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Tanvi

Department of Biotechnology,
Dr. YS Parmar University of
Horticulture and Forestry,
Nauni, Solan, Himachal
Pradesh, India

Anil Handa

Professor, Department of Plant
Pathology, Dr. YS Parmar
University of Horticulture and
Forestry, Nauni, Solan,
Himachal Pradesh, India

DK Mehta

Principle Scientist, Department
of Vegetable Science, Dr YS
Parmar University of
Horticulture and Forestry,
Nauni, Solan, Himachal
Pradesh, India

Corresponding Author:**Anil Handa**

Professor, Department of Plant
Pathology, Dr. YS Parmar
University of Horticulture and
Forestry, Nauni, Solan,
Himachal Pradesh, India

Evaluating cucumber mosaic virus in *capsicum* germplasm using ELISA

Tanvi, Anil Handa and DK Mehta

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Abstract

Surveys conducted in *capsicum* growing localities of Solan district in Himachal Pradesh revealed a high incidence of cucumber mosaic virus. Mottling, mosaic, mid vein distortion, puckering and leaf deformation were the striking symptoms observed on the leaves of *capsicum*. Due to the complexities of symptoms, appearance of visual symptoms alone is not sufficient enough to establish the identity of the causal virus. Leaves from symptomatic plants were collected and serologically detected through ELISA. A total of 70 *capsicum* varieties/breeding lines were evaluated for their reaction to CMV, out of which 14 were found to be resistant based on their optical density values. ELISA was observed to be a quick and reliable method for indexing germplasm on a large scale.

Keywords: *Capsicum*, CMV, ELISA, germplasm screening

Introduction

Capsicum (*Capsicum annuum* L.) is one of the most widely cultivated vegetable crops and has attained a status of high value crop and appreciated worldwide because of its aroma, colour and nutritive value. *Capsicum* is a major source of income for vegetable growers in India which stands fourth in world production with an area and production of 34 thousand ha and 487 thousand MT, respectively (Anonymous 2018) [3]. Major *capsicum* producing states are Andhra Pradesh, Uttarakhand, Himachal Pradesh, Maharashtra, Tamil Nadu and Uttar Pradesh. In Himachal Pradesh, annual production of *capsicum* is 59.52 thousand MT over an area of 2.59 thousand ha (Anonymous 2018) [3].

Bell pepper is infected by a large number of viruses under different agro climatic conditions resulting in huge losses in yield and quality. Pepper fields exhibit complex symptoms of mosaic, mottle, leaf distortion, vein chlorosis and stunting that cause considerable losses in yield and plant vigour. Viruses cannot be simply identified on the basis of symptoms, as these could vary with respect to the virus strain, the host cultivar, environmental conditions and co-infection with other viruses (Soleimani *et al.* 2014) [30]. Precise diagnosis and detection are the key aspects of managing virus disease and the present studies are focussed on ELISA based serological detection of viruses infecting *capsicum* germplasm.

Materials and Methods

Leaf samples exhibiting typical symptoms of viral etiology were drawn from the symptomatic marked plants in the fields and brought to the laboratory in separate polythene bags kept in ice box. Alkaline phosphate (ALP) based Direct Antigen Coating (DAC) following Handa and Bhardwaj (1994) [14] and Double Antibody Sandwich (DAS) form of Enzyme Linked Immunosorbent Assay (ELISA) were performed to detect the causal virus (es) following Clark and Adam (1977) [9].

DAC-ELISA

Infected leaf samples showing symptoms of mottling, mosaic, mid vein distortion, puckering, vein banding and cupping were drawn and brought to the laboratory. Leaves of each sample were crushed in extraction buffer (1:20 w/v) by using mortar pestle and infected sap was used for coating the wells of microtiter ELISA plates. The wells of microtiter plate were filled with 200 µl aliquots of test samples and the coated plates were kept in a humid box and incubated overnight at 4±1°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 washing was repeated 3 times and the wells were filled with 200 µl aliquots of coating antibodies diluted in 1X coating buffer. The plate was incubated in humid box for 4 hours at 30°C. The washing of the plates was done as mentioned before. ALP labelled 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 5 hours at 30°C and washed as mentioned previously. The p-nitrophenyl phosphate (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 pNPP at 405 nm wavelength in microtiter plate reader (Micro Scan MS5605A, Electronic Corporation of India Limited).

Thirty virus isolates from different *capsicum* growing localities of Solan district were collected for the serological detection of two viruses namely cucumber mosaic virus (CMV) and pepper veinal mottle virus (PVMV) through DAC-ELISA since these two viruses have been reported to be associated with *capsicum* in Himachal Pradesh. For all serological tests of ELISA, immunoreagents, buffers, positive and negative control supplied by BIORÉBA AG (Switzerland) were used as per the instructions issued by the supplier. The results of the ELISA for detection were interpreted by following Dijkstra and Jager (1998)^[10] and Hobbs *et al.* (2000)^[15] as samples were considered infected when their O.D. values at 405nm exceeded two times the mean values of respective healthy or negative control samples.

