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# Transmission relationship of whitefly vector, *Bemisia tabaci* with yellow mosaic virus disease of greengram

**Prateek Ranjan Behera, MK Mishra, Sandeep Kumar and AK Senapati**

**Abstract**
Mungbean (*Vigna radiata* L.) is a short duration pulse crop cultivated under varying environmental conditions throughout the southeast Asia and particularly in India. Yellow mosaic disease caused by yellow mosaic virus transmitted by whitefly (*Bemisia tabaci*) is an important limiting factor for the production and productivity of green gram. In the present study, different virus-vector relationships have been studied. A single whitefly could transmit 30% yellow mosaic disease in 10 days old greengram (Variety. LGG-460) after 24 hr Acquisition access period. The rate of transmissibility increased by increasing the number of viruliferous whiteflies with minimum of 13 numbers attaining 100% transmission. Minimum 15 hr acquisition access period (AAP) was required for 100% transmission of YMV in greengram plants. Also, minimum 15 hr latent period and inoculation Access Period (IAP) were required for 100% transmission of yellow mosaic virus in greengram by minimum of 13-15 number of viruliferous whiteflies. This study also revealed that maximum 100% infection in 10 days old plants and declined afterwards upto 40% transmission in 21 days old greengram plants. The PCR amplification of symptomatic leaf samples from the inoculated plants also indicated ~ 450 bp band of genomic region of yellow mosaic viruses of begomovirus genus.

**Keywords:** Yellow mosaic virus, bemisia tabaci, transmission relationship

## 1. Introduction
Mungbean commonly known as greengram, was originated in India (De Candolle, 1886) [5]. Majority of mungbean is cultivated in *rabi* and *kharif* season. The mungbean is largely cultivated in various parts of India as an intercrops or sole crop and also retains a vital role in crop rotation. It has inherent capacity to fix atmospheric nitrogen through biological nitrogen fixation and hence it is used as green manuring to substantially enhance the nutrient status of the soil. Mungbean is being grown in a land area of 30.41 lakh ha having a production of 14.24 lakh tonnes and productivity of 468 kg/ha (Anonymous, 2015) [1]. The productivity of mungbean has been challenged by insect, weeds and disease caused by virus, fungus and bacteria. Among these, the viruses act as a major devastating group of pathogens creating menace for overall production of the crop (Kang *et al.*, 2005) [6]. More than 12 viruses have been reported on mungbean out of which the yellow mosaic virus (YMV) is the most important causing significant yield loss in mungbean. The mode of transmission of the YMV was reported to be through whitefly (Nariani, 1960) [12]. The extent of transmissibility of yellow mosaic disease in greengram is influenced by number of viruliferous whiteflies, acquisition of virus by whitefly, latent period and inoculation access period. The whitefly (*Bemisia tabaci*) can transmit the soybean yellow mosaic virus to cowpea and soybean with acquisition access Period (AAP) of 24 hr (Usha Rani *et al.*, 2004) [15]. The most favourable stage for transmission of (*Mungbean* yellow mosaic virus) MYMV was reported to be the first trifoliate leaf of the plants (Chenulu *et al.*, 1979) [4]. Yaraguntaiah and Govindu (1964) [16] and Capoor and Varma (1950) [3] were reported that 24 hr and 12 to 24 hr of inoculation access period needed for transmission of dolichos yellow mosaic virus respectively. Keeping in view the above objectives experiments were conducted to determine the viruliferous whitefly number, acquisition access period, latent period and inoculation access period of whitefly for transmission of yellow mosaic disease in greengram. All the experiments were conducted in Department of Plant Pathology, Odisha University of Agriculture & Technology, Bhubaneswar, and Odisha.

