academicJournals

Vol. 12(32), pp. 5000-5009, 7 August, 2013
DOI: 10.5897/AJB2013.12012
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Characterization of *Tomato Leaf Curl New Delhi Virus* infecting cucurbits: Evidence for sap transmission in a host specific manner

Sayed Sartaj Sohrab[1,2]*, Sajjad Karim[3], Anupam Varma[2], Adel Muhammad Abuzenadah[1], Adeel Gulzar Chaudhary[1], Ghazi A Damanhouri[1] and Bikash Mandal[2]

[1]King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia.
[2]Advanced Center for Plant Virology, Division of Plant Pathology, Indian Agricultural Research Institute, Pusa, New Delhi, India.
[3]Center of Excellence in Genomic Medicine Research, King Abdulaziz University, Jeddah, Saudi Arabia.

Accepted 31 July, 2013

Sponge gourd (*Luffa cylindrica*) is an economically important vegetable crop cultivated throughout India and this crop is severely affected by yellow mosaic disease caused by begomovirus. In this study, an attempt was made to transmit the begomovirus by sap. The causal agent was easily transmitted by sap to ridge gourd, sponge gourd and *Nicotiana benthamiana.* Several factors affecting the efficient sap transmission of causal virus was identified. Use of two antioxidant (sodium sulphite and β-mercaptoethanol) and two abrasive (celite and corborundum) and application of inoculum on first true leaves and cotyledons by rubbing with cotton swab, dipped in inoculum resulted in significant higher rate of transmission. The sap inoculation protocol resulted in variable percentage of infected plants from different factors like buffer combinations, source of inoculum, age of inoculum, genotypes of test plants, and species of plants, temperature, seasons and organic materials. The most susceptible growth stage of ridge gourd plant to sap inoculation was seven days old seedlings that produced 100% infection. The sap transmission was confirmed by coat protein gene polymerase chain reaction (PCR) amplification, cloning and sequencing from infected plants. Sap transmission of begomovirus infecting luffa has not been reported previously in India.

Key words: Sap transmission, begomovirus, *Tomato leaf curl New Delhi virus* (ToLCNDV), cucurbits.

INTRODUCTION

Sponge gourd (*Luffa cylindrica*) is one of the important vegetable crops and is extensively grown in northern India. The crops were severely infected with a virus disease resulting in yellow mosaic and distortion of leaves and association of *Tomato leaf curl New Delhi virus* (ToLCNDV) with the disease was reported (Sohrab et al., 2003; Tiwari et al., 2012). The virus was easily transmitted by whitefly (*Bemisia tabaci*). The emerging disease of whitefly transmitted virus has been reviewed recently (Castillo et al., 2011). An attempt was made to transmit the virus through sap inoculation to sponge gourd and ridge gourd. Interestingly, the inoculated plant developed typical disease symptoms as it was observed in the field condition. Later replicated experiments showed that ridge gourd plants were more susceptible than the sponge gourd. The experimental transmission of

*Corresponding author. E-mail: sohrab_sartaj2@rediffmail.com or ssohrab@kau.edu.sa. Tel: +966-554627872 or +966-6400-1000. Ext 25487. Fax: +966- 6952521.

begomoviruses is reported only for whitefly. The majorities of the begomoviruses are phloem limited and are not transmitted through sap transmission. However, some begomoviruses are known to be sap transmissible. Bipartite begomovirus infecting tomato in north eastern Mexico was sap transmitted to tomato, *Nicotiana benthamiana* and common bean which induce leaf crumpling, epinasty and mottling (Paplomatas et al., 1994) and this virus does not infect pepper and appeared to be different from *Serreno golden mosaic virus* which is sap transmissible (Brown and Poulos, 1990). *Tomato leaf curl Karnataka virus* from Bangalore is reported to be transmitted by sap inoculation to tomato, *N. benthamiana* and *Nicotiana tabacum* (Chatchawankanphanich and Maxwell, 2002).

The *N. benthamiana* plant developed yellowing symptoms within seven days and were stunted by 14 days whereas *N. tabacum* developed mosaic symptoms not leaf curling and yellowing 10-14 days after inoculation (Chatchawankanphanich and Maxwell, 2002).

