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Rapid detection of a Capsicum infecting *tospovirus-Capsicum chlorosis virus* (CaCV) in asymptomatic weed reservoir hosts

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Abstract
Viral diseases rank second only after fungal diseases in terms of implicit monetary losses. Amongst emerging plant diseases around 47 percent are of viral aetiology. Historically, considerable research and emphasis have been put on the interaction between virus, host and their vectors. However, the study on weed reservoirs that significantly impacts viral epidemiology has been largely overlooked. Weeds harbouring viral vectors are not only the potential source of primary inoculum but also the source for secondary spread. More notably, the asymptomatic weeds with latent infection go unnoticed in the field and silently pose a bigger threat by contributing to the persistence of viral diseases. Incidentally, *Tospo viruses* that are ranked amongst most destructive plant viruses worldwide have a profound role of weed hosts in their persistence and spread. In recent years, bell pepper crop is reported to be infected by a novel tospo virus species i.e *Capsicum chlorosis virus* (CaCV) causing losses even up to 81 percent and the fact that CaCV is exclusively transmitted by thrips in a non-transovarial manner, the presence of ringspot symptoms at early stages of crop indicated the possibility of nearby reservoir hosts. This study aims at rapid detection of possible weed reservoirs on the basis of serological assay of associated virus via DAS-ELISA targeting the nucleocapsid (N) protein.

Keywords: Tospovirus, *Capsicum chlorosis* virus (CaCV), ELISA, weed reservoir, ringspot, latent infection

Introduction
Virus diseases are complex owing to their subtle nature. The multifarious ecological relationship between host, virus, vector and weed is an ancient one, however only in recent years the role of weeds in viral ecology and epidemiology has become the focus of research (Tahir *et al*., 2015) [1] that was otherwise largely overlooked in earlier researches studying virus diseases of plants. Weeds exhibit a high degree of adaptability and colonising competence hence are important biotic constraint of ecological systems (Anonymous, 2014; Clements *et al*., 2014) [2, 3]. Weeds not only threaten agricultural production by reducing the availability of nutrients to the cultivated crops but also serves as reservoirs of vector-borne viral diseases thereby contributing to the persistence of viral diseases in agro-ecosystems worldwide (Asala *et al*., 2014; Vafaei and Mahmoodi, 2015) [4, 5]. There have been an accumulating number of reports of virus detection on both known and unknown weed hosts in adjacent areas of crop fields (Kil *et al*., 2015; Leke *et al*., 2015; Macharia *et al*., 2016) [6, 7, 8,]. Viruses are capable of overwintering on weeds growing nearby crop fields and on wild species (Kwon *et al*., 2018) [9] in the absence of the main host, allowing for the incessant survival and spread between consecutive cropping seasons. Some weeds are predominantly important pertaining to their ability to host a variety of plant viruses thereby contributing to a greater frequency of mixed infections on cultivated hosts (Vafaei and Mahmoodi, 2015) [5]. Weeds also act as reservoirs of viral vectors, mostly for those that are of polyphagous nature (Asala *et al*., 2014 [4]. Moreover, weeds also provide vectors with sites of oviposition, thereby allowing the off-season build-up of vector populations and subsequent invasion and transmission of viruses to nearby crops (Macharia *et al*., 2016) [8]. Some vectors are known to probe into leaf surfaces regardless of whether the plant is a host or not and as a consequence may transmit viruses (Jaouannet *et al*., 2014) [10]. This behaviour is said to be responsible for the high transmission rates of viruses on non-hosts. It is evident from the studies that some vectors are capable of transmitting viruses more efficiently from infected weeds to nearby crops than straightway crop to crop transmission (Srinivasan *et al*., 2013) [11]. Studies have shown that some insect vectors have a predilection for weed hosts as reported by Srinivasan *et al*. (2013) [11] that green peach aphids had a greater preference for *Solanum sarrachoides* (hairy

nightshade) than the host potato crop, consequently enhancing vector fecundity and longevity. Further research showed that TYLCV infection on *Datura stramonium* improved vector fitness and attraction to weed hosts (Chen *et al*., 2013) [12]. This implies that certain vectors perform better on wild plants infected with viruses. In addition to the list Tobacco mosaic virus (TMV), Cucumber mosaic virus (CMV), Impatiens necrotic spot virus (INSV), Tomato spotted wilt virus (TSWV) and certain members of the Potyvirus group are among the viruses establishes in weeds and are easily transmitted to many other plant species.

