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Mechanical sap transmission of Tomato leaf curl New Delhi virus infecting ridge gourd [*Luffa acutangula* (L.) Roxb.] in south India

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Abstract

Ridge gourd (*Luffa acutangula* L.) is an important cucurbitaceous crop cultivated throughout India. The crop is severely affected by yellow mosaic disease caused by *Tomato Leaf Curl New Delhi Virus* (ToLCNDV). The symptoms of ToLCNDV consists of yellow mosaic spots on young leaves, reduction in leaf area, leaf thickening flower abortion and severe stunting of the plants. High disease dissemination under epiphytotic conditions also affects the chemical control measures and leads to heavy crop losses. Identification of resistant sources is most important tool to reduce the economic loss and maximize quality and quantity of output. Efficient and simple screening procedures are very crucial in disease resistant breeding program. In this experiment, mechanical sap inoculation of ToLCNDV has been studied in twelve ridge gourd genotypes. All the inoculated plants developed characteristics ToLCNDV symptoms and severe infection after three-week post inoculation. In all the assays ToLCNDV transmission was confirmed by PCR amplification using virus specific primer followed by sequencing of random samples. Mechanical sap transmission is easy and simple screening method which will facilitate the development of ToLCNDV resistant breeding cultivars.

Keywords: Ridge gourd, Mechanical sap inoculation, ToLCNDV, Symptoms, Screening, Susceptible

Introduction

Yellow mosaic disease caused by the *Tomato leaf curl New Delhi virus* (ToLCNDV) is a major constraint in ridge gourd production and results in considerable yield losses. ToLCNDV is more prevalent in tropical and sub-tropical regions of India (Sohrab *et al.*, 2003; Malathi *et al.*, 2017) ^[1, 2]. The symptoms on the infected plants include yellow mosaic spots on young leaves, leaf curling, vein thickening, darkening of leaf margins puckering, and severe stunting of the plant. High disease dissemination under epiphytotic conditions leads to heavy crop losses and increased cost of crop production. Increased pesticides resistance and population dynamics of whitefly (*Bemisia tabaci*, Biotype-B) is also a big issue to be addressed (Zaidi *et al.*, 2016) ^[3]. Therefore, designing appropriate integrated management strategy is most important where use of resistant variety seeds is the key tool to reduce the economic losses and maximize quality and quantity of output. Use of genetically resistant varieties is a simple and effective strategy to control ToLCNDV in affected crops and the success of any resistant breeding program depends upon the efficiency of screening methodology adopted during the experiments. Mechanical sap transmission of different isolates of ToLCNDV has been confirmed in *Cucurbita* accessions (Saez *et al.*, 2016); melon (Lopez *et al.*, 2015) ^[4, 5] and *Luffa* genotypes (Sohrab *et al.*, 2013) ^[6]. In the present work, we report mechanical sap transmission of ToLCNDV local isolate in ridge gourd under local weather conditions which will further facilitate the breeding of virus resistant ridge gourd varieties.

Materials and Methods**Plant materials**

A total of 12 ridge gourd susceptible genotypes were assayed to validate the efficiency of the selected virus inoculation method (Table 1). The selected ridge gourd genotypes were advanced inbred lines maintained at vegetable division, ICAR-IIHR, Bengaluru. Ten seeds of each genotype were soaked overnight and subsequently kept in petri plate to facilitate the germination. Seeds were directly sown in the polybags (15cm×10cm) in an insect proof net-house.

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Virus source

Virus inoculum was collected from infected ridge gourd plants from research block of ICAR-IIHR Bengaluru. The leaves were showing characteristic symptoms of ToLCNDV such as yellow mosaic on young leaves, curling, vein thickening from plants having short internodes were used as the source of inoculum. Test plants were subjected to mechanical sap inoculation to confirm the sap transmission of ToLCNDV local isolate under controlled environment conditions.