The *Tomato leaf curl Gujarat virus*-[Var] is also reported to be sap transmissible to tomato, *N. benthamiana, N. tabacum* and pepper (Chakraborty et al., 2003). *Bean Calico mosaic virus* was mechanically transmitted to bean under glass house conditions (Brown et al., 1988). Isolates of *African cassava mosaic virus* were sap transmissible to cassava cultivar, *N. Mex* 55, however, the rate of transmission was very low (Bock and Woods, 1983). A very low rate of sap transmission of *Euphorbia mosaic virus* on *Euphorbia prunifolia* and *Datura stramonium* was reported (Costa and Bennett, 1950). A geminivirus causing leaf curl and distortion symptoms in pepper in Texas, USA designated as *Pepper golden mosaic virus* was easily sap transmissible to Solanaceae species (Stenger et al., 1990). *Potato yellow mosaic virus* causing bright yellow mosaic with leaf distortion and stunting of potato in Venezuela was easily transmitted through sap inoculation to tomato, tobacco and petunia but not to potato (Buck and Coutts, 1986). *Indian cassava mosaic virus* was mechanically transmitted by using semi-purified preparation from infected leaf of cassava to *N. benthaniana* and *N. glutinosa* (Malathi and Sreenivasan, 1983). ToLCNDV from diseased potato leaves was sap transmissible to *N. benthamiana* (Usharani et al., 2003). Very low sap transmission rate was reported in the case of *Melon leaf curl bigeminivirus* from Arizona (Duffus et al., 1985). The screening of *Luffa cylindrica* against ToLCNDV has been reported (Islam et al., 2010, 2011).

Mechanical sap transmission is a convenient method for biological studies of plant viruses. Begomoviruses are known to be sap transmissible but information on sap transmission properties under various conditions is not available. In the present report, we have studied the sap transmission properties of ToLCNDV associated with sponge gourd yellow mosaic distortion disease. Effect of different extraction media, storage temperatures, seasons, host factors on transmission and physical properties of the virus were determined. The standardized protocol will be highly useful in the screening of Luffa cultivars.

MATERIALS AND METHODS

Sample collection and Identification of virus

Naturally infected leaf samples collected from various locations of cucurbits growing field showing yellow mosaic and curling symptoms and begomovirus infection was confirmed by specific amplification of coat protein gene (part of DNA A) by polymerase chain reaction (PCR) using the coat protein (CP) gene specific primers AV 30F and AV 31R. Total DNA was isolated from 100 mg leaf tissue using DNA easy plant mini kit (Qiagen Inc, USA) following the manufacturer's instructions.

The PCR reaction mixture consisted of 2.5 units of Taq DNA polymerase (MBI fermentas, USA), 5 µl of 10x PCR buffer, 1 µl of 10 mM dNTps, 1 µl of forward and reverse primers. Total reaction volume was made up of 50 µl using sterile distilled water, and PCR amplification conditions were standardized employing different temperature of denaturation, annealing and extension based on Tm value of the primers. The successful amplification of coat protein gene was obtained using the following programme: first denaturation of DNA was at 94°C for 2 min then 35 cycles of denaturation (90°C for 1 min), annealing (40°C for 30 s) and extension (72°C for 1 min). At the end of the cycles, final extension step at 72°C for 10 min was done. PCR products were analyzed on 1% agarose gel stained with ethidium bromide (0.5 µg/ml) and visualized on UV transilluminator. Only one isolate from Luffa and Pumpkin showing yellow mosaic symptom (Figure 1) was selected for cloning and sequence analysis.

Cloning and sequence analysis

The PCR amplified products of coat protein gene from Luffa and pumpkin were cloned into pGEMT easy vector (Promega) and sequenced by primer walking method using automated DNA sequencer (ABI prism-Perkin Elmer, University of Delhi, South Campus, New Delhi). The sequence data were assembled and analyzed using the software programme BioEdit. The nucleotide sequences were analyzed in CLUSTAL-W programme (http:\\www.ebi.ac.uk/clustalw). Comparisons were made with sequences obtained from GenBank nucleotide sequence database. Relationship dendrograms were constructed from the aligned sequence-using neighbor joining method and bootstrap option of the MegAlign programme of DNASTAR. The virus isolates were identified and tentatively designated as Luffa:Del and Pum:Del according to differences obtained in sequences.

Whitefly and sap transmission

Both virus isolates (Luffa and Pumpkin) were used for whitefly and sap transmission on Luffa. First of all, field collected sample was used for virus acquisition; 20 viruliferous whiteflies/plants were used for virus inoculation to healthy seedlings under insect proof glass cages. After symptom appearance, both the virus isolates were maintained on Luffa under insect proof glass house. The entire test was done by using the newly emerged infected leaves. Non-viruliferous whitefly culture was raised from the eggs of whitefly and maintained on tobacco (*N. tabacum* cv.xanthi) and egg plant (*Solanum melongena*) in insect-proof glass cages. Regular introduction of healthy plants maintained the whitefly culture after everysix to seven weeks.

For whitefly transmission, adult whiteflies were given acquisition

(a) Sponge gourd (b) Pumpkin

Figure. 1. A) Field collected Sponge gourd. B) Field collected Pumpkin plant showing yellow vein mosaic symptoms.

access period (AAP) and of 24 h on infected plants and then viruliferous whiteflies were given for inoculation access period (IAP) of 24 h on healthy test seedlings of Luffa, thereafter the white flies were killed by insecticide (Karate 0.01%) and the inoculated plants were kept in an insect-proof glasshouse for symptom development up to six weeks. For determining virus vector relationship and vector transmission efficiency of the selected virus isolates, the AAP and IAP were varied between 5 min to 24 h and the number of whiteflies per plant was also varied from 1 to 20. Other conditions of the test were similar to those described above.