Bell pepper (*Capsicum annuum* L.) that is commonly known as *Shimla Mirch* or sweet pepper is one amongst the most imperative cash crop of temperate areas particularly for the midhills of Himachal Pradesh, Uttarakhand and Jammu and Kashmir. However, the maximum attainable yield of bell peppers is undermined by several biotic and abiotic factors resulting into farmers suffering great losses every year. Besides bacterial and fungal phyto-pathogens, viruses drew considerable attention as they impose substantial production constraints affecting both yield and quality of the crop and unlike other pathogens these are difficult to manage. Investigations conducted to study the typical ringspot symptoms occurring on leaves and fruits of bell pepper rendering them unmarketable, revealed the presence of a novel *Tospovirus* i.e. *Capsicum Chlorosis Virus* (CaCV) in the hills of Himachal Pradesh (H.P.) (Bragta *et al*., 2014) [13]. Disease incidence ranged from 25 percent to as high as 51 percent in Solan district of Himachal Pradesh and upto 81 percent in some other district of HP (Bohra and Handa, 2016; Sharma and Kulshrestha, 2014) [14, 15]. The fact that virus under study is solely transmitted by thrips in a non-transovarial manner, appearance of ringspot symptoms and severe dwarfing of very young bell pepper plants indicated the possible presence of nearby reservoir hosts that retain virus inoculum in absence of cultivated host and serve as important primary source of inoculum for the crop planted in subsequent season. The identity of the collected virus isolates from bell pepper crop was established based on serology, studies on host range and molecular characterization of nucleocapsid (N) gene (Bohra and Handa, 2016) [14]. Preliminary transmission and serological assay of *capsicum* seeds ruled out the chances of seed transmission of CaCV. The potential role of some frequently and abundantly occurring weed species as reservoir host for *capsicum* infecting viruses was therefore investigated. Knowledge of the weed flora as virus reservoir is indispensable to reduce the spread of plant viruses. Therefore, keeping the things under consideration, weeds growing in and around bell pepper fields were collected and subjected to the serological detection to figure out the possible presence of even asymptomatic weed hosts carrying latent infection. Double antibody sandwich (DAS) -ELISA is able to detect virus particles in very low concentrations and can be used with viruses of different particle morphology. Because of its adaptability and high sensitivity ELISA is used in a wide range of situations, especially for indexing a large number of samples in a relatively shorter spell of time, hence aiding in rapid detection of virus under study.

Materials and methods

1. Field survey

Various bell pepper (*Capsicum annuum* L.) growing regions of Solan district in H.P. (Latitude: 30°54’16.15”N ; Longitude: 77°5’48.25” E) namely Bhajjo, Dharja, Kandaghat, Kumarhatti, Oachghat, Pandah, Naganji Farm and Vegetable Farm UHF Nauni were surveyed for investigating the presence of ring spot symptoms associated with CaCV. The heavily infested fields and poly houses were targeted for collection of nearby growing weeds at different stages of crop growth. Frequently and commonly growing weeds that were present in almost all locations in and around bell pepper fields were selected.

2. Collection of samples

However, the weeds selected were showing no typical symptoms of tospo virus infection, but the samples were collected to check out the possibility of latent infections. Weeds samples collected were mainly of *Amaranthus* sp., *Chenopodium* sp., *Cyperus* sp., *Datura* sp., *Ipomoea* sp., *Parthenium* sp. and some unidentified species. The collected samples were zip locked in polyethylene bag and immediately kept in ice in order to slower down all metabolic activities and were subjected to serological detection as early as possible.