## 2. Materials and methods

### 2.1 Rearing of whitefly and preparation of minicages for acquisition and inoculation of yellow mosaic virus by whitefly

#### 2.1.1 Rearing of whitefly

Whitefly colony were maintained on the healthy Brinjal plants (Utkal Anushree) raised in plastic pot (15x15 cm) kept in insect free aluminium cage (45x60x45 cm). Muslin cloth was covered on the three sides and the top. The front side was closed with glass fixed in two doors. The cages were kept at temperature of 25 to 30 °C in an insect free polyhouse. Whitefly individuals were collected from field by aspirator and released in the cage. They were allowed to colonize profusely on the brinjal plants in the cage. Fresh whiteflies from the newly emerged colony were collected by aspirator from the cage and used for transmission studies (Plate 1-A, B, C).

#### 2.1.2 Collection of pure cultured whiteflies

Collection of whiteflies was done with the help of aspirator having a glass tube (15x0.5x30 cm) and a rubber tube 40 cm length was employed. The whiteflies were sucked in to the glass tube by slightly turning the lower surface of the leaflets upward. The collected whiteflies were shifted to plastic tube for pre-acqisition fasting for 4 hours.

#### 2.1.3 Growing and maintenance of healthy mungbean seedlings

The seeds of the test plant (LGG-460) were procured from the Centre for Pulses Research (CPR) Berhampur, OUAT, Ganjam, Odisha. The seeds were sown on the plastic pots filled with soil and FYM (2:1), which were kept in insect free chamber covered with muslin cloth. Those healthy mungbean seedlings of 10 days old were utilized for transmission of yellow mosaic disease (Plate 1-D).

#### 2.1.4 Devising of mini cages for acquisition access feeding by whiteflies

The PVC bottles (5x5x10 cm) were cut near the base and another end tapering towards mouth were utilised. The bottom portion was covered with muslin cloth. The twigs of the infected mungbean seedlings were inserted in to the bottle through tapering end followed by plugging of that end with cotton plug to avoid escaping of the whiteflies (Plate 2-A).

#### 2.1.5 Devising of mini cages for inoculation of mungbean seedlings by viruliferous whiteflies

Plastic tubes (8x3 cm) and (5x5x10 cm) were used where the lower portion of the bottle was cut and removed and covered with the muslin cloth to prevent gathering of excess moisture inside the cage and also to facilitate aeration. A tiny hole was made in the centre of the tube to assist release of viruliferous whiteflies. The cotton plugs were used for plugging the open end of the plastic tubes (Plate 2-B).

### 2.2 Studies on the relationship between virus and vector

The relation between the vector and virus was studied for the yellow mosaic virus (YMV) employing whiteflies (*Bemisia tabaci*) allowed to colonize on the brinjal plants.

#### 2.2.1 Determination of lowest no whiteflies essential for successful transmission

Pure culture of whiteflies was allowed an AAP of 24 hr on YMV infected mungbean seedlings. The viruliferous whiteflies were permitted to 10 days old disease free mungbean seedlings at the rate of 1, 2, 3, 5, 8, 10, 13, 15, 20, and 25 number per seedling independently. For each treatment 10 healthy mungbean seedlings were inoculated. Soon after the AAP of 24 hr, the inoculated seedlings were sprayed Dimethoate (1.7 ml/lit) to kill the whiteflies. The plants were kept in insect free muslin chamber for observation of symptoms and recording of the% transmission.

#### 2.2.2 Ascertainment of minimum acquisition access period (AAP) for highest rate of transmission of YMV

Various AAP on the rate of transmission of YMV was examined by permitting groups of 13-15 whiteflies to feed for 5 min, 15 min, 30 min, 1 hr, 2 hr, 5 hr, 10 hr, 15 hr and 24 hr on YMV infected seedlings separately. For each treatment 10 healthy mungbean seedlings of 10 days old were inoculated. Soon after the IAP of 24 hr, the inoculated seedlings were sprayed Dimethoate (1.7 ml/lit) to kill the whiteflies. The inoculated seedlings were placed in an insect free muslin chamber for observation of symptoms and recording of the% transmission.

#### 2.2.3 Latent period of YMV in whitefly

The whiteflies were given 24 hr of AAP on YMV infected mungbean seedlings. Then batches of 13-15 whiteflies per plant were allowed to feed on the disease free mungbean seedlings after 5 min, 15 min, 30 min, 1 hr, 2 hr, 5 hr, 10 hr, 15 hr and 24 hr after acquisition separately. After every latent period, whiteflies are provided 24 hr of IAP. Finally, all the inoculated plants were stored in insect free muslin chamber for development of symptoms.