For sap inoculation, 1 g infected Luffa leaves were collected from both the isolates and ground in 4 ml of 0.1 M sodium phosphate buffer (pH 7.2), having 0.04% beta-merceptoethanol, 0.2% sodium sulphite and 2% celite (standard inoculum). The extract was filtered through a pad of non-absorbent cotton to remove plant material and used as the inoculum. Healthy sponge gourd plants at two fully expanded true leaves stage were pre-dusted with 600-mesh corborundum and inoculated with virus inoculum by cotton swab. After the inoculation, plants were washed with water to remove excess inoculum and kept in insect-proof micro-cages for symptom development upto six weeks. On the basis of sap transmission ability, only one isolate from Luffa (ToLCNDV-Luffa:Del) was used for further study.

Characterization of sap transmission properties

Physical properties

To determine the effect of various factors on sap transmission of the begomovirus isolated from Luffa, experiments were conducted to examine the effect of temperature, dilution and time of incubation of inoculum, effect of different plant parts as source of inoculum, effect of organic compounds, like chloroform and butanol and effects of different combinations of extraction medium.

To determine thermal inactivation point (TIP), dilution end point (DEP) and longevity *in vitro* (LIV) of the virus in crude sap, ridge gourd seedlings were inoculated using standard inoculation buffer as determined in the previous section. For thermal inactivation point, virus inoculum was prepared and incubated for 10 min at different temperatures like, ice, 25, 30, 35, 40, 45, 50, 52, 54, 56, 58, 60, 70, 80, 90 and 100°C and inoculated to healthy seedlings of ridge gourd. For dilution end point, the virus inoculum was used in extraction buffer with the following dilutions, 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128. For longevity *in vitro*, virus inoculum was prepared at 1:4 dilutions and incubated at 4°C for 0, 6, 12, 48, 72 and 96 h and then used for inoculation.

Effect of storage temperature

The symptomatic leaves of ridge gourd plants (30 days after inoculation) were stored at room temperature: 4, -20 and -80°C for one day and then used to prepare inoculum followed by inoculation on seven to 10 days old ridge gourd seedlings and kept inside insect proof glass house for symptoms development.

Effect of seasons

Large batches of healthy ridge gourd plants were inoculated in winter, summer and rainy season under greenhouse condition to determine the seasonal effect on transmission of virus, symptoms developments and severity of the disease. The virus inoculum was prepared as described in previous section from freshly harvested leaves showing mosaic symptoms and inculcation was done.

Different plant parts as source of inoculum

To determine the sap transmission efficiency of virus from different parts of the infected plants, virus inoculum (1:4 dilution) prepared from top emerging leaf, well expended leaves, older leaf, inoculated leaf, upper stem, lower stem and root of the infected plants at 15 days after inoculation and inoculation was done.

Host specificity

The differentiation of field collected virus isolates from various location were determined by whitefly and sap transmission properties of all the isolates initially transmitted to sponge gourd through whitefly from the field samples and then selected for further transmission by sap to different plant species. Only Luffa isolate was transmitted by sap to ridge gourd, sponge gourd, *N.*

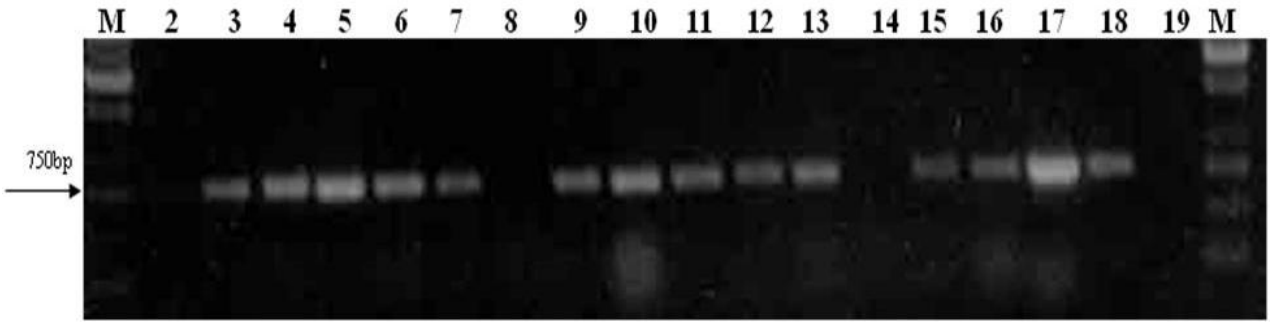

Figure 2. PCR detection of begomovirus infection in naturally infected cucurbits. L-R: Lane M: 1Kb ladder; Lane 2: Ridgegourd, Ganga Nagar; Lane 3: Ridgegourd, Delhi; Lane 4: Spongegourd, Panipat; Lane 5: Spongegourd Panipat (Ujha); Lane 6: Spongegourd, Baghpat; Lane 7: Spongegourd, Delhi; Lane 8: Healthy Spongegourd, Lane 9: Bittergourd, Ganga Nagar; Lane 10: Bittergourd, Panipat; Lane 11: Cucumber, Panipat; Lane 12: Cucumber, Delhi; Lane 13: Pumpkin, Panipat; Lane 14: Healthy Pumpkin; Lane 15: Pumpkin, Delhi; Lane 16: Watermelon, Sirsa; Lane 17: Bottlegourd, Panipat; Lane 18: Ridgegourd, Positive control, Lane 19: Healthy Bottelegourd; Lane M: 1Kb ladder.

benthamiana and tomato plants to determine the rate of transmission efficiency of the virus causing yellow mosaic disease in sponge gourd.

