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Studies on the transmission of Squash Leaf Curl China Virus causing yellow mosaic and leaf curl disease in pumpkin

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ABSTRACT

Viruses pose a significant threat to pumpkin cultivation, leading to substantial yield losses and reduced fruit quality. Understanding the transmission dynamics and disease progression of Squash Leaf Curl China Virus (SLCCV), a major virus affecting pumpkin, will contribute to developing effective management strategies for this crop. Our study aimed to develop an efficient mechanical inoculation method for SLCCV in pumpkin and investigate whitefly transmission. Mechanical inoculation was assessed using different buffers (I, II, and III) for preparing the viral inoculum. Buffer III, consisting of phosphate buffer, β-mercaptoethanol, and Na_2SO_3, showed the highest severity of symptoms and incidence of infection in the susceptible pumpkin variety Pusa Vishwas. The summer season was the most favourable for virus transmission, and plant parts such as expanded leaves, middle leaves, and stems exhibited higher infectivity than other parts. A minimum of two whiteflies per plant successfully transmitted the virus to pumpkin plants, resulting in yellow mosaic symptoms and stunted growth. The transmission efficiency increased with an increase in number of whiteflies per plant. A minimum acquisition access feeding period (AAP) of 10 min. and an inoculation access feeding period (IAP) of 24 h were optimal for whitefly-mediated transmission of SLCCV. These findings highlight the importance of vector control strategies, such as insecticides and physical barriers to prevent whitefly-mediated transmission of SLCCV in pumpkin. This is the first report of sap transmission of SLCCV in pumpkin, emphasizing the importance of developing and implementing comprehensive control strategies that address all possible routes of infection.

Key words: *Cucurbita moschata* Duchesne ex Poir., Sap inoculation, SLCCV, Transmission, Whitefly.

INTRODUCTION

Viruses pose a significant challenge to the economic viability of many horticultural crops by affecting their crop productivity. Pumpkin [*Cucurbita moschata* Duchesne ex Poir.] is highly vulnerable to these plant viruses. Among them, begomoviruses are the most widespread and economically impactful, resulting in severe losses ranging from 60 to 100% (Dhatt *et al.*, 2). The emergence of Squash Leaf Curl China Virus (SLCCV), transmitted by whitefly (*Bemisia tabaci*), is a major concern affecting squash and various other cucurbits in India and neighbouring countries, limiting their cultivation in Southeast Asia (Dhillon *et al.*, 3; Togoobat *et al.*, 14). The bipartite genome of SLCCV consists of two closed, circular ssDNA elements, namely, DNA-A and DNA-B, each with a size ranging from approximately 2.6-2.8 kb (Venkataravanappa *et al.*, 15). Infected pumpkin plants show diverse symptoms based on the timing of infection with distinct differences (Shehata *et al.*, 12). Early infections in pumpkin plants result in severe curling, impaired growth, and fruit loss, with advanced stages leading to leaf curling, young leaf yellowing, and rough texture in mature fruits (Dhillon *et al.*, 3). A diverse array of detection methods can be used for SLCCV diagnosis (Kesumawati *et al.*, 6), such as utilizing the conserved capsid protein among begomoviruses, antisera from related species, specific primer-based PCR assays and molecular hybridization using tailor-made probes for potential SLCCV identification (Desbiez, 1).

Unfortunately, control measures for SLCCV are limited. Cultural practices such as eliminating affected plants, avoiding planting susceptible crops in proximity, and controlling whitefly populations are recommended to curb the disease spread (Saha *et al.*, 11). Genetic resistance is recognized as the most effective control approach in managing viruses (Kaur *et al.*, 5). Efficient inoculation methods are vital in breeding programs for identifying resistant sources and segregating populations (Dhillon *et al.*, 3). Successful mechanical inoculation has been performed against tomato leaf curl New Delhi virus (ToLCNDV) in various cucurbit crops, enabling transmission from infected squash plants to *Cucumis* spp., *Citrullus* spp., and *Cucurbita* spp. using a specific inoculation buffer (Kil *et al.*, 7).

