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Research Article

Molecular detection and management of tomato leaf curl virus with *Pseudomonas fluorescens* and chitosan

AMIT KUMAR YADAV[1], RAMESH SINGH YADAV[1*], PRASHANT MISHRA[1], G. S. MADHU[2], ABHISHEK KUMAR[1] and AJAY KUMAR MISHRA[1]

[1]*Centre of Excellence for Sanitary and Phytosanitary (SPS), Department of Plant Pathology, Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut - 250110, Uttar Pradesh, India*

[2]*Division of Plant Pathology, IARI, New Delhi, India*

**Corresponding author E-mail:* spsprojectrkvy@gmail.com

ABSTRACT: Tomato production is affected by various fungal, bacterial and viral diseases that attribute to considerable yield losses. Among all the diseases of tomato, Tomato Leaf Curl Virus (ToLCV) disease is cited to be the most devastating both in terms of quantitative and qualitative yield losses. Often, the loss reaches to the extent of 100% during summer throughout India. The virus associated with tomato leaf curl disease was detected by PCR using coat protein specific primers. The present investigations were carried out with potent *Pseudomonas fluorescens* isolates alone and along with elicitor molecule chitosan to determine its ability to control ToLCV in tomato. Different formulations of *P. fluorescens* and chitosan were evaluated for controlling ToLCV and growth promotion of tomato. It was found that the application of *P. fluorescens* isolates Pf1 and Pf2 @ 5kg/ha in combination with chitosan @ 5% reduces the severity of the ToLCV disease by 80 – 90 %. Application of chitosan and *P. fluorescens* alone caused 60 – 70% reduction in severity of ToLCV disease. Moreover, the plants inoculated with the chitosan based formulation of *P. fluorescens* isolates Pf1 and Pf2 recorded maximum plant height, fruit number and yield/plant over the control in potted condition. The results, thus, indicate that addition of chitosan has enhanced the biocontrol efficacy of *P. fluorescens* against ToLCV.

KEY WORDS: Bio-Control, chitosan, *Pseudomonas fluorescens*, tomato, ToLCV

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INTRODUCTION

Tomato is one of the most important vegetable crops cultivated for its fleshy fruits and is considered important commercially as well as staple dietary vegetable crop. It is a rich source of minerals, vitamins, organic acids, essential amino acids, dietary fibers and also contains minerals like iron and phosphorus. It contains lycopene and beta-carotene pigments. India is the second largest tomato producing country in the world. The estimated area and production of tomato in India (during 2013-2014) was 0.88 million hectares with a total production of 18.73 million tones (productivity 21.2 mt/ha), (National Horticultural Board (NHB) Database, 2014) respectively. Tomato production is affected by various bacterial, fungal and viral diseases that can cause considerable yield losses. Viral diseases affecting tomato crop include spotted wilt, tomato big bud, tomato bunchy top, tomato mosaic and tomato leaf curl. Among all the viral diseases, tomato leaf curl disease is major constraints for tomato production and highly devastating as its incidence and severity is increasing worldwide. (Kumar *et al.,* 2012). Chakraborty *et al.* (2003) described Tomato Leaf Curl Virus (ToLCV) as a threatening virus of tomato and major constraint for the successful cultivation of tomato in tropic and subtropics. Several begomovirus species infecting tomatoes in India have been characterized (Kumari *et al.,* 2011; Chakraborty *et al.,* 2003; Tiwari *et al.,* 2010, 2013). Tomato infected by leaf curl virus in North India have bipartite genome while that of South India have monopartite genome (Borah and Dasgupta, 2012). Occurence of ToLCV in India was first reported by Vasudeva and Samraj (1948). In India, the disease is widespread in tomato, found during the summer season in southern India (Saikia and Muniyappa, 1989) and during the autumn in northern India (Banerjee and Kalloo, 1987). Tomato leaf curl virus belongs to Geminiviridae family and represents the second largest family of plant viruses and is characterized by geminate twinned particles. Geminiviruses contain four genera including Mastrevirus, Curtovirus, Topocuvirus and Begomovirus. ToLCV is shown to be transmitted by the vector, whitefly *(Bemisia tabaci* Genn.) (Varma, 1990; Muniyappa *et al.,* 1991; Padidam, *et al.,* 1995; Srivastava, *et al.,* 1995*),* in dodder and through grafting. The whiteflies and the associated plant viruses are no longer restricted to their

