

## Transmission of an Indonesian Isolate of *Tobacco leaf curl virus* (*Geminivirus*) by *Bemisia tabaci* Genn. (Hemiptera: Aleyrodidae)

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*Bemisia tabaci* Genn. is an important pest worldwide because of its ability to cause damage by direct feeding and its role as a vector of some viruses including geminiviruses. The first report of *Tobacco leaf curl virus* (TLCV), a *Geminivirus*, in Indonesia was in 1932 when the virus was found infecting tobacco plants in Central Java. The characteristic symptoms of TLCV included upward curling of the leaf edge, vein thickening, and sometimes the occurrence of enation on the underside of the leaves. Basic studies were carried out to elucidate the characteristics of TLCV transmission by its vector, *B. tabaci*. A single whitefly was able to transmit the virus and the efficiency of transmission was increased when the number of adult whiteflies was increased up to 20 per plant. Inoculation access period of 1 h could cause transmission up to 20% and the optimum inoculation access period was 12 h. Acquisition access period of 30 minutes resulted in 70% transmission while 100% transmission occurred with a 24-h acquisition access period. The virus was proven to be persistently but not transovarially transmitted. Discrete fragments of 1.6 kb were observed when polymerase chain reaction method was applied to detect the virus in viruliferous nymphs and individual adults of *B. tabaci*, while no bands were obtained from non-viruliferous nymphs and adults.

**Keywords :** *Bemisia tabaci*, geminivirus, *Tobacco leaf curl virus*, transmission, vector.

*Bemisia tabaci* (Genn.) is one of the most economically important pests in many tropical and subtropical regions (Bock, 1982). The pest can cause extensive damage in more than 500 species of crops (Greathead, 1986) through its direct feeding, and its ability to directly transmit viruses, including geminiviruses.

The geminivirus is a group of plant viruses with distinct morphological characters. Its twin isometric particles

consist of circular single-stranded DNA genomes (Bock, 1982). The first geminivirus that originated from Indonesia was reported by Thung (1932; cited in Trisusilowati et al., 1990). The virus caused leaf curling in tobacco plants. In 1984, *Tobacco leaf curl virus* (TLCV) caused serious damage in Bojonegoro, East Java, with up to 30% disease incidence (Poerbokoesoemo, 1984; cited in Trisusilowati et al., 1990). The symptoms of TLCV infection include leaf curling, vein banding, uneven leaf surface, and rigid leaves. The disease affected yield quality, especially those targeted for cigar wrapping.

Because of the widespread occurrence of TLCV epidemic in most tobacco plantations in Indonesia, knowledge of the mode of transmission of the virus from plant to plant by an insect vector *B. tabaci* is essential. Besides, rapid and specific procedures for virus detection are needed in both *B. tabaci* and plants to provide tools in epidemiological and disease management studies. One method that has become a primary means of virus detection and diagnosis was serological-based technique. This approach has been met with limited success due to the high diversity of geminiviruses and the difficulties in obtaining sufficient antisera. Detection method based on analysis of viral nucleic acid has been widely used for geminiviruses. These include nucleic acid hybridization (Bendahmane et al., 1995; Czosnek et al., 1988; Gilbertson et al., 1991; Hidayat et al., 1993; Polston et al., 1989) and polymerase chain reaction (Chiemsombat et al., 1990; Pacheco et al., 1996; Rojas et al., 1993; Roye et al., 1997; Wyatt and Brown, 1996).

This paper reports the characteristic relationship between an Indonesian isolate of TLCV and its insect vector *B. tabaci*, and the development of a polymerase chain reaction-based technique to detect the virus from its insect vector.

### Materials and Methods

**Maintenance of virus source, *B. tabaci*, and the plants.** Leaves showing TLCV symptom were collected from tobacco fields at Arjasa and Tegal Gede villages, Jember, East Java. The virus was

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maintained on tobacco plants (*Nicotiana tabacum* cv. H382) by insect transmission. Healthy tobacco plants were grown in a whitefly-proof greenhouse. Whitefly (*B. tabaci*) adults were obtained from broccoli plants in Bogor and identified using the identification key of Martin (1987). The insects were reared on tobacco and broccoli (*Brassica oleraceae* var. *Italica*) plants in whitefly-proof cages.

