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Studies on Transmission of Cucumber Mosaic Virus (CMV) by Sap Inoculation in Tomato

P. Jalender[1*], Bharati N Bhat [2], K. Anitha[3] and K. Vijayalakshmi[4]

[1 & 2]Department of Plant Pathology, PJTSAU, Rajendranagar, Hyderabad (500 030) India

[3]National Bureau of Plant Genetic Resources (NBPGR), Rajendranagar, Hyderabad (500 030) India

[4]National Institute of Plant Health Management, Rajendranagar, Hyderabad (500 030) India

*Corresponding Author E-mail: porikajalender089@gmail.com

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ABSTRACT

Tomato plants were observed free from local lesions on the inoculated leaves. However, systemic infection was noticed on the 8th day after inoculation. Electron micrographs obtained with leaf dip preparation revealed the presence of spherical particles of 28.5 nm diameter indicating the presence of CMV belonging to Cucumo virus group. DAC-ELISA results revealed that of the 31 genotypes, including Arka Vikas, all were found positive to CMV, except EC620389, which was also symptomless. All symptomatic genotypes responded positively for CMV by DAC-ELISA. Though the genotypes, EC251790 and EC625642 did not exhibit any characteristic symptoms of CMV even after second inoculation, reacted positively against CMV antiserum during DAC-ELISA.

Key words: *Kharif, Systemic Infection, Virus, Tomato.*

INTRODUCTION

Cucumber mosaic virus (CMV), the type species of the genus Cucumovirus of the family Bromoviridae, is an important plant pathogen worldwide, which infects many crops and causes yield losses. CMV is difficult to control because of its extremely broad natural host range in excess of 800 plant species, and the ability to be transmitted in a non persistent manner by more than 60 species of aphids (Zitter, 1984;[1] Palukaitis et al., 1992[2]). The characteristic field symptoms of CMV disease include stunting, yellowing, mottling of leaves, extreme filiformity or shoe stringing of leaf blades, depending on virus strain and the host (Carrere *et al.*, 1999 [3] and Emy Sulistyowati *et al.*, 2004 [4]). In India, CMV incidence was earlier recorded in tomato fields in Chittoor district of Andhra Pradesh (Kiranmai *et al*., 1997) [5], near Salem, Tamil Nadu (Sudhakar *et al*., 2006) [6], New Delhi and Aurangabad (Geetanjali *et al*., 2011) [7].

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MATERIALS AND METHODS

Sap inoculation: Young leaves showing typical CMV were collected. The collected samples were ground in a chilled mortar and pestle using inoculation buffer/phosphate buffer. The Inoculation/Phosphate buffer (0.05 M; pH 7.0) is prepared by using Potassium di-hydrogen phosphate (KH_2PO_4) 2.40 g; di-potassium hydrogen phosphate (K_2HPO_4) 5.40 g; mercaptoethanol 1.56 ml and distilled water1000 ml. One gram of tissue was ground in 10 ml buffer. After homogenizing, the pulp was squeezed through two layers of muslin cloth and the filtrate was used as inoculum. Before inoculation, small quantity of fine carborundum powder (600 mesh) was dusted on the leaves. The inoculum was rubbed on the upper surface of leaves with the help of cotton wool previously dipped in the inoculum. During inoculation, the leaves were supported from below with left-hand palm to avoid any injury and assure uniform pressure and spread of the inoculum. The inoculated leaves were washed immediately with a fine jet of distilled water using wash bottle to remove excess inoculum and carborundum powder. The inoculated plants were maintained in an insect proof glasshouse up to six weeks to observe for symptoms. Suitable uninoculated controls were also maintained.

Purification: Young tomato leaves with shoe stringing or leaf narrowing symptoms were collected and inoculated on to the plants of susceptible cv. Arka Vikas. After development of symptoms, symptomatic leaves were tested with CMV antiserum for the confirmation of presence of virus by DAC-ELISA. After confirmation, the symptomatic leaves were used as source of inoculum for purification procedures. By sap / mechanical inoculation, the virus was inoculated on to *Nicotiana rustica* plants at 2 - 4 leaves stage. For the development of prominent symptoms, the inoculated plants were kept in a moist chamber at a temperature of ≤ 240C for 12 h and in green house for another 12 h for 2 days. The well isolated local lesions from N. rustica were taken and further sap inoculated on to another set of N. rustica (1x) plants. After initiation of local lesions on *N. rustica* (1x), the procedure of confirmation of virus presence and purification was repeated by serial inoculation (up to 5x) on *N. glutinosa*. Thus the purified virus (5x) inoculum was maintained on *N. glutinosa* and used for further experiments.

Electron microscopy of the virus: The work on electron microscopy was carried out at Ruska lab, Sri Venkateshwara Veterinary University, Rajendranagar, Hyderabad, India. The virus particles were examined under electron microscope by following Brande's leaf dip method (Gibbs *et al.,* 1966; [8] Torrance and Jones, 1981 [9]) with slight modifications. Samples containing crude sap from infected tomato plant (cv. Arka Vikas) and purified CMV isolate (5x) on *N. glutinosa* were collected and ground separately in few drops of 2.5% glutaraldehyde and centrifuged at 4000 rpm for 5 min. A drop of each sample was placed on a piece of parafilm, and the carbon coated copper grid was placed on parafilm. After 5-10 min, excess sap was drained with the help of filter paper. The grid was then washed with distilled water and stained with 2% uranyl acetate. The sample on the copper grid was allowed for air drying and observed under transmission electron microscope (Model: Hitachi H-7500) at various magnifications.

