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Screening of capsicum exotic and indigenous lines for resistance against *Pepper mild mottle virus*

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ABSTRACT

Capsicum is an important offseason vegetable crop among the farmers of Himachal Pradesh (H.P.) and widely grown under protected cultivation. In recent years extensive use of hybrids from many unknown sources under the polyhouse cultivation has escalated the incidence of many viral diseases including seed-borne viruses like *Pepper mild mottle virus* (PMMoV). The use of genetic resistance is the most feasible strategy for plant virus management. In this study a panel of one hundred and seventy-seven capsicum genotypes including the local landraces, high yielding recommended cultivars and exotic collections were screened for resistance against PMMoV-HP1 isolate. The reaction of the genotypes was recorded and validated using Double antibody sandwich-Enzyme linked immunosorbant assay (DAS-ELISA) and reverse transcriptase-Polymerase chain reaction (RT-PCR). Out of 177 lines, only two accessions PI-159236 and PI-260429 have shown resistance against PMMoV-HP1. All the other accession lines were susceptible to the disease showing mottling, puckering and mild mosaic on the leaves after 10-14 days of inoculations. The DAS-ELISA assay using PMMoV specific antiserum exhibited strong reaction with an OD value at 405 nm between the range of 0.666- 1.564 which was almost 3-4 times more than the negative control (0.181) in susceptible accessions. In RT-PCR, susceptible accessions yielded an amplification product of ~730 bp, while no amplification was observed in the case of PI-159236 and PI-260429 inoculated with PMMoV-HP1. These two lines can be used in breeding programs to develop PMMoV resistant high yielding cultivar.

Keywords: Capsicum, polyhouse, PMMoV, resistance, management

Capsicum is one of the very popular vegetable crops in Himachal Pradesh (H.P.) and is being grown both under open as well as protected cultivation. In India, the major states contributing to its production are Andhra Pradesh, Karnataka, Maharashtra, Tamilnadu, Himachal Pradesh, and hilly areas of Uttar Pradesh (Sreedhara *et al*., 2014). The total area and production of capsicum in India is 24,000 ha and 3,06,000 MT, respectively (2016-17) (http://nhb.gov.in/statistics/State_Level/2016-17(Final). With the popularization of polyhouse venture in H.P., the area under capsicum in polyhouses has expanded in recent years. However, capsicum is a crop which is reported to be infected with about 68 viruses out of which 20 viruses cause serious yield losses (Kenyon *et al*., 2014). Among these, *Pepper mild mottle virus* (PMMoV), a tobamovirus is emerging as a great threat to the capsicum cultivation both in protected and open conditions in H.P. (Sharma *et al*., 2016) due to its seed borne nature and long survival in debris and soil as well (Genda *et al.*, 2005; Ikegashira *et al*., 2004).

This virus was first time recognized as a latent strain of *Tobacco mosaic virus* (TMV) in USA (McKinney, 1952) but in 1984, Wetter *et al*. identified this virus as a distinct one. PMMoV transmits through contact locally, however, long distance transmission of the virus takes place through infected seeds and soil (Genda *et al.*, 2005; Ikegashira *et al*., 2004). This virus is ubiquitous in nature as this virus has been detected from seeds, soil, waste water, drinking water, sewage water, river water, capsicum based food products and even in human stools that too in infective state (Colson *et al*., 2010; Haramoto *et al*., 2013; Balique *et al*., 2015).

In case of viral plant disease management, concentrated efforts are made to minimize the virus dissemination through chemical control of insect vectors, use of disease free planting material etc. instead of treatment of infected plants like in case of fungal and bacterial diseases (Rodrigues *et al.*, 2009).

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As chemical treatment of virus infected plants is not feasible, use of genetic resistance in viral disease management is more preferred (Garcia-Arenal and McDonald, 2003). The replication rate of viruses in resistant host is reduced when compared with the susceptible hosts, therefore the use of genetic resistance in case of plant viruses is the most viable and economical strategy for their management. The resistance against plant viruses is more durable (Harrison, 1981). In *Capsicum* spp., the resistance against tobamoviruses is goverened by *L* gene. The *L* locus has 4 corresponding alleles *viz*., L^1, L^2, L^3 and L^4 (Boukema, 1984), and based on the ability of PMMoV to attack these alleles, PMMoV has been divided into 5 pathotypes *viz*., P_0, P_1, $P_{1.2}$, $P_{1.2.3}$ and $P_{1.2.3.4}$. Based on the CP gene sequence, the pathotype of PMMoV-HP1 (KJ631123.1) was identified as P_{12} in our previous study (Rialch *et al*., 2015). Thus keeping in view the importance of virus in the state as well as the extent of yield and quality losses caused by this virus, we have screened *Capsicum* spp. germplasm to find the sources of resistance against PMMoV-HP1.

