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Identification of viruses and screening of summer squash (*Cucurbita pepo*) germplasm against viral disease under controlled conditions

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ABSTRACT

Two representative isolates (C-I and C-II) of virus infected summer squash from Himachal Pradesh were characterized and identified on the basis of their symptomatology, transmission, biophysical properties, particle morphology and serology. Isolate C-I exhibited characteristic symptoms of mosaic, inward rolling and cupping of leaves and ringspots on the surface of fruits, whereas C-II isolate produced vein clearing, mosaic, blistering and shoe-stringing in addition to wart like protrusions on fruits. Both the isolates were transmissible through sap and aphid vectors *Myzus persicae* and *Aphis craccivora.*. Biophysical properties of C-I and C-II included TIP of 65-70°C and 55-60°C, DEP of 10^{-4} -10^{-5} and 10^{-3}-10^{-4} and LIV of 10 and 5 days at room temperature and 15 and 8 days at refrigeration conditions, respectively. Serologically, C-I and C-II isolates reacted positively with antiserum of CMV and ZYMV, respectively in ELISA tests. Morphologically, C-I had isometric particles of 28.5 nm, whereas C-II had flexuous rods measuring 760x11 nm. C-II isolate was also successfully detected in RT-PCR assay by using potyvirus group specific primers (P9502 and CPUP) and desired amplicon of ~700 bp was obtained. Hence, based on these identification parameters, C-I and C-II isolates have been identified to be similar to CMV (cucumovirus) and ZYMV (potyvirus), respectively. Forty one varieties/collection/lines of *Cucurbita* spp including summer squash (15) and pumpkin (26) were evaluated in glasshouse conditions against CMV and ZYMV viruses to find out the source (s) of resistance. None of varieties/ collections/lines of *C. pepo* tested were resistant to infection of CMV and ZYMV but seven local collections of *C. moschata* were found highly resistant to both the viruses under glasshouse conditions.

Key words: Cucumber mosaic virus (CMV), ELISA, Resistance, RT-PCR, Zucchini yellow mosaic virus (ZYMV)

Summer squash (courgette, zuccchini and vegetable marrow) (*Cucurbita pepo* L.) is a warm season New World vegetable crop belonging to the family Cucurbitaceae. It is a young, soft skinned fruit that is harvested earlier in the season and used right away without storage in comparison to winter squash (Decoteau 2003). Like other crops the production of summer squash is also hampered due to various abiotic and biotic stresses including attack of plant pathogens. Amongst different plant pathogens, viral infections are responsible for causing great losses to this crop. For instance, zucchini yellow mosaic virus (ZYMV) infection at early stage of the crop could cause as much as 94 per cent reduction of marketable fruits of summer squash (Massumi *et al.* 2007).Routine surveys conducted in different vegetable growing areas of Himachal Pradesh to record the prevalence of diseases revealed that summer squash is severely affected by viral diseases and thus posing a serious threat to its cultivation. In the present study, we found the presence of potyvirus and cucumovirus in Zucchini crop. Studies on the control of this disease has been carried out by several workers using insecticides, oils, antibiotics and nylon net coverings with a view to reduce the vectors namely *M. persicae* and *A. craccivora*. These strategies check the disease to varying degree, but clearly, none is as beneficial as growing of CMV and ZYMV resistant cultivars. No commercially grown cultivars are resistant to CMV and ZYMV. The most effective and environment friendly management would be the growing of resistant or tolerant varieties especially in case of virus diseases where use of chemical in the management is a limiting factor. Various workers have screened the germplasm of *Cucurbita* spp to find out sources of resistance (Pachner & Lelly 2004, Suohoda and Palak 2004).