Mechanical Sap Inoculation Method

Mechanical sap inoculation was conducted in susceptible genotypes in an insect-proof net house. For inoculation, 1 g of infected leaves was grounded with a pestle mortar using

inoculation buffer (0.1M potassium phosphate buffer) followed by addition of beta-mercaptoethanol. The resultant homogenate was filtered through sterile cotton pads and celite (abrasive agent) was added to it. Test seedlings were inoculated at cotyledon-leaf stage (10 days after sowing) and were dusted with carborandom power 600 mesh and inoculum was gently applied on the upper surface of leaves unidirectionally with cotton swab (Plate 1). The inoculum was prepared as described by Sohrab *et al.* (2014) [7] with slight modification (0.5 M potassium phosphate buffer [pH 7.0], 0.04% mercaptoethanol, 0.2% sodium sulphite and 2% Celite) with a 1:4 proportion of infected leaves weight: buffer volume. The inoculation procedure was repeated twice to increase the virus transmission efficiency.



Plate 1: Artificial Screening (mechanical sap inoculation) of *Luffa* genotypes for ToLCNDV resistance

Inoculation of ToLCNDV on Test Genotypes

Ten plants per genotype were tested and some selected genotypes with all or most of the plants with no or mild symptoms after first week were subjected to new inoculation rounds, using the same procedure, to confirm the response. The disease response of 12 ridge gourd susceptible genotypes were used for inoculation study.

Symptoms Evaluation

Inoculated plants were kept in an insect-proof nethouse for ToLCNDV symptom evaluation. Symptom evaluation was performed at one-week post inoculation and observed upto two months. One non-inoculated control plant per genotype was kept separately to avoid virus infection. Symptoms were assessed by visual evaluation using a standardized phenotypic scale 0-5 phenotypic scale where 0= No symptoms; 1= mild mosaic symptoms; 2=moderate mosaic symptoms; 3=severe mosaic symptoms; 4= severe mosaic symptoms blistering and puckering of leaves; 5= very severe symptoms or stunted plant. To test the severity of virus infection, vulnerability index was calculated as adopted by Islam *et al* (2011) which facilitated the better comparison between genotypes. Based on per cent vulnerability index value, the genotypes were categorized into different classes such as resistant (VI= 1-25), moderately resistant (VI=26-50), moderately susceptible (VI=51-75) and susceptible (75-100). AUDPC was determined using the formula as described by Cambell and Madden (1990)^[8].

ToLCNDV Diagnosis

Inoculated plants with characteristic yellow mosaic symptoms, prominent upward curling, puckering, and thickening ToLCNDV symptoms were visually observed for symptoms development. ToLCNDV transmission was confirmed by PCR amplification using virus specific primer. In total, nineteen samples covering all the 12 genotypes along with control were used for confirmation of virus transmission. Viral DNA from apical leaves of inoculated and control plants was extracted at 30 days post inoculation (DPI) in the screening assay using the Cetyltrimethyl ammonium bromide (CTAB) method (Swarnalatha *et al.*, 2013)^[9]. DNA was quantified using a NanoDrop 1000 spectrophotometer and diluted with sterile distilled deionised water to a final concentration of 50 ng/µL. For ToLCNDV detection, 2.0 µL aliquots of total DNA (50 ng) were used as templates in PCR reactions of 0.3µL with 3U of Taq DNA polymerase, 0.5µL (20 pmol/µL) of each virus specific forward and reverse primers, 2 mM dNTPs in 10X PCR buffer and 25mM MgCl₂. Reactions were incubated for initial-denaturation at 94°C for 2 min followed by 35 cycles each consisting of denaturation at 94°C for 45 sec, annealing at 55°C for 1 min, extension at 72°C for 1:30 min with final extension at 72°C for 20 min. Amplified DNA fragments were electrophoresed in 1 per cent agarose gel. The gel piece containing amplified DNA fragment was cut from the agarose gel under UV-transilluminator. The amplified DNA fragments was eluted from the gel piece using gel extraction and DNA purification kit and the amplified fragment were sequenced at Medaxin DNA Sequencing facility, Bangalore, Karnataka, India.

Results and Discussion

The ToLCNDV was mechanically transmitted through sap inoculation in test seedlings under controlled conditions. Yellow mosaic and curling symptoms started appearing on young leaves (Plate 1). Severe curling and stunting of plants

was observed in some test entries after 30 dpi. Among all the genotypes screened through mechanical sap inoculation, mean vulnerability index was ranged from 55.00 to 77.19 per cent (Table 1). All the susceptible genotypes developed severe symptoms and the maximum vulnerability index was recorded at sixth week post inoculation. Based on mean vulnerability index, all the genotypes were moderately susceptible to susceptible (VI \geq 50%) (Table 1). Area under disease progress curve (AUDPC) value was ranged from 3010.00 (IIHRRV-6-1-7) to 4305.00 (IIHRDMVR-Sel-23) (Table 1). Most of the test entries showed very high AUDPC score (\geq 3000.00) (Table 1). AUDPC scores for all the genotypes were in close conformity with vulnerability index values where test entries with high VI were also having high AUDPC value.

