www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.03
TPI 2018; 7(10): 536-540
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www.thepharmajournal.com
Received: 21-08-2018
Accepted: 22-09-2018

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ELISA and DIBA- Efficient tools for indexing peach against prunus necrotic ringspot virus

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Abstract
Surveys conducted in 40 peach orchards of commercial peach growing orchards in the districts of Solan, Sirmour, Shimla and Kullu in Himachal Pradesh revealed incidence of prunus necrotic ring spot virus (PNRSV). Diffused chlorotic spots, necrotic rings, mid vein distortion and shot holes were the predominant symptoms. Leaves from symptomatic trees were collected for serological detection through DAC and DAS-ELISA and DIBA. All these tests resulted in the detection of PNRSV in trees marked on the basis of visual symptoms. Association of PNRSV with viral symptoms in peach was successfully confirmed by ELISA and DIBA assays in the present studies.

Keywords: Peach, PNRSV, DAC, DAS, Elisa, DIBA

1. Introduction
Peach (*Prunus persica* L.) Batch is probably the most important stone fruit grown worldwide. A member of the family Rosaceae, peach is cultivated for both table and processing purposes. Commercial cultivation of peaches is expanding at a faster pace as they come early in the market during the season (mid-April onwards) and growers can get higher returns due to the unavailability of other fresh fruits at that time of the year. Due to introduction and adaptation of low chilling cultivars, peach and plum are also now being grown commercially in certain areas of the north Indian plains. Among all stone fruits, peach is the most important in terms of production and area under cultivation.
Serological detection of a virus infecting peaches in Himachal Pradesh revealed the prevalence of PNRSV (Chandel *et al*. 2013; Kapoor and Handa, 2017a) [1, 2]. However, the virus is largely known to cause no recognizable symptoms in most of the commercial cultivars of peach (Fiore *et al*. 2008) [3]. Serological and molecular methods of detection by employing enzyme-linked immunosorbent assay (ELISA) and dot immune binding assay (DIBA) are widely used all over the world in different virus indexing and certification programmes of peach (Mink, 1992)[4]. However, for virus indexing on mass scale, ELISA is widely employed because of its rapidity, accuracy and sensitivity.

2. Materials and Methods
2.1 Serological detection of PNRSV
Leaf samples exhibiting typical symptoms were drawn from the symptomatic marked plants and brought to the laboratory in separate polythene bags in an ice box so as to keep the leaf samples fresh for serological detection of prunus necrotic ring spot virus (PNRSV) through Alkaline phosphatase (ALP) based DAC (direct antigen coating), DAS (double antibody sandwich) forms of ELISA and DIBA as per the procedure given below:

2.2 DAC-Elisa
ALP based DAC form of enzyme-linked immunosorbent assay (ELISA) was used to detect the viruses in test samples as per the protocol proposed by Handa and Bhardwaj (1994) [5]. Young leaves exhibiting typical viral symptoms were harvested and brought to the laboratory in polythene bags. Leaf extract of each sample in extraction buffer (1:20 w/v) was prepared by crushing the leaves in a tissue homogenizer (SEDIAG, France) and used for coating the wells of microtitre ELISA plates. The wells of microtitre plate were filled with 200 µl aliquots of test sample. The coated plates were kept in a humid box and incubated overnight at 4±1 ^{0}C. The plates were washed by removing suspension of samples by vigorously shaking out the plate over the wash basin. The wells were filled with 1X PBS-Tween and kept for 2 minutes

with gentle shaking and emptied the plate and in this way the washing was repeated 3 times or simply by washing in ELISA plate washer. Then the wells were filled with 200 µl aliquots of coating antibodies diluted in 1X coating buffer. The plate was incubated in humid box for 2 hours at 37 ^{0}C the washing of the plates was done as mentioned before. ALP labeled goat anti-rabbit IgG conjugate (GeNei, Bangalore) were filled in each well with 200 µl aliquots after diluting in 1X conjugate buffer. The plate was incubated in a humid box for 2 hours at 37 ^{0}C. The washing of plate was done as mentioned previously. The pNPP substrate was dissolved in 1X substrate buffer (5 mg pNPP tablet in 5 ml of substrate buffer) under dark conditions. Each well was filled with 200 µl aliquots of substrate. The plate was kept in humid box in dark at room temperature. The plate was incubated until a yellow colour was visible in the positive controls (usually between 30 and 90 minutes). If desired, the reaction was stopped by adding 50 µl of 3M NaOH to each well. 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 MS5605A, Electronic Corporation of India Limited).

