

Original Research Article

<https://doi.org/10.20546/ijcmas.2018.702.327>

## Assessment of Different Methods for Sap Transmission of Chilli Leaf Curl Virus in *Capsicum annuum* L. and Its Validation through PCR Amplification of AV1 Coat Protein Gene

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### ABSTRACT

#### Keywords

Sap transmission, Gemini virus, Coat protein, Immersion, ToLCD, ChCLV

#### Article Info

##### Accepted:

24 January 2018

##### Available Online:

10 February 2018

Chilli (*Capsicum annuum* L.) is an important spice crop severely affected by the virus causes leaf curl symptoms. Efficient transmission of Chilli Leaf Curl Virus was done by using a standardized buffer for sap inoculation to chilli plants. In present investigation, we have used three simple methods i.e. inoculation by syringe, rubbing and by immersion (dip) for sap inoculation and found reproducible when the sap from the infected plants was inoculated onto healthy plants. Out of the three methods used, syringe inoculation was found most efficient method for sap transmission. Syringe inoculation caused most severe symptoms of leaf curl followed by immersion dip after 6 weeks after the inoculation. The sap transmission of chilli leaf curl virus was confirmed by coat protein gene amplification. PCR amplification showed the presence of 320bp single DNA fragment of AV1 coat protein gene that confirmed the presence of Chilli leaf curl virus in the sap transmitted plants.

### Introduction

Chilli is most important and widely cultivated spices in a world. Viral disease constitutes one of the main causes of losses in chilli crop. The Begomoviruses belongs to family *Geminiviridae* is DNA plant viruses characterized by its twinned icosahedral particle nature, approximately 18x30nm in size (Padidam *et al.*, 1995). Begomoviruses are transmitted exclusively by the whitefly

*Bemisia tabaci*. It has been reported, that the symptoms and pattern of tomato leaf curl disease (ToLCD) and ChiLCD are often similar (Senanayake *et al.*, 2006). The typical symptoms consisting of leaf curling, rolling, puckering, blistering of intravenous areas and thickening and swelling of the veins, shortening of internodes and petioles, crowding of leaves and stunting of whole plants, older leaves may become leathery and brittle (Sinha *et al.*, 2011). According to

Matthews (1970) after artificial inoculation, virus particle stay in a single epidermal cell, from there the virus particles further spread to nearby cells and increasing the focus of infection. Morra and Petty (2000) describes that Begomovirus infection is limited to the phloem tissue of plants.

Chilli leaf curl virus (ChiLCD) can be transmitted by sap inoculation in chilli by using a standardized buffer. Mechanical sap transmission is the convenient method for biological studies of plant viruses. Factors affecting sap transmission under various conditions are not reported earlier. In the present study the various methods for sap transmission of ChLCV associated with Chilli plant. It is also reported that effect of different extraction buffer, storage temperatures, seasons, host factors on sap transmission, and physical properties of the virus indicate a difference in their transmission (Sohrab *et al.*, 2014). This standardized protocol will be highly useful for the screening of chilli. Lapidot *et al.*, (2006) developed a scale of differential hosts that enables the determination and comparison of the level of resistance to the viral disease. The sap transmission of chilli leaf curl virus was confirmed by coat protein gene amplification. The main aim of the study was. This methodology was developed for a primary screening strategy of Chilli leaf curl virus resistance putative transgenic plants.

## **Materials and Methods**

### **Sample collection and identification for virus**

Infected leaves were obtained from a chilli plant showing symptoms at vegetable research farm, Narendra Deva University of Agriculture and Technology, Faizabad, Uttar Pradesh. For the maintenance of test plant virus inoculum and for all other studies, highly

susceptible chilli cultivar Faizabadi kala was used. The healthy seedlings were raised in earthen pots of 30 cm diameter containing a mixture of sterilized soil, sand, and compost in 2:1:2 (w/w/w) ratios. The seedlings were raised in the insect-proof glasshouse. After twenty days seedlings were transplanted into 30 cm earthen pots containing soil: sand: compost (2:1:2) mixture and kept in insect-proof glasshouse.

### **Preparation of inoculums for artificial inoculation**

The inoculum was prepared from young leaves showing severe symptoms of leaf curl. 500 mg fresh leaves were washed in tap water and dried in between folds of blotting paper; leaves were then macerated using a sterilized mortar and Pestle using 1 ml of sodium phosphate buffer  $K_2HPO_4$  per gram of tissue. The buffer consisted of 0.067 M sodium phosphate of pH 7.0, containing 0.1M Tris buffer (pH 7), 0.1% B-mercaptoethanol (freshly added) transferred to 100 ml beaker gently mixed with a glass rod and the resultant pulp was squeezed through double layers of muslin cloth used as standard inoculum for all inoculation purposes (Jamsari *et al.*, 2015).