DAS-ELISA

In DAS-ELISA, wells of the microtiter plate except those of the top and bottom rows on the extreme left and right were filled with 200 µl coating antibody. The plate was incubated in humid box for 4 hours at 30 °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 and emptying the plate. The wells were filled again with PBS-Tween and the washing was repeated three times or by washing in ELISA plate washer. The leaf extract from the test samples were prepared in extraction buffer. All coated wells were filled with 200 µl aliquots of test samples besides positive control

and negative control wells. The plates were incubated in humid box overnight at 4±1°C. The washing step was repeated as mentioned earlier. 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°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 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 (pNPP) at 405 nm wavelength in microtiter/ (ELISA) plate reader (Micro Scan MS5605A, Electronic Corporation of India Limited) or through visual screening.

Screening of germplasm against cucumber mosaic virus through ELISA

Screening was undertaken to identify the sources of resistance in *capsicum* germplasm against CMV disease under insect proof glasshouse conditions in the Department of Plant Pathology, Dr YS Parmar University, Nauni. All experiments were conducted on young and healthy plants of *capsicum*. Varieties/ breeding lines available with the Department of Vegetable Science and those available in the market were raised in pot mixture and seedlings were mechanically inoculated at five to six leaf stage and visually screened for their reaction to the virus isolate under study. The disease incidence was recorded based on the visual symptoms at weekly intervals for the development of symptoms. The results of visual screening were further confirmed with DAS-ELISA assay.

Results and Discussion

Serological detection of CMV and PVMV through DAC-ELISA

A critical look at the data presented in Table 1 based on O.D. values clearly indicate the presence of CMV and PVMV. Out of 30 test plants, 23 were found to be infected with CMV and only 5 samples reacted positively to PVMV. Positive samples had O.D. values that were at least double the O.D. value of negative control. O.D. values of the samples in respect of CMV were higher than that of PVMV. ELISA plate depicting serological reaction of the virus isolates against CMV and PVMV is presented in Figure 1. Confirmation of the exact identity of the prevalent virus infecting *capsicum* plants was therefore established through DAS-ELISA.

Table 1: Serological detection of CMV and PVMV through DAC-ELISA

Isolate	O.D. Value A _{405 nm}	
	CMV	PVMV
C ₁	0.987 (+)	0.289(-)
C ₂	0.901(+)	0.313(-)
C ₃	0.960(+)	0.305(-)
C ₄	0.765(+)	0.401(+)
C ₅	1.027(+)	0.213(-)
C ₆	0.254(-)	0.118(-)
C ₇	0.886(+)	0.198(-)
C ₈	0.904(+)	0.173(-)

C ₉	0.232(-)	0.287(-)
C ₁₀	0.675(+)	0.306(-)
C ₁₁	0.119(-)	0.442(+)
C ₁₂	0.854(+)	0.158(-)
C ₁₃	0.232(-)	0.328(-)
C ₁₄	0.863(+)	0.402(+)
C ₁₅	0.982(+)	0.212(-)
C ₁₆	0.745(+)	0.168(-)
C ₁₇	0.298(-)	0.170(-)
C ₁₈	0.873(+)	0.216(-)
C ₁₉	0.895(+)	0.443(+)
C ₂₀	0.604(+)	0.513(+)
C ₂₁	0.855(+)	0.302(-)
C ₂₂	0.284(-)	0.220(-)
C ₂₃	0.984(+)	0.196(-)
C ₂₄	0.243(-)	0.189(-)
C ₂₅	0.612(+)	0.231(-)
C ₂₆	0.541(+)	0.318(-)
C ₂₇	0.629(+)	0.300(-)
C ₂₈	0.750(+)	0.206(-)
C ₂₉	0.845(+)	0.159(-)
C ₃₀	1.119(+)	0.184(-)
Positive Control	1.241	1.185
Negative Control	0.203	0.176

Sample C₃₀ recorded the maximum O.D. values of 1.119 followed by sample C₅ with O.D. value of 1.027 for CMV whereas in case of PVMV, sample C₂₀ recorded maximum O.D. value of 0.513. The results of the present findings are in concurrence with those of Arogundade *et al.* 2012; Fatogoma

et al. 2014^[21, 12]. Besides, a number of other workers have also reported the presence of CMV in mixed infection with *Potyvirus* on the basis of DAC-ELISA tests (Verma *et al.* 2004; Ryu *et al.* 2009; Mascia *et al.* 2010; Aliyu *et al.* 2014; Appiah *et al.* 2014; Ayo-John *et al.* 2017)^[23, 25, 19, 2, 4, 7].