**Virus-vector relationship.** TLCV transmission experiments with *B. tabaci* were conducted using cylindrical cages with mesh tops, which were inverted over individual leaves. *Bemisia tabaci* adults were introduced into the cage through a hole which was sealed afterwards. The insects were given access to TLCV-infected tobacco plants in separate whitefly-proof cages. After acquisition access period, the whiteflies were re-collected individually using an aspirator and transferred to separate cages of healthy tobacco plants for inoculation access periods. After inoculation access period, the whiteflies were removed, and the plants were sprayed with an insecticide and held for symptom development in an insect-proof screen house.

The minimum acquisition access period required for transmission of TLCV was determined by allowing *B. tabaci* adults access to TLCV-infected tobacco plants for 0.5, 1, 3, 6, 12, and 24 h before transferring them to healthy tobacco plants for a 48-h inoculation access period. Ten *B. tabaci* adults per plant were used for each acquisition access period. Fifteen plants were used for each acquisition access period and the percentage of virus infection was calculated from plants showing TLCV symptoms after 10-25 days.

To determine the minimum inoculation access period, *B. tabaci* adults were given a 24-h acquisition access period on TLCV-infected tobacco plants and inoculation access periods of 1, 3, 6, 12, 18, and 24 h on healthy tobacco plants. Ten *B. tabaci* adults per plant were used for each acquisition access period. Fifteen plants were used for each acquisition-access period and the percentage of virus infection was calculated from plants showing TLCV symptoms after 10-25 days.

The effect of vector number on the relative efficiency of virus transmission was determined by allowing 1, 3, 5, 10, 15, and 20 *B. tabaci* adults a 48-h inoculation access period on healthy tobacco plants after a 24-h acquisition access period on TLCV-infected tobacco plants. Fifteen plants were used for each treatment.

TLCV acquisition by *B. tabaci* nymphs was determined by collecting 4<sup>th</sup> instars from TLCV-infected tobacco plants using an insect pin. Nymphs were placed on petri dishes until they complete their development. The adults were then removed and fed on healthy tobacco plants for 24 h.

Serial transmission was conducted to determine persistence of the virus on its insect vector. One *B. tabaci* adult was given a 24-h acquisition access period on TLCV-infected tobacco plants and viruliferous insects were transferred serially every 24 h to healthy tobacco plants. Serial transmission was conducted until the insects die.

The ability of the virus to be transovarially transmitted was determined by rearing viruliferous *B. tabaci* in insect-proof screen cages. The insects were allowed to lay eggs on broccoli plants, which are not a host of TLCV, for 7 days before removing

them from the cages. The eggs were maintained until they hatched, then the adults were allowed to feed on healthy tobacco plants for 24 h.

**Detection of TLCV in *B. tabaci* by polymerase chain reaction.** *Bemisia tabaci* adults and nymphs were collected after a 24-h acquisition access period. Sets of 1, 5, 10, 20, 30, and 50 *B. tabaci* were subjected to DNA extraction following the method of Goodwin et al. (1994), while DNA extraction from tobacco plants was conducted using the method of Dellaporta (1983). The method of Rojas et al. (1993) was used for amplification of viral DNA from *B. tabaci* extracts by polymerase chain reaction (PCR). Amplified DNA fragments were electrophoresed in 1% agarose minigels in TBE buffer and detected with UV light after staining in ethidium bromide (Maniatis et al., 1982).

## Results

**Acquisition feeding period.** After a 30-minute acquisition access period, ten *Bemisia tabaci* adults were able to cause 70% transmission of TLCV (Table 1). The transmission rate increased as the acquisition access period was prolonged and reached a maximum at 24-h acquisition access period. The TLCV symptoms were upward curling of the leaves and vein banding with crinkle-like symptom.