native habitats or contained by natural geographic boundaries. Among the viruses transmitted by whitefly, Tomato Leaf Curl Virus Diseases (ToLCVD) have seriously hampered the cultivation and production of tomato.

Several approaches have been taken to manage the tomato leaf curl virus. For management of whiteflies generally farmers use high doses of chemical pesticides that can remain as residue in fruits and also can create imbalances in microbial community. Use of potential bio inoculants represents an attractive alternative approach for disease management and growth promotion of crops.

In the present study combined application of *Pseudomonas fluorescens* and the elicitor molecule chitosan strategy was tested for management of tomato leaf curl virus. Chitosan is one of the most studied elicitiors which regulate the expression of resistance genes and induces jasmonate synthesis (Doares *et al*., 1995). It is known to have eliciting activities leading to a variety of defense responses in host plants in response to microbial infection, inducing the accumulation of proteinase inhibitors, phytoalexin, callose formation, Pathogen-Related (PR) proteins and lignin synthesis. Chitosans are naturally-occurring compounds that have potential to control plant diseases. They have been reported to be having potential to suppress viruses, bacteria and other pests (Abdelbasset *et al*., 2010). A number of antagonistic microorganism such as *Trichoderma* spp. *Steptomyces* spp. *Bacillus* spp. *Gliocladium* sp. and *Pseudomonas fluorescens* have potential to control a variety of plant diseases. Several researchers have implicated plant growth promoting rhizobacteria for management of viral diseases in many crops such as TMV in tomato (Kirankumar, 2007), sunflower necrosis virus (Srinivasan *et al*., 2005), banana bunchy top virus (Kavino *et al*., 2003) and tomato spotted wilt virus (Kandan *et al*., 2003). In other studies, beneficial microorganisms were combined with a natural compound such as chitin (Sid Ahmed *et al*., 2003) or chitosan (Benhamou *et al*., 1998) to improve their biocontrol efficacy.

MATERIALS AND METHODS

Collection of infected plants

Tomato plants showing chlorosis, leaf curling, distortion, yellowing and/or stunted growth were collected from the tomato growing farmer's fields near the University campus.

Maintenance of infected plants

Some infected plants were selected on the basis of severity of the symptoms and were uprooted from the fields with some soil attached to their roots. The uprooted plants were immediately placed in plastic pots containing prepared soil. All such pots were covered with mosquito net specially prepared to cover each pot. All the pots thus prepared were labeled and were maintained in a net house (specially built for keeping the infected plants) near the Department of Plant Pathology situated at the main campus of Sardar Vallabhbhai Patel University of agriculture and Technology, Meerut (Uttar Pradesh).

Total DNA extraction from virus infected tomato plant

Total DNA was extracted from the infected and healthy leaves by Cetyl Trimethyl Ammonium Bromide (CTAB) method Dellaporta *et al.* (1983) modified by Sharma *et al.* (2003) 200 mg of leaf tissue (avoid midrib and try to took the tip of the leaf) homogenized with pre-warmed (at 60˚C) 2% CTAB DNA extraction buffer (1.4 M Nacl, 100mM TrisHCl, 20 mM EDTA, 0.2% β- mercaptaethanol, 2% CTAB). The resultant homogenate was transferred into 1.5 ml eppendorf tube and incubated at 60˚C for 1hr, in a water bath. During incubation, occasionally the homogenate was mixed by gentle swirling. After incubation, an equal volume of chloroform isoamylalcohol (24:1) - was added, mixed by gently inversion and centrifuged at 15,000 rpm for 10 min. The resulting supernatant was transferred to a new eppendorf tube and DNA was precipitated by using 0.6 volume of isopropanol. DNA was pelleted by centrifugation at 12,000 rpm for 5 min. The pellet was washed with 70% ethyl alcohol and dried at room temperature for 20 min until all traces of ethanol disappeared. The Pellet was dissolved in TE buffer [pH 8.0] and stored at -20˚C for further use. All extracted DNAs were diluted 10-fold in sterile distilled deionised water before amplification through PCR as recommended by Reddy *et al.* (2005).