#### 2.2.4 Determination of minimum inoculation access period (IAP) for highest rate of transmission of YMV

The whiteflies were allowed AAP for 24 hr on YMV infected seedlings separately. Batches of 13-15 viruliferous whiteflies were then shifted to 10 days old healthy mungbean seedlings for IAP for 5 min, 15 min, 30 min, 1 hr, 2 hr, 5 hr, 10 hr, 15 hr and 24 hr. Ten healthy seedlings were inoculated per each treatment. The inoculated seedlings were sprayed Dimethoate (1.7 ml/lit) to kill the whiteflies. The inoculated seedlings were placed in an insect free muslin chamber for observation of symptoms and recording of the % transmission.

#### 2.2.5 Impact of age of the test seedlings on transmission of YMV

The Whiteflies were independently inoculated to each mungbean seedlings on 7, 10, 14, 18 and 21 days old plants. Groups of 13-15 whiteflies were allowed to feed on 10 test seedlings per treatment. After allowing the whiteflies for 24 hr both during AAP and IAP, the inoculated seedlings were sprayed Dimethoate (1.7 ml/lit) to kill the whiteflies. The inoculated seedlings were placed in an insect free muslin chamber for expression of symptoms and recording of the% transmission.

### 2.3 Molecular detection of the virus causing yellow mosaic disease in mungbean seedling after whitefly transmission

#### 2.3.1 Extraction of the total genomic DNA from the test seedlings employing the CTAB (Cetyl trimethyl ammonium bromide) method

The total DNA was extracted from the healthy and

symptomatic leaves of inoculated mungbean seedlings according to the method reported by Rouhibakhsh *et al.*, (2008) [14]. Freshly collected leaf tissue (150 mg) was ground employing sterile mortar and pestle with liquid nitrogen and then the ground tissue was shifted into a 1.5 ml eppendorf tube. 1500 µl of extraction buffer (CTAB) pre-warmed up to 65 °C was mixed with the ground sample present in 1.5 ml eppendorf tube. Incubation of the whole crude sap was done for ½ hr at 60 °C in a water bath with intermittent mixing. 750 µl of the supernatant was shifted into a new 1.5 ml eppendorf tube and mixed with same volume (750 µl) of Phenol: chloroform: isoamyl alcohol (25:24:1) by vertexing. The whole sample was allowed to a micro centrifuge for centrifugation at 1300 rpm for 10 minutes, followed by the supernatant was collected in a new 1.5 ml eppendorf by discarding the solid palette settled at the bottom of the Eppendorf. The mixture of 30 µl of 7.5 M ammonium acetate + 300 µl of chilled isopropanol was mixed with aqueous supernatant by inversion and hence the DNA was got precipitated. The Eppendorf tube was centrifuges for 10 minutes at 13000 rpm and the pellet formed was washed with 70% ethyl alcohol followed by dried in a vacuum drier for 10 min until alcohol odour disappears. Then it was re-suspended with 40 µl of $T_{10}E_{0.1}$ buffer (0.1 mM EDTA of pH 8.0 and 10 mM Tris-HCl of pH 8.0) and stored in deep freezer at -20 °C. All the extracted DNA were additionally diluted from 1:10 to 1:40 with double distilled water (DDW) before amplification employing the thermocycler. The quality and quantity of DNA was examined at 280 and 260 nm by employing UV spectrometer and the ratio of $OD_{260}$ to $OD_{280}$ was found to be 1.9-2.0 range.

