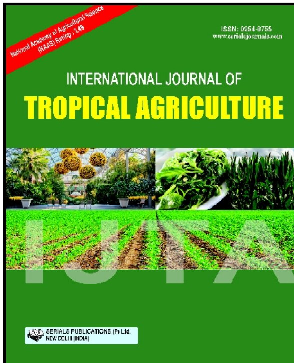

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Incidence of Apple Stem Grooving Virus and Apple Chlorotic Leaf Spot Virus of Pear in North Western Himalayan Region

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Abstract: *Apple chlorotic leaf spot virus* (ACLSV) and *Apple stem grooving virus* (ASGV) are important viruses of pome fruit in India. RT-PCR protocol was developed to detect both the viruses of pear. A survey was carried out to know the status of ACLSV and ASGV infecting pear in North Western Himalayan region of India. These samples were screened by ELISA and RT-PCR; it was found that, out of 59 germplasm, 13 were positive for ACLSV and 9 for ASGV. This is a first extensive study on the distribution of ACLSV and ASGV in pear orchards in India.

Keywords: Pear, DAS-ELISA, RT-PCR, ACLSV, ASGV

INTRODUCTION

Pear (*Pyrus communis*) is an important temperate fruit crop after apple in acreage, production and varietal diversity in India. It is cultivated in 40000 ha area in India with 323000 MT production and 8 t/ha productivity (Anonymous, 2017). Relatively low productivity of pear is due to several biotic and abiotic factors. Among biotic factors diseases caused by viruses play an important role. Several viruses which infects pear are *Apple Chlorotic leaf spot virus* (ACLSV), *Apple mosaic virus* (ApMV), *Apple stem grooving virus* (ASGV) and *Apple stem pitting virus* (ASPV).

ACLSV is a type member of the genus *Trichovirus* (Martelli *et al.* 1994) of *Betaflexiviridae* family (Adams *et al.* 2004). ASGV is also a member of the

plant virus family *Betaflexiviridae* and the type member of the genus *Capillovirus*. ACLSV is an economically important virus of pome fruits because it is distributed worldwide. Although most strains are latent in fruit trees, while others could be responsible for russetting, top working disease and lethal decline. ASGV is also economically important and common pathogen in commercial cultivars (Welsh and Van der Meer, 1989). Its infection could cause 12 to 30 percent reduction in yield (Cembali *et al.* 2003).

ELISA is generally used for routine virus indexing of planting material in pome fruits. It is simple, robust and cost-effective but often fails because of low virus titres or the inhibitory effects of plant polysaccharides or phenolic compounds. Reverse transcription (RT) coupled with Polymerase Chain Reaction (PCR) assay has emerged as a most reliable method for detection of RNA viruses. It provided rapidity and sensitivity to potentially overcome some of the problems mentioned above. Hence we developed RT-PCR protocol for the detection of ACLSV and ASGV in pear germplasm. Many reports are available on the incidence of apple viruses in India but very few studies were carried out on pear viruses. Keeping this in view we planned this study to record the incidence of ACLSV and ASGV in pear orchards of the north-western Himalayan region.

During the routine survey of pear orchards in the North-Western Himalayas, plants showing symptoms of virus-like diseases were marked. Leaf samples from such suspected plants were collected for laboratory assay. Pear germplasm maintained at Dhanda farm of ICAR-Indian Agricultural Research Institute, Regional Station, (IARI-RS) Shimla (HP) and ICAR-National Bureau of Plant Genetic Resources, Regional Station, (NBPGR-RS) Shimla were indexed for the presence of ACLSV and ASGV. Lab-based techniques were carried out in the Division of Plant Protection, ICAR-Central Potato Research Institute (CPRI) Shimla, HP (India). Visual inspection of specific symptoms of virus and viroid infections was also carried out during the field surveys.

MATERIALS AND METHODS

Leaves collected from different pear plants were then serologically subjected to detection of ACLSV and ASGV through DAS-ELISA. For ELISA test, reagents, buffers and controls supplied by the BIOREBA AG (Switzerland) were used as per the instructions of the manufacturer. ELISA results were interpreted as per Lemmetty, (1988) and Dijkstra and Jager, (1998), wherein the samples were considered infected when their OD values at 405nm (A_{405}) exceeded two times the mean values of respective healthy and negative control samples.

