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Diagnosis of Important Virus Diseases of Ornamental *Anthurium* in Screen- Houses in Tehran Province

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ABSTRACT

Introduction: One of the limiting factors for *Anthurium* production is its infection with various viruses. During the year 2014-2015, a total of 504 symptomatic ornamental *Anthurium* leaf samples with virus like symptoms including leaf and flower blister, dwarfing, mosaic, yellowing, leaf marginal chlorosis and deformity were collected from Varamin and Pakdasht (Tehran province) Screen-houses.

Aim: Samples were tested for infection to *Cucumber mosaic virus* (CMV),*Tomato spotted wilt virus* (TSWV), *Impatiens necrotic spot virus* (INSV) and *Dasheen mosaic viruses* (DaMV).

Method: Biological, serological (ELISA) and molecular methods (RT-PCR) were performed for detection and analysis of the important viral agents infecting *Anthurium* ornamental plants.

Results: According to ELISA test results the prevalence of CMV were (31.54%) followed by TSWV (25%) and DaMV (7.93%) viruses respectively. Results of mechanical inoculation and host range reaction of CMV, TSWV and DaMV isolates were also recorded (fig 2). Coat protein gene sequence of Iranian CMV isolate showed highest similarity (97%) with a CMV isolate *Cucurbit pepo* (Acc. No.GU327368.1) from South Korea. DaMV on ornamental *Anthurium* is reported for the first time in this research from Iran.

Conclusion: Despite the detection of CMV, TSWV and DaMV in this study, viral pathogens are one of the most economically damaging agents in the ornamental *Anthurium* plant. Development of research methods for, detection and study of different isolates of these agents from different hosts, study of the characteristics of each of these isolates has always been a scientific need.

Key Words: CMV, TSWV, DaMV, Serology, Molecular test, Ornamental Anthurium

INTRODUCTION

Anthurium anderadum Lind. (Araceae) is one of the most important cut flowers, which blooms all year round. Ninety-five percent of the world's flower exports in 2012 were *Anthuriums*.[1] The sale of 40 *Anthurium* flowers can be worth a barrel of oil, and if the acceptable global parameters (health, quality and marketability) are provided, the export of flowers and ornamental plants can replace.[2] One of the limiting factors for *Anthurium* production is its infection with various viruses.[3] In some cases, the rate of *Anthurium* infection with viral diseases in greenhouses in the country has been estimated up to 70%.[4] Infection with viral diseases, not only reduced the quality, but also leads to leaf and flower blistering, dwarf plant , mosaic, marginal leaf chlorosis, leaf entanglement, deformity or lack of flower formation.[5] Among the most important viruses reported in the world from *Anthuriums* incuding: Orthotospo viruses of *tomato spotted wilt virus* (TSWV)[3,6], *Ground-nut bud necrosis virus* (GBNV) and*Impatiens necrotic spot virus* (INSV)., *Dasheen mosaic virus* (DaMV)[7], and *cucumber mosaic virus* (Cucumber virus) CMV [3,5,8] are present. *Cucumber mosaic virus*-CMV belongs to the genus Cucumovirus of the family Bromoviridae is one of the most important among these viruses which is transmitted by aphids, infected seeds, grafting and mechanical transmission.6,9 Also different types of weeds are considered as a source of infection and the cause of the spread of this virus. TSWV

