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Original Article

An Investigation of *Apple Chlorotic Leaf Spot* (ACLSV) and *Tomato Ring Spot* (ToRSV) in Some Iranian Pear Gardens

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Abstract

Background and Aims: In spite of a long tradition of fruit-tree growing in all provinces of Iran, information on pear (*Pyrus communis*) tree viruses is scant. In order to identify of the nature of the virus like symptoms and decline in pear trees, we carried out a survey for the occurrence of four important viruses.

Materials and Methods: Leaf samples of pear showing virus like symptoms were collected in three provinces, Iran, in 2013 and 2014 and tested for *Apple chlorotic leaf spot* (ACLSV), *Apple stem pitting virus* (ASPV), *Apple stem grooving virus* (ASGV) and *Tomato ring spot* (ToRSV) infection by ELISA, using specific antibodies.

Results: 39 out of 112 leaf samples were found to be infected by at least ToRSV and ACLSV. ToRSV was detected in the symptomatic samples from all surveyed provinces with infection ranging from 32.4% up to 35.0%; however, the infection of ACLSV was differed between 9.9 to 18.2 %. Infections of ELISA positive samples were confirmed by biological assay. DNA fragments of 580 and 700 bp in size were RT-PCR amplified using specific primers designed according to RdRp and CP genes of ToRSV and ACLSV, respectively which indicated 96 and 92 % highest identities with available sequences of ToRSV and ACLSV isolates in GenBank, respectively.

Conclusion: This analysis provides, to our knowledge, the first demonstration of pear trees infection with ACLSV and ToRSV in Iran. Early detection of viral pathogens and accurate diagnosis, are undoubtedly essential for controlling the spread of virus and ensuring production of healthy plant reproductive material for further cultivation.

Keywords: Detection; ACLSV; ToRSV; RT-PCR

Introduction

The pear (*Pyrus communis*) is native to Western Asia and Eastern Europe, especially the north-west Iran and the Caucasus mountains. Pear trees have been cultivated in China for approximately 3000 years, and now this plant is cultivated in many countries and is the second wide-spread garden tree, after apple in the world and Iran. Pear is considered as one of the oldest fruit trees in Iran. According to FAO the harvested pear area in Iran is 15,000 ha with an approximate annual production of 147,000 tones, and 9.8 tone product per ha (1). The productivity of pear plants is greatly constrained by pests and diseases that cause yield reduction, among which viruses are a worldwide responsible for substantial economic losses. Pear plant is host

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for a large number of economically important and common RNA plant viruses belonging to different genera. Vegetative propagation methods of pear probably favored the spread of viruses which have increased management difficulties. In addition, these can lead to spread of viral infections and significant losses because of undesirable changes in the plant phenotype.

Among the virus diseases affecting pome fruit trees, those caused by *Tomato ringspot virus* (ToRSV, genus *Nepovirus*, subgroup C, family *Secoviridae*) and *Apple chlorotic leaf spot virus* (ACLSV, genus *Trichovirus*, family *Betaflexiviridae*) (2), are the most economically important ones (3). ToRSV is among the plant pathogens which has been shown to cause mild to severe economic losses in many perennial fruit crops (3-5) and is an agent of concern for many countries that produce or import nursery stock (6). The widespread occurrence of ToRSV in pome fruits including pear trees in Jordan, with the highest rate infection in nurseries has been shown by Salem *et al* (7).

ACLSV is one of the most widespread and economically important latent viruses, which has a broad host range that includes most, if not all, Prunoideae (apricot, cherry, peach, and plum) and Maloideae (apple, pear, and quince) fruit tree species (8). Although it is mostly symptomless in many of its host plants, ACLSV may also cause serious diseases (3).

The incidence of ToRSV and ACLSV recently, has been reported in Iran (9, 10). Despite the important role of these viruses in Iran, limited information is available on the occurrence of them in pear trees. In the present study the occurrence and partial genome analysis of ToRSV and ACLSV were investigated.

