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Establishing serological host range of major temperate fruit viruses

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Abstract
Young plants of standard and new cultivars of apple, peach, pear, plum, apricot, kiwifruit and strawberry growing in an experimental orchard in Solan district of Himachal Pradesh were visually indexed for observing typical symptoms caused by major viruses like apple mosaic virus (ApMV), apple chlorotic leafspot virus (ACLSV), apple stem pitting virus (ASPV), apple stem grooving virus (ASGV) and prunus necrotic ringspot virus (PNRSV) in these commercial fruit crops. With the objective of characterizing the associated viruses, samples were drawn from symptomatic plants for serological detection of these viruses in infected plants. Since majority of the viruses in temperate fruit crops are of latent nature, appearance of visual symptoms alone is not sufficient enough to be considered as an affirmative criteria for establishing the identity of major viruses in these crops. These studies observed DAS-ELISA to be an efficient tool for confirming the association of these viruses. Results of DAS-ELISA revealed that ApMV was detected in all temperate fruits, ACLSV in apple, pear and strawberry, ASPV in apple, pear, peach, plum and strawberry and PNRSV in peach, plum and strawberry. However, ASGV was not found in any test sample. The findings of these studies present an overview of the status of major viruses of temperate fruits in this region.

Keywords: Temperate fruits, viruses, DAS-ELISA

Introduction
Temperate fruits comprising of apple, pear, peach, plum, apricot, cherry, strawberry and kiwifruit account for a major part of the economic returns from horticulture sector in Himachal Pradesh. Apple is the largest contributor in terms of area and production though other temperate fruits do not contribute much towards the total income generated by the horticulture sector, these still have a key role in diversification of horticulture in the state. Total area under temperate fruits in Himachal Pradesh is around 1, 37,453 Ha with a production of 6,68,810 MT (Anonymous, 2016) [1].

Viruses of temperate fruit crops are of high significance owing to their worldwide occurrence and distribution in a number of temperate crops and a large number of viruses affect temperate fruit crops resulting in losses in fruit yield and quality of planting material. Impact of viruses in temperate fruit crops are largely unnoticed as the role of these pathogens in adversely affecting the productivity is witnessed over a period of time.

Accurate diagnosis and detection are the key aspects of managing viruses in an economically viable temperate fruit production system. Reliable detection of viruses in propagative material is a pre-requisite for the production of healthy planting material. These studies are primarily focussed on DAS-ELISA based serological detection of major viruses infecting temperate fruits to identify virus free healthy plants for taking budwood for raising future nurseries.

Material and Methods
Leaf samples drawn from different temperate fruits including kiwifruit and strawberry were collected from an experimental farm in Solan. On the basis of symptoms on leaves, plants were marked as positive or negative. Positively marked plants were further indexed by DAS-ELISA to confirm the presence of one or more of major five viruses considered for present studies.

DAS-ELISA
Infected leaves showing symptoms of mosaic, mottle, deformation, yellowing, shot holes, chlorotic and necrotic rings, distortion of mid vein, oak leaf pattern, puckering, cupping and

reduced leaf size collected from a single plant were considered as one composite sample and brought to the laboratory in ice bucket for conducting DAS-ELISA tests as per the protocol given by Clark and Adams (1977) [2]. Wells of the microtitre plate (NUNC certified microplates) except those of the top and bottom rows and rows on the extreme left and right, were filled with 200µl aliquots of coating antibodies diluted in 1x coating buffer (1:1000 ratio v/v). The plate was incubated in humid box for 4 hours at 30 ºC. The coating antibody suspension was removed by shaking out the plate over the wash basin. The wells were filled with 1x PBS-Tween and kept for 2 minutes with gentle shaking. The plate was emptied and filled again with PBS-Tween. The washing was repeated three times. The test samples were grounded in 1x extraction buffer (1:10 ratio v/v). All coated wells were filled with 200µl aliquots of test samples (each sample in duplicate) besides positive and negative control wells. The plate was incubated in humid box overnight at 4±1 ºC. The washing steps were repeated as mentioned above. Alkaline phosphate (ALP) conjugated antibodies were filled in each well with 200µl aliquots after diluting it in 1x ECI (enzyme conjugated immunoglobin) buffer at a (ratio of 1:1000 v/v). The plate was incubated in humid box for 5 hours at 30 ºC. The washing was done as mentioned above. P-nitrophenyl phosphate (pNPP) substrate was dissolved in 1x substrate buffer by dissolving 5mg pNPP tablet in 5ml of 1x substrate buffer. Each well was filled with 200µl aliquots of the substrate. The plate was kept in humid box in dark condition at room temperature until a yellow colour was clearly visible in the positive control (usually between 30-60 minutes). The results were assessed by measurement of the absorbance value of the hydrolysed substrate (p-nitrophenyl) at 405 nm wavelength in a microtitre plate reader (Micro Scan MS 5608A, Electronics Corporation of India Limited, Hyderabad). The results of ELISA for the detection were interpreted as per Dijkstra and Jager (1998) [3] as samples were considered infected when their absorbance values (A_{405}nm) exceeded two times the mean values of respective healthy control samples.

