

Serological Confirmation of Chilli (*Capsicum annuum* L.) Lines for Their Resistance Against Cucumber Mosaic Virus

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ABSTRACT

One hundred and eleven chilli lines were evaluated by mechanical virus inoculation and resistance to Cucumber mosaic cucumovirus (CMV) - chilli isolate was examined by visual examination for symptoms and confirmation by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). Six chilli lines *viz.*, AVPP 0906, AVPP 1110, Aparna, Susanjoy, Phule Jyothi and ADR Driver were remained free of infection and catalogued as highly resistant. Rest of the lines exhibited characteristic symptoms like mosaic, vein banding, mottling, vein clearing, leaf distortion, rat tailing and dwarfing depending upon tested lines. Among these lines, Six lines were categorized as highly resistant, nine as resistant, three as moderately resistant, 19 as moderately susceptible and 74 as susceptible based on disease incidence (DI). These highly resistant and resistant lines could be used by breeders in developing new chilli hybrid resistant lines to CMV.

Keywords: Screening, chilli, CMV, disease incidence (DI), ELISA

CHILLI (*Capsicum annuum* L.) is one of the most important members of solanaceous vegetables and spice crop. India is one of the major producers and largest exporters of dry chilli and its derived products. During 2012–13, the total value of export was about USD 410 million (Reddy *et al.*, 2014). The major growing areas of chilli have tropical and subtropical climates, which favour the high incidence of sucking pests, and thus resulted in the transmission of viruses.

The pungency in pepper is due to an alkaloid known as capsaicine ($C_{18}H_{27}O_3N$) and peppers are characterized as sweet, hot or mild depending on capsaicine content. It is rich sources of vitamins A and C. Chilli contains more ‘vitamin C’ than any other vegetable crop (Saha *et al.*, 2007). Among the various factors limiting to chilli production, viruses appear to be significant production constraints (Kreuze and Valkonen, 2017). Among these viruses, Cucumber mosaic virus (CMV) causes severe / economic yield reduction in chilli crop during a prolonged rain-free period (Naresh *et al.*, 2016).

CMV is a member of the Cucumo virus genus, from the family Bromoviridae and has a wide host range, infecting over 800 plant species and causing severe damage in most many species. More than 60 aphid species are known to be vectors for this virus.

CMV is tri-partite, single-stranded, positive sense RNA virus. CMV subgroup II reported to be infect Chilli in India (Biswas *et al.*, 2013) and exhibit complex symptoms of mosaic, mottle, leaf distortion, vein chlorosis and stunting causing considerable loss in yield and plant vigor (Rashid *et al.*, 2007). CMV is easily transmitted mechanically through sap inoculation and naturally transmitted (non persistently) by 80 aphid species (Chandankar *et al.*, 2013).

As cucumber mosaic virus is one of the major virus that is known to have broad host range so it is not easy to control it. Usually the conventional measures like cross protection, eradication of infected plants, crop rotation, use of virus free plants and use of chemicals against vectors has been practiced since a long time to control or manage the plant viral diseases (Hull, 2014). But in the field existence of virus species could not be predicted as these viruses occur in combination with other viruses *i.e.*, Tobacco Mosaic Virus (TMV) & Poty virus (PVY). A good deal of research work has been directed to identify resistant sources under diverse environmental conditions and screening of available genotypes and new germplasm, which constitutes the basis of this work suggested by several research workers

(Ashfaq *et al.*, 2007; Ashfaq *et al.*, 2008 and Ashfaq *et al.*, 2014).

Use of disease resistant varieties is regarded as an economical and durable method for controlling plant diseases, especially those caused by viruses. Therefore, to identify resistant sources for CMV, One hundred and eleven different chilli germplasms / varieties / hybrids were screened through mechanical inoculation. The level of resistance to CMV in chilli leaf tissues was evaluated serologically through enzyme-linked immunosorbent assay (ELISA) in addition to visual symptoms.

MATERIAL AND METHODS

Maintenance of CMV: The chilli leaf samples showing CMV infection symptoms were collected from chilli plants grown in experimental field Department of Genetics and Plant Breeding, UAS, GKVK, Bangalore. The virus was propagated through sap inoculation and maintained on susceptible chilli variety Pusa Jwala at Main Research Station, Hebbal, Bangalore. The confirmation of virus in the chilli plants with CMV specific antisera through ELISA and the same was used as a virus source for mechanical inoculation.

