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BIOLOGICAL CHARACTERIZATION OF PAKISTANI ISOLATES OF CUCUMBER MOSAIC CUCUMOVIRUS (CMV)

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Abstract

Cucumber mosaic virus (CMV) can be transmited mechanically as well as biologically through insect vector (*Aphis gossypii*). CMV has a wide host range particularly it infects Solanaceous crops. Sixty one different hosts were tested against Pakistani isolates of CMV, among them only 33 hosts were found to be infected with CMV upon inoculation while rest remained asymptomatic and showed negative response to ELISA test. Weed flora was also tested as an alternate host of CMV. *Datura stramonium, Datura metal, Portulaca olercea* (Kulfa), *Cyprus rotundus* (Deela) and *Trianthema pentandra* (itsit) showed positive reaction to CMV infection. Different inocula of CMV were applied on test plants/indicator hosts and only *Chenopodium quinoa* was found to be susceptible to CMV infection while *Nicotiana tabacum cv. samsum* remained resistant to each tested isolate under controlled conditions.

Introduction

Chilli pepper of the family *Solaneaceae*, is both a vegetable and spice crop of significant economic value in Pakistan (Khan *et al*, 2006). It is considered as most important vegetable ranked after potato and tomato (Berke, 2002). CMV is an isometric virus and has the widest host range among all plant viruses (Edwarson *et al.*, 1991) in the temperate region of the world (Kaper & Waterworth, 1981), including some monocotyledonous and a great number of dicotyledonous host plants (Palukaitis *et al*., 1992). About 20 economically important viruses have been detected in cucumber (*Cucumis sativus* L.) crop (Brunt *et al.*, 1996). CMV reported to infect 1287 plant species including cucurbits, solanaceous crops, cereals, fruits, vegetables, ornamentals (Roossinck *et al*., 1999) and also other several crops viz., tomato, pepper, cucumber, melons, squash, spinach, celery, beets, petunia, chickweed, mustard, sowthistle, nightshade, *Musa* spp. and several weeds are host of CMV (Hord *et al.*, 2001). CMV was detected in leguminous, ornamental and tomato plants in Lithuiana (Zitikaite & Staniulis, 2006). Plant viruses are dependent on vectors for their horizontal transmission, and aphids are the most common and important group of plant virus vectors. CMV is efficiently transmitted in a non-persistent manner by more than 75 species of aphids (Palukaitis *et al*., 1992). The coat protein (CP) of CMV is a primary determinant of aphid transmission (Chen *et al.*, 1990). The Fny isolate of CMV is efficiently transmitted by both *Aphis gossypii* Glover and *Myzus persicae* Sulzer (Perry *et al.*, 1998). Weed hosts function as a reservoir for the virus and serve as primary source of inoculum for the development of disease epidemics (Grube *et al.*, 2000). Transmission through planting materials, seeds and weed hosts are also significant in some crop (Hsu *et al.*, 2000). The present study was conducted to identify whether the virus of the same strain infect the same crop or not.

Material and Methods

Characterization of CMV

Mechanical transmission: CMV infected leave tissues were harvested and homogenized (1:3 w/v) in sterilized pre-cold pestle and mortar in chilled 0.05M Potassium Phosphate buffer, pH 7.2 containing 1% Sodium sulphite (Na_2SO_3) and was strained/ passed through double layer of muslin cloth as described by Noordam, (1973) and Hill, (1984). The mechanical inoculation was carried out according to the protocol described by Noordam, (1973). The *Capsicum* plants at 2-3 two leaf stages were rub-inoculated on the upper surface of the leaves with the slurry, using carborundum powder, 600 mesh as abrasive. After inoculation, the plants were rinsed with distilled water to remove superfluous inoculum and kept in an insect free glasshouse (25^{o}C temperature and 70% humidity). The un-inoculated plants (healthy plants) of each test genotype were maintained as control. Plants were observed daily for the symptom development and ELISA was performed up to four weeks post inoculation as described by Clark & Adam (1977) and Verma *et al*., (2005).