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MATERIALS AND METHODS

The leaf samples of summer squash showing virus disease symptoms from different localities in Solan, Una, Hamirpur and Kullu districts were brought to the laboratory and the virus(es) carried by them were transformed to the zucchini seedlings grown in the glasshouse at solan. Various types of symptoms caused by the viruses were recorded and then two isolates showing distinct symptoms of zucchini were selected. The virus cultures were maintained on *Cucurbita pepo* (zucchini) and *Nicitiana glutinosa* depending on the season. Sap inoculations were done by triturating leaf tissues with 0.1M phosphate buffer (pH 7.2), and rubbing the extract on carborundum dusted leaves of diagnostic species. Biophysical properties namely thermal inactivation point (TIP), dilution end point (DEP) and longevity *in vitro* (LIV) were also studied by employing the conventional methods described by Noordam (1973). Aphid transmission was conducted with *Myzus persicae* Sulz. and *Aphis craccivora* Koch. The aphids were given pre acquisition fasting for one hour and then they were allowed for acquisition access probes of five minute on an infected symptomatic zucchini leaf. Healthy zucchini leaves were used as control. Then viruliferous aphids were placed an each of these zucchini plants for inoculation feeding for ten minutes and then killed by insecticidal spray. The inoculated plants were maintained over a period of three weeks in an insect proof glasshouse and then assayed by DAC- ELISA for the presence of potyvirus and cucumovirus. Commercial polyclonal antibodies of zucchini yellow mosaic virus (ZYMV), cucumber mosaic virus (CMV), watermelon mosaic virus (WMV), papaya ring spot virus (PRSV) and squash mosaic virus (SQMV) were purchased from BIOREBA. Samples were ground in 1X extraction buffer (1:10 w/v), microtitre plates were incubated in humid box for overnight at 4°C. Virus specific immunoglobulin were used at 1:1000 (200 µl/well) and the plates were incubated at 37°C for 2 hours, goat antirabbit IgG alkaline phosphatase conjugate has been used at 1:1000 dilution and incubated for two hours at 37°C. Reaction was observed 20 minutes after addition of paranitrophenyl phosphate (pNPP) substrate. The virus isolates from zucchini were purified from systemically infected leaves of pumpkin 2-3 weeks after inoculation by the method of Christie *et al.* (1987). Leaf dip and purified preparations of the viruses were stained with uranyl acetate (2%, pH 4.5) and examined under JEOL 100S transmission electron microscope at 80 KV. Immunosorbent electron microscopic studies were done as described by Garg *et al.* (2000). Molecular detection of one of the isolate belonging to a potyvirus group was carried out by employing reverse transcription-polymerase chain reaction (RT-PCR) assay. RT-PCR assay was conducted by using potyvirus group specific primers (P9502 as downstream primer and CPUP as degenerate upstream primer) which amplify part of the coat protein gene and 3' UTR of the viral genome, previously used by Vander Vlugt *et al.* (1999). The germplasm of summer squash collected from different sources were screened to evaluate their susceptibility or resistance to the virus infection under glasshouse conditions. Germplasm of pumpkin (*C. moschata*) collected from different districts of Himachal Pradesh as listed below was also tested to find out the source(s) of resistance.

Districts and place of collection		Collection code
Solan	Nauni	LC-1
	Nagali	LC-2
	Jaunaji	LC-3
	Kotla	LC-4
	Kandaghat	LC-5
	Kasoli	LC-6
	Jabli	LC-7
Sirmour	Dilman	LC-8
	Shinaghat	LC-9
	Dodo Deori	LC-10
	Narag	LC-11
	Rajgarh	LC-12
	Mangarch	LC-13
Shimla	Rohru	LC-14
	Jubbal	LC-15
	Chopal	LC-16
	Kotgarh	LC-17
	Junga	LC-18
	Kiari	LC-19
Mandi	Baggi	LC-20
	Patli	LC-21
	Nalsor	LC-22
Hamirpur	Bhoranj	LC-23
Chamba	Thalil	LC-24
Una	Santoshgarh	LC-25
Kangra	Bagli	LC-26

The seeds of each collection were sown in earthen pots having sterilized soil mixture under insect proof glasshouse conditions. Sap inoculation of the viruses were done at the cotyledonary stage. Observations to record the incidence of virus were made 45-55 days after inoculation. Number of healthy and diseased plants were recorded in each case and per cent disease incidence was calculated. Reaction of different varieties/collections/lines was recorded by using following scale.