Germplasm lines: One hundred and eleven different chilli germplasms / varieties / hybrids were obtained from different institutes / companies such as Dept. of Genetics and Plant Breeding, UAS, GKVK, Bangalore (65 no's); Indian Institute of Horticultural Research (IIHR), Bangalore (5 no's); AVRDC Taiwan (14 no's); Namdhari Seeds Pvt Ltd. Bangalore (5 no's); Nongwoo Seed India Pvt. Ltd., Bangalore (16 no's) and I & B Seeds Pvt. Ltd, Bangalore (5 no's). Fifteen seeds of each line were sown in pro-trays contained coco-peat and maintained upto two leaf stage, then these seedlings were transplanted to polythene covers (12"×6") contained a sterilized soil mixture composed of peat, clay and sand, mixed in equal ratio of 1:1:1 under greenhouse conditions and inoculated mechanically to the two leaves just above the cotyledon leaves.

Inoculum standardization: A known quantity of leaf tissue obtained from CMV infected chilli plants was macerated in chilled pestle and mortar using

sodium phosphate buffer (pH 7.0, 0.1M), containing 1 per cent Na₂SO₃. The resulted sap was diluted upto 10⁻⁶ to know the optimum virus concentration in particular dilution using DAS-ELISA as described by Nagaraju (1996).

Mechanical inoculation: The chilli plants at the two leaf stage were rub-inoculated with standard sap extract as described by Ashfaq *et al.* (2010). After inoculation, the plants were rinsed with distilled water to remove superfluous inoculum and kept in an insect free greenhouse (25°C temperature and 70 per cent humidity). The chilli *var.* Pusa Jwala was also inoculated as a susceptible check. Grouping of these lines were made (Table I) after six weeks after inoculation based on disease incidence and on the basis of host reaction (Shah *et al.*, 2011).

TABLE I
Disease rating scale used for CMV reaction

Disease rating scale	Category	Disease incidence (DI %)
1	Highly resistant (HR)	0 - 10
2	Resistant (R)	11 - 20
3	Moderately resistant (MR)	21 - 30
4	Moderately susceptible (MS)	31 - 40
5	Susceptible (S)	> 60

(Shah *et al.*, 2011)

Serological assay: DAS-ELISA (Double Antibody Sandwich-ELISA) test was employed (Clark and Adam, 1977) for investigation of virus in leaves of chilli lines after four weeks of inoculation. Polystyrene plates were coated with anti-CMV antibodies (LOEWE, Germany), diluted 1:200 in coating buffer and incubated for four hours at 37 °C. Sap was extracted by grinding leaves in the extraction / sample buffer in pestle and mortar and then centrifuged at 8000 rpm for 5 min. Exactly 200µl of the extracted sap of each sample was then added to the coated polystyrene plate and incubated overnight at 4 °C. Alkaline phosphatase-conjugated anti-CMV antibody (LOEWE, Germany) was added in 1: 200 dilution and incubated for four hours at 37°C, followed by incubation with p-nitrophenyl phosphate (5 mg/5ml) (AGDIA, India) at room temperature for 1 h.

The absorbance values (405 nm) were measured with an Automatic ELISA Reader (TECAN, Australia). Samples were considered positive for CMV infection when the ELISA absorbance value was equal to two times or higher than the average of absorbance value of the healthy tissue as well as negative control.

RESULTS AND DISCUSSION

Virus inoculum was standardized for the mechanical inoculation of chilli lines. Among the different dilutions made from standard extract of CMV infected chilli leaf, the virus concentration was found similar upto 10^{-2} dilution. Whereas, there was a sudden decline in virus load in 10^{-3} dilution and further showed gradual decrease in virus load as the sap was diluted upto 10^{-6} (Table II). Therefore, the sap at 10^{-2} dilution was used as a standard for screening of Chilli lines for CMV through mechanical inoculation.