3. Serological detection

Seeds obtained from previous year harvest of infected bell pepper crop and samples from asymptomatic weeds growing in and around bell pepper fields were subjected to the serological detection of *Tospovirus* for checking out the possibility of seed transmission and identification of reservoir host. Detection was carried out by following double antibody sandwich- enzyme linked immunosorbent assay (DAS-ELISA) technique. Commercially available immuno reagents (M/s BIOREBA AG, Switzerland), following the protocol of suppliers of ELISA Kits with little modification were used in the present investigation.

3.1 Detection of virus in the bell pepper seeds through DAS-ELISA

Seeds from severely infected bell pepper fruits were harvested and were subjected to DAS-ELISA to check out whether the virus is capable of getting transmitted through seeds. The protocol of DAS-ELISA as described below was followed with some minor modifications.

3.2 Detection of virus in the symptomless weeds through DAS-ELISA

Serological detection of virus through DAS-ELISA was carried out by following the protocol of manufacturers of ELISA reagents (M/s BIOREBA AG, Switzerland). Wells of the microtitre plate (NUNC maxisorp certified micro plates) were filled with 200 µl aliquots of coating antibodies diluted in coating buffer (1:1000 ratio v/v). The plate was incubated in humid box for 4 hours at 30°C. The coating of antibodies suspension was removed shaking out the plate over the washbasin. The wells were filled with PBS-Tween (washing buffer) and plate was emptied and filled again with PBS-Tween. The washing was repeated three times. ELISA plate washer was also used to eliminate the manual error. The test samples were ground in extraction buffer (1:10 w/v). All coated wells were filled with 200 µl aliquots of test sample (plates in triplicates) besides positive control, negative control and buffer control wells. The plates were incubated overnight at 4±1°C. The washing step was repeated thrice as mentioned above. The alkaline phosphate (ALP) conjugated antibodies were filled in each well with 200 µl aliquots after diluting it in ECI (enzyme conjugated immunoglobulin) buffer at ratio of 1:1000 v/v. The plate was incubated in humid box for 5 hours at 30°C. The washing was done three times as mentioned

above. The para-nitrophenyl phosphate (pNPP) substrate was dissolved in substrate buffer by dissolving 5 mg pNPP tablet in 5 ml of substrate buffer. ELISA plate wells were 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 control (usually between 30 to 120 minutes). If found necessary, the reaction was stopped by adding 50 µl of NaOH to each well. The results were assessed by recording the absorbance in ELISA Plate Reader (Micro scan MS 5605A, Electronics Corporation of India Limited, Hyderabad) for interpretation of the results at 405 nm. The results of ELISA for the detection were interpreted as per Dijkstra and Jager (1998). Test samples were considered infected with if their absorbance values (A_{405} nm) exceeded two times the mean values of the respective healthy (negative) control and buffer control samples.

Results

Typical ringspot sypmtoms in bell pepper leaves and fruits were observed and its serological and molecular detection was carried out that revealed the identity of associated virus as a novel tospo virus CaCV (Bragta *et al*., 2014) [13]. The foliar symptom of CaCV observed on bell pepper crop is presented in figure 1.

Serological detection of virus in bell pepper seeds

None of the seeds harvested from previous year infected bell pepper fruits yielded positive reaction in DAS-ELISA. All seed samples yielded absorbance values very close to relative absorbance value of negative control at 405 nm (A_{405}) indicating the absence of CaCV in seeds.