PCR detection of tomato leaf curl virus

A set of ToLCV specific coat protein primers, forward sequence - 5' GGT CCC CTC CAC TAA ATCAT 3' (20nt) and reverse sequence -5' CAG TTG GTT ACA GAA TCG TAG AAG 3' (24nt) were used to amplify virus DNA in the samples. Polymerase Chain Reactions (PCR) was performed in 25 µl reaction mixtures containing 2 µl of template DNA, 0.5 units of *Taq* polymerase, 1.5mM $MgCl_2$, 0.2mM dNTPs, 5pmol of each forward and reverse primers in 1X reaction buffer (*Taq* buffer B). The amplification was carried out using thermal cycler PCR system. Amplification programme consisted of one initial cycle of denaturation at 94˚C for 1 min, annealing at 52˚C for 1min 30sec and extension at 72˚C for 2 min. After the initial cycle, 38 cycles of PCR were performed of denaturation (94˚C for 50sec), annealing (at 52˚C for 45sec) and extension (at 72˚C for 1min 30sec). After that amplification programme was continued for 10 min. at 72˚C. Amplified products were electrophoresed through 1.5% (w/v) agarose gels in 1X Tris Acetic acid EDTA (TAE) electrophoresis buffer and was visualized under UV transilluminator following ethidium bromide staining of the

gel (30min in0.5mg/ml ethidium bromide). The molecular weight of the amplified products was calculated using standard molecular weight DNA markers.

Isolation of *Pseudomonas fluorescens*

The sample were collected from rhizosphere soils of rice and tomato plants for isolation of *Pseudomonas fluorescens.* All the samples were processed using the soil dilution plate method Waksman, (1922). For isolation, one gram of each soil sample was placed in a 100 ml of Sterilized Distilled Water (SDW) and mixed thoroughly. Suspension was serially diluted up to 10^{-8}. The selective medium (King's B medium) was used for the isolation of *P. fluorescens*. The diluted soil suspension (1 ml) of dilutions five onwards were inoculated from each dilution on King's B media and incubated at 28 ± 2^0C for 48 h. Pigmented yellow green and blue white colonies of *P. fluorescens* were picked after viewing under UV light.

***Pseudomonas fluorescens* treatment**

The bacteria were inoculated in King's B broth medium and incubated on a shaker (150 rpm) for forty eight hours. After incubation the bacterial culture was centrifuged at 10,000 rpm for 5 min and the pellet mixed with sterile Carboxy Methyl Cellulose (CMS) suspension (1%). Tomato seeds were surface sterilized with mercuric chlorite solution, placed in CMC cell suspension, air dried inside a sterile chamber and the biocoated seeds sown in pots. For soil application, the lignite based culture was applied to soil @ 5kg/ha before sowing seeds and mixed well. For foliar application, the lignite based culture was filtered through a muslin cloth and sprayed @ 1% (w/v) at 10 Days after Sowing (DAS) and 20 DAS. Control plants in pots were maintained without application of rhizobacteria. All treatments were replicated thrice and arranged in a Randomized Block Design (RBD).