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tospoviruses and INSV of the Bunyaviridae family are spreading viral diseases that are economically important pathogens worldwide and are distributed by mechanical transmission and several species of thrips (Thripidae-Tysanoptera).[10] Potential carriers of TSWV is onion thrips (*Thrips tabaci*) and western flower thrips (*Frankliniella occidentalis*). This virus is the most important harmful and predominant virus in ornamental plants in Iran and has been reported from various ornamental species in the country.[10,11,12] DaMV belongs to the genus Potyvirus and infects plants of the *Araceae* family. The virus causes mosaic and deformities in ornamental plants of families, including *Anthuriums*, *agglomerates*, *Diphenbachia*, and filigrees. *Dasheen mosaic virus*-DaMV is spreading worldwide and has been reported from the United States, Europe, Egypt, India, Japan and Oceania regions. The virus is transmitted by aphids in an unstable manner and by infected plant sap mechanically.[13] Due to world wide spread of theseviruses in ornamental plants of *Diphenbachia*, *Filigrees*, *Anthurium* and *Agglonaema*, and reportson prevalence in Asian countries such as Japan and India [14,15], the observed signs of suspected infection with the viral agents in ornamental hosts in Iran and the extensive import of ornamental plants from foreign countries, this investigation in Iranis brought in to focus. Therefore, in this study, and in accordance with the importance of CMV and DaMV viruses in the ornamental plant for *Anthurium*, these two viruses mainly were tested serologically and also detected by RT-PCR test. The general strategy for controlling viral diseases in flowering ornamental plants includes rapid identification, vectors control managements and elimination of viral contaminants in ornamentals production areas. The purpose of this study was to accurately identify important and harmful viruses in ornamental *Anthurium* main production screen houses in Tehran province.

MATERIAL AND METHODS

Sampling

Six main greenhouses producing *Anthurium* ornamental plants in Varamin and Pakdasht regions (Tehran provinces) were visited and a total of 504 samples suspected of viral infection with symptoms of leaf and flower blistering, dwarfing, mosaic, marginal chlorosis, leaf complexity, Deformity or lack of flower formation were collected from important commercial *Anthurium* cultivars available in the market (Simba, Calore, Carnaval, Angel and Calisto). Specimen characteristics, symptoms, location and date of collection were recorded and the samples were transferred to the laboratory on ice bucket for examination and further tests.

Serology

To evaluate the contamination of *Anthurium* samples with CMV virus, enzyme linked immunosorbent assay (ELISA) test by indirect method of ELISA based on [16] using antiserums provided by ICARDA Center(in Syria), for INSV, TSW and DaMV direct DAS-ELISA test was performed by polyclonal antibodies (Bioreba company, Switzerland)method described by Clark MF and Adams AN.[17] Leaf samples were extracted in the ratio of one gram of tissue with 5 ml of extraction buffer and 100 µl was added to each well (Elisa plate) at each stage. In each test, two replicates of healthy sample extract (negative control), two replicates of infected extract (positive control) and three replications were considered as blank well. One hour after adding the substrate solution containing Paranitro-phenyl phosphate, the light absorption of the wells at 405 nm was measured using a microplate reader (Multiscan 334 Lab system, Finland).

Biological tests:

For all four viruses CMV, TSWV, INSV and DaMV, samples which showed infection with only one virus in ELISA were selected and leaf extracted (in a ratio of one to five) by cold phosphate buffer of 0.01 M containing 0.15% antioxidant 2-mercaptoethanol pH = 7, were mechanically inoculated to specific indicator plants of *Chenopodium amaranticolor*, *C. quinoa*, *Nicotiana rustica*, *Capsicum annum*, *Datura metel* and *Vigna unguiculata*. Inoculated plants were kept in greenhouse free of vector insects with temperature conditions of 23-25° C, natural light for 14 hours, light and relative humidity of 80-60%, and symptoms appearance were studied and recorded. Twenty days after inoculation, the onset of systemic symptoms was assessed by ELISA. Symptoms of each virus on specific hosts were recorded Table 2, Fig 1 and 2.

Molecular diagnosis-DaMV

Since *Anthurium* infection with DaMV and CMV viruses is economically very important and there was a report of high damage to *Anthurium* in green houses (up to 70%)[7] and so far no research has been done on this virus in Iran,[4] RT-PCR, Molecular test was performed for samples with single infection with these two viruses (based on positive serological and biological reaction). Amplification of the CMV coat protein gene and DaMV related NIb encoding gene for positive samples by reverse transcription-polymerase chain reaction (RT-PCR) carried outusing specific primers for the two viruses (Table 1). Five samples were selected by positive reaction to each of the two viruses in ELISA test and total RNA of samples were extracted by commercial solution RNXTM -plus (SinaGen Company, Iran) according to the method recommended by the manufacturer. RNA extracted using specific primers with two steps RT-PCR. In the case of

DaMV, descriptive primers were of[18] and for CMV, specific primers of[19] were used (Table 1).