Total RNA was isolated from leaves of pear plants by using Spectrum™ Plant Total RNA kit (Sigma-Aldrich, USA) as per manufacturer's instructions. The isolated total RNA was quantified by Thermo Scientific Nanodrop 2000 and stored at -80 °C until further use. First strand complementary DNA (cDNA) was synthesized by using cDNA synthesis kit as per manufactures instructions (Promega, Madison, USA).

Virus-specific primers were designed by aligning CP gene sequences of ACLSV and ASGV obtained from National Centre for Biotechnology Information (NCBI). Three pair of primers were designed for ACLSV and one pair for ASGV. Primers were selected by analysing GC ratio, primer dimer formation, self-complementary of primers and confirmed its specificity using BLAST (Table 1). Selected primers were synthesized at Integrated DNA Technologies, Inc. (IDT), USA. To avoid false negative results, primers for internal control designed by Menzel *et al.* (2002) i.e., sense: 5'-gatgcttcttggggcttcttgtt-3' and antisense: 5'-ctccagtcaccaacattggcataa-3' giving 181 base pair amplification were used in the present study.

Table 1
Details of primers

S.N.	*Code*	*Sequence*	*Polarity*	*Position of primer within CP gene*	*Product size (bp)*
1	ACLSV 1F	GAATTCATGGCGGCAGTGCT	Sense	1-20	582
	ACLSV 1R	CTCGAGTTAAACGCAAAGATCAGTT	Antisense	598-579	
2	ACLSV 2F	GAATTC ATGGCGGCAGTGCTGAATC	Sense	1-25	582
	ACLSV 1R	CTCGAGTTAAACGCAAAGATCAGTT	Antisense	598-579	
3	ACLSV 3F	ATGGCGGCAGTGCTGAATCTT	Sense	1-21	582
	ACLSV 3R	TTAAACGCAAAGATCAGTTGTAAC	Antisense	582—559	
4	ASGV 1F	TGCTGCCACTTCCAGGCAGAAC	Sense	396-417	303
	ASGV 1R	AGATTGCTCTCCGAACCCGCCT	Antisense	698-677	

For standardization of protocol for ACLSV and ASGV, PCR was carried out in thin walled 1.25 ml tubes in GeneAmp PCR 9700 system (applied biosystems, USA). The reaction mixture of 20 ml containing 2.8 µl of 10 X dream Taq buffer (thermo scientific), 2 µl of 2.5 mM dNTP mix (GeNei, Bangalore, India), 0.5 µl of 10 pM upstream and downstream primers each, 0.4 ml of 5 U/µl of dream Taq DNA polymerase (thermo scientific), 2.0 µl of cDNA and volume was made up to 20 ml with DEPC treated water. Amplification was carried out by following PCR conditions with primer pairs along with an internal control. Denaturation was performed at 95°C/30 s annealing temperature/30 s followed by extension at 72°C /1 min. for 40 cycles along with a final elongation step at 72°C for 10 minutes. After PCR, about 10 ml of the reaction mixture from each tube was loaded onto 1 % agarose gel alongside 1 kb DNA ladder as molecular weight marker was also loaded. Electrophoresis was carried out at 80 V, the buffer used was 1x TAE at pH 8.0. The DNA bands in the gel were visualized on a UV-transilluminator and primer pair showing expected size was selected. For optimization of annealing temperature same PCR mix and PCR conditions were used (as mentioned above) on varying temperatures ranging from 54 °C to 60 °C (54 °C, 55 °C, 56 °C, 57 °C, 58 °C, 59 °C and 60 °C). Once annealing temperature was standardized, the optimized parameters were used for further studies. After standardization of RT-PCR parameters the protocol was used for the detection of ACLSV and ASGV in the samples collected from different locations.

RESULT AND DISCUSSION

Surveys were conducted for monitoring viral diseases of pear in North Western Himalayan region. Leaves showing the variety of symptoms (Fig 1) including uneven lamina, mottling, line patterns, vein yellowing, chlorosis, mosaic etc. were observed. Leaf samples were collected from all the symptomatic trees for laboratory analysis. Twenty-eight commercial cultivars, as well as thirty-one exotic collection of pear, were serologically tested for the presence of ACLSV and ASGV. OD value-based serological detection of viruses revealed that out of 28 commercial cultivars 6 and out of 31 exotic collections 3 were found positive for ACLSV, whereas the rest of the cultivars were found free from infection. In case of ASGV, 5 commercial cultivars and 2 exotic collections were found positive (Table 2).