Chitosan treatment

Chitosan was dissolved in 100 mM acetate buffer (pH 6.5). The bacterial pellet was mixed well with chitosan solution (5%). Surface sterilized tomato seeds were soaked in chitosan cell suspension and shaken for 3 hr at 28^0C. The biocoated seeds were dried inside a laminar flow chamber. At 25 DAS, both upper and lower surfaces of the leaves were sprayed with the chitosan solution (1 mg/ml) prepared in 100 mM acetate buffer (pH 6.5)

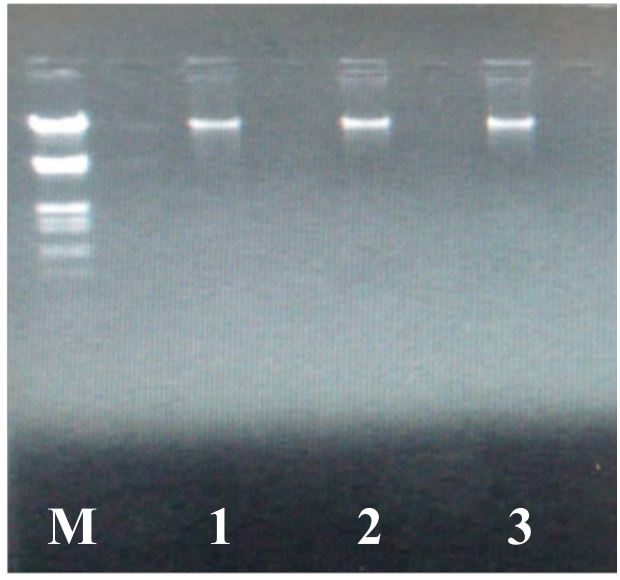
Statistical analysis

The data were subjected to analysis of variance, and treatment means were differentiated using Fischer's T test. The data taken into percentage were first, transformed into angular value and then analyzed for test of significance (Gomez, 1996; Chandel, 2002).

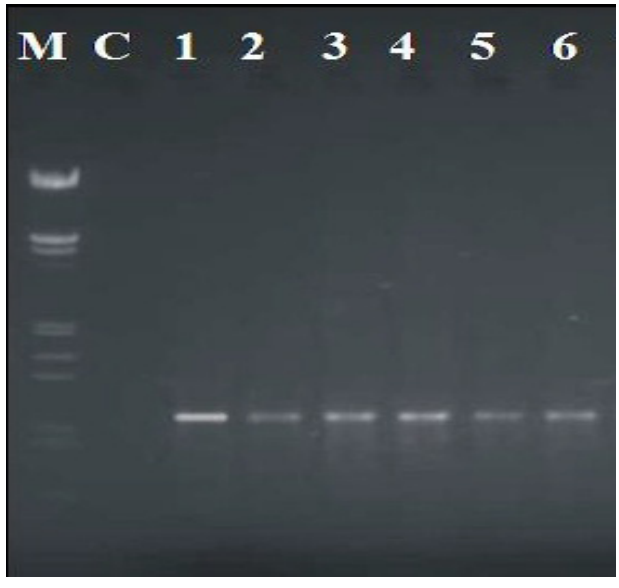
RESULTS AND DISCUSSION

Detection of tomato leaf curl virus through Polymerase Chain Reaction (PCR)

A total of three ToLCV infected samples were used in this study. All three samples were subjected for genomic DNA isolation and PCR amplification by using coat protein specific primers. The quality of DNA was confirmed through 0.8% agarose gel electrophoresis by visualizing bands in gel documentation unit. The genomic DNA bands have been presented in (Fig.1). Further PCR assay was carried out using set of a ToLCV specific coat protein primers, forward sequence



(A)



(B)

Fig. 1. (A) Total DNA of ToLCV with tomato plant. (B) PCR analysis of ToLCV in tomato. Lane M: Double digest marker (ECoRI+Hind III). Lane C: control, Lane 1-6: Infected tomato plant sample.