Amplification and Sequencing

In order to determine the nucleotide sequence of the fragment for isolation of CMV from *Anthurium*, 20 microliters of PCR product with specified primers (with a concentration of 50 picomol) was sent to the representative of Kiagen Biotech Korea in Iran. The fragment was sequenced and the sequences obtained from PCR products were compared with the information and sequences in the Gene Bank (NCBI) by BlastN software at the nucleotide level. The nucleotide data sequenced for the CMV virus were then multiple-aligned with virus isolates already available in the NCBI database using MEGA5 and ClustalX software, the progeny analysis was based on nucleic acid using the Neighbor-Joining method. MEGA5 software. The offspring tree was drawn in 1000 boot strap using MEGA5 bioinformatics software.[20,21] All branches merged with a bootstrap value of less than 70%. In this analysis, *Peanut stunt virus* (PSV) ER isolate (U15730) was used as an out-group with *cucumber mosaic virus* sequences.

RESULT

ELISA test

A total of 504 *Anthurium* samples collected from 6 main *Anthurium* producing greenhouses in Varamin and Pakdasht counties with symptoms of systemic mosaic, malformation, blistering, marginal chlorosis, leaf complexity, dwarfing, malformation, or lack of flowering. In ELISA serological test the highest infection (159 samples (34.54%)) were related to CMV, followed by TSWV (126 samples (25%), DaMV (40 samples (7.93%)) and no sample were found positive to INSV infection. (Fig. 1). 20 symptomatic specimens were recorded as mix infection with CMV and DaMV. Due to the importance of CMV and the highest percentage of infection of samples with this virus, the infection of seven important commercial cultivars of *Anthurium* were preliminary evaluated with CMV infection. Among the cultivars tested in this study, Calore with 88.2% has the highest level of infection and then Carnaval (75%), Calisto (55.5%), Angel (36.6%), Simba (30.7%), Pistache (7.6%) and Essencia (8.3%). Based on the results of ELISA, *Anthurium* cultivars with red flowers were more infected, pink cultivars were moderately infected and white cultivars were less infected by CMV respectively. Based on the observations and results of this study, severe mosaic symptoms due to CMV virus infection, deformity and dwarfing by DaMV virus and blistering due to TSWV virus were recorded in *Anthurium* Fig 1.

Reaction of test plants

The results of test plants inoculated with samples tested positive in ELISA with only one of the three viruses CMV, TSWV and DaMV is presented in Table 2.

Molecular test

Five isolates from 40 samples which tested positive for DaMV in ELISA serological test were examined by a specific primer pair of potyviruses in RT-PCR test. The results showed DNA fragment with an expected size of about 350 bp belonging to part of the gene encoding intracellular organelles (NIb) of potyviruses which observed in four samples (Figure 3). Also, five samples of *Anthurium* cultivars Calore, Carnaval, Angel, Calisto and Simba, which tested positive for CMV on the ELISA test, were examined by a pair of virus-specific primers in the RT-PCR test, resulting in amplification of a DNA fragment. The approximate length of 540 bp corresponded to part of the CMV protein coat gene region in the samples, Figure 4.

Considering the frequency and importance of *cucumber mosaic virus*-CMV among the other viruses studied in this study, the envelope protein sequences of five CMV isolate in Pakdasht region was determined and compared with other sequences in the Gene Bank (Table 3). The nucleotide sequence length of the isolates was 540 nucleotides. High similarity between the sequenced samples with the *Cucurbit pepo* (GU327368) samples from South Koreawere observed in the Gene Bank.Recently, the CMV S-I has been divided into two subgroups, S-IB (Asian isolate) and (other isolates) S-IA, based on genome sequencing.[22,23] The *Cucurbit pepo* (GU327368) specimen from South Korea is in the S-IA subgroup and Pakdasht *anthurium* specimen is most similar to CMV S-IAin this study.