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Unravelling SLCCV resistance sources in cucurbit crops relies on a robust mechanical inoculation method and precise diagnostic techniques, yet research on the sap transmission of SLCCV in cucurbits, specifically pumpkin, is lacking. Our study aims to fill this research gap by developing a sap inoculation method for SLCCV transmission. The present study primary goals involve developing the inoculation procedure and assessing whitefly transmission in pumpkins.

MATERIALS AND METHODS

During June 2022, naturally virus-infected pumpkin plants exhibiting symptoms like vein yellowing, mild curling, puckering, and the presence of greenish yellow mosaic patches in young leaves were selectively collected from the IARI Vegetable Experimental Field in New Delhi (longitude 77°12' E; latitude 28°40' N) (Fig. 1A). The presence of different viruses in the surrounding area was tested in the plants using PCR with specific primers for different begomoviruses like SLCCV, tomato leaf curl New Delhi virus (ToLCNDV), tomato leaf curl Palampur virus (ToLCPalV), tomato leaf curl Gujarat virus (ToLCGV), and tomato leaf curl Joydebpur virus (ToLCJoV) (Table 1) and ELISA against Papaya ringspot virus (PRSV). The infected leaves were diced and thoroughly mixed before testing with three different buffers. Buffer I contained 0.1 M sodium phosphate buffer, 0.2% sodium sulfite, and 2% Celite. Buffer II was composed of 15 mM NaCl, 7.5 mM Na_2HPO_4, 0.38 mM NaH_2PO_4, and 25 mM polyvinylpyrrolidone. Buffer III was formulated as a phosphate buffer (0.1 M Monobasic, 0.1 M Dibasic) at a pH of 6.5, and it also contained 0.02% β-mercaptoethanol, 0.2% sodium sulphite, and 2% Celite. For sap inoculation of SLCCV, infected pumpkin leaves were ground with one of the three inoculation buffers. The resulting homogenate was used to gently rub the adaxial surface of the leaf for inoculation.

A begomovirus-free whitefly colony was established by rearing pumpkin plant-collected whiteflies on healthy cotton plants. Pumpkin seedlings were infected with the SLCCV isolate through the inoculation of 10 virus-free whiteflies, and infected leaves were obtained with severe symptoms for inoculum preparation. Susceptible pumpkin plants were used, and the inoculum was prepared from seeds treated with 5% sodium hypochlorite and cultivated in Petri dishes at 25°C in an insect-free climatic chamber. The acquisition access feeding period (AAP) and inoculation access feeding period (IAP) for SLCCV were determined by allowing whiteflies potentially carrying the virus to feed on pumpkin plants for different periods to ascertain virus acquisition and transmission requirements.

Symptom evaluation was conducted at various time points, and PCR amplification was used to validate sap transmission. The impact of different seasons on virus transmission and disease severity was assessed, and the inoculum was prepared using different parts of infected plants. Subsequent inoculations took place 15 days following the initial inoculation. For each combination of the days of post-inoculation (DPI) and the buffer solutions, we computed the percentage of plants that exhibited symptoms using the formula: $100 \times [(n_1 + n_2 + n_3 + n_4) / (n_0 + n_1 + n_2 + n_3 + n_4)]$. We also calculated the mean symptom score as $(n_1 + 2n_2 + 3n_3 + 4n_4) / (n_0 + n_1 + n_2 + n_3 + n_4)$, where n_0, n_1, n_2, n_3, and n_4 represent the number of plants with symptom scores of 0, 1, 2, 3, and 4, respectively, as per López *et al*. (8). This

Table 1. PCR primers used to confirm the virus associated with diseased pumpkin plants.