Age of infected plant as source of inoculum

To determine the age of plant as inoculum source on virus transmission, symptomatic leaves of ridge gourd plants were harvested from 15 days, 1, 2, 3, 4, 5 and 6 months old plants and inoculation was done as described earlier.

Cultivars of ridge gourd

Different cultivars of ridge gourd were grown in 6 inches pots (one plant/pot) in the green house during August-September. For each cultivar, 13 to 21-ridge gourd seedlings were inoculated by sap at 2 true leaf stages. Two to five plants for each cultivar were kept as non-inoculated healthy control and observations were made till 60 days after inoculation. The severity grade (scale 1 to 5) was determined on the basis of symptoms appearance on inoculated seedlings.

Confirmation of sap transmission by PCR

The sap transmission was performed on young seedlings of Luffa by applying inoculum prepared from freshly harvested leaf and standardized sap transmission protocol. The sap transmission was confirmed by using the total DNA isolated from symptomatic leaves and PCR amplification of coat protein gene by using specific primers.

RESULTS

Identification of virus

Begomovirus infection was confirmed by specific amplification of coat protein gene (AY309957, AY686500) by polymerase chain reaction (PCR) and using begomovirus coat protein gene specific primers AV 30 F and AV 31R as described earlier for naturally infected cucurbits samples (Figure 2).

Cloning and sequence analysis

The PCR amplified products of coat protein gene (750 bp) from Luffa and pumpkin isolates were separately cloned and sequenced. Pair wise comparisons were made by using the nucleotide sequences obtained from clones of ToLCNDV-Luffa (AY309957) and SqLCV-[Pum:Del](AY686500). The BLAST result showed highest homology with ToLCNDV isolates and produced the evidence that the sap transmissible virus is highly similar to *Tomato leaf curl New Delhi virus* and designated as ToLCNDV-Luffa. Comparison of the nucleotides sequence of Luffa:Del isolate (AY309957) with selected begomoviruses showed highest (97.5%) identity with ToLCNDV (AY939926) and 96.1% with ToLCNDV-Luffa (AF102276) followed by 95.1% identity with ToLCNDV-[Svr] (U15015) and the lowest identity (55%) with *Squash mild leaf curl virus*-[Imperial Valley] (NC_004645- ICTVdB Management-2006) and 87.7% with SqLCV-[Pum:Del] (AY686500) was observed. These results showed that the virus associated with the Luffa:Del and Pum:Del isolate are the variants of ToLCNDV and *Squash leaf curl china virus* (SqLCV), respectively. These isolate were therefore designated as ToLCNDV-[Luffa:Del] and SqLCV-[Pum:Del] (Table 1), but for convenience, these were referred to as Luffa:Del and Pum:Del. In the phylogenetic tree derived from complete DNA A nucleotide sequences, Luffa:Del and Pum:Del isolates formed separate clusters with ToLCNDV isolates and SqLCV isolates (Figure 3).

Optimization of sap transmission

The Luffa:Del isolate could be transmitted by sap inoculation using sap extracted in 0.1 M sodium phosphate buffer pH 7.2, even without the application of any abrasive. Addition of abrasive (2% celite and a pinch of corborundum powder) in the sap extracted with buffer resulted in increased efficiency of sap transmission up to

Table 1. Nucleotides sequence identity matrix of Luffa Del isolate (AY309957) with selected begomoviruses.

Accession number	Acronym	Identity matrix (%)
AY686500	SqLCV-[Pum: Del]	87.7
NC_004042	BGMV	54.1
AF224760	CuLCrV	54.8
AF509729	LYMV	79.9
AF325497	MCLCuV	54.9
AF416742	MYMIV	58.0
DQ285019	SqLCV[Cai]	55.0
AB027465	SqLCV	85.5
AY184487	SqLCV-[Pum:Coim]	87.9
NC_004645	SMLCV	55.0
NC_003891	ToLCBV	71.5
U15016	ToLCNDV-[Mld]	89.6
U15015	ToLCNDV-[Svr]	95.1
AJ012081	WmCSV	65.6
AF102276	ToLCNDV-Luffa	96.1
AY939926	ToLCNDV-Luffa	97.5