The performance of 111 chilli lines against CMV are given in Table III, IV and V. The results of the study revealed that there had the variability among the lines in respect of phenotypic reaction against CMV. The disease incidence (DI) of all lines were ranged from 0.0-100 per cent. The mean percentage of lines falling in the categories were 0.00, 18.52, 26.67, 40.00 and 76.67, respectively. Six lines *viz.*, AVPP 0906, AVPP 1110, Aparna, Susanjoy, Phule Jyothi and ADR Driver were categorized as highly resistant, nine lines *viz.*, AVPP 0302, AVPP 1111, AVPP 0508, A. Khyati, Byadagi dabbi, Assam 2, Ujwala, LCA 960 and HMT 1 as resistant, three lines *viz.*, AVPP 0904, Wakako long and Utkal Rashmi as moderately resistant, 19 as moderately susceptible and 74 as susceptible (Table VI). Naresh *et al.* (2016) screened

fifty capsicum genotypes for CMV resistance through mechanical inoculation and categorised the lines into different reaction types *viz.*, immune (18 no's), highly resistant (8 no's), resistant (5 no's) and moderately resistant (2 no's) based on the DI and severity of symptoms.

Out of 111 Chilli lines screened against CMV disease, showed systemic symptoms upon mechanical inoculation except highly resistant lines. However, in some of the chilli lines, expression of symptoms were observed within 15 days after inoculation, but in some cases, the symptoms were masked at initial stages and expressed systemic symptoms around 30 days after inoculation. At initial stages of infection, the plants showed mosaic types of symptoms later as the severity of the infection increases, plants exhibited different kind of symptoms include vein clearing, vein banding, mosaic, mottling, leaf distortion, rat tailing, and dwarfing (Fig. 1). Similar kind of symptoms were



Fig. 1: Chilli plants showing different types of symptoms of CMV observed by Iqbal *et al.* (2011) where CMV infected plants manifested yellowing, stunted growth, mosaic and narrowing of the leaves. DAS-ELISA results confirmed the different disease reaction types in these

TABLE II
Standardization of inoculum for mechanical transmission of CMV through DAS-ELISA

No. of replications	Standard extract	OD value @ 405 nm					
		Dilutions					
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
1.	2.42	2.38	1.88	1.20	0.91	0.76	0.52
2.	2.53	2.41	1.96	1.32	0.86	0.61	0.49
3.	2.19	2.15	1.82	1.13	0.88	0.73	0.54
4.	2.30	2.23	1.90	1.36	0.96	0.79	0.61

TABLE III
Confirmation of chilli hybrids for resistance reaction to CMV based on disease incidence and serological assay

Chilli Hybrids	15 DAI	30 DAI	45 DAI	Disease Incidence(%)	OD value @ 405nm	Disease reaction
CH 01	M	M	M, VB	40.00	0.84	MS
CH 23	M	VB	M, VB, LD	93.33	0.99	S
CH 3011	M	M	M, LD	100.00	1.36	S
CH 31	M	VB	M, RT, VB	40.00	0.96	MS
CH 37	M	M	M, m	66.67	1.03	S
Arka Meghana	M	M	M, VB	46.67	0.86	MS
A. Haritha	M	M, VB	M, VB	73.33	1.09	S
A. Khyati	M	M, VB	M, VB	20.00	0.70	R
A. Sweta	NS	M	M, m	66.67	1.08	S
A. Suphala	NS	M	M, m	100.00	1.56	S
NW 01	M	VB	M, VB, LD	100.00	1.98	S
NW 02	NS	M	M, VC	33.33	0.89	MS
NW 03	M	M	M, LD	53.33	1.06	S
NW 04	M	M, m	M, m, LD, RT	80.00	1.42	S
NW 05	M	M, D	M, LD, RT	73.33	1.16	S
NW 06	M	M, m	M, m, LD	60.00	1.04	S
NW 07	M	M, LD	M, LD, RT	80.00	1.35	S
NW 08	M	M, VB	M, VB, m	80.00	1.23	S
NW 09	M	M	M, VB	86.67	1.34	S
NW 10	M	M, m	M, m, RT	60.00	0.97	S
NW 11	M	M, VB	M, VB, m	93.33	1.09	S
NW 12	M, m	M, m, LD	M, m, LD, RT	80.00	1.01	S
NW 13	M	M, VB	M, VB, LD	80.00	1.42	S
NW 14	M	M, VB	M, VB, LD	60.00	1.12	S
NW 15	NS	M	M, VB	53.33	0.96	S
NW 16	M	M, m	M, m, D	60.00	0.92	S
NS 211	M	M, VB	M, VB, m	66.67	1.66	S
NS 1840	NS	M	M, m	40.00	0.96	MS
NS 238	NS	M	M, VC	40.00	0.85	MS
NS 1701 LG	NS	M	M, VB	46.67	0.99	MS
NS 230	M	M, VB	M, VB, m	86.67	1.06	S
Healthy					0.54	
Buffer					0.45	