Results and Discussion

In the present study, attempts were made to serologically detect major viruses of temperate fruits like apple mosaic virus (ApMV), apple chlorotic leafspot virus (ACLSV), apple stem pitting virus (ASPV), apple stem grooving virus (ASGV) and prunus necrotic ringspot virus (PNRSV) in symptomatic plants of available cultivars of temperate fruits growing in the experimental orchard. Since symptoms on these fruit crops were observed to be of complex nature resulting due to the latent nature and low concentration of some of these viruses, the exact identity of the viruses could not be established on the basis of symptomatology alone. DAS-ELISA was therefore conducted to positively identify the viruses associated with these crops and to mark virus free plants for further use in healthy plant production system. The most prominent and striking symptoms of these viruses were in the form of mosaic, mottle, deformation, yellowing, shot holes, chlorotic and necrotic rings, distortion of mid vein, oak leaf pattern, puckering, cupping and reduced leaf size (Fig 1-4).

DAS-ELISA based detection of viruses in temperate fruits

Owing to theirlatent nature, ApMV and PNRSV remains symptomless in most of the temperate fruits even though these viruses invoke some striking symptoms like yellow mosaic, flecking, diffused chlorotic spots, brown lines, rings and leaf curling (Brunt *et al.* 1996 [4]; Hammond 2011 [5]; Almaraz *et al.* 2014 [6]) Leaf damage in the form of mosaic, flecking, yellow mottle, chlorotic rings, linear pattern and oak leaf patterns were observed in peach and apple by a number of workers (Halk *et al.* 1984 [7]; Imed *et al.* 1997 [8], Sanchez *et al.* 2004 [9]; Kapoor and Handa, 2017 [10]). Similar type of symptoms on many cultivars of apple and peach cv. July Elberta were observed in the present studies. Mixed infection by viruses was found to be of common occurrence in these temperate fruit crops. Similar observations have been made by a number of workers from other parts of the world as well (Di Terlizzi *et al.* 1992 [11]; Aouane, 2003 [12]; Polak, 2007 [13]; Matic *et al.* 2008 [14]; Scott, 014 [15]; Pallas *et al.* 2012 [16]).

Trees marked positive on the basis of visual indexing were screened by DAS-ELISA during active growing season for the presence of apple mosaic virus (ApMV), apple chlorotic leafspot virus (ACLSV), apple stem pitting virus (ASPV), apple stem grooving virus (ASGV) and prunus necrotic ringspot virus (PNRSV) in all temperate fruits. Since concentration of temperate fruit viruses vary in young and older leaves, composite samples of young and older leaves exhibiting variety of symptoms were used for virus detection (Fig. 1, 2 and 3). Virus concentration in leaves was assessed during spring season and OD values (nm) were recorded (Table1). The data in Table1 indicated that all viruses except for ASGV were present in one or more fruit crops. It is evident from the OD values recorded at A_{405} nm that ApMV was found in detectable limits in all cultivars of these temperate fruits except for Red Velox cultivar of apple. ASPV was observed to be the most prevalent virus after ApMV as shown by the OD values but not in apricot cultivar New Castle. ACLSV was found only in apple cultivar Scarlet Spur, strawberry cultivar Sweet Charlie and pear cultivar Gelato whereas none of the other cultivars of these temperate fruits had infection of ACLSV according to the OD values. A number of workers have also found that these viruses are capable of infecting several hosts in addition to apple (Isac *et al*. 2008 [17]; Negi *et al*. 2009 [18]; Wu *et al*. 2010 [19]; Rana *et al.* 2011 [20]; Piero *et al.* 2012 [21]). PNRSV, the most prominent virus in stone fruits was found to be associated with peach cultivar July Elberta, apricot cultivar New Castle, plum cultivars Duarte and Shiro besides strawberry cultivar Sweet Charlie. These results are in line with the findings of Salem *et al*. 2003 [22]; Myrtra *et al*. 2001 [23]; Chandel *et al.* 2013 [24]; Kapoor and Handa, 2017 [`10]) who reported the occurrence of PNRSV using DAS-ELISA in temperate fruits. ApMV was detected in strawberry by Tzanetakis and Martin (2005) [25] and similar results were observed in present studies. However, there are no reports of ASPV, PNRSV and ACLSV infections in strawberry.