Virus name	Primer name	Primer sequence (5' to 3')	Temp (°C)	Target region	Target size (bp)
Begomovirus generic	BM783F	CCCCTGTCCGTGAATCCGT	58	Partial AV2	538
	BM784R	SDVTBCMGCTGCGCGGCC			
ToLCPalV	BM798F	AGACTTGCTCACCAAGC	56	Partial AV2	647
	BM799R	GAAATCTTGTGGCGCAC			
ToLCGV	BM800F	GAAGCGACCAGCAGATATGC	60	Partial AV2	396
	BM801R	GTGTTGGTGTGGTTCTTCAC			
ToLCJoV	BM802F	AGAATGTGCATCGTGACAGG	62	Partial AV2	732
	BM803R	TCGTCGCGTTGACCTGGAC			
ToLCNDV	ToL-F	GGGTATGGGAGTAGGTGGA	56	Partial BC1	202
	ToL-R	AACCTGACTGCGATATTCTAG			
SLCCV	SLCCV-F	GGAGGTCACTCTGACGTTC	52	Partial BC1	513
	SLCCV-R	CGTAACGATCTTGAACTGTG			

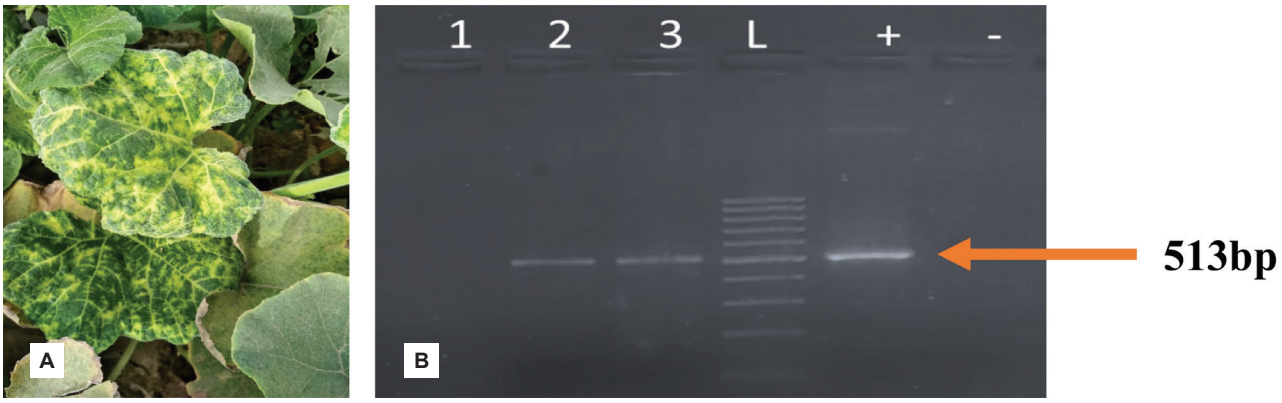

Fig. 1. **(A)** Symptomatic pumpkin samples from the field of IARI. **(B)** PCR validation of identified virus from pumpkin sample: Agarose gel (1%) electrophoresis of PCR amplified products of SLCCV (513 bp); Lane 1, 2, 3: Infected pumpkin samples; Lane +: Positive control using PCR product of respective virus; Lane -: Negative control (reagent control); Lane L: 100 bp plus DNA ladder (Thermo Fisher Scientific, USA).

mean score serves as an average measurement of the symptoms for a specific treatment, ranging from 0 to 4, and varies proportionally with the vulnerability index (VI), where the maximum VI value is 100% (López *et al*., 8). In our study, we considered five symptom scores and calculated the Vulnerability Index (VI) as 25 times the mean symptom score. To determine the incidence of symptomatic plants and the average mean score for each buffer treatment, we relied on observations conducted on twenty individual plants.