The mechanical sap transmission is a recently reported ToLCNDV inoculation method and therefore, ToLCNDV infection in test genotypes was confirmed by symptoms scoring and specific PCR amplification with virus specific primers (Plate 2). Random symptomatic plants were selected for PCR amplification using specific primers. All the selected symptomatic plants resulted in positive simplification of ~750bp DNA bands (Plate 2), confirming the association of a ToLCNDV with the disease. However, no such amplicon was obtained in non-inoculated plants taken as negative control. BLASTN analysis of nucleotide sequence data of the PCR amplified fragments revealed 97 per cent nucleotide homology with ToLCNDV- ridge gourd isolate RG 3 [Ridge Gourd: India] (KT426905.1) (Plate 3) which confirm the successful transmission of the virus through mechanical sap inoculation.

It has been observed that successful virus transmission of ToLCNDV depends upon number of external factors such as inoculation buffer properties, prevailing weather conditions, age of the infected plants for inoculum and age of the test seedlings. Sohrab *et al.* (2014)^[7] reported that the effectiveness of inoculum was significantly increased with the addition of sodium sulphite, celite, beta-mecaptaethanol and dusting carborundum powder on the young leaves. In addition to inoculum composition, various climatic and genetic factors influenced the transmission of virus in the ridge gourd seedlings. Maintenance of virus culture, plant growth stage, inoculum preparations, inoculation techniques, inoculation time and prevailing weather conditions are very important steps in screening experiment. Sohrab *et al.* (2013)^[6] conducted experiment on mechanical sap transmission of ToLCNDV isolate (*ToLCNDV- Luffa: Del*) in ridge gourd [*L. acutangula* (Roxb)] and ToLCNDV isolate from *Luffa* plants was transmitted to ridge gourd seedlings with high efficiency after 15 dpi. Lopez *et al.* (2015)^[5] observed that ToLCNDV was mechanically sap transmitted to 4 genera and 13 species of the *cucurbitaceae* family including all the commercial crops such as cucumber, watermelon, pumpkin and melon. Also, transmission was confirmed in wild species and landraces.

Among the three different inoculation buffers *viz.* used by Sohrab *et al.*, (2014) (1:4), COMAV buffer (1:4) and COMAV buffer (1:10), COMAV buffer (1:10) was most effective to transmit ToLCNDV to test seedling after 24 dpi. Virus transmission was confirmed though PCR amplification of inoculated sample followed by amplified products sequencing. Following the same procedure, Saez *et al.* (2016)^[4] reported that severe symptoms and maximum ToLCNDV viral content was found after 30 days post inoculation in various *Cucurbita* species accessions. Following the Procedure of Sequiera and Harrison (1982)^[10] for cassava

mosaic virus, Sangeetha *et al.* (2018) [11] infected different cucurbits plants with ToLCNDV isolates. They extracted the crude sap from infected leaves in 100mM Tris (pH 8.0) and 10mM EDTA (pH 8.0) with 0.2 percent beta mercaptoethanol. ToLCNDV symptoms were observed in watermelon, cucumber, bottle gourd, bitter gourd and ridge gourd after 9-15 dpi. Symptoms were confirmed through PCR amplification with ToLCNDV-specific primers.

In the present experiment, inoculation at cotyledons-leaf stage, high temperature and repeated inoculation played a significant role in transmission of the virus. However, more germplasm entries are required to evaluate the genotypic effect on the screening methods. Mechanical sap transmission is easy and simple screening methods. Studies on the factors affecting sap inoculations will further facilitate the large-scale screening of the germplasm rapidly.