Vector transmission

Source of aphid: Aphids were collected from chilli field of Vegetable Program (HRI) and also from pre-established chilli nursery in a NARC Green House. The aphids were picked with fine paint brush and were reared on more susceptible chilli variety in insect proof cages and kept under the optimal conditions i.e., 20-25^o and 70% climatic humidity for the proper aphid growth. Aphid species was identified at Insect Pest Management Programme, NARC, Islamabad and also from the Department of Entomology, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi.

Aphid transmission: Aphid's colony was reared on healthy chilli pepper plant at 3-4 leaf stages in insect proof cages. The cages were placed in the growth room at temperature 15-22^{o}C and provide a photoperiod of 8-10 hours. After 3 weeks of rearing, colonies of aphid developed, the aphids were picked up by slight disturbing so that they withdraw their stylet through gentle breath and collected in a Petri dish with the help of moist brush. Aphids were starved for one hour. Then transferred to infected plants and allowed an acquisition feeding period for 2-3 minutes so that they would acquire the CMV virus. Aphids were allowed transmission feeding period for one hour on the test plants in insect proof cages. After one hour,

the insecticide (Karate) @ 1% solution was sprayed to kill all aphid vectors. The plants were observed daily for the symptom development. After 2-4 weeks of inoculation, symptoms were noted and ELISA was performed to confirm the presence of CMV in the test plants.

Biological characterization of CMV

Inoculation of different isolates on test plants for host range studies: The seeds of all indicator host plants (*Cucumus sativus,* peanut (*Arachis hypogea*), *Lycoprescicon esculentum, Chenopodium murale, Chenopodium quinoa, Nicotiana gulutinosa, Nicotiana tabaccum cv. Samsum, Datura metal* and *Datura strmonium*) were sown in small clay pots which contained a sterilized soil mixture composed of peat, clay and sand, mixed in equal ratio of 1:1:1 under green house conditions (25^{o}C temperature and 70% humidity). Seedlings were then transplanted in separate plastic pots having a sterilized soil as described earlier.

Revival of isolate and differential studies: All host plants were kept in the glass house for the proper establishment of roots and shoots. Host plants were inoculated by the different isolates of CMV which were isolated from different hosts plants (Sunflower *(Helianthus annuus L.),* Cucumber (*Cucumis satvius*), Methi (*Trigonella foenumgraecum*), Chillies, POG, Tobacco (*Nicotiana glutinosa*) and Onion (*Allium cepa*) plants. After inoculation, all plants were kept under controlled conditions. All host plants were daily observed for the symptoms development up to four weeks after inoculation. Symptoms were recorded on daily basis and ELISA was performed against CMV in each test plant after 3-4 weeks.

Host range studies: Sixty one host plants of different genotype were obtained from the Horticulture Research Institute (HRI), Crop Disease Research Program (CDRP), NARC and also from Pir Mehr Ali Shah, Arid Agriculture University Rawalpindi (PMAS AAUR). Ten seeds of each test host plants were raised in the green house as described earlier. Weed flora of chilli pepper was also tested to determine alternate hosts of CMV.

Two plants of each host were mechanically inoculated with CMV as described earlier. The host species tested include spinach (*Spinacia oleracea), Chenopodium album, Chenopodium quinoa,* potato (*Solanum tuberosum),* tomato (*Lycopersicon esculentum*), mash *(Vigna radiata),* mung *(Vigna mungo),* pumpkin (*Cucurbita spp*.), cucumber (*Cucumis sativus),* soybean *(Glycine max*), sunflower *(Helianthus annuus*), okra *(Hibiscus esculentus*), fenugreek (*Trigonella foenumgraecum,* local name Methi), radish (*Raphanus sativus*) , sesam (*Sesamum indicum*), red beans (*Vigna angularis*., *Phaseolus vulgaris*) garlic *(Allium sativum),* chickpea (*Cicer arietinum*), maize (*Zea mays*), peas *(Pisum sativum),* eggplant (*Solanum melongena*), bean BS-75, alsi (*Linum usitatissimum*), onion *(Allium cepa),* sounf (*Pimpinella anisum)* , mint (*Mentha arvensis)* , Cucurbits (tori,tar, tenda) *Cucurbita* sp.) celery (*Apium graveolens*) , tobacco (*Nicotiana benthamina,* and *Nicotiana glutinosa*) and certain chilli (*Capsicum* sp.) genotypes like PGRI, CV-1, C-9, C-2, C-10, C-11, C-1, C-8, C-6, C-5, C-4, C-21 and also weed hosts Datura (*Datura stramonium, Datura metal),* Hogweed *(Boerhaavia diffusa),* Horse grass (*Setaria spp.*), Itsit (*Trianthema pentandro*), kulfa (*Portulaca oleracea*) and Deela *(Cyprus rotundus)*. Plants were observed daily for the symptom development and DAC- ELISA was performed after four weeks of post inoculation for the detection of virus.