Table 1: DAS-ELISA detection of major viruses in temperate fruits

Crop	Cultivar	O.D. Values (A_{405}nm)				
		ApMV	ACLSV	PNRSV	ASPV	ASGV
Apple	Gibson Golden	0.457(+)	0.279(-)	0.299(-)	0.305(-)	0.237(-)
	Early Red I	0.375(+)	0.246(-)	0.285(-)	0.217(-)	0.202(-)
	Gale Gala	0.357(+)	0.260(-)	0.271(-)	0.225(-)	0.207(-)
	Scarlet Spur	0.506(+)	0.423(+)	0.432(-)	0.250(-)	0.362(-)
	Golden Delicious	0.408(+)	0.313(-)	0.318(-)	0.370(+)	0.206(-)
	Anna	0.399(+)	0.233(-)	0.216(-)	0.277(-)	0.214(-)
	Granny Smith	0.362(+)	0.268(-)	0.227(-)	0.306(-)	0.203(-)
	Jeromine	0.338(+)	0.237(-)	0.198(-)	0.205(-)	0.118(-)
	Red Velox	0.313 (-)	0.219(-)	0.238(-)	0.218(-)	0.152(-)
Pear	Gelato	0.410(+)	0.395(+)	0.331(-)	0.442(+)	0.247(-)
Plum	Shiro	0.485(+)	0.380(-)	0.391(+)	0.308(+)	0.231(-)
	Shiro	0.544(+)	0.279(-)	0.335(-)	0.347(+)	0.264(-)
	Duarte	0.492(+)	0.283(-)	0.676(+)	0.376(+)	0.223(-)
Apricot	New Castle	0.439(+)	0.375(-)	0.369(+)	0.261(-)	0.297(-)
Peach	July Elberta	0.454(+)	0.265(-)	0.389(+)	0.322(+)	0.227(-)
Kiwi	Monty	0.334(+)	0.260(-)	0.270(-)	0.492(+)	0.175(-)
	Allison	0.400(+)	0.237(-)	0.250(-)	0.210(-)	0.194(-)
Strawberry	Sweet Charlie	0.549(+)	0.425(+)	0.385(+)	0.388(+)	0.289(-)
Positive Control		0.820	0.722	0.792	0.705	0.810
Negative Control		0.159	0.216	0.180	0.115	0.200

Fig 1: Yellow mosaic and flecking in apple-ApMV

Fig 2: Puckering and severe deformation of apple leaves-ACLSV

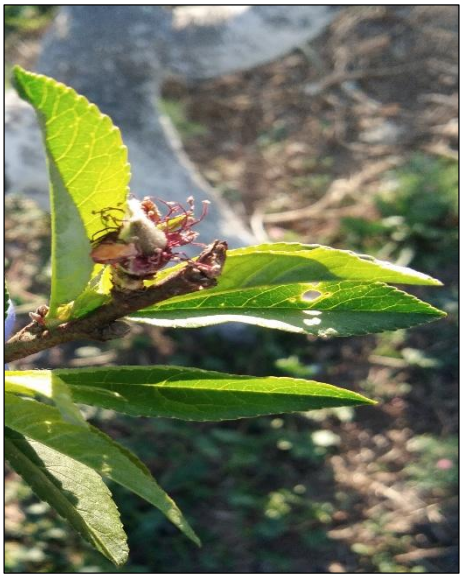

Fig 3: Necrotic shot holes on peach cv. July Elberta leaves-PNRSV

Fig 4: Yellowing and reduction of leaf size in apricot-ApMV

Fig 5: Oak leaf pattern in strawberry-ApMV

Fig 6: Shot holes and leaf deformastion in plum-PNRSV

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