Methods

Sampling and serological assays

Leaf samples were collected in the growing seasons of 2013 and 2014 from pear orchards that showed virus like symptoms such as chlorotic spots and/or mosaic, vein clearing, chlorosis, reduced growth and decline (Table 1; Fig. 1). The surveyed regions included 33 gardens in three major pear growing provinces in Iran, including Alburz, Ghazvin and Tehran provinces. Composite samples of ten to twelve apical leaves were collected from branch tips throughout the canopy of individual trees to determine the occurrence of viruses. In total, 112 pear trees were sampled and assayed for the presence of ACLSV, *Apple stem pitting virus* (ASPV), *Apple stem grooving virus* (ASGV) and ToRSV by ELISA (11) using specific antibodies from Bioreba AG (Reinach, Switzerland). A portion of 10-12 stacked leaves was torn, ground in phosphate buffer saline supplemented with 10 mM sodium sulfite, 1% polyvinylpyrrolidone 40, 1% Tween 20 and powdered egg albumin (2 g/l) at a 1:10 ratio (w:v), and tested according to the antibody manufacturer's instructions. Substrate hydrolysis was determined at 405nm with an absorbance microplate reader MultiScan 340 (Labsystems, Finland). Samples were considered positive if their optical density (OD 405nm) readings were at least twice those of healthy controls.

Host range studies

Leaf tissue from the symptomatic plants was used to mechanically inoculate appropriate herbaceous indicator plant species belonging to different families including *Chenopodiaceae* (*Chenopodium quinoa*, *Ch. amaranticolor*, *Ch. mural*), *Solanaceae* (*Nicotiana occidentalis*, *N. clevelandii*, *N. benthamiana*, *N. tabacum* cv. White Burley, *N. tabacum* cv. Xantia, *N. tabacum* cv. Samsun, *N. rustica*), *Lamiaceae* (*Ocimum basilicum*), *Cucurbitaceae* (*Cucumis sativus*), *Fabaceae* (*Phaseolus vulgaris*, *Vigna unguiculata*), and *Amaranthaceae* (*Gomphrena globosa*). In mechanical transmission experiments, 0.01 M K-phosphate buffer (pH 7.0) containing 2% nicotine was used as a general inoculation buffer. A total of four plants of each experimental species were inoculated and bioassay was repeated three times. Furthermore, graft transmission was done using scions that were excised from symptomatic infected pear trees. Inoculation was done by side cleft grafting on pear plants. The graft insertion side was taped tightly with Parafilm (Bemis Co., WI., USA) to prevent desiccation and the grafted plant was protected

from excessive evaporation by a plastic bag cover, which was removed after two to three days. The indicator plants were kept in an insect-free greenhouse with about 15 hours light, at 25±5°C and 50% to 70% relative humidity, and were regularly observed during one to four weeks or longer after inoculation. Four weeks post inoculation all symptomatic and asymptomatic plants were assayed by ELISA and RT-PCR.

RNA extraction and RT-PCR

Pear leaf samples were assayed for ACLSV and ToRSV by RT-PCR with total RNA and appropriate primers. Total RNA was extracted from leaf tissue (100 mg) using the RNeasy Plant Mini kit (Qiagen, Valencia, CA, USA). A segment of the ToRSV RNA-1-encoded RNA dependent RNA polymerase (RdRp) gene was amplified with primers ToRSV-R (5'-CCACACACACTCCACCTAC-3') and ToRSV-F (5'-ACTTCTGAAGGCTACCCGTT-3') (12). A primer pair specific to the ribulose 1,5-bisphosphate carboxylase chloroplast (*Rbc1*) ribosomal gene [Rbc1-R 5'-CTGCATGCATTGCACGGTG- 3' and Rbc1-F 5'- TACTTGAACGCTACTGCAG-3' (13)] was used as internal control to amplify the corresponding mRNA in pear leaf tissue in RT-PCR. For ACLSV, a segment of the coat protein gene was amplified from pear leaf tissue with primers ACLSV-R (5'-AAGTCTACAGGCTATTTATTATAAGTCTAA-3') and ACLSV-F (5'-GACCCCTTCATGGAAAGACAGGGG-3') (14). First-strand cDNAs were synthesized using 5 µl of template RNA (1.5 µg), one µl of the reverse primer (20 pmol/µl) and one µl of RevertAid^{TM} M-MuLV reverse transcriptase (200 unit/µl) (Fermentas, Lithuania) in 20 µl reaction volume at 42°C for 60 min and then at 72°C for 10 min to inactivate the enzyme, according to manufacturer's instructions. For the amplification reaction cDNA was used as a template in PCR using *Pfu* DNA polymerase (Cinnaclone, Iran) and ACLSV and ToRSV specific primers. PCR program included 95°C for 10 minutes, 35 cycles of 95°C for 45 seconds, 48°C for 1 minute, 72°C for 1 minute, with the final extension step at 72°C for 10 minutes.