### **Inoculation of plants**

Three methods were used for inoculation viz., by syringe, rubbing and by immersion (dip). Inoculation by syringe involves only 2 $\mu$ l injected volume sap injected by using sterilized syringe with 4.5x13mm needle at just below the stem apex and syringe less inoculated leaves were washed with water to remove excessive inoculation. Rubbing which engages mechanical inoculation was carried out by rubbing the upper surface of two or three young leaves of the tested plant with a small piece of sterilized absorbent cotton wool soaked in the inoculum. Prior to inoculations, carborundum 0.037 mm powder serves as an

abrasive was is lightly dusted to injured leaf. To avoid enzymatic activities, inoculated leaves were gently rinsed with water from a wash bottle and excess of inoculum remaining on the surface of the inoculated leaves.

Help with cardboard for support after inoculation into leaves were washed with water to remove excessive inoculation. Inoculated plants were kept in glass house for observation. For immersion of sap from infected leaves freshly extracted in phosphate buffer as above dilute serially from  $10^{-1}$  to  $10^{-8}$ . Then immersed in the sap for 10 minutes maintained at  $20^{\circ}\text{C}$  for 2-3 days for retain moisture. All inoculated and control (non - inoculated) plants were shifted to greenhouse for scoring of symptom development.

### **Disease scoring**

After inoculation, plants were kept in the greenhouse for symptom development. Leaves were regularly observed for lesion formation. After days of inoculation, the overall disease incidence and severity was scored and rated by a severity scale from 0 to 5 (Banerjee and Kalloo, 1987) with slight modification.

The modifications includes—(0) no visible symptoms, inoculated plants show same growth and development as non-inoculated ones; (1) 0-5% curling and clearing of upper leaves and very slight yellowing of leaflet margins on apical leaf; (2) 6-25% curling and clearing of leaves and swelling of veins minor curling of leaflet ends; (3) 26-50% curling puckering and wide range of yellowing, curling, and cupping, yet plants continue to develop; (4) 51- 75% very severe leaf curling and stunted plants growth and blistering of internodes pronounce cupping and curling, plant stop growing. (5) More than 75% curling and deformed small leaves, stunted plant growth with a small flower and no or small fruit set.

### **PCR for confirmation of viral particles in inoculated plant after sap inoculation**

Young leaves of infected chilli plant after 3 weeks of the sap transmission were collected. The sap transmission was confirmed using the total DNA isolated by following CTAB methods from symptomatic leaves and PCR amplification by using specific primers of AV1 CP gene. Total genomic DNA was isolated by using a simplified CTAB method. Leaves from treated plants of each method were collected in an ice box and the collected leaf samples were used to extract DNA following the CTAB method (Murray and Thompson, 1980). 500 mg of leaves were cut into bits with the help of sterile scissors and transferred to mortar.

The leaf tissues were frozen in liquid nitrogen and ground into fine powder. The tubes were added with 500 ul of 2X CTAB buffer (100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2 % CTAB, 2 % PVP and 0.2 %  $\beta$  mercaptoethanol) and mixed by inverting the tubes. The tubes were incubated in water bath at  $65^{\circ}\text{C}$  for 30 min with 2–3 mixing by inverting the tubes. Then 500ul of chloroform was added into the tubes and mixed by inverting. The tubes were centrifuged at 14,000 rpm for 10 min at room temperature. The upper aqueous layer was then transferred carefully into new autoclaved microcentrifuge tubes, 0.6 volume of isopropanol was added and mixed well by inverting the tubes. The tubes were incubated at  $-20^{\circ}\text{C}$  for 1 h followed by centrifugation at 14,000 rpm for 10 min at  $4^{\circ}\text{C}$ . The DNA pellet was washed with 70 % ethanol and dried at RT for 15 min. Finally the DNA was dissolved in 100 ul of sterile water and stored at  $-20^{\circ}\text{C}$  for subsequent PCR analysis. PCR amplification was performed with the against AVI viral coat protein primers Chilli Leaf curl virus AV1 F (5'-TCTAGAGCGTTTCTGTGTTAAGTCTGTGT-3') and Chilli leaf curl virus AV1 R (5'-

GGATCCGTCAAACATGTTGAACACCTC  
TC-3').

### Results and Discussion

In the present study, we reported that the begomovirus causing chilli leaf curl disease is highly sap transmissible. Observation of the leaf curl symptom of chilli plants inoculated by three methods (syringe, rubbing and immersion dip) showed that inoculated plants developed very good symptoms of leaf curling followed by cupping, stunting and curling at a later stage. Phenotype changes in diseased leaves due to lowered chlorophyll content were observed in yellowish color as compared to more natural green color in the healthy leaf. Late but severe symptoms were acquired in 4-

5 weeks by the plants those were treated with the syringe inoculation and immersion dip. Rubbing method of sap inoculation caused only 6-25% curling and clearing of leaves, swelling of veins and minor curling of leaflet ends even after 6 weeks of inoculation. Among three methods applied for sap transmission, syringe infiltration showed most severe symptoms of chili leaf curl virus after 6 week after inoculation followed by immersion dip. However for the development of highly efficient sap transmission protocol, standardize buffer with or without abrasive, the age of source plants, storage temperatures, growing inoculated plants, type of tissue and susceptibility of the genus type/plant species also gave different responses (Fig. 1–3).