Leaf samples from fifty symptomatic trees were collected from the symptomatic trees for serological detection of two ilarviruses namely apple mosaic virus (ApMV) and prunus necrotic ring spot virus (PNRSV) through DAC-ELISA since these two viruses have been reported to be associated with stone fruits in India. For all serological tests of ELISA, immunoreagents, buffers, positive and negative control supplied by BIOREBA AG (Switzerland) were used as per the instructions issued by the supplier. The results of the ELISA for detection were interpreted by following Lemmetty (1988) [6] and Dijkstra and Jager (1998) [7] as samples were considered infected when their OD values at 405nm exceeded two times the mean values of respective healthy and negative control samples.

2.3 Das-Elisa

Alkaline phosphatase based direct double antibody sandwich enzyme-linked immunosorbent assay (ELISA) was used to detect the virus as per the protocol of Clark and Adams (1977) [8] with slight modifications. Leaf samples from twenty symptomatic trees which were found positive in DAC-ELISA were collected for further serological detection through DAS-ELISA. The detailed procedure used for DAS-ELISA based serological detection of PNRSV is described hereunder:

In DAS-ELISA, wells of the microtitre plate except those of the top and bottom rows on the extreme left and right were first filled with 200 µl coating antibody. The plate was incubated in humid box for 4 hours at 30 ^{0}C. The coating antibody suspension was removed by vigorously shaking out the plate over the wash-basin. The wells were filled with 1X PBS-Tween and kept for 2 min with gentle shaking emptied the plate and filled again with PBS-Tween. The washing was repeated three times or by washing in ELISA plate washer. The leaf extract from the test samples were prepared in buffer. All coated wells were filled with 200 µl aliquots of test samples (each sample at least in duplicate) besides positive control and negative control wells. The plates were incubated in humid box overnight at 4±1 ^{0}C. The washing step was repeated as mentioned above. The specific alkaline phosphatase (ALP) based conjugated antibodies were filled in each well with 200 µl aliquots. The plate was incubated in humid box for 5 hours at 30 ^{0}C. The washing was done as mentioned above. The p-nitrophenyl phosphate (pNPP) substrate was dissolved in 1X substrate buffer by dissolving 5 mg pNPP tablet in 5 ml of 1X substrate buffer under the dark conditions. Each well was filled with 200 µl aliquots of substrate. The plates were kept in humid box in the dark condition at room temperature after giving a brief incubation of 15 minutes at 30 ^{0}C. The plates were incubated until a yellow colour was clearly visible in the positive controls (usually between 30 and 90 minutes). If desired the reaction was stopped by adding 50 µl of 3M NaOH to each well. The results were assessed either by measurement of the absorbance value of the hydrolyzed substrate (p-nitrophenyl) at 405 nm wavelength in a microtitre/(ELISA) plate reader (Micro Scan MS5605A, Electronic Corporation of India Limited) or through visual screening.