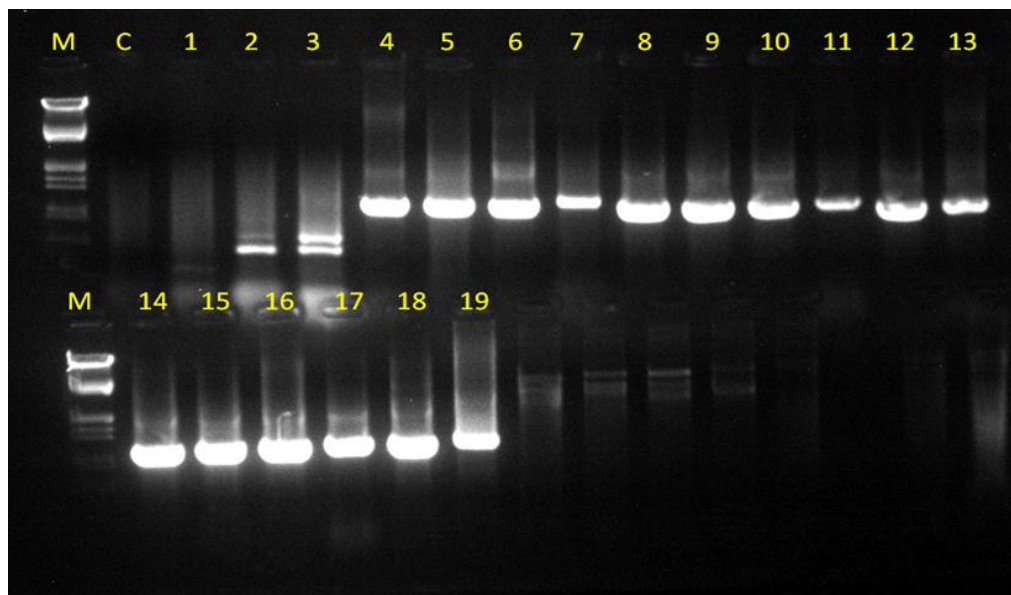


Plate 2: PCR Confirmation of the ToLCNDV transmission through mechanical sap inoculation Lane 1-19: highly susceptible plant samples (M: Lambda DNA/EcoRI+HindIII Marker, C: Control)