Direct Antigen Coated Enzyme-Linked Immunosorbent Assay (DAC-ELISA): The DAC-ELISA procedure (Hobbs *et al.,* 1987) [10] was used to detect the virus in the test plants. Test samples, healthy sample, positive control and negative control were ground separately in carbonate buffer @ 1ml of carbonate buffer per 100 mg (1:10 w/v) of sample using sterile, separate sets of pestle and mortar to prevent cross contamination. After grinding, the sap drawn in eppendorf tubes was centrifuged @13000 rpm for 2 min, and the supernatant @ 100 µl/well was dispensed into the 96-well, polysterene ELISA plates (Make: Costar). The plates were incubated at 37 °C for 45 min. After incubation for 45 min, the ELISA plate was washed with PBS Tween, three times by allowing 3 min between each wash. After 3 washes, it was ensured that the plate was

absolutely dry without any air bubbles before proceeding to the next step. Then, blocking buffer (100 µl) was added to each well, and the plate was incubated at 37 °C again for 45 min. Washing procedure as detailed before was repeated at the end of 45 min incubation. Meanwhile, the antiserum of CMV (source: IARI, New Delhi), which was diluted at 1:500 dilution in antibody buffer, was cross-adsorbed with healthy tomato plant extract @ 0.5 g / 10 ml of buffer and filtered through two layers of cheese cloth. Cross-adsorption was done to prevent the false positive reaction, which might occur due to the host proteins present in the polyclonal antiserum. In the third step, the cross-adsorbed antiserum @100 µl/well was dispensed into each well of ELISA plate. The plates were incubated at 37 °C for 45 min and washed three times with PBST, as described earlier. Alkaline phosphatase conjugate (ALP; Source: Sigma), diluted in antibody buffer, was added to each well @ 100 µl, which was prepared by adding 10 ml antibody buffer to 1µl ALP (1:10,000 dilution). Incubation, washing and drying procedures were repeated as mentioned earlier. In the final step, 100 µl of substrate buffer was added to each well and the ELISA plate was kept in dark for 15-20 min. The colourless para-nitrophenyl phosphate in each well turns to yellow colour indicating positive reaction to the homologous antiserum. Absorbance values were recorded at 405 nm with Biotek - ELISA micro plate reader. Samples were considered 'positive' when the absorbance value exceeded 2 times of that of the negative control.

RESULTS AND DISCUSSION

Sap / Mechanical transmission: Leaf extracts for sap inoculation were prepared by using phosphate buffer (0.05 M; pH 7.0) and inoculated on tomato cultivar, Arka Vikas. Local lesions were not observed on the inoculated leaves of tomato plant. However, systemic infection was noticed on the 8th day after inoculation as the symptoms were initiated on the new, upcoming leaves as mosaic, mottling, followed by leaf narrowing. These symptoms were advanced further to give typical shoe string symptoms by the end of 14 days. All the plants exhibiting typical CMV symptoms were found positive to CMV when tested against CMV antiserum in ELISA. The results are in conformity with the findings of Kiranmai *et al.* (1997) [5], Akhtar *et al.* (2010) [11], Mohamed (2010) [12] and Mahjabeen *et al.* (2012) [13].

Purification: After confirmation of pathogenicity on tomato cv. Arka Vikas, upcoming, fresh leaves showing CMV symptoms were collected and used for further inoculation on to the leaves of *Nicotiana rustica* for purifying the virus isolate. *N. rustica* exhibited necrotic local lesions within 5 days after inoculation. The well isolated local lesions from the infected *N. rustica* plant were taken and further inoculated on to *N. rustica* (1x). Necrotic local lesions appeared on *N. rustica* (1x) after 6 days of inoculation. *N. glutinosa* (2x) exhibited chlorotic spots within 5 days after inoculation followed by mosaic mottle symptoms in 12 days, similar symptoms were produced on *N. glutinosa* (3x) at 3 and 11 days, respectively. *N. glutinosa* (4x) and *N. glutinosa* (5x) exhibited systemic mosaic symptoms within 2 days after inoculation.

Electron microscopy: Leaf dip preparations of sap from infected tomato plant (cv. Arka Vikas) and purified CMV isolate (5x) on *N. glutinosa* were examined under transmission electron microscope (TEM). The study revealed that the negatively stained virus preparation contained numerous spherical particles in the crude sap (Plate 4.4), and few, well isolated spherical particles with a diameter of 28.5 nm in the purified sample indicating the presence of *Cucumber mosaic virus* of cucumo virus group. Similar particle size, morphology and average diameter of 29-30 nm were reported earlier by Lockhart and Fischer (1976) [14], Bidari and Reddy (1990) [15], Holcomb and Valverde (1991) [16], Kiranmai *et al.* (1997) [5], Doomar Singh *et al.* (1999) [17] and Jagadeeshwar (2004) [18].

DAC-ELISA: Results of DAC-ELISA revealed that out of 31 genotypes, including Arka Vikas, all were found positive to CMV,

except EC620389 (*Solanum lycopersicum*), which was also symptomless. All symptomatic genotypes responded positively for CMV by DAC-ELISA. Though the genotypes, EC251790 (*S. peruvianum*) and EC625642 (*S. lycopersicum*) did not exhibit any characteristic symptoms of CMV even after second inoculation, reacted positively against CMV antiserum during DAC-ELISA.

CONCLUSION

Sap inoculated Arka Vikas plants developed mosaic, mottling and shoe string symptoms after 7 days of inoculation. The inoculum was purified up to 5x on *Nicotiana glutinosa* and used for further studies of host range and screening studies. Electron micrographs obtained with leaf dip preparation revealed the presence of spherical particles of 28.5 nm diameter indicating the presence of CMV belonging to *Cucumo* virus group.

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