2.4 Dot Immuno Binding Assay (DIBA)

Peach trees testing positive for PNRSV were further analyzed using Dot immuno binding assay (DIBA). Composite leaf samples from healthy and PNRSV infected peach trees were used for DIBA wherein leaves from top, middle and bottom branches were collected and brought to the laboratory. The leaves were further rolled longitudinally to form a tight scroll and a sharp razor blade was used to cut them at the center and cut ends of such leaves were immediately pressed on the surface of a nitrocellulose membrane (0.45µm pore size, Bio-Rad). The sample tissues were then homogenized with extraction buffer (1:100) and 2 µl of the mixture transferred onto a nitrocellulose membrane. The membrane was air dried and blocked for 30 min with TBS-milk buffer consisting of 10 mM Tris–HCl, (pH 7.4), 0.15 M NaCl and defatted milk powder (50 g/l). The membranes were then washed thrice with distilled water. After that, membrane was incubated for 60 min in 1 µg/ml MAbs diluted with PBS-milk buffer and washed for 5 min with PBST (PBS + Tween-20, 3g/l) followed by two washes with distilled water. The membrane was incubated for 60 min in 1:5000 dilutions in PBS-milk buffer with appropriate goat anti-mouse alkaline phosphatase (ALP)-conjugated antibody (BIOREBA AG, Switzerland). Washing of the membrane was again repeated as before and was equilibrated in substrate buffer (0.1 M Tris–HCl, pH 9.5) for 5 min and polyvinylidene fluoride (PVDF). Observations were made on the basis of the development of brown color which was indicating positive reaction for the presence of PNRSV in test samples.

3. Results and Discussion

Leaf samples from the marked trees exhibiting typical viral symptoms in peach orchard were collected and brought to the laboratory in separate polythene bags in an ice box. These samples were further serologically detected for the presence or absence of ilarviruses (PNRSV or ApMV) through the use of DAC-ELISA. Further confirmation of the exact identity of the virus was established through DAS-ELISA. The results pertaining to serological detection are presented in Tables 1 and 2.

3.1 Serological detection of ilarviruses in peach orchard through DAC-ELISA

Alkaline phosphatase based DAC-ELISA was performed to detect the presence of ilarviruses (PNRSV or ApMV) in the leaf samples drawn from 50 symptomatic trees. The data on OD values and serological reactions set out in Table 1 clearly indicate the positive detection of both ilarviruses in test

samples. Positive samples had OD values that were at least double the OD value of negative control. ELISA plate depicting serological reaction of the virus isolates against ilarviruses in DAC-ELISA is presented in Fig 1. DAC-ELISA results confirmed the presence of *Ilarvirus* in the orchard surveyed. However, OD values of the samples in respect of PNRSV was higher than that of ApMV. Therefore, further investigations were carried out using DAS-ELISA to confirm whether PNRSV was present alone or in combination with ApMV as mixed infection.

Table 1: Serological detection of Ilarviruses (PNRSV or ApMV) in peach orchard through DAC-ELISA

Tree No.	OD Value (A_{405}nm)/ Serological Reaction	
	PNRSV	ApMV
1	0.102 (-)	0.009 (-)
2	0.333 (+)	0.267 (+)
3	0.008 (-)	0.123 (-)
4	0.144 (-)	0.156 (-)
5	0.356 (+)	0.265 (+)
6	0.036 (-)	0.020 (-)
7	0.045 (-)	0.097 (-)
8	0.445 (+)	0.319 (+)
9	0.367 (+)	0.266 (+)
10	0.044 (-)	0.038 (-)
11	0.377 (+)	0.236 (+)
12	0.099 (-)	0.077 (-)
13	0.353 (+)	0.256 (+)
14	0.056 (-)	0.087 (-)
15	0.279 (+)	0.234 (+)
16	0.061 (-)	0.054 (-)
17	0.178 (-)	0.163 (-)
18	0.301 (+)	0.259 (+)
19	0.126 (-)	0.132 (-)
20	0.093 (-)	0.075 (-)
21	0.323 (+)	0.267 (+)
22	0.045 (-)	0.064 (-)
23	0.146 (-)	0.137 (-)
24	0.280 (+)	0.258 (+)
25	0.068 (-)	0.074 (-)
26	0.308 (+)	0.251 (+)
27	0.280 (+)	0.209 (+)
28	0.124 (-)	0.098 (-)
29	0.142 (-)	0.128 (-)
30	0.106 (-)	0.115 (-)
31	0.278 (+)	0.234 (+)
32	0.053 (-)	0.065 (-)
33	0.098 (-)	0.049 (-)
34	0.297 (+)	0.269 (+)
35	0.105 (-)	0.089 (-)
36	0.333 (+)	0.287 (+)
37	0.109 (-)	0.112 (-)
38	0.067 (-)	0.100 (-)
39	0.290 (+)	0.258 (+)
40	0.103 (-)	0.068 (-)
41	0.114 (-)	0.126 (-)
42	0.312 (+)	0.276 (+)
43	0.095 (-)	0.076 (-)
44	0.116 (-)	0.104 (-)
45	0.290 (+)	0.257 (-)
46	0.278 (+)	0.289 (+)
47	0.126 (-)	0.134 (-)
48	0.067 (-)	0.059 (-)
49	0.299 (+)	0.301 (+)
50	0.097 (-)	0.065 (-)

