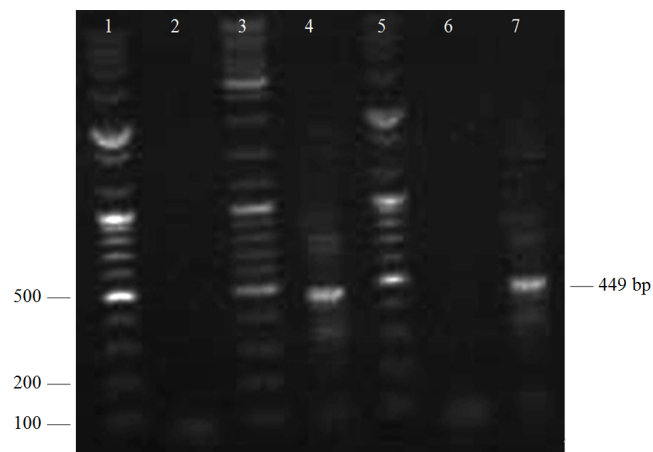


Fig. 4. Electrophoresis pattern of RT-PCR products of Tomato ring spot virus. Lane 1-3-5, Gene ruler DNA ladder 100 bp; Lane 2-6, Healthy ornamental plants; Lane 4-7, *Ipomoea batatas* (L.) Lam. samples infected

DISCUSSION

Tomato ring spot virus (ToRSV), that can infect tomatoes and a variety of plants in Iran, is among the most damaging and important viruses. In this study, the prevalence of ToRSV in parks, gardens, and streets of the cities in North Khorasan Province was investigated (Bojnord and Shirvan). In the past years, the presence of virus has been reported among the fruit trees of the province (Moini, 2010; Moini *et al.*, 2008), but the results of this study are the first evidence of the presence of ToRSV in ornamental plants in the north-east of the country. The prevalence of ToRSV in ornamental plants can be justified given the suitability of environmental conditions for the activity

of nematodes, inefficient use of certified virus-free seedlings, and lack of laboratory facilities in the quarantine posts and contamination reports of fruit trees in Khorasan Province to ToRSV,

In this study, the presence of the virus in the ornamental plant of *Ipomoea batatas* (L.) Lam. was confirmed using classical and molecular biological methods. Although various researchers in Iran have performed the molecular detection of virus on different crops, this work is the first report of molecular detection of ToRSV in ornamental plants in Iran. RT-PCR test is more sensitive than ELISA. Also, RT-PCR is simpler than other molecular methods and, thus, a large number of samples can be tested simultaneously. The quality of total nucleic acid and extraction method is very important as well (Gumus and Paylan, 2013). In this study, there were infected samples with positive ELISA test results, but in most cases, in RT-PCR test, there is no proliferation using specific primers of virus. The non-positive results of RT-PCR using ToRSV-specific primers can have a variety of reasons such as low quality of nucleic acid, inappropriate RNA extraction method, and even false coloring of the wells in ELISA test for various reasons. It seems that the use of more advanced molecular methods, especially IC-PCR method in future research, can reduce the above-mentioned problems due to the use of both serological and molecular methods in one-step. In addition, due to the high concentration of the virus, IC-PCR method can be useful in the detection of the virus with the same symptoms on the test plants.

Although ELISA is better than RT-PCR test due to such advantages as no need for special education and economic factors, RT-PCR method is important and can contribute to viral disease management programs owing to the unique characteristics such as simplicity, fastness, and traceability of virus even at low concentrations in genetic engineering for determining viral pathogens. In the mechanical inoculation of ToRSV on the test plant of *Cucumis sativus* L., Sattary *et al.* (2012) observed the clearing of veins, which corresponded to the results of the cucumbers test plant in response to ToRSV in this study. Also, a large number of symptomatic plants did not react to the antibodies of ToRSV. The lack of positive reaction might be due to contamination with other common viruses such as TMV, TuMV, BYMV, or latent viruses (Ghotbi and Shahraeen, 2012). Damages caused by viral infections in plants, can vary from 10 to 100% depending on the composition of the virus and host (Loebenstein *et al.*, 1995). The most important approach against viral diseases is to prevent their spread. Since the propagation method of most plants is vegetative, in this regard, secure and virus-free seedlings, bulbs, and vegetative parts are the effective method for preventing the spread of viral diseases in ornamental plants. Therefore, as an ongoing need, there is a need to launch and develop a virus-free seedling production system in the country by producers.

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