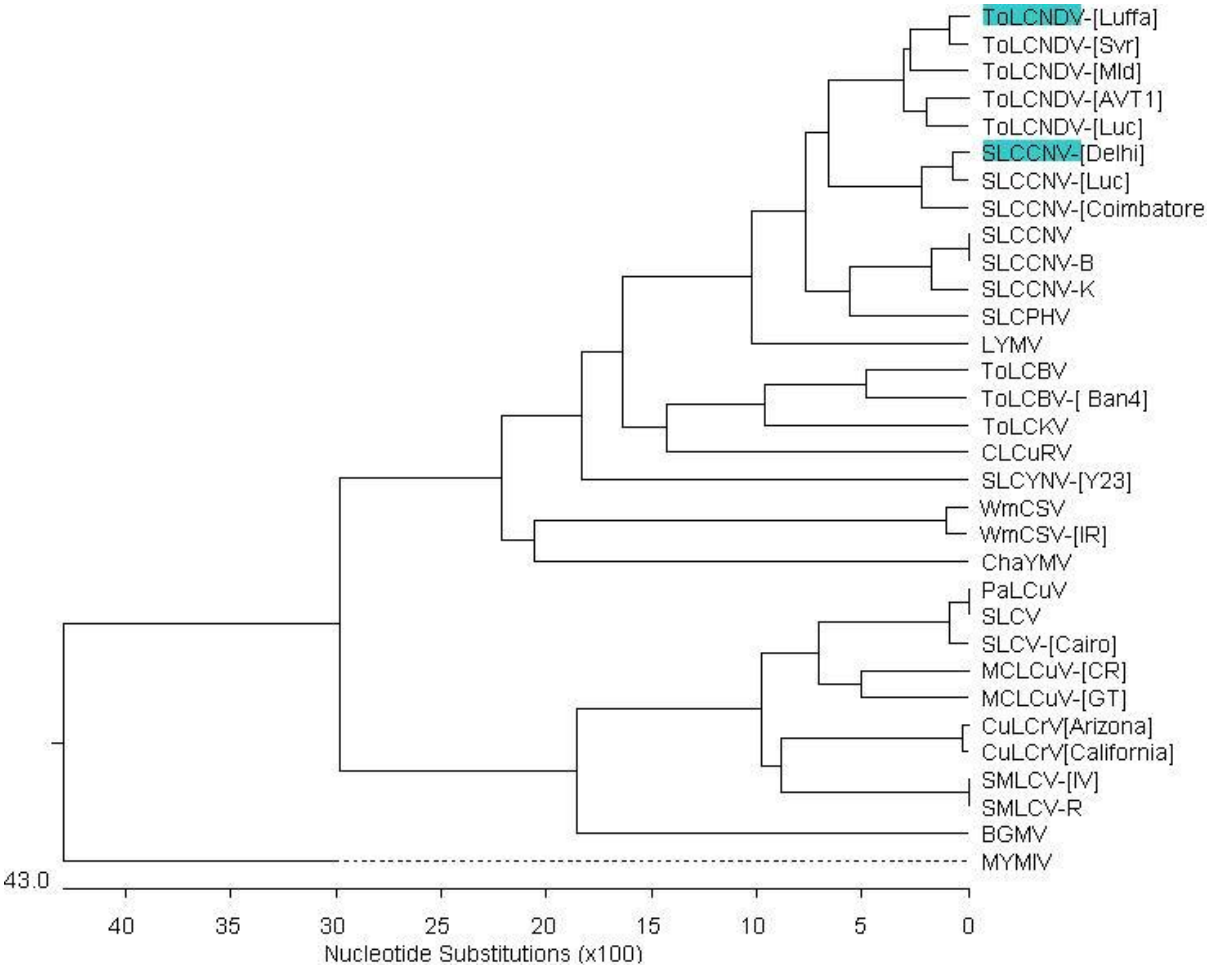

Figure 3. Phylogenetic relationship based on nucleotide sequences of the DNA A of Luffa:Del and Pum:Del isolates with selected begomoviruses using the ClustalW algorithms the MegAlign program of DNASTAR.

Table 2. Effect of extraction medium and additives on sap transmission of Luffa-Del isolate.

Extraction medium	Number of plants infected/inoculated	DAI	Symptom
Water	0/10	30	No symptoms
Water + celite + Corborundum powder	0/6	30	No symptoms
Buffer only	2/6	7–13	Yellow dots followed by mosaic
Buffer followed by 10% Chloroform	6/7	7–13	--- do---
Buffer followed by 10% Butanol	5/6	7–13	--- do---
Buffer + ME([b)]	3/6	7-13	--- do---
Buffer+ME+ Sodium sulphite.	1/5	7- 13	--- do---
Buffer + ME+ Sodium sulphite + celite	5/6	7-13	--- do---
Buffer +ME ,Sodium sulphite + Corborundum powder	6/6	7-13	--- do---
Buffer +celite + corborundum powder	6/7	7-13	Yellow dots followed by mosaic
Liquid nitrogen	7/10	7-13	Mild mosaic

DAI+ Days after inoculation; [b]ME: beta mercaptoethanol.