RESULTS AND DISCUSSION

The pumpkin plants were tested to ascertain the presence of other viruses that are frequently encountered in the surrounding region using ELISA against PRSV and PCR with specific primers for SLCCV, ToLCNDV, ToLCPalV, ToLCGV, and ToLCJoV (Table 1). All tests, except for SLCCV, yielded negative results (Fig. 1B). Buffer III induced more severe symptoms than buffer I, with 85.0% of Pusa Vishwas plants showing symptoms at 8 DPI compared to 65.0% with buffer II. All plants inoculated with buffer III displayed symptoms by 24 DPI, while buffer I took an additional 6 days. Buffer III is recommended for the mechanical inoculation of SLCCV based on the findings in the highly susceptible pumpkin variety Pusa Vishwas, where buffer III resulted in more severe symptoms than other buffers (Table 2). Buffer III consistently resulted in higher mean symptom scores compared to buffer II at all time points, with a notable difference observed at 7 DPI, where buffer III had a mean symptom score more than twice as high (1.65) compared to buffer I (0.85) (Table 2). Mastrochirico *et al.* (9) achieved better results with a COMAV buffer (similar to buffer III but with a different pH) than buffer I. This implies that polyvinylpyrrolidone (PVP) may significantly impact virus infectivity, as it binds polyphenols, reduces oxidase activity, and maintains virus stability (Singh *et al.*, 13). Buffer, I had lower symptom incidence and mean scores than the other two buffers, with a maximum infection rate of 20% at 16 DPI. However, when treated with Buffer II, infected plants exhibited severe mosaic symptoms (score 4) 24 days after inoculation (Table 2). The absence of β-mercaptoethanol in Buffer I suggests that it plays a role in facilitating infection by maintaining inoculum infectivity for a longer duration. Buffers II and III efficiently facilitate SLCCV infection, with buffer III resulting in a higher percentage of symptomatic plants and more pronounced symptoms in less time (Table 2). Consequently, the preferred option is to utilize buffer III for the mechanical inoculation of SLCCV (Fig. 2). In the summer (May-June), maximum infection rates were observed, gradually declining with the lowest infection proportion in December. Expanded leaves, middle leaves, and pumpkin stem showed higher infectivity, while the lower stem was insensitive to infection (Table 3).

Yellow mosaic disease symptoms in whitefly-inoculated pumpkin plants (Fig. 3) resembled field observations, with successful transmission leading to a 100% infection rate in 8-day-old seedlings, exhibiting initial yellow mosaic symptoms after 8-10 days of incubation and subsequent mild leaf curling, serving as the basis for further experiments.

The successful transmission of SLCCV required a minimum of two whiteflies (Asia-II biotype) per pumpkin plant (Table 4). However, augmenting the whitefly count from 2 to 25 per plant improved the transmission efficiency of SLCCV, with the highest transmission observed when eight or more whiteflies were present, resulting in symptom development within 8-10 days, while control plants remained symptomless.

Table 2. Incidence and severity of symptoms caused by SLCCV in pumpkin plants (cv. Pusa Vishwas) after mechanical inoculation with different inoculation buffers (I, II and III).

Symptom score[a]	Inoculation buffer I				Inoculation buffer II				Inoculation buffer III			
	8 DPI[b]	16 DPI	24 DPI	30 DPI	8 DPI	16 DPI	24 DPI	30 DPI	8 DPI	16 DPI	24 DPI	30 DPI
0	17	16	16	16	7	6	3	0	3	2	0	0
1	2	1	0	0	8	7	6	4	5	3	3	0
2	1	2	0	0	4	5	4	3	8	6	6	2
3	0	0	1	0	1	1	3	6	4	5	4	7
4	0	1	3	4	0	1	4	7	0	4	7	11
Symptomatic plants (%)	15.0	20.0	20.0	20.0	65.0	70.0	85.0	100.0	85.0	90.0	100.0	100.0
Mean symptom score	0.20	0.45	0.75	0.80	0.85	1.20	1.95	2.8	1.65	2.30	2.75	3.45

[a]Symptom score values: 0-absence of symptoms; 1-mild symptoms; 2-moderate symptoms; 3-severe symptoms; 4-very severe symptoms.
[b]Incidence and severity assessments at 8-, 16-, 24- and 30-days post-inoculation (DPI). Twenty plants were evaluated for each inoculation buffer treatment.