Table 2
Virus indexing of pear cultivars through ELISA and RT-PCR against ACLSV and ASGV

Sr no	*Name of cultivar*	*Botanical name*	*Origin*	*Place of collection*	*Altitude (meter)*	*Virus indexing*			
						ACLSV		*ASGV*	
						ELISA	*RT-PCR*	*ELISA*	*RT-PCR*
1	Red Bartlet	*P. communis*	USA	Kullu	1216	-	-	-	-
2	Max Red Bartlet	*P. communis*	USA	Kullu	1216	-	-	-	-
3	Starkrimson	*P. communis*	USA	Kullu	1216	+	+	+	+
4	Williams	*P. communis*	USA	Manali	1893	+	+	+	+
5	Red Bartlet	*P. communis*	USA	Rohru, Shimla	1590	-	+	-	-
6	Williams	*P. communis*	USA	Rohru, Shimla	1590	-	-	-	-
7	Doyenne Du Comice	*P. communis*	France	Rohru, Shimla	1590	-	+	-	-
8	Moti Dandi	*P. communis*	-	Kotkhai, Shimla	1831	-	-	-	-
9	Conference	*P. communis*	Britain	Kotkhai, Shimla	1831	+	+	+	+
10	Carmen	*P. communis*	Italy	Kotkhai, Shimla	1831	-	+	-	-
11	Packham	*P. communis*	Australia	Kotkhai, Shimla	1831	+	+	+	+
12	Kashmir Pear	*P. pyrifolia*	India	IARI-RS, Shimla	1936	-	-	-	-
13	Fertility	*P. communis*	Britain	IARI-RS, Shimla	1936	-	-	-	-
14	Fertility	*P. communis*	Britain	IARI-RS, Shimla	1936	-	-	-	-
15	Fertility	*P. communis*	Britain	IARI-RS, Shimla	1936	-	-	-	-
16	Fertility	*P. communis*	Britain	IARI-RS, Shimla	1936	-	-	-	-
17	Fertility	*P. communis*	Britain	IARI-RS, Shimla	1936	-	-	-	-
18	Dr Jules Guyot	*P. communis*	France	IARI-RS, Shimla	1936	-	-	-	-
19	Dr Jules Guyot	*P. communis*	France	IARI-RS, Shimla	1936	-	-	+	+
20	Dr Jules Guyot	*P. communis*	France	IARI-RS, Shimla	1936	-	-	-	-
21	Bartlett	*P. communis*	USA	IARI-RS, Shimla	1936	-	-	-	-
22	Bartlett	*P. communis*	USA	IARI-RS, Shimla	1936	-	-	-	-
23	Bartlett	*P. communis*	USA	IARI-RS, Shimla	1936	-	-	-	-

contd. table 2

Sr no	Name of cultivar	Botanical name	Origin	Place of collection	Altitude (meter)	Virus indexing			
						ACLSV		ASGV	
						ELISA	RT-PCR	ELISA	RT-PCR
24	Bartlett	*P. communis*	USA	IARI-RS, Shimla	1936	-	-	-	-
25	Virod Anglis	*P. communis*	-	NBPGR, Shimla	1900	-	-	-	-
26	Beurre Hardy	*P. communis*	France	NBPGR, Shimla	1900	-	-	-	-
27	Flemish Beauty	*P. communis*	Belgium	NBPGR, Shimla	1900	-	-	+	+
28	Nijisseiki	*P. pyrifolia*	Japan	NBPGR, Shimla	1900	+	+	+	+
29	Pearce	*Pyrus* sp.	USA	NBPGR, Shimla	1900	-	-	-	-
30	Devoe	*P. communis*	Canada	NBPGR, Shimla	1900	-	-	-	-
31	Severyanka	*P. elaeagrifolia*	USSR	NBPGR, Shimla	1900	-	-	-	-
32	PI 264694	*P. communis*	USA	NBPGR, Shimla	1900	-	-	-	-
33	PI 541931	*Pyrus pyrifolia*	USA	NBPGR, Shimla	1900	-	-	-	-
34	Santya Brasakaya	*Pyrus* sp.	USSR	NBPGR, Shimla	1900	+	+	+	+
35	PI 282935	*P. communis*	USA	NBPGR, Shimla	1900	-	-	-	-
36	*Pyrus ussuriensis*	*P. ussuriensis*	Korea, Japan	NBPGR, Shimla	1900	-	-	-	-
37	Keiffer	*P. communis* × *P. pyrifolia*		Japan	NBPGR, Shimla	1900	-	-	--
38	Chinese Sandy Pear	*P. pyrifolia*	China	NBPGR, Shimla	1900	-	-	-	-
39	Moon Glow	*P. communis*	USA	NBPGR, Shimla	1900	-	+	-	-
40	Bergiffer	*Pyrus* sp.	USSR	NBPGR, Shimla	1900	-	-	-	-
41	Manning Elizabeth	*Pyrus* sp.	Belgium (USA)	NBPGR, Shimla	1900	-	-	-	-
42	Alozwed Hardee	*P. communis*		NBPGR, Shimla	1900	-	-	-	-
43	Leconte	*P. communis* × *P. pyrifolia*	Japan	NBPGR, Shimla	1900	-	-	-	-
44	Hood	*P. communis*	USA	NBPGR, Shimla	1900	-	-	-	-
45	Doynee Bussarch	*P. communis*	-	NBPGR, Shimla	1900	-	-	-	-
46	Kings Pear	*P. communis*	-	NBPGR, Shimla	1900	-	-	-	-