- 5' GGT CCC CTC CAC TAA ATCAT 3' (20nt) and reverse sequence -5' CAG TTG GTT ACA GAA TCG TAG AAG 3' (24nt) successfully amplified DNA fragments of ~530 bp of CP gene of tomato leaf curl virus have been presented in (Figure 2). The amplified coat protein gene was confirmed that tomato plants were infected by tomato leaf curl virus.

Effect of *Pseudomonas fluorescens* with chitosan on growth parameters of tomato plant and disease severity of ToLCV was studied. The tomato seed and seedlings were initially treated with *P. fluorescens* and chitosan and followed by soil application and foliar spray. The results indicated that there was a significant increase in plant height, number of fruits, yield per plant and reduction of disease severity to the tomato plants treated with *Pseudomonas* and chitosan compared to the untreated control plants.

Effect of *Pseudomonas fluorescens* and chitosan on bio-control of ToLCV

The growth parameters of tomato plants under treated and untreated condition have been presented in (Table 1). The results showed that maximum plant height was recorded as 52.83cm, number of fruit per plant 21.33, and fruit yield per plant 916.67 gm in T_6 (Chitosan + *Pseudomonas fluorescens* (Pf–1) + *Pseudomonas fluorescens* (Pf–2) followed by T_4 Chitosan + *P. fluorescens* (Pf–1) 48.16cm, 18.33, 816.67gm respectively and in chitosan treated (T_7) plant height was 39.83 cm, number of fruit per plant 8.00 and fruit yield per plant 416 gm. The lowest parameters were recorded in control 24.67cm, 7.33, and 155gm respectively.

The per cent disease severity reduction was recorded at 45 and 75 days after inoculation of ToLCVf for all the treatments and is presented in Table 2. The result revealed that there was a tremendous decrease in disease severity. The highest disease severity reduction of 89.95% at 45 days and 85.93% at 75 days was observed in T_6Chitosan + *Pseudomonas fluorescens* (Pf–1) + *P. fluorescens* (Pf–2) followed by T_4 Chitosan + *P. fluorescens* (Pf–1) 86.12% at 45 days and 78.29% at 75 days. The lowest disease severity reduction of 69.97% at 45 days and 63.66% at 75 days was observed in T_7 Chitosan.

Table 1. Effect of *Pseudomonas fluorescens* and chitosan on growth and yield parameter of tomato plant

Sl. No.	Treatment details	Plant height (cm)	Number of fruit per plant	Fruit Yield / plant (gm)
1	*Pseudomonas fluorescens* (Pf–1)	43.17	14.00	655.00
2	*Pseudomonas fluorescens* (Pf–2)	40.50	10.67	545.67
3	*Pseudomonas fluorescens* (Pf–1) + *P. fluorescens* (Pf–2)	46.50	15.00	726.33
4	Chitosan + *Pseudomonas fluorescens* (Pf–1)	48.17	18.33	816.67
5	Chitosan + *Pseudomonas fluorescens* (Pf–2)	42.67	12.33	613.00
6	Chitosan+ *Pseudomonas fluorescens* (Pf–1) + *P. fluorescens* (Pf–2)	52.83	21.33	961.67
7	Chitosan	39.83	8.00	416.00
8	Control (Untreated)	24.67	7.33	155.00
CD at 5%		2.164	2.037	4.054

Table 2. Effect of *Pseudomonas fluorescens* and chitosan on reduction of disease severity of tomato leaf curl virus

Sl. No.	Treatment details	Disease severity		Disease severity control (%)	
		45 DAI	75 DAI	45 DAI	75 DAI
1	*Pseudomonas fluorescens* (Pf–1)	15.61	20.34	76.43	71.33
2	*Pseudomonas fluorescens* (Pf–2)	17.49	22.25	73.59	68.64
3	*Pseudomonas fluorescens* (Pf–1) + *P. fluorescens* (Pf–2)	10.16	16.97	84.65	76.08
4	Chitosan + *Pseudomonas fluorescens (Pf–1)*	9.19	15.40	86.12	78.29
5	Chitosan + *Pseudomonas fluorescens* (Pf–2)	13.38	19.40	79.79	72.66
6	Chitosan + *Pseudomonas fluorescens* (Pf–1) + *P. fluorescens* (Pf–2)	6.65	9.98	89.95	85.93
7	Chitosan	19.95	25.78	69.97	63.66
8	Control (Untreated)	66.23	70.96	00.00	00.00
CD at 5%		2.38	2.01		