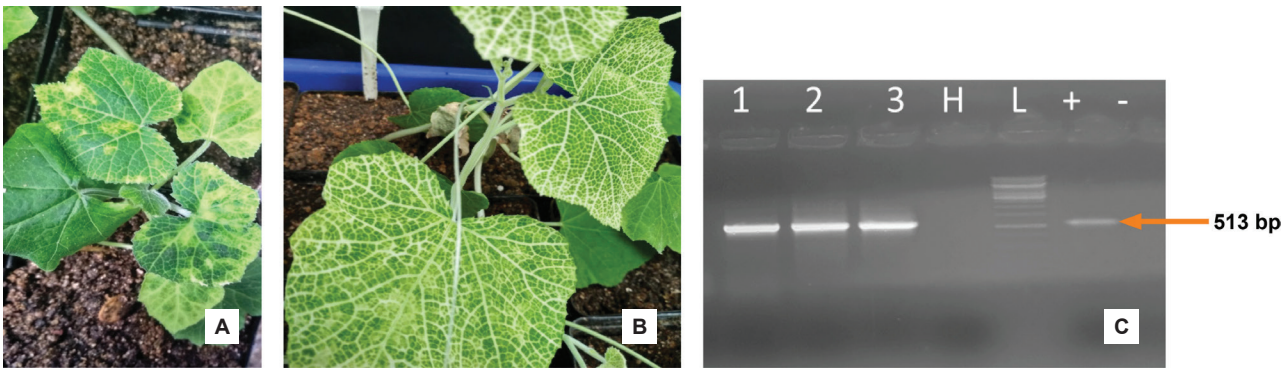

Fig. 2. Symptoms indicative of begomovirus infection in the artificially sap-transmitted Pumpkin test plants under greenhouse experimental conditions. **(A)** green-yellow mosaic, interveinal yellowing, yellow spots in leaves. **(B)** Yellow vein at 24 days post inoculation (dpi) and **(C)** the PCR-based confirmation of the association of squash leaf curl China virus (SLCCV)- amplicon size 513 bp; Lane 1, 2, 3: sap inoculated Pumpkin samples; Lane H: Healthy pumpkin sample; Lane +: Positive control using PCR product of respective virus; Lane –: Negative control (reagent control); Lane L:100 bp plus DNA ladder (Thermo Fisher Scientific, USA).

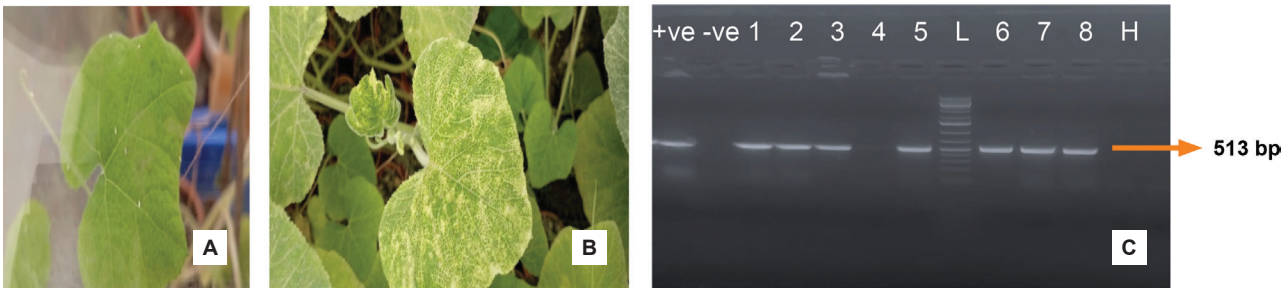

Fig. 3. (**A and B)** Symptoms indicative of begomovirus infection in the artificially whitefly-transmitted pumpkin test plants under greenhouse experimental conditions. **(C)** PCR-based confirmation of the association of squash leaf curl China virus (SLCCV)- amplicon size 513 bp; Lane 1 to 8: whitefly inoculated pumpkin samples; Lane H: Healthy pumpkin sample; Lane +ve: Positive control using PCR product of respective virus; Lane -ve: Negative control (reagent control); Lane L: 100 bp plus DNA ladder (Thermo Fisher Scientific, USA).

A minimum acquisition access feeding period (AAP) of 10 min was essential for whiteflies (Asia-II biotype) to acquire and transmit SLCCV to assay plants. Remarkably, prolonging the AAP to 24 h resulted in complete transmission. Similarly, a 24 h inoculation access feeding period (IAP) resulted in 100% transmission of SLCCV to pumpkin seedlings by eight adult-viruliferous whiteflies. In comparison, a 10 min. IAP led to 10% transmission, with symptom development observed in successfully infected plants (Table 5). This recurring pattern has been extensively described in prior studies (Fiallo-Olive *et al.*, 4;

Table 3. Influence of different plant parts on the sap transmission of pumpkin cv. Pusa Vishwas.