Per cent disease incidence	Rating
5.0	Highly resistant (HR)
5.1-10.0	Resistant (R)
10.1-20.0	Moderately susceptible (MS)
20.1-30.0	Susceptible (S)
Above 30	Highly susceptible (HS)

RESULTS AND DISCUSSION

Symptomatology

The characteristics symptoms exhibited by C-I isolates were noticed in the form of cholorotic spots, yellow mosaic, inward rolling and cupping of leaves and ringspots on the surface of fruits (Fig 1). Whereas the zucchini plants inoculate with the C-II isolates showed prominent veinal chlorosis followed by severe mottling, vein-banding and leaf malformation such as blistering and shoe strings similar to the symptoms observed in the field. Malformation, discoloration from green to yellow and blistering on fruits of infected plants made them unmarketable (Fig 2). Symptoms observed under study are consistent with reports of CMV and ZYMV (Ghosh and Mukhopadhyay 1979, Sharma *et al.* 1984, Bashir *et al.* 2006, Lisa *et al.* 1981, Kim *et al*. 1995 and Chalam *et al.* 2003).

Fig 1 Symptoms produced by CMV on leaf and fruits of *Cucurbita pepo*

Fig 2 Symptoms produced by ZYMV on leaves and fruits of *Cucurbita pepo*

ZYMV isolates have been reported (Provvidevti *et al*. 1984) to fall into biotypes, those represented by the Florida isolates that produce milder symptoms on squash and the Connecticut isolate that produce severe yellowing symptoms. The virus under study produced very severe mosaic, filiformy, distortion and yellowing of the leaves like that of Connecticut isolate but unusual symptoms on fruits makes it different from the ZYMV reported from other countries.

Biophysical properties

The dilution end point of the C-I isolate was 10^{-3}-10^{-6}, thermal inactivation point between 65-70 °C and longevity *in vitro* was found 10 days. While in case of C-II the DEP, TIP and LIV were 10^{-4}-10^{-5}, 55-60°C and 5 days.

Aphid transmission

Both the isolates were transmitted in a non-persistent manner by two aphid species, viz. *Myzus persicae* Sulz and *Aphis craccivora*. Aphid vectors differed in their transmission efficiency depending on the isolates. However, *M. persicae* transmitted both of them most efficiently followed by *A. craccivora*. Similar findings were reported by (Purcifull *et al.* 1984).

Electron microscopy and serology

Electronic microscopic studies of C-I isolate revealed that it had isometric particles measuring 28.5 nm in diameter in comparison to isolate C-II which had flexuous rod shaped particles with modal dimension of 760 × 11 nm (Fig 3). Virions of C-I and C-II isolates showed good clumping with CMV and ZYMV antiserum, respectively in immuno-sorbent electron microscopic (ISEM) studies which indicated a serological identity of C-I and C-II isolates with CMV and ZYMV, respectively

Cucumber mosaic virus reacted strongly with CMV antiserum whereas the ZYMV was serologically related with antiserum of ZYMV but not with WMV and PRSV. Similar

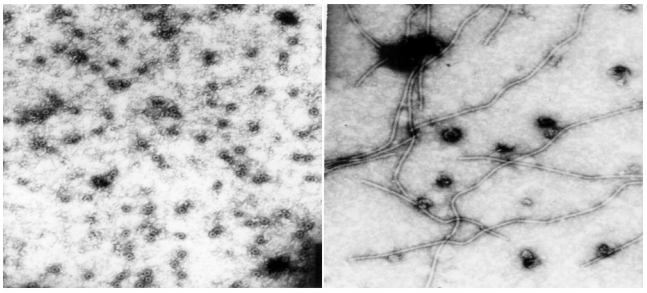

Fig 3 Electron micrograph showing virus particles of CMV and ZYMV

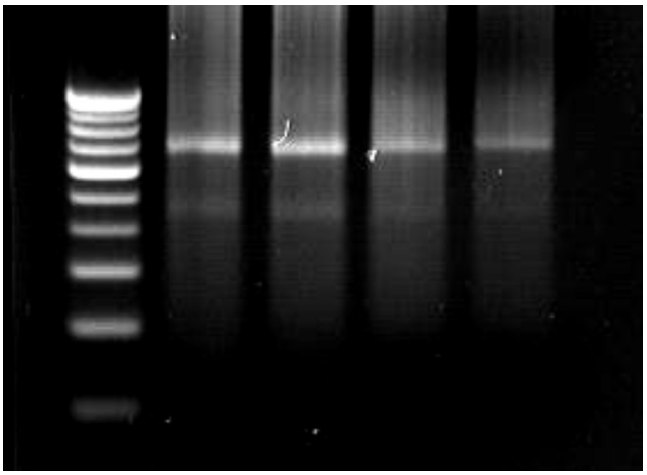

Fig 4 RT-PCR detection of C-II isolate
Diseased sample showing amplified product (~700bp) of ZYMV