In present studies Tomato Leaf Curl Virus (ToLCV) was detected by PCR using coat protein specific primers. Three virus affected samples from the different locations of the area were tested. All three samples were subjected for genomic DNA isolation. ToLCV coat protein gene was amplified in PCR using coat protein specific primer. All the samples showed positive PCR reaction amplifying desired size, ~530 bp sequence fragment of CP gene ToLCV. The results of the study confirmed the presence of ToLCV causing tomato leaf curl disease. Similarly Reddy *et al.* (2005) extracted total DNAs from 69 infected tomato plants and amplified coat protein gene (AV1) from 29 infected tomato plants using Coat Protein (CP) gene specific primers (CRv301 and CRc1152). Maruthi (2007) reported that at least five distinct species of ToLCV infected tomato and other host plants in Bangladesh. Phylogenetic analysis of their nucleotide sequences (~530 bases) from the intergenic region and capsid protein of DNA-A indicated the existence of five distinct begomoviruses. Noha *et al*, (2014) isolated the virus from infected tomato and pepper plants grown under natural conditions of different areas in Egypt. The isolates was characterized using various techniques such as biological tests, serology, electron microscopy, and Polymerase Chain Reaction (PCR) by using degenerate primers were used to amplify partial sequences of Coat Protein gene (Cp) of begomovirus from samples of diseased plants.

The studies have shown that chitosan can be effectively used as biologically viable elicitor for the management of viral disease caused by ToLCV by enhancing the activity of beneficial organisms, thereby increasing the resistance against broad range of pathogens. Thus, pest management practices are to be replaced by wide range of naturally occurring and introduced biocontrol agents. The biocontrol ability of rhizobacterial isolates were evaluated under natural conditions with high virus - vector pressure in plant population. We demonstrated the biocontrol efficacy of chitosan treatment along with the strains of *Pseudomonas* spp., Pf-1, and Pf-2. The observation period of 75 days was selected in order to notice the observed changes after the treatment period with or without chitosan in the viral inoculated plants. Our results showed that there was a significant reduction in the disease severity and increase in growth parameters i.e. plant height, number of fruit per plant and yield per plant in comparison to chitosan alone or untreated control. The results of the experiment revealed that highest plant height, number of fruit per plant, fruit yield per plant were recorded (52.83cm), (21.33), (916.67gm) in combination with Chitosan + *Pseudomonas fluorescens* (Pf–1) + *P. fluorescens* (Pf-2) followed by Chitosan + *P. fluorescens*(Pf–1) (48.16cm), (18.33), (816.67gm). The highest disease severity reduction at 45 days 89.95% and 75 days 85.93% was again noticed in Chitosan + *P. fluorescens* (Pf–1) + *P. fluorescens* (Pf–2) compared to the untreated control. Postma *et al*. (2009) observed reduction of number of *Pythium aphanidermatum* infected cucumber plants by 50–100 percent, after a combined application of *Lysobacter enzymogenes* strain 3.1T8 and chitosan. Mishra *et al.* (2014) reported that combined application of *Pseudomonas* sp. and chitosan (elicitor molecules) increased plant growth and reduces the severity of the ToLCV disease by 80–90 percent. The plants inoculated with the combination of chitosan and *Pseudomonas* sp. based formulations recorded the highest activity of Induced Systemic Resistance (ISR) molecules and recorded maximum total biomass, plant height, fruit number, chlorophyll content and yield over the diseased control.

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