MATERIALS AND METHODS

Capsicum germplasm comprising of local landraces, recommended cultivars and exotic accessions procured from various sources including National Bureau of Plant Genetic Resources (NBPGR), New Delhi were screened against PMMoV under greenhouse conditions. The nursery of test genotypes was raised in the plugged trays containing mixture of vermiculite, perlite and cocopeat (3:1:1). Five plants of each accession were sap inoculated at 4-5 leaf stage using standard leaf rub method with PMMoV-HP1 (pathotype P_{12}) inoculum maintained on susceptible capsicum variety "California wonder" in green house. One plant of each accession inoculated with sterilized water was kept as control. The plants were kept in the green house maintained at $25\pm2^{o}C$ and observed for the appearance of disease symptoms for about one month after inoculation. Plants showing no symptoms were back inoculated on healthy susceptible plants for symptomless carrier. The observations were recorded on symptom expression and plants showing disease symptom were graded as susceptible and plants with no disease symptom were graded as resistant.

The symptomatic plants selected at random from test genotypes and asymptomatic plants of two accessions PI-159236 and PI-260429 were subjected to DAS-ELISA using commercially avalaible PMMoV-coat protein (CP) specific antibodies (Bioreba) as described by Clark and Adam (1977) to confirm the presence or absence of virus. The absorbance was recorded using ELISA reader (MULTISKAN FC ELISA Reader, Thermo Scientific) at 405 nm wavelength. The test samples showing two to three times higher absorbance values over negative control sample were rated as positive. To further authenticate the DAS-ELISA results, Reverse transcriptase-Polymerase chain reaction (RT-PCR) was performed using the cDNA synthesized from total RNA isolated from young leaves of inoculated capsicum accessions with PMMoV-CP specific primers (CP-F 5'-CCAATGGCTGACAGATTACG-3' and CP-R 5'- CAACGACAACCCTTCGATTT-3') as described by Rialch *et al*. (2015). The samples yielding negative results in both the serological as well as nucleic acid based assays were regarded as resistant.

RESULTS AND DISCUSSION

A panel of 177 accessions of capsicum and chili comprising of local landraces, indigenous and exotic accessions screened under greenhouse conditions against P_{12} pathotype of PMMoV showed resistance only in two exotic accessions PI-159236 (*C. chinense*), PI-260429 (*C. chacoense*) (Table 1). Remaining 175 accessions exhibited susceptible reaction producing virus like symptoms consisting of mild mottling, puckering, leaf upward cupping, yellow/ green mosaic and chlorosis which were more prominent on young leaves after 10-14 days of inoculation (Fig. 1). The inoculated plants remained stunted when compared with the control plants. The fruits from the infected plants were small, distorted and lumpy in appearance with colour variations. However, no such symptoms were observed on the inoculated plants of PI-159236 and PI-260429.

The plants showing virus symptoms (randomly) as well the inoculated plants of accessions PI-159236 and PI-260429 (all) without any visible symptoms were subjected to DAS-ELISA. The OD values of test samples are given in Table 2. All the samples with typical virus symptoms yielded OD value 2-3 times greater than the negative control at 405 nm,

Table 1. Screening of capsicum germplasm for resistance to PMMoV-HP1 under greenhouse conditions