Note: *No. of plants tested: 15

M: Mosaic; m: Mottling; VB: Vein Banding; LD: Leaf Distortion; VC: Vein Clearing; RT: Rat tail; D: Dwarfing; NS: No Symptoms

TABLE IV
Confirmation of chilli varieties for resistance reaction to CMV based on disease incidence and serological assay

Chilli Varieties	15 DAI	30 DAI	45 DAI	Disease Incidence(%)	OD value @ 405nm	Disease reaction
AVPP 0105	NS	NS	M	46.67	1.12	MS
AVPP 0302	NS	M	M	13.33	0.60	R
AVPP 0514	NS	M	M, LD	66.67	1.26	S
AVPP 0904	NS	M	M, LD, m	26.67	0.78	MR
AVPP 0906	NS	NS	NS	0.00	0.58	HR
AVPP 1106	NS	M	M, m	80.00	1.30	S
AVPP 1108	NS	M	M, LD, VB	46.67	0.98	MS
AVPP 1109	NS	M, LD	M, LD, RT, D	100.00	1.16	S
AVPP 1110	NS	NS	NS	0.00	0.49	HR
AVPP 1111	NS	M	M, VB	20.00	0.62	R
AVPP 9813	NS	M	M, VB	60.00	1.32	S
AVPP 9905	NS	M	M, m	66.67	1.13	S
CO 5573	NS	M	M, LD	40.00	0.86	MS
AVPP 0508	NS	M	M, VB	13.33	0.65	R
Chitrachamba	NS	M	M, VB	33.33	0.85	MS
Utkal Rashmi	NS	M	M	26.67	0.64	MR
Shelyavi-b	NS	M	M	53.33	0.91	S
Shelyavi-a	NS	M	M, VB	73.33	0.95	S
Utkal awa	NS	M	M, VB	100.00	0.96	S
Jayanthi	M	M, VB	M, VB, LD	100.00	0.96	S
Pant C-1	M	M, m	M, m, LD	53.33	1.24	S
Tiwari	M	M	M	60.00	0.91	S
Ujwala	NS	M	M	20.00	0.69	R
Anugraha	NS	M	M, VB	40.00	0.94	MS
Aparna	NS	NS	NS	0.00	0.56	HR
Phule Jyothi	NS	NS	NS	0.00	0.55	HR
Pusa sadabahar	NS	M	M, VB	33.33	0.87	MS
Arka sapal	NS	M	M, VB	53.33	1.25	S
KHPH 248	M	M, VB, LD	M, VB, LD, RT	86.67	1.93	S
KHPH 272	NS	M	M, VB, LD	46.67	0.94	MS
Healthy					0.54	
Buffer					0.45	

Note: *No. of plants tested: 15

M: Mosaic; m: Mottling; VB: Vein Banding; LD: Leaf Distortion; VC: Vein Clearing; RT: Rat tail; D: Dwarfing; NS: No Symptoms

TABLE V
Confirmation of chilli germplasms for resistance reaction to CMV based on disease incidence and serological assay