Serological detection of virus in bell pepper seeds

The weeds collected were namely *Amaranthus* sp. (Pig weed), *Chenopodium* sp., *Cyperus rotundus* (Purple nutsedge), *Dhatura* sp. (Jimson weed), *Ipomoea purpurea* (Morning glory) and *Parthenium hysterophorus* (Congress grass/Carrot weed). Double Antibody Sandwich Enzyme linked immunosorbent assay (DAS-ELISA) was conducted using Tospo (I, II, III) antisera directed against nucleocapsid (N) protein of virus. Development of yellow color being indicative of positive antigen-antibody interaction and absorbance at 405nm as shown in DAS-ELISA results (Table 1), suggested that only *Amaranthus* sp. and *Datura* sp. reacted with mild positive reaction yielding O.D. values 0.573 and 0.519 as compared to 0.251 of negative control at A_{405}. All other known and unknown weed species reacted negatively with the antisera against Tospo (I, II, III) establishing *Amaranthus* and *Datura* species to be the natural reservoir weed hosts of the virus under study even themselves being symptomless (Figure 2).

Table 1: Serological reaction of weeds in DAS-ELISA test

Weed species	O.D value (A_{405nm}) against *Tospovirus* antisera
Amaranthus sp.	**0.573(+)**
Chenopodium sp.	0.358(-)
Cyperus rotundus	0.260(-)
Dhatura sp.	**0.519(+)**
Ipomoea purpurea	0.293(-)
Parthenium hysterophorus	0.255(-)
Positive control	1.629(+++)
Negative control	0.251(-)
Buffer control	0.263(-)

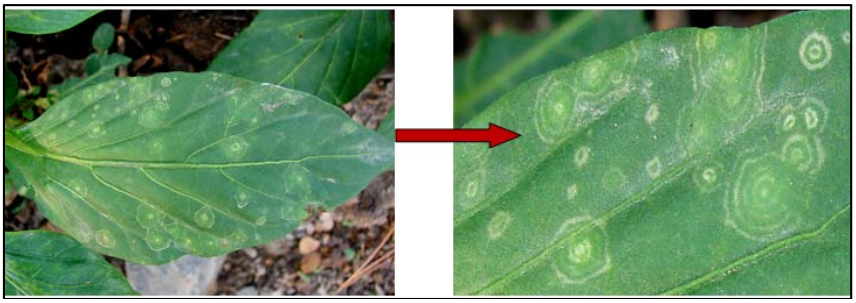

Fig 1: Typical ring spot symptom on bell pepper leaf due to CaCV infection, closer view of the symptom.

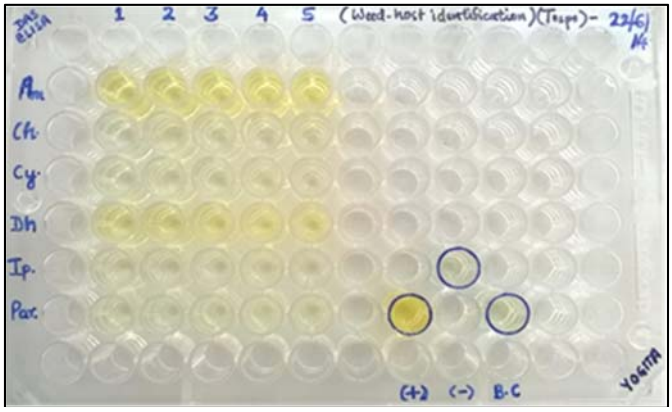

Fig 2: ELISA plate showing reaction of tested weeds to Tospo (I, II, III) antisera (Am= *Amaranthus* sp.; Ch= *Chenopodium* sp.; Cy= *Cyperus* sp.; Dh= *Datura* sp.; Ip= *Ipomoea* sp.; Par= *Parthenium* sp.; (+) = positive control; (-) = negative control; B.C= buffer control)

Discussion

Tospo viruses are obstinately hampering cultivation of bell pepper throughout the world. *Capsicum chlorosis virus*, a new member of group Tospovirus has also been reported to infect various bell pepper growing regions in India (Kunkalikar *et al*., 2007; Krishnareddy *et al*., 2008; Sharma and Kulshrestha, 2014) [15, 16, 17]. Diffused circular yellow ring spots on fruits with bronzing and necrotic spots on leaves of tomato growing in the vicinity of bell pepper fields observed during investigations were suspected to be associated with the virus under study and were found to react positively with polyclonal antisera against *Tospovirus*. Kunkalikar *et al*. (2010) [18] also reported the disease in chilli and tomato that exhibited spotted-wilt-like and ringspot symptoms.