Fig 1: DAC-ELISA detection of viruses (CMV and PVMV) in *capsicum* plants

Serological detection through DAS-ELISA

It is evident from the data in Table 2 and Figure 2 that majority of the isolates reacted positive with antibodies against CMV. None of the isolates recorded positive results

against PVMV in DAS-ELISA thereby indicating that the causal virus associated with the symptoms was CMV and not PVMV.

Table 2: Serological detection of CMV and PVMV through DAS-ELISA

Isolate	O.D. Value A _{405 nm}	
	CMV	PVMV
C ₁	0.854(+)	0.176(-)
C ₂	0.742(+)	0.145(-)
C ₃	1.005(+)	0.126(-)
C ₄	0.898(+)	0.201(-)
C ₅	0.945(+)	0.180(-)

C ₆	0.200(-)	0.195(-)
C ₇	1.211(+)	0.219(-)
C ₈	0.874(+)	0.196(-)
C ₉	0.198(-)	0.154(-)
C ₁₀	0.783(+)	0.185(-)
C ₁₁	0.216(-)	0.264(-)
C ₁₂	0.765(+)	0.208(-)
C ₁₃	0.199(-)	0.190(-)
C ₁₄	0.967(+)	0.250(-)
C ₁₅	0.914(+)	0.216(-)
C ₁₆	1.006(+)	0.203(-)
C ₁₇	0.228(-)	0.185(-)
C ₁₈	1.231(+)	0.162(-)
C ₁₉	0.965(+)	0.212(-)
C ₂₀	0.690(+)	0.228(-)
C ₂₁	0.854(+)	0.199(-)
C ₂₂	0.289(-)	0.176(-)
C ₂₃	0.973(+)	0.276(-)
C ₂₄	0.253(-)	0.209(-)
C ₂₅	0.756(+)	0.180(-)
C ₂₆	0.669(+)	0.266(-)
C ₂₇	0.850(+)	0.218(-)
C ₂₈	0.974(+)	0.196(-)
C ₂₉	0.821(+)	0.160(-)
C ₃₀	0.763(+)	0.246(-)
Positive Control	1.189	1.204
Negative Control	0.211	0.198

Sample C₁₈ recorded the maximum O.D. value of 1.231 closely followed by sample C₇ with O.D. value of 1.211 whereas sample C₉ recorded minimum O.D. value of 0.198 for CMV. DAS-ELISA results in the present study are in line with the findings of Eiras *et al.* 2004; Arli-Sokmen *et al.* 2005; Bernaik *et al.* 2009; Tamarzizi *et al.* 2013; Kapoor *et al.*

2018 [11, 18, 8, 29, 17] who have also detected CMV. Afouda *et al.* (2013) [1] reported mixed infection of CMV, PVMV and PVY in pepper grown in Alibori in northern Benin. However, Arogundade *et al.* (2016) [5] detected CMV and PVMV using DAS-ELISA besides many viruses but CMV and PVMV were not found in mixed infection.

Fig 2: DAS-ELISA detection of CMV in capsicum leaves

Screening of germplasm against cucumber mosaic virus through ELISA

After visual screening, the available germplasm of *capsicum* was screened serologically to ascertain the sources of resistance against cucumber mosaic virus. Leaf samples from different cultivars of *capsicum* grown under insect proof glasshouse were collected for ELISA based screening.

A total of seventy varieties /breeding lines were screened and O.D. values based on serological detection of the virus isolate are presented in Table 3. The data reveals that out of seventy varieties/ breeding lines screened, fifty six tested positive in DAS-ELISA for Cucumber mosaic virus (CMV). *Capsicum* variety Nirmal Karol variety recorded the least O.D. value of

0.074 at A₄₀₅ nm followed by UHF BP-5 (0.098) and SV 1865-PV (0.101). *Capsicum* variety Dollar was found to be most susceptible variety as it recorded the maximum O.D. value of 0.684 closely followed by variety Meenakshi (0.638) and Solan Bharpur (0.613). Germplasm screening through DAS-ELISA is often used as an efficient tool for the identification of sources of resistance against *capsicum* viruses (Shifiss and Cohen 1989; Hobbs *et al.* 1996; Zhang *et al.* 1998; Rashid *et al.* 2007; Yao *et al.* 2013; Ashfaq *et al.* 2014; Rahman *et al.* 2016) [26, 16, 28, 24, 27, 6, 22]. Grube *et al.* (2000) [13] and Naresh *et al.* (2016) [20] also screened accessions of *Capsicum* and identified sources of resistance against cucumber mosaic virus (CMV).