Table 3. Transmission of Luffa Del and Pum Del isolates by sap and whiteflies.

Plant species inoculated	Whitefly inoculation						Sap inoculation (Luffa Del)		
	No of plants infected / inoculated		Symptom development (DAI)		Percent Infection		No of plants infected / inoculated	Symptom development (DAI)[a]	Percent Infection
	Luffa Del	Pum Del	Luffa Del	Pum Del	Luffa Del	Pum Del			
Pumpkin	9/10	8/10	7-10	7-8	90	80	9/10	8-10	90
Ridge gourd	45/50	9/10	6-7	6-11	90	90	9/10	7-10	90
Sponge gourd	45/50	6/9	6-7	7-10	90	90	8/10	11-13	80
Tomato	30/50	5/10	7-10	6-12	60	50			

[a]DAI, Days after inoculation.

100%. The virus was found to be stable in organic solvents as the buffer extract clarified by 10% chloroform or butanol and resulted in 85 and 83% infection, respectively (Table 2).

Whitefly and sap transmission

Both isolates, ToLCNDV-[Luffa:Del] and SqLCV-[Pum:Del] were transmitted efficiently by even single whitefly, which resulted in infection of 90% plants by Luffa:Del isolate and 80% by Pum:Del isolate. The symptoms developed, however, took slightly longer time when single whiteflies were used for inoculation as compared to 20 whiteflies per plants. The efficiency of transmission varied with the variation in AAP and IAP. For a single whitefly, the minimum AAP required to transmit the virus was 60 min for both Luffa:Del and Pum:Del isolates, whereas, for a group of 20 whiteflies, the minimum AAP required for transmission was 15 min. With the increase in AAP, percent transmission increased. Similar trend was obtained for IAP. Minimum IAP required was 15 min, but the efficiency of transmission increased with the increase in IAP.

In sap transmission, the Luffa:Del isolate was transmitted with greater efficiency by sap inoculation to both ridge gourd and sponge gourd and this isolate was further used in this study. It was observed that Pum:Del isolate was not sap transmissible.

Sap transmission from ridge gourd to pumpkin, ridge gourd and sponge gourd resulted in 80 to 90% transmission, whereas no sap transmission was obtained in tomato which was infected up to 50 to 60% by whitefly inoculation by both isolates. Back inoculation from the sap and whitefly inoculated ridge gourd and tomato plants to ridge gourd seedlings was positive for ridge gourd but not of tomato (Table 3).

Characterization of sap transmission: Physical properties

Plants through sap inoculation with buffer were found to have about 25% infectivity expressed in proportion of plants inoculated. Infectivity further increased to 50% with the addition of βME and sodium sulphite to the buffer and

Table 4. Effect of plant parts on the sap transmission of Luffa Del isolate.

Plant parts used as source of inocula*	No.of symptomatic plants out of 10 inoculated (%)	Incubation period
Top leaves	4 (40)	6-16
Well expanded leaves	8 (80)	6-7
Older leaves	6 (60)	6-10
Inoculated leaves	8 (80)	7-10
Upper stem	8 (80)	6-16
Lower stem	0 (0)	30
Root	4 (40)	7

*The plant parts were drawn from symptomatic ridge gourd plants at 15 days after inoculation. Incubation period: The time that elapsed between exposure to a virus and when a symptom and sign first appeared on inoculated seedlings.

with the addition of celite maximum infectivity which was observed.

Dilution end point (DEP), thermal inactivation point (TIP) and longevity in vitro (LIV)

Eight fold dilution has been found to be optimum dilution end point as infectivity was found to fall abruptly after 1/8 dilution. Exposure at various temperature above 25°C has been found to increase sharply as measured by thermal inactivation percent. Maximum inactivation in terms of temperature rise was noted at 40°C as indicated by maximum slope and complete inactivation occurrence at temperature of 54°C. Longevity of the virus particles was found to decrease with the passage of time as infectivity of the samples reduced by almost 20% for every 12 h and by 48 h, infectivity was lost.

Effect of storage temperatures

When the plant samples were kept at different temperatures conditions, infectivity was found to decrease with reduced storage temperature. Maximum infectivity was 100% at room temperature followed by 4°C where proportion of infection was 80%. Storage at sub zero temperatures, -20 and -80°C infectivity was found to drastically decrease.

Effect of seasons

Infectivity test has shown that summer period is the optimum season for maximum infection afterward infectivity was found to decrease gradually and minimum proportion of infection was noted during the month of December.

Effect of different plant parts

Infectivity on expanded, middle leaves and stem of ridge gourd was comparatively higher as top leaves and roots have shown lower proportion of infection. However, lower stem was found to be insensitive to infection (Table 4).

Host specificity

The differentiation of virus isolates were determined by whitefly and sap transmission properties and it was observed that all the seven field collected virus isolates were readily transmitted by whitefly but differed in sap transmissibility. Whereas, from the field samples, only the isolates from ridge gourd and sponge gourd were sap transmissible, after one passage through whitefly to sponge gourd the isolates from water melon and bitter gourd were also found to be sap transmissible. Except for the two isolates from sponge gourd, they could also infect *N. benthamiana* by sap inoculation (Table 5).