MKMD3258_1455R-G01.ab1	1	---	GAGTGGCG	TGTTTATCTG	GACCGTTCAA	TCCCTGTAT	TTCAAAAAT	TGAAACACGA
MKMD3259_1455R-H01.ab1	1	---	GAGTGGCG	TGTTTATCTG	GACCGTTCAA	TCCCTGTAT	TTCAAAAAT	TGAAACACGA
MKMD3260_1455R-A02.ab1	1	---	GAGTGGCG	TGTTTATCTG	GACCGTTCAA	TCCCTGTAT	TTCAAAAAT	TGAAACACGA
KT426905.1	Tomato leaf curl Ne	1	GGGAGGAACG	TATGCTCTA	GGGAG--CAA	GCATTAGTTA	GGAAAGTTGT	TAGGGTTAAT
KT426906.1	Tomato leaf curl Ne	1	GGGAGGAACG	TATGCTCTA	GGGAG--CAA	GCATTAGTTA	GGAAAGTTGT	TAGGGTTAAT
MH816959.1	Tomato leaf curl Ne	1	GGGAGGAACG	TATGCTCTA	GGGAG--CAA	GCATTAGTTA	GGAAAGTTGT	TAGGGTTAAT
MN594504.1	Squash leaf curl Ch	1	GGGAGGAACA	TATGCTCTA	GGGAG--CAA	GCATTAGTTA	GGAAAGTTGT	TAGGGTTAAT
MH577759.1	Tomato leaf curl Ne	1	GGGAGGAACG	TATGCTCTA	GGGAG--CAA	GCATTAGTTA	GGAAAGTTGT	TAGGGTTAAT
MKMD3258_1455R-G01.ab1	99	CAACTACAGT	TCAACCACAA	CTCGGGAGAG	CCCTGGGGCT	TACACAGTGT	TTTCTAAGTT	TTTCTAAGTT
MKMD3259_1455R-H01.ab1	99	CAACTACAGT	TCAACCACAA	CTCGGGAGAG	CCCTGGGGCT	TACACAGTGT	TTTCTAAGTT	TTTCTAAGTT
MKMD3260_1455R-A02.ab1	99	CAACTACAGT	TCAACCACAA	CTCGGGAGAG	CCCTGGGGCT	TACACAGTGT	TTTCTAAGTT	TTTCTAAGTT
KT426905.1	Tomato leaf curl Ne	99	ATACTGAAAA	GGCATTAAATG	TTATGGCCTG	TACTCATGCA	TCAAACTGT	TATGCGACTT
KT426906.1	Tomato leaf curl Ne	99	ATACTGAAAA	GGCATTAAATG	TTATGGCCTG	TACTCATGCA	TCAAACTGT	TATGCGACTT
MH816959.1	Tomato leaf curl Ne	99	ATACTGAAAA	GGCATTAAATG	TTATGGCCTG	TACTCATGCA	TCAAACTGT	TATGCGACTT
MN594504.1	Squash leaf curl Ch	99	ATACTGAAAA	GGCATTAAATG	TTATGGCCTG	TACTCATGCA	TCAAACTGT	TATGCGACTT
MH577759.1	Tomato leaf curl Ne	99	ATACTGAAAA	GGCATTAAATG	TTATGGCCTG	TACTCATGCA	TCAAACTGT	TATGCGACTT
MKMD3258_1455R-G01.ab1	199	CTTGAGGSET	TTTAGAACGC	AGSTCCCTAA	TACCTAAATT	GTTTGGTGT	GTGGAAATTT	GTGGAAATTT
MKMD3259_1455R-H01.ab1	199	CTTGAGGSET	TTTAGAACGC	AGSTCCCTAA	TACCTAAATT	GTTTGGTGT	GTGGAAATTT	GTGGAAATTT
MKMD3260_1455R-A02.ab1	199	CTTGAGGSET	TTTAGAACGC	AGSTCCCTAA	TACCTAAATT	GTTTGGTGT	GTGGAAATTT	GTGGAAATTT
KT426905.1	Tomato leaf curl Ne	199	ATTATTGCTC	TTTATATCAT	ATGTTGCCAA	TACATCAACT	GTTTTATGCA	ATCATTATCT
KT426906.1	Tomato leaf curl Ne	199	ATTATTGCTC	TTTATATCAT	ATGTTGCCAA	TACATCAACT	GTTTTATGCA	ATCATTATCT
MH816959.1	Tomato leaf curl Ne	199	ATTATTGCTC	TTTATATCAT	ATGTTGCCAA	TACATCAACT	GTTTTATGCA	ATCATTATCT
MN594504.1	Squash leaf curl Ch	199	ATTATTGCTC	TTTATATCAT	ATGTTGCCAA	TACATCAACT	GTTTTATGCA	ATCATTATCT
MH577759.1	Tomato leaf curl Ne	199	ATTATTGCTC	TTTATATCAT	ATGTTGCCAA	TACATCAACT	GTTTTATGCA	ATCATTATCT
MKMD3258_1455R-G01.ab1	299	TTCATATAAA	CA-GTTGAG	TA-TTGGCAA	CATATGATAT	AAAAATATTT	ATTAATTTGT	ATTAATTTGT
MKMD3259_1455R-H01.ab1	299	TTCATATAAA	CA-GTTGAG	TA-TTGGCAA	CATATGATAT	AAAAATATTT	ATTAATTTGT	ATTAATTTGT
MKMD3260_1455R-A02.ab1	299	TTCATATAAA	CA-GTTGAG	TA-TTGGCAA	CATATGATAT	AAAAATATTT	ATTAATTTGT	ATTAATTTGT
KT426905.1	Tomato leaf curl Ne	299	CTACACCAAG	CATATTTAGG	TACTTAAGGA	CCTGGGTTCT	AAAACCCCTCA	AGAAAAATCC
KT426906.1	Tomato leaf curl Ne	299	CTACACCAAG	CATATTTAGG	TACTTAAGGA	CCTGGGTTCT	AAAACCCCTCA	AGAAAAATCC
MH816959.1	Tomato leaf curl Ne	299	CTACACCAAG	CATATTTAGG	TACTTAAGGA	CCTGGGTTCT	AAAACCCCTCA	AGAAAAATCC
MN594504.1	Squash leaf curl Ch	299	CTACACCAAG	CATATTTAGG	TACTTAAGGA	CCTGGGTTCT	AAAACCCCTCA	AGAAAAATCC
MH577759.1	Tomato leaf curl Ne	299	CTACACCAAG	CATATTTAGG	TACTTAAGGA	CCTGGGTTCT	AAAACCCCTCA	AGAAAAATCC
MKMD3258_1455R-G01.ab1	397	TATACAGGAT	TTGATGCATG	AGTACAGGCC	ATATACAACA	TTAATCGGTT	TTGATGATGA	TTGATGATGA
MKMD3259_1455R-H01.ab1	397	TATACAGGAT	TTGATGCATG	AGTACAGGCC	ATATACAACA	TTAATCGGTT	TTGATGATGA	TTGATGATGA
MKMD3260_1455R-A02.ab1	397	TATACAGGAT	TTGATGCATG	AGTACAGGCC	ATATACAACA	TTAATCGGTT	TTGATGATGA	TTGATGATGA
KT426905.1	Tomato leaf curl Ne	399	AACACTTGTG	AAGCCCCAGG	GCTCTCCGCA	GGTTGTGGTT	GAACTGTATT	TGGACTTTGA
KT426906.1	Tomato leaf curl Ne	399	AACACTTGTG	AAGCCCCAGG	GCTCTCCGCA	GGTTGTGGTT	GAACTGTATT	TGGACTTTGA
MH816959.1	Tomato leaf curl Ne	399	AACACTTGTG	AAGCCCCAGG	GCTCTCCGCA	GGTTGTGGTT	GAACTGTATT	TGGACTTTGA
MN594504.1	Squash leaf curl Ch	399	AACACTTGTG	AAGCCCCAGG	GCTCTCCGCA	GGTTGTGGTT	GAACTGTATT	TGGACTTTGA
MH577759.1	Tomato leaf curl Ne	399	AACACTTGTG	AAGCCCCAGG	GCTCTCCGCA	GGTTGTGGTT	GAACTGTATT	TGGACTTTGA