Accession Number/ Name	Reaction
Local landraces: Him Chili-9, Him Chili-43, Him Chili-11, Him Chili-3, Him Chili- 14, Him Chili-41, Him Chili-8, Him Chili-35, Him Chili-15, Him Chili-1, Him Chili-17, Him Chili-39, Him Chili-18, Him Chili-13, Him Chili-16, Him Chili-4, Him Chili-25, Him Chili-24, Him Chili-30, Him Chili-21	Susceptible
Indigenous collections: IC-545656, IC-545671, IC-545730, IC-545648, IC-545681, IC-545674, IC-545653, IC-545727, IC-545679, IC-545680, IC-545731, IC-545649, IC-545728, IC-545729	Susceptible
Exotic collections: EC-322727, EC-57996, EC-631752, EC-631755, EC-631750	Susceptible
Recommended cultivars: Indira, Nandi, Ujala, Nandita, Phule Jyothi, Natasha, Indam Bharath, Orobelle, Indam Supergold, California Wonder, Indian F1 hybrid, Spinx+, Bomby, Indam Laxmi, Inspiration, Arka Gaurav, Grauda, Vaishali, Tiwari, Surajmukhi, Paladin, MacKang, Indresh, Mahabharata, Toronto, US, Swarna CM-344, Daftari, Susan's Joy, Jinsjoy, Indonesia Local, PBC-322728, IHR-63, Kashmiri Chili, PMR-57, Devanur Deluxe, CW, Chili LLS, SJM, Sel-69-1-1-4, Yellow Capsicum, PBC 375, PBC-80, LAM-960, Byadi-Kaddi, UHF-494, Chili- Punjab-Surkh, Darl-03, Darl-05, Darl-10, Ferroz, UHFSP-11, Solan Bharpur, Local Collection-1, CVV-116, CM-344, Kandaghat Selection-9, Kashmiri Selection-1, Selection-9, PS-04, Nellore-Tomato-Chili, RC-01, Darl-02, PC-02, ARCH-19, PBC- 81, Blocky Pepper, Darl-09, ARCH-09, MS-12, SH-KC-17, SH-KC-9, SC-1003-3, SP-608-4, C.W. PL-2, Palam Bell, Arka Basant, YW PL-2, Nishat-PL-2, Harit+ Red Fruit, R.Y.+ PL-1, KTPL-19, Pusa Sadabahar, Chili Sonal, Chili Pant C-1, Punjab Gucchedar, DPC-2015-66, DPC-2015-58, DPC-2015-10, DPC-205-71, DPC-2015-55, DPC-2015-10, DPC-2015-71, DPC-2015-55, DPC-2015-43, DPC- 2015-19, DPC-2015-74, Chili-1020132, DPC-11-2-5-1-3-1, DPC-2015-47, DPC-2015-39, Chili-01, Chili-02, DPC-2015-27, DPC-2015-36, DPC-2015-08, DPC-2015-07, DPC-2015-16, DPC-3-4-1-1, DPC-2015-41, DPC-2-3-5-1-1, DPCH-6, SHKC-07, SHKC-09, Bacterial Wilt Resistant Lines: DPCBWR-14-1, DPCBWR- 14-2, DPCBWR-14-3, DPCBWR-14-4, DPCBWR-14-7, DPCBWR-14-9, DPCBWR-14-11, DPCBWR-14-12, DPCBWR-14-16, DPCBWR-14-24, DPCBWR- 14-25, DPCBWR-14-26, DPCBWR-14-27, DPCBWR-14-28, DPCBWR-14-29, DPCBWR-14-30, DPCBWR-14-31, DPCBWR-14-32, DPCBWR-14-35, DPCBWR- 14-36, DPCBWR-14-39	Susceptible
PI-159236, PI-260429	Resistant

Fig. 1. Symptoms on capsicum accessions produced after inoculation with PMMoV-HP1 isolate

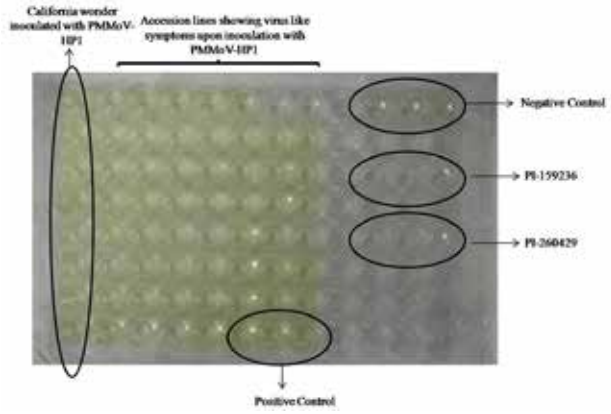

Fig. 2. Indexing of symptomatic and asymptomatic capsicum accessions after inoculation with PMMoV-HP1 through DAS-ELISA