Data presented in Table 1 on the basis of OD values clearly indicate the presence of ilarviruses (PNRSV and ApMV) as some of the samples reacted positively for both PNRSV and ApMV in DAC-ELISA. Out of 50 symptomatic trees, only 20 were found to be infected with ilarviruses as indicated by the OD values. Tree No. 8 recorded the maximum OD values of 0.445 followed by Tree No. 11 and 9 with OD values of 0.377 and 0.367, respectively for

PNRSV whereas in case of ApMV, Tree No. 8 recorded maximum OD value of 0.319 followed by Tree No. 49 and 46 with OD values of 0.301 and 0.289, respectively. DAC-ELISA has been used widely for the detection of plant viruses (Ramiah *et al*. 2001; Sujitha *et al.* 2015; Rageshwari *et al.* 2017) [9, 10, 11]. Successful detection of ilarviruses under present studies is in conformity with the findings of Roussel *et al*. (2004) [12] and Salem *et al.* (2004) [13] who have also used DAC-ELISA for the detection of ilarviruses in temperate fruits. Besides, a number of other workers have also reported the association of ilarviruses with stone fruits on the basis of DAC-ELISA tests (Digiaro *et al*. 1991; Mink, 1992; Hammond, 2003; Bashir *et al*. 2017) [14, 4, 15, 16].

3.2 Serological detection of PNRSV and ApMV in peach orchard through DAS-ELISA

Alkaline phosphatase based DAS-ELISA was performed for the detection of PNRSV and ApMV in the leaf samples drawn from 20 symptomatic trees marked in peach orchard and confirmed positive in DAC-ELISA test. Observations recorded on OD values are presented in the Table 2.

Table 2: Serological detection of PNRSV and ApMV in peach orchard through DAS-ELISA

Tree No.	O.D. Value A_{405}nm	
	PNRSV	ApMV
1	0.345 (+)	0.102 (-)
2	0.321 (+)	0.068 (-)
3	0.467 (+)	0.112 (-)
4	0.328 (+)	0.079 (-)
5	0.279 (+)	0.057 (-)
6	0.378 (+)	0.146 (-)
7	0.296 (+)	0.131 (-)
8	0.304 (+)	0.115 (-)
9	0.386 (+)	0.102 (-)
10	0.265 (+)	0.098 (-)
11	0.411 (+)	0.056 (-)
12	0.260 (+)	0.109 (-)
13	0.312 (+)	0.089 (-)
14	0.294 (+)	0.111 (-)
15	0.285 (+)	0.102 (-)
16	0.342 (+)	0.076 (-)
17	0.279 (+)	0.132 (-)
18	0.342 (+)	0.126 (-)
19	0.299 (+)	0.094 (-)
20	0.345 (+)	0.106 (-)

It is evident from the data in Table 2 and Fig 2 that the *Ilarvirus* associated with test samples is PNRSV and not ApMV as leaf samples from all 20 symptomatic marked trees reacted positively with antibodies against PNRSV and failed to record positive reaction with antibodies against ApMV. Tree number 3 recorded the maximum OD value of 0.467 followed by tree number 11 and tree number 9 with OD values of 0.411 and 0.386, respectively.