Age of plant as source of inoculum

Infectivity of Luffa:Del isolate was decreased when inoculum was prepared from 15 to 180 days old plants. Infectivity in 15 days old source of inoculum was as high as 100% and afterward decreased and showed only 40% infection when inoculum was used for 180 days old plants.

Cultivars of ridge gourd

All the 21 genotypes of ridge gourd tested were infected by Luffa:Del isolate by sap inoculation. The severity of the disease however indicated that, ridge gourd varieties: Bio-Kaveri, BRG-31 and DRG-1 were moderately resistant as compared to the varieties; RHRG-2, SVRGH-54, VRGH-2 and VRGH-336, which were highly susceptible. The remaining varieties gave intermediate reaction these results indicate lack of resistance to Luffa:Del isolate in ridge gourd (Table 6).

Table 5. Differentiation of virus isolates by sap and whiteflies transmission.

	RG-(Raj)		WM-Sirsa		BG-Panipat		Luffa Del		Bit-G (Raj)		SG- Panipat		Pum Del	
	Sap	WF	Sap	WF	Sap	WF	Sap	WF	Sap	WF	Sap	WF	Sap	WF
Ridge gourd	+	+	-	+	-	+	+	+	-	+	+	+	-	+
Sponge gourd	+	+	+	+	-	+	+	+	+	+	+	+	-	+
Pumpkin	-	+	-	-	-	-	-	+	-	+	-	+	-	+
Tinda	-	+	-	+	-	+	-	+	-	+	-	+	-	+
Bottle gourd	-	+	-	-	-	+	-	+	-	+	-	+	-	-
Water melon	-	-	-	+	-	-	-	-	-	-	-	-	-	-
Cucumber	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Musk melon	-	+	-	+	-	+	-	-	-	-	-	-	-	+
Tomato	-	+	-	+	-	+	-	+	-	+	-	+	-	-
N. benthamiana	-	+	-	-	-	-	+	+	-	+	+	+	-	-
N. tabacum	-	+	-	+	-	-	-	+	-	+	-	+	-	-

(-) Negative result, (+) positive result; virus isolates were first transmitted by whiteflies on sponge gourd crops and then the leaf showing freshly developed symptoms of viral infection was used as inoculum for whiteflies and sap transmission. Whiteflies were given AAP and IAP of 24 h each and 20 whiteflies were used for inoculation of each plant. RG, Ridge gourd Rajasthan; WM, watermelon-Sirsa; BG, bottle gourd-Panipat; Luffa, Del- Luffa Delhi; Bit- G Raj, bitter gourd-Rajasthan; SG-Panipat, sponge gourd-Panipat; Pum-Del, Pumpkin-Delhi; WF- whiteflies.

Table 6. Sap transmission of Luffa Del isolate on ridge gourd cultivars.

Name of variety	No of plants infected/inoculated	Percent Infection	Local lesions on Inoculated leaf	Time taken for systemic symptom development (DAI)[a]	Average severity grade
Aarti	10/15	66.6	1	10	3.4
Bio-Kaveri	12/20	60.0	0	12	2.0
Bio-R2	10/13	76.9	3	10	3.6
BRG31	12/21	57.1	5	12	2.0
DRG-1	7/10	70.0	3		2.5
DRG-2	11/18	61.1	0	11	3.0
HRGH-9	10/15	66.6	4	10	3.5
Mahima	14/21	66.6	0	14	3.0
NBRGH-602	10/14	71.4	3	10	3.5
NRGH-1	16/20	80.0	6	16	3.0
NRGH-9	14/21	66.6	1	14	3.0
NRGH 21	14/20	70.0	1	14	3.25
NRGH-22	17/19	89.4	2	17	4.3
Pusa Nasdar	10/13	76.9	3	10	3.5
RHRG-1	8/10	80.0	0	8	3.5
RHRG-2	14/19	73.6	4	14	4.5
SVRGH-54	15/18	83.3	1	15	4.0
VRGH –2	12/20	71.4	0	12	4.5
VRGH101	10/14	71.4	2	10	3.9
VRGH-336	11/14	78.5	0	11	4.5

[a]DAI; Days after Inoculation. Average severity grade scale: 0: No symptoms, 2: yellow dots, 3: yellow dots fused and mosaic, 4: yellow mosaic and leaf curling, 5: leaf curling and stunted growth of plant.