Plant part used as source of inocula*	No. of symptomatic plants out of 10 inoculated (%)#	Incubation period (day)
Top leaf	5 (50)	8-15
Well expanded leaves	9 (90)	8-9
Older leaves	6 (60)	8-10
Inoculated leaves	9 (90)	8-10
Upper stem	8 (80)	8-15
Lower stem	0 (0)	30
Root	4 (40)	8

*The plant samples were obtained from symptomatic pumpkin plants 15 days after the inoculation. The incubation period refers to the duration between virus exposure and the initial appearance of symptoms and signs on the inoculated seedlings.
#The percentage of plants that exhibited symptoms out of 10 inoculated plants.

Table 4. Impact of insect quantity on SLCCV transmission and incubation period in pumpkin with 24-hour virus acquisition and inoculation feeding.

No. of whiteflies per plant	No. of plants infected/ inoculated	Transmission[a] (%)
0	0/10	0
1	0/10	0
2	1/10	20
3	3/10	30
5	5/10	50
7	7/10	80
10	9/10	100
12	10/10	100
15	10/10	100
20	10/10	100
25	10/10	100

[a]Positives are confirmed by PCR
Ten viruliferous flies were released on each plant with 24 h AAP and IAP each

Table 5. Assessment of minimum AAP and IAP for whitefly-mediated transmission of SLCCV among pumpkin plants.

Determination of minimum AAP[a]				Determination of minimum IAP[b]			
AAP[a]	IAP[b]	Transmission[c]		AAP[a]	IAP[b]	Transmission[c]	
		Plants infected/ inoculated	% of plants infected			Plants infected/ inoculated	% of plants infected
0 min	10 h	0/10	0	10 hr	0 min	0/10	0
5 min		0/10	0		5 min	0/10	0
10 min		1/10	10		10 min	1/10	10
15 min		2/10	20		15 min	2/10	20
20 min		2/10	20		20 min	2/10	20
25 min		3/10	30		25 min	3/10	30
30 min		4/10	40		30 min	4/10	40
45 min		5/10	50		45 min	5/10	50
1 h		5/10	50		1 h	5/10	50
2 h		6/10	60		2 h	6/10	60
4 h		6/10	60		4 h	6/10	60
8 h		7/10	70		8 h	7/10	70
12 h		8/10	80		12 h	8/10	80
16h		9/10	90		16h	9/10	90
24 h		10/10	100		24 h	10/10	100

Ten viruliferous flies were released on each plant with 24 h AAP and IAP each
[a]Acquisition access period; [b]Inoculation access period; [c]Positives are confirmed by PCR

Venkataravanappa *et al.*, 15). A 100% transmission efficiency with a 24 h acquisition access period (AAP) suggests fulfilling the latent period within or less than 24 h, aligning with previous studies on begomoviruses (Rodriguez *et al.*, 10).

In conclusion, we developed the sap inoculation method specifically for Squash Leaf Curl China Virus (SLCCV), which involves using the β-mercaptoethanol-containing buffer III for the virus inoculum and has demonstrated high efficiency in pumpkin plants. These findings provide valuable insights for successful sap inoculation and detection of SLCCV, aiding in germplasm screening and material selection in breeding programs. Our research has also revealed that two whiteflies are sufficient enough to transmit SLCCV in a single pumpkin plant. Mechanical sap transmission provides a convenient screening method, and further studies on factors influencing sap and whitefly-mediated inoculations will enhance large-scale germplasm screening in pumpkin.

AUTHORS' CONTRIBUTION

Conceptualization of research (AKS, SS, PKD, ADM); Designing of the experiments (AKS, BMR); Contribution of experimental materials (AKS, SS, PKD, ADM); Execution of field/lab experiments and data collection (BMR); Analysis of data and interpretation (BMR); Preparation of the manuscript (AKS, BMR).

DECLARATION

The authors declare that they do not have any conflict of interest.

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