PCR amplification were conducted in 50 µl reaction solution containing 5 µl DNA template, 1 µl of each primers (20 pmol/µl), 5 µl of 10X reaction buffer (200mM Tris-HCL, 500 mM Kcl, pH 8.4), 1.5 µl $MgCl_2$ (50 mM), 1 µl dNTPmix (10mM) and 1 µl *Pfu* DNA polymerase (Cinnaclone, Iran). The PCR products were analyzed by electrophoresis in a 1 % agarose gel. DNA fragments with the expected size obtained after RT-PCR with RNA templates of ToRSV and ACLSV isolates were excised from the gel, and cleaned by the QIAquick Gel Extraction Kit (QIAGEN, USA) according to the manufacturers' instructions. Nucleotide sequences of DNA amplicons from each isolate were determined. DNA sequencing was done in both directions using the BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit and an Applied Biosystems Genetic Analyser DNA model 310 (Applied Biosystems, Foster City, CA, USA). Nucleotide sequence data were compared with other sequences available in Genbank using BLAST tool.

Results and Discussion

In spite of a long tradition of fruit-tree growing in all provinces of Iran, information on pear

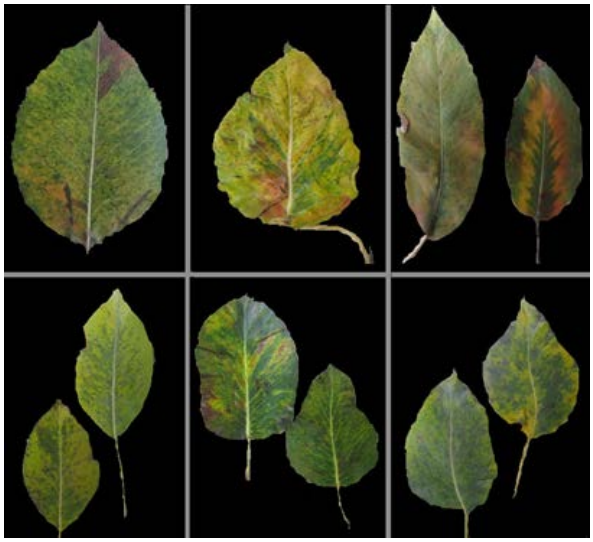

Fig. 1. Different virus-like symptoms including deformation, dark and reddish lesions, mottle, mosaic, vein clearing and chlorosis in pear leaf samples.

tree viruses is scant. In order to identify of the nature of the virus like symptoms and decline in pear trees, we carried out a survey for the occurrence of viruses. Early detection of viral pathogens and accurate diagnosis of diseases that caused by them, are undoubtedly essential for controlling the spread of virus diseases and ensuring production of healthy plant reproductive material for further cultivation.

According to ELISA test results, among 112 composite pear leaf samples tested, 39 (34.82%) were infected by at least one of ACLSV or ToRSV viruses. ToRSV was detected in symptomatic leaf samples of all three surveyed provinces with infection ranging from 20.6% in Alburz up to 31.0% in Tehran provinces (Table 1). ASGV and ASPV were not detected in any of the tested samples. Among the eight leave samples infected with ACLSV, five ones showed mixed infection with ToRSV with characteristic leaf deformation and vein yellows symptoms. Also, ACLSV was detected through in all visited districts in Alburz, Guilan and Tehran provinces with a mean infection rate of 2.67% in symptomatic samples. ToRSV showed more infection (27.67%) than ACLSV (2.67%) in the samples tested, however the incidence was differed among symptomatic samples collected from the provinces surveyed (Table 1). ToRSV were also detected in two samples collected from traditional nurseries in Alborz province.