contd. table 2

Sr no	Name of cultivar	Botanical name	Origin	Place of collection	Altitude (meter)	Virus indexing			
						ACLSV		ASGV	
						ELISA	RT-PCR	ELISA	RT-PCR
47	Berlautsa	*Pyrus* sp.	USSR	NBPGR, Shimla	1900	-	-	-	-
48	Monarch	*P. communis*	-	NBPGR, Shimla	1900	-	-	-	-
49	Chugeryanshiki	*P. communis*	-	NBPGR, Shimla	1900	-	-	-	-
50	Korean giant pear	*P. pyrifolia*	Korea	NBPGR, Shimla	1900	-	-	-	-
51	Baldwin pear	*P. communis*	USA	NBPGR, Shimla	1900	-	-	-	-
52	Columbus red pear	*P. communis* × *P. pyrifolia*	-	NBPGR, Shimla	1900	-	-	-	-
53	Bruce	*Pyrus* sp.	-	NBPGR, Shimla	1900	+	+	-	-
54	Viva Gold	*P. communis*	Canada	NBPGR, Shimla	1900	-	-	-	-
55	Doyenne DU Comice	*P. communis*	France	NBPGR, Shimla	1900	-	-		
56	Red Bartlet	*P. communis*	USA	Yangpa, Kinnaur	2493	+	+	-	-
57	Bartlett	*P. communis*	USA	Katgaon, Kinnaur	2226	-	-	-	-
58	Kieffer	*P. communis* × *P. pyrifolia*	Japan	Pangi, Kinnaur	2760	+	+	+	+
59	Starkrimson	*P. communis*	USA	Nako, Kinnaur	3626	-	-	-	-

Figure 1: Symptom of viral diseases of pear

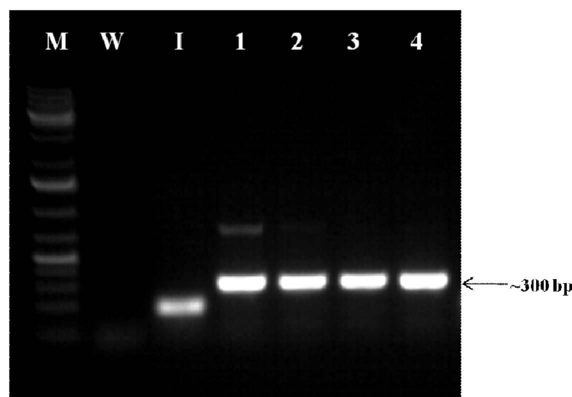

Figure 2: Optimization of annealing temperature for ASGV. Lane M: 1 kb DNA ladder, Lanes W: water control, I: Internal control, 1 to 4: ASGV +ve samples at different annealing temperature (56 °C, 58 °C, 60 °C and 62 °C)

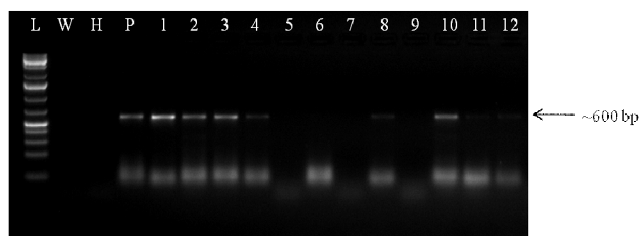

Figure 3: Validation of RT-PCR protocol for ACLSV by screening pear germplasm. Lane M: 1 kb DNA ladder, Lanes W: water control, H: Healthy control, P: positive control, 1 to 12: pear germplasm