### 2.3.2 Primers used for the amplification

RUGEMF1 and RUGEMR1 have been used which is specific to the conserved region of Coat protein (AV-1) region of the yellow mosaic viruses belonging to begomoviruses (Ramesh *et al*, 2016) [13]. The sequence of the primers was detailed below.

| Primers | Sequence (5'- 3') |
|---|---|
| RUGEMF1 | TGTGAGGGACCATGTAAAGTTC |
| RUGEMR1 | GCATGAGTACATGCCATATAC |

### 2.3.3 Master mixture of PCR

The master mixture needed was prepared from the Banglore Genei and the Eppendorf, Germany was used. The reaction mixture was dispensed into 0.2 ml of PCR tubes and the total volume was made up to 20 µl with double distilled water (DDW) as detailed below.

| Components | Volume (µl) |
|---|---|
| Taq buffer (10 X) | 2.00 |
| dNTP (1 mM) | 1.00 |
| Forward Primer (5 pM) | 1.00 |
| Reverse Primer (5 pM) | 1.00 |
| DNA template (40 ng) | 1.00 |
| Taq polymerase (5 u/µl) | 0.50 |
| Double distilled water | 13.50 |
| Total | 20.00 |

### 2.3.4 Thermal cycler program for PCR amplification

The programme for PCR consists of single cycle of initial denaturation of DNA at 94 °C for 5 min followed by 35 cycles each having denaturation at 94 °C for 40 sec, annealing at 57 °C for 30 sec and an extension of primer at 72 °C for 30 sec. The final extension at 72 °C for 5 min was given for 1 cycle and finally hold at 4 °C for 30 min. After PCR amplification, the PCR tubes were collected and stored at -20 °C until the gel electrophoresis was completed.

### 2.3.5 Agarose gel electrophoresis

One gram of agarose was taken in a 250 ml conical flask having 100 ml of 1X TAE buffer followed by heating of the solution was done in a hot air oven by uniform stirring in order to dissolve the agarose completely. The solution was allowed to cool to bring its temperature to about 50 °C followed by 2-3 drops of ethidium bromide (0.5 µg/ml) was mixed with the solution. The solution was poured onto the gel casting plate after placing the comb in the trough and care was taken to ensure the solution remain bubble free. The agarose solution was allowed to solidify and then the comb was removed after keeping the casting plate in electrophoretic apparatus comprising enough 1X TAE (Stock 50X TAE) buffer to cover the wells thoroughly. The amplified PCR products (5 µl) to be examined were loaded carefully into the wells present on the casting plate after mixing with loading dye (6x) by using the micropipette. During the electrophoresis, voltage was fixed at 80 volts for 45 minutes. The DNA bands stained with the Ethidium bromide were seen under the UV-transilluminator and documented employing gel documentation system (UviTec Cambridge, England)

**Note**: 20 ml of 1X TAE was made up to 1 lit (1000 ml) by adding sterile distilled water.

## 3 Result and Discussion

### 3.1 Effect of number of whiteflies for transmission of YMV in mungbean

The studies revealed increased rate of transmission of YMV in mungbean by increased number of viruliferous whiteflies. It was found that singe whitefly can transmit the YMV successfully however the percent transmission was upto 30%. The percent transmission increased with the increase in the number of whiteflies per plant for inoculation. Hundred% transmission was recorded when 13 or more number of whiteflies were inoculated per plant (Table 1). Similar results were also reported by Manjunath *et al*, 2013 [9] with one indigenous and B-biotype whitefly able to transmit the MYMV. A minimum of 15 viruliferous whiteflies per plant were reported to be essential for 100% transmission of MYMV (Chenulu *et al.*, 1979) [4] and croton yellow mosaic virus (CYMV) in croton (Mandal, 1989).

### 3.2 Effect of Acquisition access period (AAP) on transmission of YMV

At least 30 mins of AAP was needed for whiteflies to become viruliferous and could successfully transmit the YMV at a% transmission of 10%. The% transmission was found to be increased to 50% when 10 hr of AAP was given to the whiteflies. Hundred% transmission of YMV was attained by 15 and 24 hr of AAP (Table 2). The% transmission and AAP were noticed to be positively correlated with each other. With increase in AAP, the% of whitefly becoming viruliferous increased, as a consequence the transmission efficiency also was enhanced. The present results were in agreement with the findings recorded by (Nair and Nene, 1974) [11] for MYMV and (Muniyappa and Reddy, 1976) [10]. On greengram seedlings the transmission of MYMV was reported to be successfully transmitted with just 15 min of AAP (Capoor and Verma, 1948) [2].