Results and Discussion

Characterization of CMV

Mechanical transmission: The virus was found mechanically transmissible to the plant tested in this study. CMV transmitted through the infected sap, as a result plant manifested yellowing, stunted growth, mosaic and narrowing of the leaves. Inoculation at cotyledon stage provides better results than inoculation on to the older leaves. Symptom appeared at the newly emerged young leaves as shown in Fig. 1.

Potassium phosphate buffer (0.05M) was used as inoculation buffer for maceration /homogenization of the infected plant material. Inoculation was successful when phosphate buffer was used. Potassium phosphate buffer destabilizes the plant virus during extraction, for this purpose Sodium sulphite was added to the buffer which helps in preventing oxidation and inactivates CMV infectivity in sap (Hull, 2002). The virus was readily transmitted mechanically onto the chilli plants and produced characteristic systemic and local symptoms of CMV (Khan *et al*., 2007; Madhubala *et al*., 2005).

Vector transmission: The aphid transmitting CMV was identified as *Aphis gossypii* that belongs to order Homoptera class *Aphididae*. *Aphis gossypii* transmitted the virus in a non-persistent manner with 2-3 min of inoculation feeding period. It has wide host range and chilli pepper is also one of them. The plants inoculated through aphid (Fig. 2) mostly showing yellowing, chlorosis and mosaic symptoms of CMV that were also confirmed through ELISA test. All of these finding showed that insect vectors play an important role in the transmission of CMV within a plant population even certain weeds act as a reservoir for the CMV inoculums in the field and also harbour aphid colony as an alternate source for secondary infection. Aphids play an important role in the natural spread/ transmission of CMV with in an area. Aphid acquired virus with in 1 minute of feeding on an infected plant, but the aphid ability to transmit the virus quickly decline and is lost within hours. Transmission efficiency varies with the aphid spp., environmental conditions and time of the year. About 90% of *Nicotiana glutinosa* plants were found to be infected with the CMV upon inoculation with A*phis gossypii*. Aphid inoculated plants produced systemic mosaic symptoms and similar results regarding aphid transmission of CMV have also been previously reported by Palukaitis *et al.,* (1992). The presence of the virus in the plants was also confirmed by RT-PCR (Data is not given). As it is confirmed that CMV does not persist in crop debris or in soil even it is not readily transmitted by handling infected plants and besides this CMV is also not transmitted through seeds of infected chilli plants.

Fig.1. Chilli plants showing symptoms after mechanical inoculation with CMV.

Fig. 2. Aphid feeding on infected chilli plant.

Biological Characterization of CMV

Inoculation of different isolates on test plants: Six groups of different indicator plant were prepared and each group was inoculated with different CMV isolates isolated from different host plants and results are given in Table 1.

Tomato, *Chenopodium murale*, *Chenopodium quinoa* and *Nicotiana glutinosa* showed localized chlorotic symptom and was confirmed by ELISA when these were inoculated with onion isolate of CMV. Similarly, *Chenopodium murale, Datura* sp., *Cucumis sativus, Arachis hypogea and Chenopodium quinoa* showed masked symptom and were ELISA positive when inoculated with tobacco isolate of CMV. *Chenopodium murale*, Tobacco *White Burley cv tabaccum* and *Chenopodium quinoa* showed the presence of CMV when inoculated with chilli isolate of CMV. *Chenopodium quinoa* and *Cucumis sativus* showed presence of CMV when inoculated with POG isolate of CMV, *Chenopodium murale, Chenopodium quinoa* and *Arachis hypogea* were found positive to CMV infection when inoculated with sunflower isolate of CMV. *Datura*, *Cucumis sativus* and *Chenopodium quinoa* showed positive reaction to CMV when inoculated with methi isolate of CMV. All these above findings showed that each isolate of CMV has its own characteristic properties with different degree of infection i.e. masked symptom/latent infection (Fig. 3). However, from these observations it seems that these isolates are heterogenous with different levels of variability. Thus each isolate belongs to a different group so that one group can infect limited number of plants but not infect those plants which belongs to different group as CMV actually has 2 groups viz., Group1A, Group1B and Group II as reported by Verma *et al*., (2005). These results were further confirmed through RT-PCR (Data is not given).