Table 1: Provinces, number of collected pear leaf samples and number of ELISA positive samples with *Apple cholorotic leaf spot virus* and *Tomato ring spot virus* and their infection rate (%).

Province	Samples	No. Garden visited	No. Collected Sample	Virus detection			Total no of positive samples
				ACLSV	ToRSV	ACLSV+ ToRSV	
Alburz	Charbagh	1	16	0 [a] (0.00) [b]	5 (31.30)	0 (0.00)	5 (31.3)
	Shahrestanak	2	11	2 (18.20)	1 (9.10)	1 (9.10)	4 (36.4)
	Nazarabad	2	7	0 (0.00)	1 (14.30)	1 (14.30)	2 (28.6)
	Total	5	34	2 (5.90)	7 (20.60)	2 (5.90)	11 (32.40)
Tehran	Abali	5	13	0 (0.00)	7 (53.84)	0 (0.00)	7 (53.84)
	Bidak	7	23	0 (0.00)	7 (30.40)	1 (4.30)	8 (34.78)
	Vadan	5	12	0 (0.0)	3 (25.0)	1 (8.33)	4 (33.33)
	Sarbandan	1	5	0 (0.00)	1 (20.00)	0 (0.00)	1 (20.00)
	Ochonak	5	5	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
	Total	23	58	0 (0.00)	18 (31.00)	2 (3.44)	20 (34.48)
Guilan	Emamzadeh Hashem	1	4	0 (0.00)	2 (50.00)	0 (0.00)	2 (50.00)
	Lahijan	1	11	1 (9.09)	4 (36.36)	0 (0.00)	5 (45.45)
	Rasht	3	5	0 (0.00)	0 (0.00)	1 (20.00)	1 (20.00)
	Total	5	20	1 (5.00)	6 (30.00)	1 (5.00)	8 (35.00)
Total		33	112	3 (2.67)	31 (27.67)	5 (4.46)	39 (34.82)

a: number of ELISA positive samples. ASGV and ASPV were not detected in any of the samples.
b: Infection rate (%)

ToRSV isolates induced systemic reaction on different indicator plants belonged to *Chenopodiaceae*, *Cucurbitaceae* , *Fabaceae* and *Solanaceae* families) (Table 2). The reaction of ToRSV-B2 isolate on *Nicotaina tabacum* cv. White burley (Fig 2a), *N. occidentalis* (Fig. 2b), *Cucumis sativus* (Fig. 2c) was shown in Fig 2. No infection was found on *Ocimum basilicum* (*Lamiaceae*, family). Meanwhile, systemic reaction including vein clearing and mottle were observed on *Ch. quinoa* (Fig. 2d), those were inoculated with the ACLSV-infected pear samples (Table 2). The herbaceous host range of ACLSV and ToRSV isolates from pear were similar to those previously reported for corresponding isolates from other crops (15, 16). Inoculation assays of different ToRSV isolates collected from various sources, showed four biological groups using *N. benthamian* (17). Inoculation of Iranian ToRSV-B2 isolate on *N. bethamiana* induced necrotic local lesions on the inoculated leaves fallowed by mottle and mosaic, and it seems that ToRSV-B2 has different biological reaction from those reported by Li *et al* (17). Four weeks post inoculation all symptomatic and asymptomatic plants were assayed by ELISA (data not shown). Young pear plants showed mottling in upper new leaves six to eight weeks after graft inoculation by ToRSV and infection was confirmed using ELISA.