Table 1 Reaction of summer squash germplasm to virus disease (s) under glasshouse conditions

Collections/Variety	CMV		ZYMV	
	Per cent disease incidence	Reaction	Per cent disease incidence	Reaction
Cucurbita pepo				
Varieties				
Australian Dark Green	100	HS	100	HS
Pusa Alar	100	HS	100	HS
Hybrids				
Cheongma Zucchini	80	HS	70	HS
Romolus	80	HS	70	HS
Himanshu	90	HS	80	HS
Cora-F_1	80	HS	90	HS
Monitor	60	HS	80	HS
Dark Green Zucchini	70	HS	60	HS
Bulum House	90	HS	70	HS
Yellow Zucchini	100	HS	80	HS
Lines				
Pb 16026	100	HS	80	HS
Pb 16006	100	HS	80	HS
Pb 16010	80	HS	70	HS
Pb 16008	70	HS	80	HS
Pb 16022	90	HS	90	HS
Cucubita moschata				
Local Collection				
LC-1	0	HR	0	HR
LC-2	0	HR	0	HR
LC-3	0	HR	0	HR
LC-4	0	HR	0	HR
LC-5	0	HR	0	HR
LC-5	0	HR	0	HR
LC-6	0	R	0	HR
LC-7	10	R	40	HS
LC-8	10	HR	40	HS
LC-9	0	HR	0	HR
LC10	10	R	0	R
LC-11	20	MS	10	R
LC-12	20	MS	10	R
LC-13	30	S	30	S
LC-14	25	S	30	S
LC-15	20	MS	20	MS
LC-16	30	S	30	S
LC-17	25	MS	30	S
LC-18	20	S	25	MS
LC-19	30	S	30	S
LC-20	70	HS	80	HS
LC-21	80	HS	70	HS
LC-22	90	HS	80	HS
LC-23	100	HS	90	HS
LC-24	80	HS	100	HS
LC-25	90	HS	70	HS
LC-26	60	HS	70	HS

results have been reported by Davis (1986), Desbiez and Lecoq (1997).

Molecular detection through RT-PCR

Reverse transcriptase polymerase chain reaction (RT-PCR) assay was performed to detect potyvirus isolate, i e ZYMV on the original host (C*ucurbita pepo* var Australian Dark Green) of the virus by using potyvirus group specific primers (C9502 and CPUP). Total RNA isolated was used for cDNA synthesis and further PCR amplification was done. RT-PCR amplification resulted in the amplified product of 700 bp (Fig 4) in a diseased sample of *Cucurbita pepo* var. Australian Dark Green, the original host of the virus. This also confirmed the identity of this isolate to be a potyvirus. However, in case of sample drawn from healthy summer squash, no amplification was obtained.RT-PCR has also been used previously to detect other potyviruses by using the same primers derived from a highly conserved domain of potyvirus coat protein gene amplifying 3' terminal region of potyviruses (Vander Vlugt *et al.* 1999).

Hence, based on different identification criteria particularly particle morphology, serological relationship and RT PCR detection, isolate C-I and C-II have been identified to be similar to CMV and ZYMV as described by Brunt *et al.* (1996).

Screening of the germplasm

Forty one varieties/collection/lines of *Cucurbita* spp including summer squash (15) and pumpkin (26) were evaluated in glasshouse conditions against viruses under investigation. For this, infected and healthy plants of each variety/collections/lines were counted after inoculation and per cent disease incidence was calculated. Data presented in Table 1 revealed that different varieties/collection/lines of *Curcurbita* spp tested showed variable reactions to virus from highly susceptible (HS) to highly resistant (HR). All the 15 varieties/lines/hybrids of summer squash under investigations showed highly susceptible reactions to both the viruses (CMV and ZYMV) under glasshouse conditions. However, in pumpkin, reactions ranging from highly susceptible to highly resistant were observed in different collections screened. Out of 26 collections of pumpkin, seven namely, LC-1, LC-2, LC-3, LC-4, LC-5, LC-6 and LC-9 were highly resistant to CMV and ZYMV.

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