Use of DAS-ELISA for detecting viruses in peach is widely practiced and findings of the present studies are in line with

those of a number of workers who have observed DAS-ELISA to be an effective technique for detecting PNRSV in peach (Ghanem, 2000; Scott *et al*. 2001; Myrtra *et al*. 2001; Salem *et al.* 2003; Virscerk and Mavric, 2005; Szyndel *et al*. 2006; Salam *et al.* 2007; Almaraz *et al*. 2008; Chandel *et al*. 2013; Vemulapati *et al*. 2014; Kapoor and Handa, 2017 a &b; Kapoor and Handa, 2018) [17, 18, 19, 20, 21, 22, 23, 24, 1, 25, 2, 26, 27].

3.3 Detection of PNRSV using dot immuno binding assay (DIBA)

Under present investigations, an attempt was made to further substantiate the finding of DAS-ELISA results with dot immune binding assay (DIBA). Composite leaf samples drawn from top, middle and bottom branches of PNRSV infected 10 peach trees were loaded onto a nitrocellulose membrane and leaf samples from healthy trees were used as control. Extracted sap from both infected and healthy trees was used at a concentration of 1:100.

Table 3: Reaction of peach trees in Dot immune binding assay (DIBA)

Tree No	Status of PNRSV
1	Positive
2	Positive
3	Positive
4	Positive
5	Positive
6	Positive
7	Positive
8	Positive
9	Positive
10	Positive

The results depicted in Fig 3 and Table 3 clearly indicated that healthy trees had no zonation in the blot assay whereas samples from PNRSV infected trees had clear zonation and pronounced coloration effect around the dots. A critical look at the coloration effect in the dots in different test samples revealed that there was variation in the intensity of colour. Samples 2 and 8 had the maximum coloration around the dots thereby indicating that these samples had the highest concentration of the virus whereas sample 5 had the least virus concentration as indicated by faint coloration around the dot. Though DIBA is mainly used to reconfirm ELISA results for the detection of plant viruses (Bhat an Maheshwari, 2017) [28], the technique has an advantage over ELISA as it is economical and less time consuming. Under present studies also, DIBA test was performed to further confirm the results obtained in DAS-ELISA. There are a few reports on the use of DIBA for the detection of PNRSV in stone fruits and some weeds (Aparico *et al*. 1999; Herranz *et al*. 2013; Sabaghian *et al.* 2013; Kumlachen, 2015; Kapoor, 2018) [29, 30, 31, 32, 33].

Fig 1: DAC-ELISA detection of ilarviruses in peach leaves

Fig 2: DAS-ELISA detection of PNRSV and ApMV in peach leaves

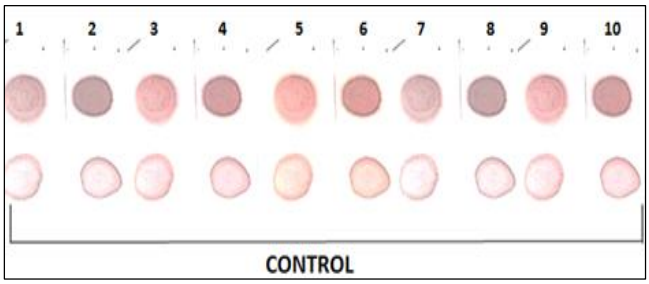

Fig 3: Detection of PNRSV in dot immune binding assay (DIBA)

4. Conclusion

Peach is the leading stone fruit crop of Himachal Pradesh, however, its production is hampered by a number of diseases particularly those of viral etiology which are of unique nature in their importance. Prunus necrotic ring spot virus (PNRSV) was found to be associated with a number of peach orchards in Himachal Pradesh. Under the present investigations, studies were conducted for serological characterization of PNRSV in peach using DAC and DAS forms of ELISA and DIBA. DAC-ELISA confirmed the association of an *Ilarvirus* with peach infected plants and DAS-ELISA test further conclusively proved that the *Ilarvirus* associated with infected plants to be PNRSV. To further substantiate the findings of DAS-ELISA, Dot immune binding assay (DIBA) was performed on symptomatic trees and the findings reconfirmed the prevalence of PNRSV in infected peach trees.

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