DISCUSSION

Viral pathogens are one of the most economically damaging agents in the ornamental *Anthurium* plant. *Cucumber mosaic virus*, with a host range of about 1000 species from 85 different plant families, is one of the most important *Anthurium* infecting viruses in Iran and the world.[4,5,24] In this study, a high percentage of *Anthurium* cultivars were infected with CMV, using serological and molecular tests, the virus was detected in some samples (Fig 1 and 4). In the present study, CMV isolate from *Anthurium*, unlike field isolates, was found on cowpea (*V. unguicullata*), mung bean (*V. radiata*), cucumber (*C. sativus*), tomato (*L. esculentum*), bean (*Vicia faba*) and the button flower (*Gomphrena globosa*) did not cause any symptoms. Despite the cultivation of *Anthurium* in indoor greenhouses, unfortunately, the lack of awareness of growers about some common diseases of

ornamental and other fieldcrops (e.g.,*Cucurbit* plants) may lead to mechanical transmission of the viral agentsinto the greenhouse, Fig 6.

Therefore, planting cultivars resistant (modified and marketable) to important and common viral agents is one of the best ways to reduce damage and prevent the spread of viral disease in an area.[25] DaMV has also been reported from other crops and orchards. Decreased production of up to 60% due to virus infection in *Colocasia esculenta* has been reported as a plant species of the family Aroid (*Aracea*).[26] *Ground nut bud necrosis virus* (GBNV) is another important virus of the ornamental plant *Anthurium* recently reported from India.[3]According to this, rapid and timely detection of viral agents of ornamental plants and the establishment and development of their detection systems in the country is of particular importance.

CONCLUSION

Collecting more samples from other greenhouses on other parts of the country will give us more information about the viral agent infecting and limiting *Anthurium* production in the country. The best way to control viral pathogens in the ornamental *Anthurium* is to know the symptoms of the disease and prevent the spread of viral agents in the screen houses. Regular examination of greenhouses for infection by plant viruses, isolation and introduction of viruses identified in each area, removal of suspected weed and infected plant materials, stored in greenhouses or surrounding areas. Proper and timely use of insecticides is one of the useful strategies in controlling spread of viral diseases in ornamental plants, including *Anthuriums*.[27,28]

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CONFLICT OF INTEREST:

There are no undisclosed conflicts of interest for the Authors.

AUTHORS' CONTRIBUTION

Shahraeen Nooh -1) Implementation of the project; 2) Intellectual content review of the article, and 3) final version approval for publication

Tabassom Ghotbi-1) Analysis of the results; 2) conclusions;3) drafting the article, and 4) final version approval for publication

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Table 1: Specifications of primers used for amplification of intercellular organelles gene of DaMV and Coat protein gen of CMV

(Reference)	Rt-PCR product size (bp)	Tm	Nucleotide sequence	(Primer)
			ACTATCTAGAGCGGCCGCTTT(16)	Oligo dT (FP)
Zheng et al., 2010	350bp	43	GTNTGCGTCGACGACTTCAACAA	DaMV-F(FP)
			TCAACAACAGTAGAAGGCTGACC	DaMV-R(RP)
Zittkaite and Samuitiene, 2009			GTA GAC ATC TGT GAC GCG A	CMV-F
	540bp	54	GCG CGA AAC AAG CTT CTT ATC	CMV-R

Table 2: Response of indicator plants inoculated by CMV, TSWV and DaMV

(Virus)			(Family)	(Scientific name)
DaMV	TSWV	CMV		
CLL, C	NLL	CLL	Chenopodiaceae	*Chenopodium amaranticolor*
Mt*, C	NLL, CLL	CLL, Mt		*Ch. quinoa*
NI	NI	M*	*Cucrbitacea*	*Cucumis sativus*
NI	NLL, CS*	M*	*Solanaceae*	*Nicotiana tabacum*
NI	NLL	NI		*Datura metel*
NI	NLL	NLL	*Fabaceae*	*Vigna unguiculata*

C: chlorosis, CLL: chlorotic local lesion, CS: systemic chlorotic spots, Mt: mottling, NI: no infection, NLL: necrotic local lesion. M:mosaic.