Chilli Varieties	15 DAI	30 DAI	45 DAI	Disease Incidence(%)	OD value @ 405nm	Disease reaction
Shankeshwar	M	M	M, RT	100.00	1.62	S
Japanilong	NS	M	M, VB	53.33	1.06	S
Wakako long	NS	M	M	26.67	0.63	MR
Vangara	NS	M	M, VB	100.00	1.36	S
Periyakulam	NS	M	M, VC	53.33	1.10	S
PBC 142	M	M, VB	M, VB, m	53.33	0.89	S
Byadagi kaddi	M, VB	M, VB, RT	M, VB, RT, D	100.00	1.30	S
Byadagi dabbi	NS	M	M	20.00	0.68	R
PBC 80	NS	M	M	66.67	1.05	S
CA 14	NS	M	M, VB	66.67	1.41	S
LCA 424	M	M, VB, m	M, VB, m, LD	53.33	1.82	S
Lamong local short	NS	M	M, VB	100.00	0.87	S
Bird eye chilli	M	m	M, m, LD	100.00	1.75	S
Jatni local	M	M, VB	M, m, VB	100.00	1.23	S
Assam 1	M	M, VB	M, VB, RT	73.33	1.03	S
Bolangir local 1	NS	M	M, VB	40.00	0.92	MS
African accession	NS	M	M, VB	53.33	0.88	S
Assam 2	NS	M	M	20.00	0.67	R
Warangal Chappata	M	M, VB	M, VB, m	66.67	1.02	S
PBC 81	NS	M	M	53.33	0.99	S
Bhooth Jalokia	M	M, VB	M, VB, LD	60.00	1.12	S
LCA 305	M	M, VB	M, VB, D	100.00	1.36	S
LCA 235	M	M, VB	M, VB, LD	100.00	1.56	S
Susanjoy	NS	NS	NS	0.00	0.54	HR
Gowribidanur	NS	M	M, VB	73.33	1.26	S
G 4	M	M, VB	M, VB, LD	80.00	1.63	S
G 3	NS	M, m	M, m, RT	100.00	1.70	S
LCA 639	NS	M, VB	M, VB, m	86.67	0.90	S
LAM 333	M	M, VB, LD	M, VB, LD, RT	60.00	0.98	S
LCA 960	NS	NS	M	20.00	0.76	R
LCA 655	M	M, VB	M, VB, LD	33.33	1.06	MS
LCA 625	NS	M	M, VB	73.33	0.88	S
LCA 436	NS	M	M, VB	73.33	0.91	S
LCA 353	NS	M	M, VB, m	73.33	1.03	S
LCA 336	M	M, VB	M, VB, m	66.67	0.96	S

Chilli Varieties	15 DAI	30 DAI	45 DAI	Disease Incidence(%)	OD value @ 405nm	Disease reaction
LCA 334	NS	M	M, VB	100.00	0.87	S
LCA 206	NS	M	M, VB	100.00	0.83	S
ADR Driver	NS	NS	NS	0.00	0.54	HR
DAC 71	M	M, VB	M, VB, LD	73.33	1.34	S
HMT 1	NS	M, LD	M, LD	20.00	0.78	R
DAC 93	M	M, VB	M, VB, RT	60.00	1.53	S
AR 75	M	M, m	M, m, LD	60.00	1.00	S
G 5	NS	M	M, m	33.33	0.87	MS
LCA 620	NS	M	M, m	40.00	0.82	MS
CMS 10A	NS	M	M, VB	60.00	1.71	S
CMS 8A	NS	M	M, VC, m	100.00	1.73	S
CMS 6A	NS	M	M, LD	100.00	1.91	S
CMS 9A	NS	M	M, m	80.00	1.81	S
CMS 7A	NS	M	M, VB	40.00	0.95	MS
Pusa Jwala	M	M, VB, m	M, VB, m, RT	93.33	1.96	S
Healthy					0.54	
Buffer					0.45	

Note : *No. of plants tested: 15

M: Mosaic; m: Mottling; VB: Vein Banding; LD: Leaf Distortion; VC: Vein Clearing; RT: Rat tail; D: Dwarfing; NS: No Symptoms