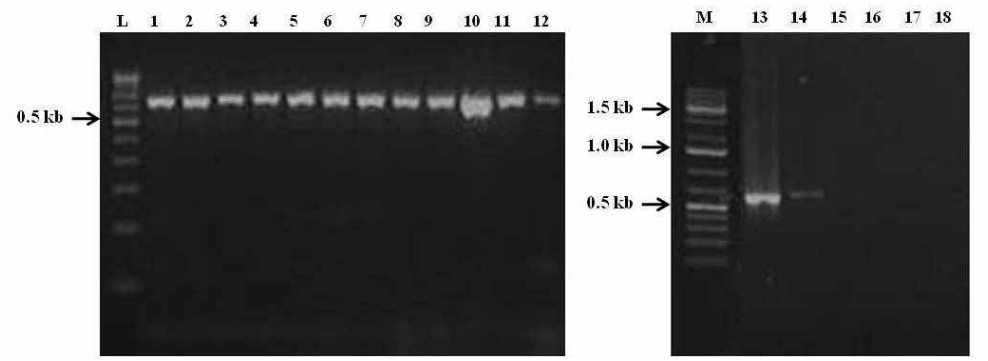

Fig. 3. RT-PCR amplification of inoculated capsicum accessions. Lane 1: 100 bp ladder, Lane 1-12: Susceptible accessions, Lane 13: positive control (California wonder inoculated with PMMoV-HP1), Lane 14-15: PI-159236, Lane 16-17: PI-260429, Lane 18: Negative control (water used as template)

Table 2. Indexing of capsicum accessions inoculated with PMMoV-HP1 through DAS-ELISA

Reaction	Absorbance at 405nm
Susceptible lines	0.666- 1.564
California wonder	1.067- 1.840
PI-159236	0.190 - 0.358
PI-260429	0.131-0.278
Positive control	0.541
Negative Control	0.181

however, the OD value in case of leaf samples from the inoculated plants of accessions PI-159236 and PI-260429 were at par with the negative control. The results of DAS-ELISA confirmed that the inoculated plants of accessions PI-159236 and PI-260429 were not the symptom less carriers for PMMoV-HP1. The results of DAS-ELISA were further validated through RT-PCR, all the samples showing positive reaction in DAS-ELISA amplified a product of 730 bp while the samples with OD value at par with the negative control did not yield any amplification (Fig. 3). Thus based on DAS-ELISA and RT-PCR results two capsicum accessions *viz*., PI-159236 and PI-260429 were regarded as resistant to pathotype P_{12}.

There are few reports on screening of capsicum for resistance against PMMoV alone (Marte *et al.*, 1992; Suzuki *et al*., 2003) or along with other capsicum viruses (Stoimenova *et al*., 2005; Cezar *et al*., 2009). In studies to find a source of resistance against PMMoV performed by Marte *et al*., (1992), Suzuki *et al*., (2003); Cezar *et al*., (2009), *C. chacoence* PI 260429 line (type accession of L^4 gene) or crosses derived from this line and *C. chinense* PI 152225 (type accession of L^3 gene) line showed resistance to PMMoV which suggested the pathotype of PMMoV used for screening was P_{12}. Marte *et al*. (1992) evaluated 28 lines constituted of open pollination progeny of *C. chacoence* PI 260429 and hybrids resulted from the crosses of *C. annuum* and *C. chinense*. Only 11 out of 28 PI 260429 lines were found to be resistant. In an another attempt to find a source of resistance against PMMoV made by Cezar *et al*. (2009), a single line PI 152225 of *C. chinense* was found to be a potential source of resistance against PMMoV in the breeding programmes. Choi *et al*. (2014) used SCAR and CAPS markers linked to *L* gene along with bioassay to identify resistant lines against PMMoV. Ozkaynak *et al*. (2014) pyramided 3 resistant genes against PVY, TSWV and PMMoV in a sweet Charleston pepper line 'Y-CAR'. Genetic markers linked to L^4 gene in Capsicum was used by the workers for marker assisted selection during the pyramiding programme.

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