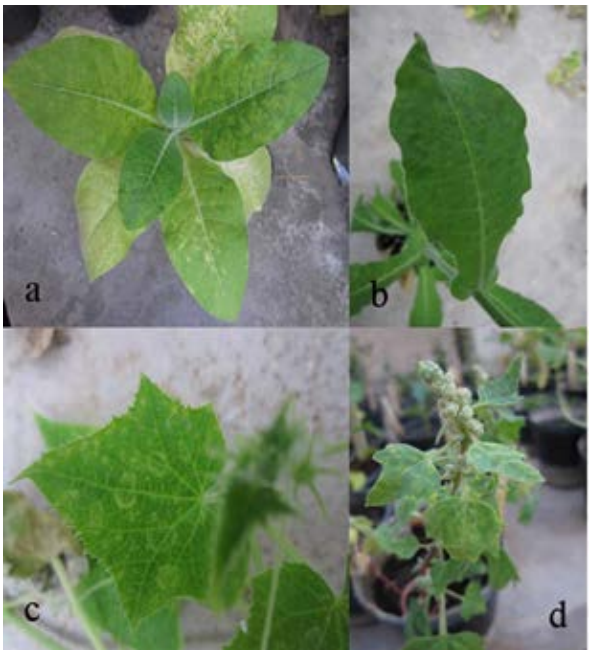

Fig. 2. Symptoms induced by *Tomato ring spot virus* isolate B2 on *Nicotaina tabacum* cv. White burley (a), *N. occidentalis* (b), *Cucumis sativus* (c), and *Apple chlorotic leaf spot virus* isolate on *Chenopodium quinoa* (d) indicator plants.

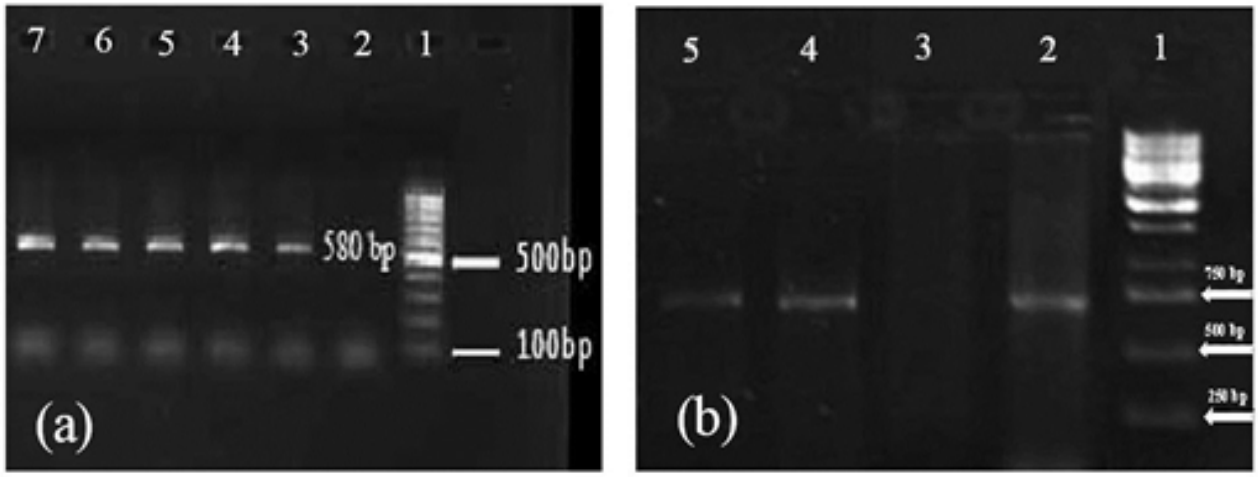

Fig. 3. Gel electrophoresis of PCR products, (a) *Tomato ring spot virus* detection using RdRp specific primers. Lane 1, 100 bp DNA Ladder (Ferments, Lithuania); Lane 2, Negative control; lane 3, Positive control; lanes 4-7, positive samples with ToRSV (a DNA fragment about 580 bp were amplified). (b) *Apple chlorotic leaf spot virus* detection using CP specific primers. Lane 1, 1 Kbp DNA Ladder (Ferments, Lithuania); Lane 2, Positive control; Lane 3, Negative control; lanes 4 and 5, positive samples with ACLSV (a DNA fragment about 700 bp were amplified).

Table 2: Symptoms induced by *Apple chlorotic leaf spot virus* and *Tomato ringspot virus* isolates on indicator plants.