**Table 1.** The effect of various acquisition feeding periods of *Bemisia tabaci* (10 adults per plant) on transmission and incubation period of TLCV on tobacco plants after a 48-h inoculation feeding period

Acquisition feeding period (h)	Number of infected plants (%)	Incubation period (days)
0.5	70	6-13
1	70	8-30
3	90	9-30
6	90	6-30
12	90	6-30
24	100	4-13

**Table 2.** The effect of various inoculation feeding periods of *Bemisia tabaci* (10 adults per plant) on transmission and incubation period of TLCV on tobacco plants following a 24-h acquisition feeding period

Acquisition feeding period (h)	Number of infected plants (%)	Incubation period (days)
1	20	20-28
3	60	9-28
6	80	15-24
12	90	11-27
18	80	13-24
24	90	10-24

**Table 3.** The effect of number of *Bemisia tabaci* on transmission and incubation period of TLCV on tobacco plants after a 24-h acquisition feeding period and a 48-h inoculation feeding period

Number of insects	Number of infected plants (%)	Incubation period (days)
1	50	13-29
3	50	13-29
5	70	12-29
10	70	13-29
15	70	12-18
20	100	11-16

**Inoculation feeding period.** One-hour inoculation access period following a 24-h acquisition access period was sufficient to cause 20% transmission by 10 *B. tabaci* adults. The number of infected tobacco was increased as the inoculation access period was prolonged (Table 2).

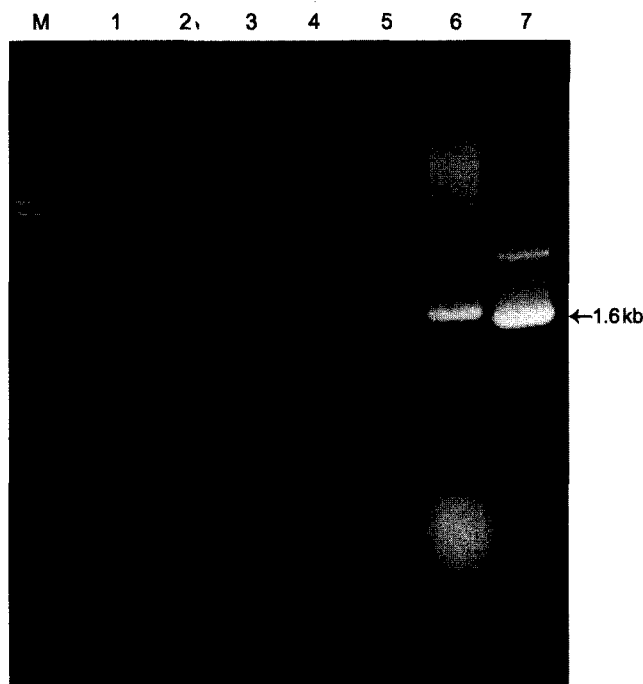
**Effect of the number of *B. tabaci* adults on transmission efficiency.** Given a 24-h acquisition access period and a 48-h inoculation access period, a single *B. tabaci* adult was able to cause 50% transmission. The efficiency of transmission increased as the number of *B. tabaci* adults was increased and reached a maximum (100% transmission) when 20 *B. tabaci* adults were used. Incubation period of TLCV on infected tobacco plants tended to be shorter as the number of *B. tabaci* adults was increased (Table 3).

**Acquisition of TLCV by nymphs.** *Bemisia tabaci* adults

**Table 4.** Persistence of TLCV on *Bemisia tabaci* after a 24-h acquisition feeding period and 24-h inoculation feeding period

Serial transmission (days)	<i>B. tabaci</i> <sup>a</sup>		
	1	2	3
1	+	-	+
2	-	+	-
3	-	+	+
4	-	+	-
5	+	+	-
6	+	+	+
7	+	+	+
8	+	+	-
9	+	+	+
10	+	+	+
11	+	-	-
12	D	+	+
13		+	+
14		+	+
15		+	+
16		D	+
17			+
18			D

<sup>a</sup>+ = successful transmission; - = no transmission; D = death of insect.