Table 1. Response of indicator/test plant to CMV isolate.

S. No.	Indicator/test plant name	Onion isolate of CMV	Tobacco isolate of CMV	Chilli isolate of CMV	POG isolate of CMV	Sunflower isolate of CMV	Methi isolate of CMV
1.	*Lycopersicon esculentum*	++	-	-	-	-	-
2.	*Nicotiana samsum*	-	-	-	-	-	-
3.	*Nicotiana glutinosa*	++	-	-	-	-	-
4.	*Cucumis sativus*	-	++	-	++	-	++
5.	*Datura stramonium*	-	++	-	-	-	++
6.	*Chenopodium murale*	++	++	++	-	++	-
7.	*Chenopodim quinoa*	++	++	++	++	++	++
8.	*Arachis hypogea*	-	++	-	-	++	-
9.	*Solanum tuberosum*	-	-	-	-	-	-
10.	*Nicotiana tabaccum cv* Tobacco white burley	-	-	++	-	-	-

Host Range Studies: Out of 61 host plants only 30 hosts showed positive response to CMV infection that was confirmed through ELISA (Table 2). Some host plant manifested characteristic systemic symptoms after 7 days of inoculation. *Chenopodim* spp., manifested localized chlorotic lesion on inoculated leaves. The symptomatic plants were then further confirmed through DAC-ELISA whereas; uninoculated plants of these hosts did not manifest any symptom and were negative during ELISA test. Plants that showed positive reactions to CMV includes Chick pea, garlic, lentil, tomato (*Lycopersicon esculentum*), sunflower (*Helianthus annuus*), methi (*Trigonella foenumgraecum*), urdbean, red beans, cucumber (*Cucumis sativus*), datura *(D. metal and D. stramonium*), Onion (*Allium cepa*), potato (*Solanum tuberosum*), *Chenopodim quinoa, Nicotiana glutinosa*, celery and chilli varieties as shown in Fig. 4 (a-g). Certain weeds were also tested to confirm as alternate hosts of CMV. Some weeds found positive when tested through ELISA test as shown in Table 3. CMV has a wide host range infecting over 1000 plant species and virus has also the ability of adapting successfully to a new hosts and environment (Roossinck, 2002) and these results are also in an agreement to Madhubala *et al*., (2005) who also reported almost similar host range for CMV from India.

(A) Datura plant affected by tobacco isolate of CMV.

(B) *Nicotiana glutinosa* affected byonion isolate of CMV.

(C) Cucumber is affected by tobacco isolate of CMV.

(D) Cucumber is affected by Methi isolate of CMV.

(E) *Chenopodium murale* is affected by all isolates of CMV.

Fig. 3. (A-E): Masked symptoms produced by different Pakistani isolates of CMV in (A) Datura, (B) Tobaco, (C, D) Cucumber and (E) *Chenopodium* plants.

Table 2. List of host species tested through mechanical inoculation against CMV host range under glass house conditions.