Family	Scientific name	Virus	
		ACLSV	ToRSV
Amaranthaceae			
	Gomphrena globosa	-	Nrs
Chenopodiaceae			
	Chenopodium quinoa	Cll, Vc,Mo, Ld	Cll, Vc,Mo, Ld
	Ch. amaranticolor	Cll, Nll, VC,Mo, Ld	Cll, Nll, VC,Mo, Ld
	Ch. murale	Cll,Vc, Mo	Cll,Vc, Mo
Cucurbitaceae			
	Cucumis sativus	-	Cls, Vc, Snrs
Fabaceae			
	Phaseolus vulgaris	-	Nll, Tn
	Vigna unguiculata	-	Nll, Mo
Lamiaceae			
	Ocimum basilicum	-	-
Solanaceae			
	Nicotiana occidentalis	-	Snrs, Vc, Mo
	N. clevelandii	-	Cll, Vc
	N. benthamiana	-	Snrs, Vc, Mo
	N. tabacum cv. White Burley	-	Snrs
	N. tabacum cv. Xantia	-	Snrs
	N. rustica	-	Mo

Cll: chlorotic local lesion; Cls: chlorotic local spot; Ld: leaf deformation; Mo: mottle; Nll: necrotic local lesions; Nrs: Necrotic ring spot; Srsn: systemic necrotic ring spot;Tn: Top Necrosis; Vc: vein clearing; -: without infection

The RT-PCR analysis of total RNA extracts from samples using specific primers resulted to amplification of two DNA fragments with the expected or close to the expected size of approximately 580 bp (Fig. 3a) and 700 bp (Fig. 3b), which confirmed the presence of ToRSV and ACLSV infections, respectively. BLAST analysis and sequence alignment of partial nucleotide sequence of RdRp gene revealed 96% identity with ToRSV isolate 13C280 from *Prunus* sp., USA (Accesion no. KM083890). Also, BLAST analysis using partial sequence of Iranian ACLSV isolate obtained in this study from pear showed 92% nucleotide identity with Turkish ACLSV-KP2 isolate (Accesion no. AY730558).

Serce and Rosner (18) showed that the ACLSV isolates were classified into three main groups, which the Turkish peach isolate (ACLSV-KP2) fell in subgroup I that belonging to phylogenetic group III, with tow apple isolates from Hungary (18). This group containing isolates of variable characteristics was divided into two subgroups, I, including mixed European isolates, and II, including central European isolates (18). They also, showed a correlation between nucleotide sequence divergence and the geographic origin of the ACLSV isolates, and this may give an indication of extraction of the Turkish isolates (18). ACLSV has been previously reported from apple in Iran (10), but this is the first report on detection of ACLSV in pear using biological, serological and molecular assays, in Iran.

ToRSV infects a broad range of herbaceous and perennial plants, and causes serious diseases like peach stem pitting and yellow bud mosaic, prune brown line, and grapevine decline. Multiple sequence alignment indicated a high variability in coat protein of ToRSV isolates; however, the 3'UTR region is highly conserved (17). In addition, phylogenetic analysis using 3'UTR sequences showed two main groups that most of the isolates except two of them (PV-100 and RIB9001 from grapevine and redcurrant, respectively) clustered in group I. But, no correlation was found between the 3'UTR sequences and symptom expression on *N. benthamiana*, host plant or geographic origin of ToRSV isolates (17).

Previous studies have shown the occurrence of ToRSV in some deciduous fruit trees,

grapevine, and field crops such as soybean and tomato in Iran (9; 19; 20; 21), however, this analysis provides the first demonstration, to our knowledge, of occurrence of ToRSV in pear trees in Iran. Beside the strict quarantine regulations to be applied to new imports, the need for a national wide certification program is highly desirable. Certification program should be conducted using field inspections and clinical laboratory tests. However, associating defined field symptoms with the ACLSV and/or ToRSV infection was generally difficult due to the weak conditions of growth in many gardens, mainly because of global warming and water shortage in recent years. The occurrence of ToRSV in samples collected from nurseries confirmed the necessity for such certification program. It would be interesting to further characterize the genetic variability of different ToRSV and ACLSV isolates from pear using complete genome sequences to advance our knowledge of diversity in these viruses.

Conclusion

This analysis provides, to our knowledge, the first demonstration of pear trees infection with ACLSV and ToRSV in Iran. Early detection of viral pathogens and accurate diagnosis, are undoubtedly essential for controlling the spread of virus and ensuring production of healthy plant reproductive material for further cultivation.

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