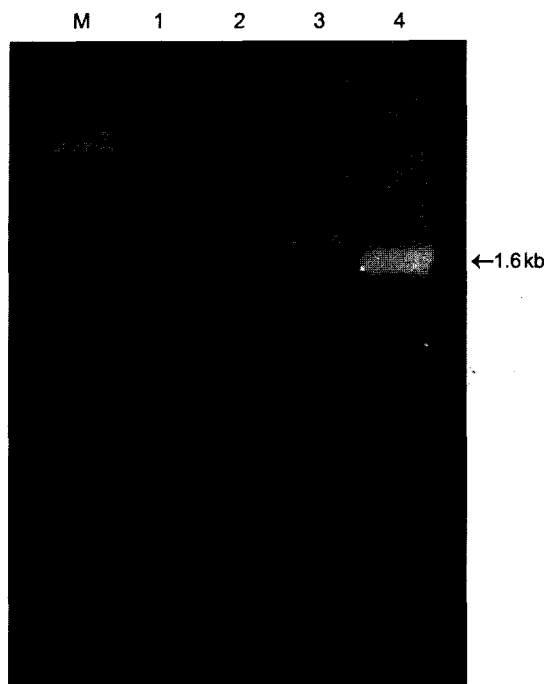
**Fig. 1.** Polymerase chain reaction-amplified product of TLCV from viruliferous *B. tabaci*. Amplification of 1.6-kb fragments with the primer pair PAL1v 1978 and PAR1c 715 (Rojas et al., 1993). A 10- $\mu$ l aliquot of each polymerase chain reaction mixture was analyzed in a 1.0% agarose gel. Lane M is 1-kb ladder size marker. The samples are 1) 1 viruliferous individual of *B. tabaci* with TLCV; 2) 5 individual viruliferous *B. tabaci* with TLCV; 3) 10 individual viruliferous *Bemisia tabaci* with TLCV; 4) 30 individual viruliferous *B. tabaci* with TLCV; 5) 10 individual non-viruliferous *B. tabaci*; 6) TLCV-infected tobacco; 7) DNA clone of *Bean golden mosaic (Geminivirus)*-Brazil.

emerging from nymphs that were reared on TLCV-infected tobacco transmitted TLCV after 48-h inoculation access period. All tobacco plants tested showed typical symptoms of TLCV within 8-22 days.

**Transovarial transmission of TLCV and persistence of TLCV on *B. tabaci*.** Adult progenies of viruliferous *B. tabaci* were unable to transmit TLCV after they emerged from the eggs. This result proved that TLCV could not be transmitted through eggs.

Serial transmission with a single *B. tabaci* given a 24-h acquisition access period and 24-h transmission interval showed that TLCV was persistent in the insect vector up to the end of their life cycle (Table 4).

**Detection of TLCV in *B. tabaci* by PCR.** PCR-amplified fragments of the predicted size from the annealing positions of the primers were obtained from individuals, as well as, from groups (5, 10, 30) of viruliferous *B. tabaci* (Fig. 1). The same results were obtained when 50 viruliferous nymphs of *B. tabaci* were tested (Fig. 2). PCR-amplified fragments of -1.6 kb also showed the concentration of



**Fig. 2.** Polymerase chain reaction-amplified product of TLCV from viruliferous nymphs of *Bemisia tabaci*. Amplification of 1.6-kb fragments with the primer pair PAL1v 1978 and PAR1c 715 (Rojas et al., 1993). A 10- $\mu$ l aliquot of each polymerase chain reaction mixture was analyzed in a 1.0% agarose gel. Lane M is 1-kb ladder size marker. The samples are: 1) 50 viruliferous nymphs of *B. tabaci* with TLCV; 2) 20 viruliferous nymphs of *B. tabaci* with TLCV; 3) TLCV-infected tobacco; 4) DNA clone of *Bean golden mosaic Geminivirus-Brazil*.

TLCV on insect vector. Virus concentrations of 1 and 5 adults, or 10 and 30 adults were not significantly different, but virus concentration of 1 and 5 adults was significantly different from that of 10 and 30 adults. Moreover, concentration of the virus on viruliferous nymphs was much lower than that on the adults.

## Discussion

The probability of subsequent transmission of circulative viruses by insect vectors generally increases with increasing acquisition access period until all insects that are able to do so have acquired the virus (Swenson, 1967). Virus acquisition by insect vectors may depend on the virus titer in the infected plant, the ability of the insect to ingest the virus, and the passage of the virus through the midgut wall and subsequent survival in the insect vector. The higher levels of TLCV transmission efficiency after extended acquisition access in this study are supportive of a circulative mode of transmission.