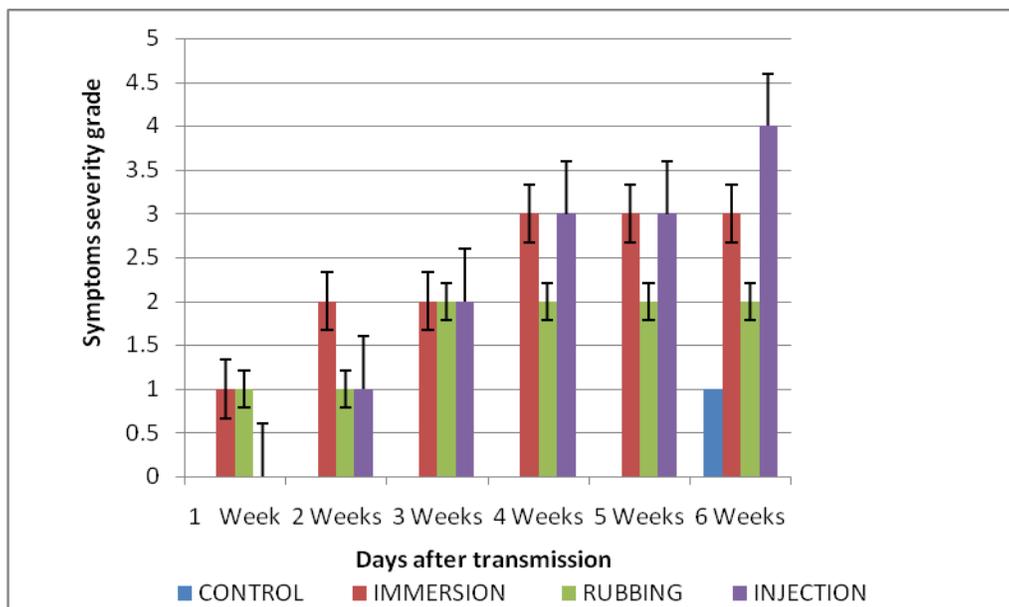
**Fig.1** Sap preparation (b) from severely virus-infected leaves (a) at the stem (c), just below the stem apex of the chilli plant



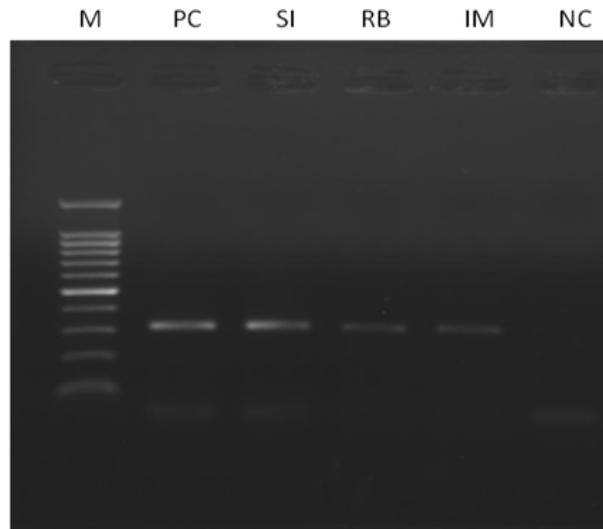
**Fig.2** Sap transmission in chilli plants A: Immersion in prepared sap, B: by rubbing of a cotton swab, C: transmission by syringe directly below the stem apex. Inoculated plants have newly emerged infective leaves with the severe disease symptoms characteristic



**Fig.3** Differential disease scaling grade for symptom development of chilli leaf curl disease by using different methods after sap transmission



**Fig.4** PCR analysis shows the 320 bp PCR CP product, indicating *Geminivirus* particle in inoculum sample, PCR products of samples from three successful treatments



M = 100 bp ladder, B: Blank, PC: Positive control, SI: Syringe inoculation, RB: BY Rubbing Sap and IM: Immersion, NC: Negative control are putative successfully infected samples

### Confirmation of sap transmission by PCR

The sap transmission of chili leaf curl virus into inoculated plants was confirmed by specific amplification of CP gene using specific primers. An amplicon of 320 bp was amplified from infected leaves shows the sap transmission of ChCLV into Chilli inoculated plants. Successful PCR amplification was shown by the presence of an about 320bp single DNA fragment of AV1 coat protein gene. It confirms the presence of ChCLV virus in the sap transmitted plants.

Sap transmission is an effective and efficient method of inoculation to develop a primary screening strategy for the selection of transgenic developed for ChLCV resistance. This methodology can also be required extended for the use for virulence resistance analysis of other plant viruses.

### Acknowledgments

Financial support from Department of Biotechnology is thankfully acknowledged

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**How to cite this article:**

Tanvi Chauhan, Amit Choudhary, Dharmendra Kumar and Singh, K.N. 2018. Assessment of Different Methods for Sap Transmission of Chilli Leaf Curl Virus in *Capsicum annuum* L. and Its Validation through PCR Amplification of AV1 Coat Protein Gene. *Int.J.Curr.Microbiol.App.Sci.* 7(02): 2697-2703. doi: <https://doi.org/10.20546/ijcmas.2018.702.327>