Confirmation of sap transmission by PCR

Inoculated plants developed very good symptoms of yellow dots followed by mosaic and curling at later stage. The sap transmission was confirmed by specific amplification of coat protein gene by using specific primers. An amplicon of 750 bp was amplified from infected leaves showing the sap transmission of

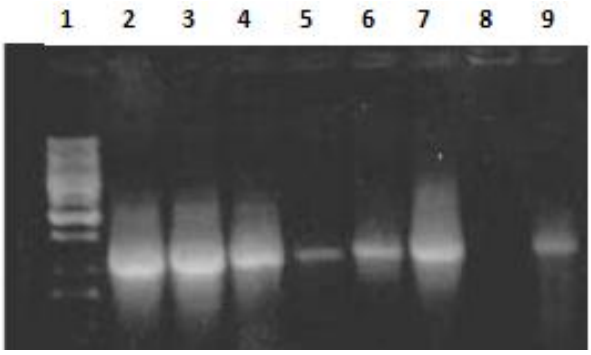

Figure 4. PCR amplification of Coat Protein gene from sap inoculated Ridge gourd plant showing systemic mosaic (Lanes 2-4) curling of lanes (5-7) and local lesions on inoculated leaf (Lane 9) Lane 1: Marker Lane 8: Healthy control.

ToLCNDV-Luffa isolate to Luffa plants (Figure 4).

DISCUSSION

In the present study, we reported that the begomovirus causing yellow mosaic diseases in Luffa is highly sap transmissible. Sap and whitefly transmission of ToLCNDV:Luffa:Del and SqLCV:Pum:Del isolates was conducted. Both virus isolate was easily transmitted by whiteflies but only ToLCNDV-Luffa:Del isolate was sap transmissible. Sap transmission for most of the begomovirus is difficult, except a few. ToLCNDV is a bipartite virus and due to having the DNA B genes (involved in virus movement), such viruses tend to not be phloem limited and thus likely to be mechanically transmissible. Recently, a New World begomovirus lacking a DNA-B component was associated with tomato leaf curl disease (ToLCD) in Peru and named Tomato leaf deformation virus (ToLDeV) have been reported which show that isolates of ToLDeV associated with ToLCD in Ecuador and Peru have a single, genetically diverse, genomic DNA that is most closely related to DNA-A components of New World bipartite begomoviruses (Melgarejo et al., 2013). A begomovirus infecting tomato in north eastern Mexico was sap transmitted to tomato, *N. benthamiana* and common bean which induce leaf crumpling, epinasty and mottling (Paplomatas et al., 1994) and this virus does not infect pepper and appeared as different from *Serreno golden mosaic virus* which is sap transmissible (Brown and Poulos, 1990). *Tomato leaf curl Karnataka virus* from Bangalore has also been reported to be transmitted by sap inoculation to tomato, *N. benthamiana* and *N. tabacum*. The sap inoculation was done by using inoculum prepared in 0.5 M phosphate buffer pH 8.0 from the symptomatic tomato leaves. The *N. benthamiana* plant developed yellowing symptoms within seven days and were stunted by 14 days (Chatchawankanphanich and Maxwell, 2002) but when sap transmission of the virus associated with sponge gourd distortion disease was done on *N. benthamiana*, the sap inoculated plants developed yellowing with severe leaf curling seven days after inoculation and stunted by 15 days. The *Tomato leaf curl Gujarat virus*-[Var] is also sap transmissible to tomato, *N. benthaniana, N. tabacum* and pepper (Chakraborty et al., 2003). In the case of *Tomato leaf curl Gujarat virus*-[Var], sap transmission was done by using potassium phosphate buffer of 0.1 M pH 7.0 (1:2 w/v) containing 0.15% sodium sulphite. But in our study, it was observed that when sap transmission was done by using various types of 0.1 M sodium phosphate buffer like only buffer, buffer with sodium sulphite, and buffer in combination with additives (pH 7.2, 1:4 w/v) using sodium sulphite, corborundum powder and celite, affected the rates of infection and 100% plant developed symptoms when standardized buffer (buffer + sodium sulphite + corborundum + celite) was used, and this is the important parameter which enhanced the infection rate.

Evaluation of several factors affecting the sap transmission of ToLCNDV-Luffa:Del isolates, such as buffer with or without antioxidant and abrasive, temperatures of storage and growing inoculated plants, age of source plants, type of the tissue and susceptibility of the genus type/plant species resulted in the development of highly efficient transmission protocol. The standardized protocol will be highly useful for the screening of Luffa cultivars. The molecular data revealed that the virus is a variant of ToLCNDV. The reason for high level sap transmission of ToLCNDV-Luffa:Del in Luffa species has not been studied under the present investigation however, it is possible that the Luffa species have some important factors which are helpful in sap transmission of the begomovirus causing disease. It is also possible that the virus associated has ability to efficiently multiply and move in Luffa species. Inoculation method described here would help in the rapid evaluation of ridge and sponge gourd lines for the source of resistance and also to characterize the new emerging sap transmissible begomoviruses in the near future. The begomovirus sap transmission properties indicates the changing pattern and nature of disease causing ability and it is possible to have broader host ranges and disease spread to other crops. This requires further details study to determine the molecular mechanism and factors responsible for efficient sap transmission.

ACKNOWLEDGEMENT

Financial support from Indian Council of Agricultural Research (National Professor's scheme) is thankfully acknowledged.

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