Sr. No.	Host name		No of plant tested	ELSIA +ve/-ve
	Common name	Scientific name		
1.	Chick pea	*Cicer arietinum*	10	+
2.	Lentil	*Lens culinaris*	10	+
3.	Garlic	*Allium oleraceum*	10	+
4.	Tomato	*Lycopersicon esculentum*	10	+
5.	Sunflower	*Helianthus annuus*	10	+
6.	Radish	*Raphanus sativus*	10	-
7.	Methi	*Trigonella foenugracum*	10	+
8.	Spinach	*Spinacia oleracea*	10	-
9.	Urdbeans	*Vigna mungo*	10	+
10.	Kidney bean	*Phaseolus* sp.	10	+
11.	Cucumber	*Cucumis sativus*	10	+
12.	Datura	*Dature metal.*	10	+
13.	Onion	*Allium cepa*	10	+
14.	*Jawar*	*Sorghum bicolor*	10	-
15.	Maize	*Zea mays*	10	-
16.	Carrot	*Daucus pusillus*	10	-
17.	Potato	*Solanum tuberosum*	10	+
18.	Luffa	*Luffa cylindrical*	10	-
19.	Soybean	*Glycin max*	10	-
20.	Zucchini	*Cucuerbita* spp.	10	-
21.	Alsi	*Linum usitatissimum*	10	-
22.	Egg plant	*Solanum melongena*	10	-
23.	Coriander	*Coriandrum sativum*	10	-
24.	Cucumber	*Cucumis sativus*	10	-
25.	Lady finger	*Hibiscus esculentus*	10	-
26.	Watermelon	*Citrullus lanatus*	10	-
27.	Gourd	*Luffa octanglata*	10	-
28.	Pinto bean	*Phaseolus vulgaris*	10	-
29.	Hogweed	*Boerhaavia diffusa*	10	-
30.	Cowpea	*Vigna unguiculata*	10	-
31.	Chenopodium spp.	*Chenopodium capitatum*	10	-
32.	Wheat	*Tritichum aestivum*	10	-
33.	Black pepper	*Piper nigrum*	10	-
34.	Red bean	*Vigna angularis*	10	-
35.	Chenopodium spp	*Chenopodium quinova*	10	+
36.	Bean BS-75	*Phaseolus* spp.	10	-
37.	Chilli pepper cv. Delikatess Robusta	*Capsicum* sp.	10	+
38.	Guvar	*Citrullus gradis*	10	-
39.	Chillies	*Capsicum annum*	10	+
40.	Tobacco	*Nicotiana tabacum* cv.Tobacco white burley	10	+
41.	Tobacco	*N. tabacum cv.samsum*	10	+
42.	Tobacco	*N. glutinosa*	10	+
43.	Mung bean	*Vigna radiata*	10	+
44.	Aniseed (Sounf)	*Pimpinella anisum*	10	-
45.	Chenopodium	*Chenopodium foliorcum*	10	-
46.	Peas	*Pisum sativum*	10	-
47.	PGRI chilli variety	*Capsicum* genotype	10	-
48.	Chilli genotype viz.,C-2	*Capsicum* genotype	10	+
49.	C-10	*Capsicum* genotype	10	+
50.	C-11	*Capsicum* genotype	10	+
51.	C-1	*Capsicum* genotype	10	-
52.	C-8	*Capsicum* genotype	10	+
53.	C-6	*Capsicum* genotype	10	+
54.	C-5	*Capsicum* genotype	10	+
55.	CV-1	*Capsicum* genotype	10	-
56.	C-4	*Capsicum* genotype	10	-
57.	Celery	*Apium graveolens*	10	+
58.	Tar	*Cucurbita* spp.	10	-
59.	Mint	*Mentha arvensis*	10	+
60.	Lily	*Lilium longiflorum*	10	+
61.	Banana	*Musa* spp.	10	+

Abbreviations: Infected with virus (+ve). Not infected with virus (-ve)

(a) Datura plant showed necrotic/ chlorotic lesions.

(b) Severe yellowing and stunted growth of the chilli plant.

(c) Chilli plant infected with CMV under natural field conditions with yellowing and stunted growth symptoms.

(**d)** Celery infected with CMV under natural field conditions.

(e) Onion affected by CMV showing mosaic symptoms.

(**f)** *Nicotina glutinosa* infectd by CMV.

(g) Chick pea is showing lateral shoots from its node side.

Fig. 4(a-g). Differential response of different host species to CMV infection in host range study.

Table 3. Weed host tested against CMV under glasshouse conditions

Sr. No.	Weed name	Scientific name	No. of plants tested	ELISA +ve/-ve
1.	Datura spp.	*Datura stramonium*	2	+
2.	Datura spp.	*Datura metal*	2	+
3.	Hogweed	*Boerhaavia diffusa*	5	-
4.	Horse grass	*Setaria* sp.	4	-
5.	Itsit	*Triathema pentandra*	4	+
6.	Kulfa	*Portulaca oleracea*	4	+
7.	Deela	*Cyprus rotundus*	4	+

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