TABLE VI
Grouping of chilli lines based on disease reaction

Disease Reaction	No. of lines	Germplasm/variety/hybrid
Highly Resistant (HR)	06	AVPP 0906, AVPP 1110, Aparna, Susanjoy, Phule Jyothi, ADR Driver
Resistant (R)	09	AVPP 0302, AVPP 1111, AVPP 0508, A. Khyati, Byadagi dabbi, Assam 2, Ujwala, LCA 960, HMT 1
Moderately Resistant (MR)	03	AVPP 0904, Wakako long, Utkal Rashmi
Moderately Susceptible (MS)	19	AVPP 0105, AVPP 1108, CO 5573, CH 01, CH 31, Arka Meghana, NW 02, NS 1840, NS NS 238, NS 1701 LG, Chitrachamba, Bolangir local 1, Anugraha, Pusa sadabahar, LCA 655, KHPH 272, G 5, LCA 620, CMS 7A
Susceptible (S)	74	AVPP 0514, AVPP 1106, AVPP 1109, AVPP 9813, AVPP 9905, CH 23, CH 3011, CH 37, A. Haritha, A. Sweta, A. Suphala, NW 01, NW 03, NW 04, NW 05, NW 06, NW 07, NW 08, NW 09, NW 10, NW 11, NW 12, NW 13, NW 14, NW 15, NW 16, NS 211, NS 230, Shankeshwar, Japanilong, Vangara, Periyakulam, Jayanthi, Pant C-1, PBC 142, Byadagi kaddi, PBC 80, CA 14, LCA 424, Lampong local short, Shelyavi-b, Bird eye chilli, Jatni local, Shelyavi-a, Assam 1, African accession, Tiwari, Utkal awa, Warangal Chappata, PBC 81, Bhooth Jalokia, LCA 305, LCA 235, Gowribidanur, G 4, G 3, LCA 639, LCA 625, LCA 436, LCA 353, LCA 336, LCA 334, LCA 206, Arka sapal, LAM 333, KHPH 248, DAC 71, DAC 93, AR 75, CMS 10A, CMS 8A, CMS 6A, CMS 9A, Pusa Jwala

lines. The absorbance value of highly resistant lines was ranged from 0.49-0.58, whereas, resistant and moderately resistant lines showed 0.60-0.78 and moderately susceptible 0.82-1.12, susceptible chilli lines showed 0.83-1.98 OD value.

Six lines *viz.*, AVPP 0906, AVPP 1110, Aparna, Susanjoy, Phule Jyothi and ADR-Driver did not manifest any symptom were found negative to CMV antisera and are on far with negative and healthy samples and therefore catalogued as highly resistant against CMV (Fig. 2). ELISA technique has been used



Fig. 2 : Chilli lines showing highly resistant reaction to CMV

to detect CMV and other viruses resistant chilli lines and other crops by many workers. Rashid *et al.* (2007) was employed ELISA to detect CMV infection and didn't detect any infection in C-1, C-2, C-5, C-7, C-9, C-11 except the pepper lines C-4, C-8, C-9 and local check which did show positive reaction to CMV. Similarly, Akhtar *et al.* (2010) evaluated sixty-nine tomato genotypes representing nine *Solanum* spp. for the source of resistance to Cucumber mosaic virus (CMV) by mechanical inoculation, six genotypes (TMS-1 of *S. lycopersicum*, LA1963 and L06049 of *S. chilense*, LA1353, L06145 and L06223 of *S. habrochaites*) were found resistant and six genotypes (L06188 and L06238 of *S. neorickii*, L06219 of *S. habrochaites*, L05763, L05776 and L06240 of *S. pennellii*) were found tolerant.

It is apparent from the above results that all the lines developed by Namdhari Seeds Pvt. Ltd.,

Bangalore; Nongwoo Seed India Pvt. Ltd., Bangalore and I & B Seeds Pvt. Ltd., Bangalore lines were found susceptible to CMV infection. However, six lines (AVPP 0906, AVPP 1110, AVPP 0302, AVPP 1111, AVPP 0508 and AVPP 0904) from AVRDC were showed highly resistant, resistant and moderately resistant reaction to CMV, where as other lines showed susceptibility to CMV when inoculated under greenhouse conditions. Ashfaq *et al.* (2014) reported that, all local genotypes were susceptible to CMV infection except PBC-385 that showed high resistant response to CMV and all Mexican genotypes *viz.*, M-2001, CM-2001, M-97, and CP-328 were remained highly resistant to CMV infection except GM-2001. Whereas, AVRDC lines *i.e.*, C-2, CV-2, CV-5, BSS-269 and PGRI were resistant to CMV. From this study, it could be concluded that, chilli lines showing highly resistant, resistant and moderately resistant reaction to cucumber mosaic virus could serve as a potential source for resistance in breeding programme. The major problem in germplasm evaluation is that some lines found resistance at one location turn out to be susceptible at another place therefore environmental-genotype interaction should also be studied for durable resistance in future.

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