Plate 3: Confirmation of the ToLCNDV transmission through mechanical sap based on sequence of amplified fragment

Table 1: Vulnerability index (VI) and area under disease progress curve (AUDPC) of different *Luffa* genotypes for reaction against ToLCNDV disease under artificial screening (mechanical sap inoculation)

Sl. No.	Genotypes	Vulnerability Index (%)								Mean VI	AUDPC	Disease reaction
		1 WPI	2 WPI	3 WPI	4 WPI	5 WPI	6WPI	7 WPI	8 WPI			
1	IIHRRV-6-1-7	30.00	45.00	50.00	50.00	60.00	65.00	70.00	75.00	55.63	3010.00	MS
2	IIHRDM-16-134-1	33.33	46.66	46.66	66.66	73.33	80.00	100.00	100.00	68.33	3709.83	MS
3	IIHRDMVR-Sel-20	0.00	45.00	75.00	85.00	95.00	100.00	100.00	100.00	75.00	4200.00	MS
4	IIHRDMVR-Sel-23	5.00	45.00	75.00	92.50	100.00	100.00	100.00	100.00	77.19	4305.00	S
5	IIHRDMVR-Sel-62	0.00	28.00	40.00	88.00	96.00	96.00	96.00	96.00	67.50	3780.00	MS
6	IIHRDMVR-Sel-21	12.00	20.00	28.00	72.00	88.00	96.00	96.00	96.00	63.50	3514.00	MS
7	IIHRDMVR-Sel-10	8.57	8.57	80.00	91.43	94.29	100.00	100.00	100.00	72.86	4050.03	MS
8	IIHRDMVR-Sel-25	16.66	16.67	70.00	86.67	96.67	100.00	100.00	100.00	73.33	4048.38	MS
9	IIHRDMVR Sel-12	20.00	20.00	60.00	100.00	100.00	100.00	100.00	100.00	75.00	4130.00	S
10	IIHRDMVR Sel-46	0.00	0.00	40.00	60.00	80.00	86.67	86.67	86.67	55.00	3080.07	MS
11	IIHRDMVR-Sel-11	12.50	26.67	46.67	95.56	95.56	100.00	100.00	100.00	72.12	3994.97	MS
12	IIHRDMVR Sel-45	0.00	13.33	53.33	93.33	93.33	100.00	100.00	100.00	69.17	3873.24	MS

WPI - Week post infection, VI - Vulnerability index, AUDPC - Area under disease progress curve, MS - Moderately Susceptible and S-Susceptible

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