Table 3: Serological screening of *capsicum* germplasm

S. No.	Variety/Breeding line	Symptoms	O.D. value(405 nm)
1	Solan Bharpoor	Mottle, mid vein distortion, mosaic	0.613 (+)
2	SB-3	Mosaic and puckering	0.495 (+)
3	Yolo Wonder (S)	Mottling and mosaic	0.512 (+)
4	Yolo Wonder 1	Mosaic and puckering	0.497 (+)
5	SB × YW	Mottling	0.583 (+)
6	California Wonder	Vein banding, cupping and mottling	0.581 (+)
7	California Wonder (SST)	Mottling and leaf puckering	0.531 (+)
8	California Wonder-11	Mosaic and leaf deformation	0.396 (+)
9	Sweet Banana	Mottling and cupping of leaves	0.596 (+)
10	Yellow Purple Macroni	No symptoms	0.121 (-)
11	<i>Capsicum</i> Green	Puckering and leaf deformation	0.513 (+)
12	Red-1	Mosaic and puckering	0.473 (+)
13	White Exotic	Mosaic	0.429 (+)
14	KTC-12	Mottling	0.431 (+)
15	KTC-15	No symptoms	0.198 (-)
16	KTC-31	Mottling	0.422 (+)
17	KTC-181	Mottling and leaf deformation	0.553 (+)
18	KTC-182	Mottling	0.397 (+)
19	KC-10	Mosaic and mottle	0.384 (+)
20	KC-11	Mosaic and vein distortion	0.425 (+)
21	KC-12	Puckering and mottling	0.395 (+)
22	N-1	Mosaic	0.462 (+)
23	N-1-2	Puckering and vein banding	0.537 (+)
24	CK1(AP-3)	No symptoms	0.261 (-)
25	CK2	Mosaic	0.519 (+)
26	K-1	Mottle, mosaic and leaf deformation	0.505 (+)
27	Kdz-1	No symptoms	0.183 (-)
28	UHF-BP-3	Mottling	0.433 (+)
29	UHF-BP-3-2	Mottle and leaf deformation	0.486 (+)
30	UHF-BP-5	No symptoms	0.098 (-)
31	UHF-BP-6	Mottling and leaf deformation	0.438 (+)
32	CW × SB	Mosaic, mottle and mid vein distortion	0.392 (+)
33	Arka Gaurav	Mosaic, puckering and leaf deformation	0.416 (+)
34	Arka Basant	Mottle and mosaic	0.524 (+)
35	Solan Wonder	Mosaic and puckering	0.538 (+)
36	Solan Local	Mottling and mid vein distortion	0.468 (+)
37	Dark Green	Vein banding and mosaic	0.431 (+)
38	Solan Selection-1	No symptoms	0.217 (-)
39	ACC-16	No symptoms	0.151(-)
40	Dilman Collection	Mottling	0.424 (+)
41	Kadar	Mosaic	0.388 (+)
42	Nirmal Karol	No symptoms	0.074 (-)
43	Dyarag Selection	Mosaic	0.396 (+)
44	Deothi Selection	Mid vein distortion and mottling	0.443 (+)
45	Selection-9	Mosaic and puckering	0.418 (+)
46	Dollar	Mottling and mid vein distortion	0.684 (+)
47	Paladin	Mottle and mosaic	0.512 (+)
48	Asha	Mosaic and yellowing	0.435 (+)
49	Indra	Chlorotic spots	0.379 (+)
50	Dina-2020	Mottling and mosaic	0.391 (+)
51	Orobelle	Puckering and mottling	0.513 (+)
52	Kaveri	Mid vein distortion and stunting	0.526 (+)
53	Sakata Excel	Mosaic and yellowing	0.419 (+)
54	11367	No symptoms	0.119 (-)
55	Spinx	Chlorotic spots and puckering	0.385 (+)
56	Bomby	Mosaic and mottling	0.413 (+)
57	Indiraget	Mottling	0.382 (+)
58	SV-1865 PV	No symptoms	0.101 (-)
59	Aishwarya (BSS89)	Mottling and mosaic	0.398 (+)
60	Solan Hybrid-2	No symptoms	0.129 (-)
60	Swarup	Mosaic and yellowing	0.482 (+)
61	Bharath	Mottle and leaf deformation	0.474 (+)
62	Green Gold	Mosaic and puckering	0.469 (+)
63	ArkaMohini	Mosaic	0.381 (+)
64	Meenakshi	Mid vein distortion, mottling and stunting	0.638 (+)
65	Variety-367	Mottle and puckering	0.582 (+)

66	Feroz	No symptoms	0.133(-)
67	Ghalai Selection	Mottle	0.521 (+)
68	PC-2	Mottle and yellowing	0.498 (+)
69	Chinese Giant	No symptoms	0.184 (-)
70	KandaghatSel-9	No symptoms	0.176 (-)
Positive control			1.021
Negative control			0.189

The studies emphasize the need for ELISA based screening of *capsicum* germplasm for identifying sources of resistance with the objective of inclusion in future breeding programmes aimed at developing resistance against viruses in *capsicum*.

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