Serodiagnosis is often considered a predominant method for detection of tospo viruses in plants and thrips (German *et al*., 1992; Mumford *et al*., 1996) [19, 20]. This is undoubtedly due to the high sensitivity, reliability and capability of ELISA technique to detect the viruses rapidly. Amongst various forms of ELISA, Double Antibody Sandwich (DAS) and Direct Antigen Coating (DAC)-ELISA are the most commonly used techniques for the detection of plant viruses

(Rajasekharam, 2010) [21]. Initially ELISA formed the basis of distinct grouping of various members of tospo viruses. On the basis of nucleocapsid (N) protein serology, tospo viruses have been classified into different sero groups like TSWV, IYSV and WSMoV sero groups (Moyer, 2000) [22]. The present investigations have also concluded that direct DAS-ELISA is capable of detecting *Tospovirus* in the reservoir hosts. Immunoassay studies using polyclonal antisera against the virus isolates revealed that symptomatic tomato fruit samples and asymptomatic weed hosts reacted positively with Tospo (sero group I, II, III) antisera though variable O.D values were recorded with the samples from different localities.

Experimental finding showed that the subjected virus is not transmissible through seeds and it goes in line with the results established by Sherwood *et al*. (2003) [23]. Moyer (2000) [22] also stated that the seed transmission of all Tospo viruses is not known to occur, but some of these viruses are commonly spread in the vegetatively propagated crops via infected propagation material.

Weeds were earlier known to act as alternate hosts of plant viruses, however, their role in virus ecology and epidemiology was thought to be insignificant (Mubin *et al*., 2009) [24] and their role was hence often overlooked (Prajapat *et al*., 2014) [25]. Recent studies, however, show that weeds play a pivotal role in virus distribution, overwintering, sites of oviposition and as initial sources of inoculum (Asala *et al*., 2014) [4]. Rapid detection of possible weed reservoirs showing no symptoms of infection was essential to devise efficient management strategies. Studies for the identification of reservoir hosts revealed that *Amaranthus* sp. and *Datura* sp. growing in and around bell pepper fields might be serving as reservoir weed hosts of the causal virus under study on the basis of O.D. values obtained in DAS-ELISA studies. Tate *et al*. (1991) [26] and Fletcher (2001) [27] also conducted studies on possible reservoir hosts in New Zealand and revealed that *Amaranthus* sp., *Chenopodium album*, *Datura stramonium*, *Solanum diflorum*, *Solanum nigrum* and *Solanum oleraceus* are important weed hosts for tospo viruses. Sharma and Kulshrestha (2014) [15] also reported *Amaranthus* sp. as a natural host of CaCV on the basis of serological study.

Conclusion

Even though virological research is now encompassing the study of weeds, much is still unknown about its impact on virus epidemiology. Weeds harbouring both viruses and the vectors that transmit them ensures a persistent reservoir of vectors and vector-borne diseases in the vicinity of crop fields. It is imperative that stringent cultural control practices as a part of an integrated pest management programme, be employed to reduce the impact of weeds on the incidence of vector-borne diseases because the presence of infected weeds throughout the year means that they are an important reservoir and source for secondary spread. Due to the lack of effective weed management strategies in agricultural settings, virus infections in crop fields are exacerbated. Since no other easy control measures are yet available for managing plant viruses besides resistance genotypes, the elimination of alternate and possible reservoir weed hosts is crucial.

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