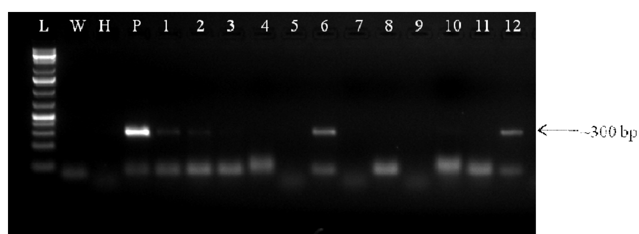

Figure 4: Validation of RT-PCR protocol for ASGV by screening pear germplasm. Lane M: 1 kb DNA ladder, Lanes W: water control, H: Healthy control, P: positive control, 1 to 12: pear germplasm

For RT PCR standardization and detection of the ACLSV three primer pair were designed and used (Table 1). The feasibility of the primers was checked according to the target virus. Among three primer pairs amplification of c-DNA fragments was obtained with ACLSV 2F and ACLSV 1R. An amplification of 582 bp was obtained. Among different annealing temperatures (54, 55, 56, 57, 58, 59 and 60 °C) tested 57 °C was found ideal and hence selected for further studies. For ASGV 1F and 1R gave expected size amplification (303 bp) without any multiple bands. Among different annealing temperature (56 °C, 58°C, 60°C and 62°C) 60 °C was found ideal (Fig. 2).

RT-PCR protocol standardized for detection of ACLSV and ASGV were validated by screening leaf samples collected from various locations ranging from 1590-3600 m height in North-Western Himalayan region (Fig 3 and 4). These germplasm originated from different countries and imported in India, out of 59 cultivars, 21 were from USA, 06 from France, 05 from Japan, 04 from USSR, 2 from Korea, Britain, Belgium, Canada and one each from, Italy, Australia, India and China. Among 59 germplasm indexed, 13 were positive for ACLSV and 9 were positive for ASGV. In case of ACLSV ELISA showed only 9 positive germplasm in contrary to

RT-PCR results where 13 germplasm were found positive (Table 2). It indicates RT-PCR is more sensitive and reliable technique than ELISA.

Survey of pear orchards in north-western Himalaya indicated presence of virus diseases. Pear leaves showed variety of symptoms viz., uneven lamina, mottling, line patterns, chlorosis, mosaic etc. similarly Wood, 1972 and Sertkaya, 2010 observed symptoms of virus diseases in pear in New Zealand and Turkey respectively. We report here the development of RT-PCR protocol for the detection of ACLSV and ASGV in pear. By using the conditions described, we amplified a 582 bp fragment specific for coat protein region of ACLSV from leaves of pear tree. Similarly Kinard *et al.*1996, Watpade *et al.*2012, Watpade *et al.*2013 were successful in amplifying fragments specific for ACLSV in apple by RT-PCR. Likewise by using optimised protocol for ASGV, fragment of 303 bp specific to ASGV coat protein gene was also amplified. Similarly Clover *et al.*2003, Foissac *et al.*2005, Zheng *et al.*2005, Zheng *et al.*2006 reported RT-PCR protocol for detection of ASGV in apple, kiwi, pear etc. In all the reactions of RT-PCR a primer set amplifying apple mitochondrial NADH dehydrogenase subunit 5 (nad 5) gene was used as an internal control.

After standardization of protocols for RT-PCR we screened the commercial cultivars collected from farmer's field, mother plant of commercial cultivars maintained at ICAR-IARI RS Shimla (India) and exotic germplasm maintained at ICAR-NBPGR, RS Shimla (India). These germplasms originated from different countries (Table 2) either European pear or Asian pear. Some of the cultivars like Keiffer, Leconte etc. are inter-specific hybrids and they were also screened. Based on results obtained we can conclude that among 59 different germplasm 15 were found infected with tested viruses (ACLSV and ASGV). Results imply that given primer set can be used to screen presence of ACLSV and ASGV in different germplasm of pear. Polak *et al.*(1997) from Czeck Republic reported detection of ACLSV through ELISA in apple and pear orchard as well as they studied distribution of ACLSV in Czeck Republic. In India this is the first extensive study on distribution of ACLSV and ASGV in pear orchards. From the results we can conclude that ACLSV and ASGV are present in pear orchards and indexing of mother plants is necessary to manage viral diseases in India. Based on the study we can conclude that RT-PCR is sensitive, reliable and fast procedure for detection of ACLSV and ASGV in pear. ACLSV and ASGV are present in pear orchards in north-western Himalayan region of India and indexing of mother plants is necessary to manage viral diseases.

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