The ability of insect vector to transmit TLCV is affected by the inoculation access period. Percent transmission

increased as the inoculation access period was prolonged (Fig. 2), and reached a maximum (100%) after a 24-h inoculation access period. Shorter inoculation access period, i.e., 12 h, was achieved with tomato yellow leaf curl virus as reported by Mehta et al. (1994), and with chino del tomate virus (Brown and Nelson 1988).

Transmission of TLCV was observed with one adult *B. tabaci*, but the efficiency of transmission increased almost two-folds as the number of adults was increased to five per plant. Maximum efficiency of 100% was reached when 20 adults per plant was used in the transmission experiment (Table 3). According to Trisusilowati et al. (1990), five plants showed infection of isolate 2 of TLCV (severe leaf curling and vein banding) and only two plants showed infection of isolate 1 of TLCV (severe leaf curling) when the insects used in the transmission experiment were given acquisition access period on infected tobacco showing mix infection of TLCV isolates 1 and 2. Furthermore, Trisusilowati et al. (1990) reported that one individual insect was unable to transmit isolate 1. This may suggest the high concentration of TLCV isolate 2 in the infected-tobacco.

The use of PCR technique confirmed the results of the transmission experiment. Viral DNA fragments of expected size of 1.6 kb was amplified from a single adult of viruliferous *B. tabaci*. As the number of insects was increased, the amount of viral DNA amplified in the polymerase chain reaction became higher, as shown by the brightness of the DNA fragment in the gel electrophoresis (Fig. 1).

Nymphs of *B. tabaci* reared in infected tobacco were viruliferous and the adults that emerged were still viruliferous. TLCV DNA was detected from the nymphs that were reared on TLCV-infected tobacco using PCR technique (Fig. 2). Similar results were shown by Butter and Rataul (1977) with tomato leaf curl virus, as well as, Cohen and Nitzany (1966) and Mehta et al. (1994) with tomato yellow leaf curl virus. Transmission experiment with *B. tabaci* adults emerging from viruliferous nymphs showed that insect molting (nymph-pupa-adult) did not eliminate the virus from the insect body, therefore, the transmission was persistent and transtadial (Table 1). Bos (1983) reported that transmission efficiency is not affected by molting for insect vector transmitting the virus persistently, and virus can be detected in the haemolymph.

TLCV is not transmitted through the eggs. *B. tabaci* adults emerging from eggs of viruliferous insects were not able to cause infection. The same results were reported by Butter and Rataul (1977), Mehta et al. (1994), and Idris and Brown (1998) with tomato leaf curl virus, tomato yellow leaf curl virus, and sinaloa tomato leaf curl geminivirus, respectively. Furthermore, Costa (1969) stated that virus transmitted by *B. tabaci* is not transovarial.

As stated by Varma (1963), symptom development of

TLCV on tobacco plants inoculated serially via viruliferous *B. tabaci* showed that TLCV persisted in the insect body through the end of the insect's life cycle (Table 1). Persistency of virus in the insect body varies, for example, 1-15 days for tomato yellow leaf curl virus (Cohen and Nitzany, 1966), 8-55 days for tomato leaf curl virus (Butter and Rattaul 1977), 9 days for sinaloa tomato leaf curl geminivirus (Idris and Brown 1998), and the whole life cycle for zinnia yellow net disease (Srivastava et al., 1977).

Detection of TLCV from *B. tabaci* using PCR technique showed that the concentration of the virus increased as the number of insect was increased (Fig. 1). This is in agreement with the experiment on the effect of insect number on TLCV transmission. The more insects inoculated to tobacco plants, the higher the transmission rate and the shorter the incubation period obtained (Table 3). The PCR technique was also able to amplify viral DNA from 50 nymphs of *B. tabaci* after acquisition access period on infected tobacco (Fig. 2). Thus, nymphs of *B. tabaci* could acquire the virus and become viruliferous. The PCR-based detection procedure could be used to detect TLCV from a single insect and groups of nymphs, making it a potential tool for the study of the epidemiological aspect of the disease.

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