*: Systemic infection confirmed by ELISA.

Table 3: Samples used for phylogenic tree

Percent similarities	Access No In GeneBank	Country	Host	Sample name In phylogenic tree
%97	GU327368.1	S Korea	*Cucurbit pepo*	1E2
%87	JX993912.1	China	*Solanum lycopercicum*	2E2
%90	AJ890465.2	India	*Lilium tigrinum*	3E2
%91	DQ018288.1	Poland	*Cucumis sativus*	4E2

Figure 1: Symptoms of *Anthurium* plants infected with three viruses. a: CMV (Mosaic, complexity and swelling of veins) b:TSWV (Blistering of leaf surface) c: DaMV (leaf deformity and asymmetry.

Figure 2: Results of mechanical inoculation of virus infected *Anthurium* on indicator plants. a: Local necrotic spots on *C.amaranticolor* by CMVb: Local necrotic ring spots on *V. unguiculata* by TSWV c: Local yellow spots on *C.amaranticolor* by DaMV.

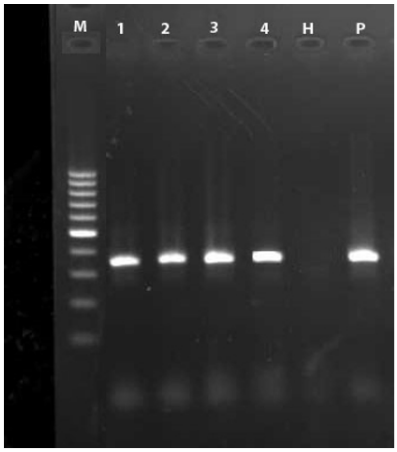

Figure 3: Gel electrophoresis pattern of amplified partial NIb gene using specific primers of DaMV in agarose gel. M: 1 Kbp molecular weight marker (Fermentas-Lithuania); H: negative control; 1&2&3&4: *Anthurium* samples infected by DaMV; P: positive control.

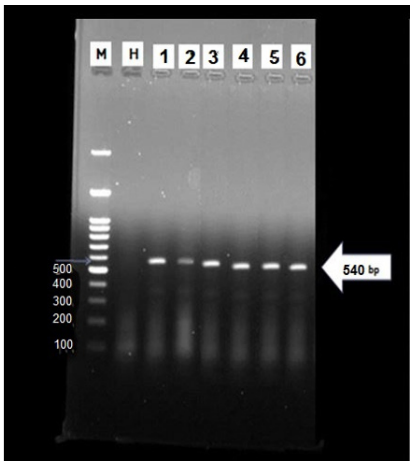

Figure 4: Gel electrophoresis pattern of amplified partial coat protein gene using specific primers of CMV in agarose gel. M: 1 Kbp molecular weight marker (Fermentas-Lithuania); H: negative control; 1: Simba & 2: Calisto & 3: Angel & 4: Carnaval & 5: Calor cultivar 6: positive control.

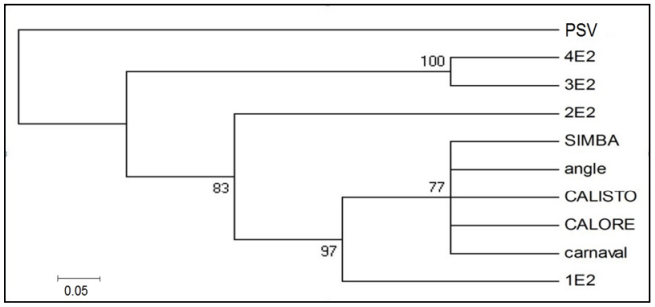

Figure 5: Constructed phylogenetic tree using nucleic acid sequence of coat protein gene of CMV isolates from *Anthurium*using CLUSTAL X with four other Genbank isolates. Coat protein gene sequence of BCMV was used as outgroup.

Figure 6: CMV infected cucumber plants outside and nearby entrance of *Anthurium* greenhouse in Varamin (Pakdasht area).