### 3.3 Latent period of yellow mosaic virus in whitefly

The results indicated that minimum of half an hour period of waiting (latent period) was enough for 10% transmission. Hundred% transmission was achieved after the whiteflies were given a latent period of 15 and 24 hr (Table 3). Hence, there was enhanced transmission of YMV by whitefly after increase in the latent period. Similar trends were found by Manjunath *et al.*, 2013 [9]. They reported 66.67 and 53.30 percent transmission after 24 hr of latent period in B-biotype and Indigenous whiteflies which were given 1 hr of AAP.

### 3.4 Effect of inoculation access period (IAP) on transmission of YMV

A minimum of 30 min of IAP was found to be enough for viruliferous whiteflies for successfully transmit the virus to 10%. The percent of transmission showed 60% after an IAP of 10 hr and 100% after an IAP of 15 hr and more (Table 4). The consequences indicated that with increase in IAP, transmission of YMV by whitefly was increased. Similar virus-vector relationship studies were reported by Manjunath *et al.*, 2013 [9] for the MYMV in mungbean seedlings by indigenous and B-biotype whiteflies.

### 3.5 Effect of age of the mungbean seedlings on transmission of yellow mosaic virus (YMV) in mungbean by Whitefly

Mungbean seedlings of different age group were utilized for examining the effect on the percent transmission of YMV by viruliferous whitefly. Maximum transmission of YMV was found in 7 to 10 days old greengram seedlings and declined afterwards in 14 to 21 days old seedlings of mungbean (Table 5). It could be because of the more succulent and tenderness of young seedling, which was favourable for the whiteflies to feed more and transmit the virus with higher efficiency. This result was found similar with the observations in other whitefly borne viruses such as Cotton leaf curl virus and Euphorbia mosaic virus (Costa and benett, 1950). With increase in the age of the seedlings, the percent transmission decreased. The development and expression of symptoms on the inoculated mungbean seedlings is shown in plate 3.

### 3.6 Molecular detection of the inoculated virus in greengram

The molecular detection of the virus associated with the yellow mosaic disease on the inoculated mungbean seedlings was done through PCR amplification of the conserved coat protein genomic DNA. The healthy samples also used along with one positive control to ensure the primers have attached to the genomic DNA strand and one negative control to ensure there is no other unwanted DNA contaminant in the PCR reaction mixture. The gel documentation of five samples collected from inoculated mungbean seedlings showed bands at ~ 450 BP having 100 BP DNA ladder (Plate 4). Ramesh *et al.*, 2016 [13] also have got bands of 450 BP specific to conserved region of coat protein region of Legume infecting YMV. The coat protein (CP) genomic region of YMV which ascertained the ability of transmission of the viruses through whitefly (*Bemisia tabaci*). The CP gene is highly conserved among begomoviruses (Mahesh *et al.*, 2010) [7]. Hence in the present study primers targeting the CP region of the YMV belonging to begomoviruses have been employed.

**Table 1:** Effect of number of whiteflies (Bemesia tabaci) on transmission of yellow mosaic virus (YMV) in mungbean

| Number of whiteflies[1] | Number of Plants | |
|---|---|---|
| | Infected/Inoculated[2] | Percent Transmission (%) |
| 1 | 3/10 | 30 |
| 2 | 3/10 | 30 |
| 3 | 5/10 | 50 |
| 5 | 6/10 | 60 |
| 8 | 7/10 | 70 |
| 10 | 9/10 | 90 |
| 13 | 10/10 | 100 |
| 15 | 10/10 | 100 |
| 20 | 10/10 | 100 |
| 25 | 10/10 | 100 |

[1] AAP & IAP- 24 hr

[2] Variety- LGG-460 (10 days old)


**Plate 1:** Rearing of healthy whitefly on brinjal plants placed in insect free chamber (a, b) whitefly colonics developed on the underside of the leaflet (c) & raising of healthy mungbean test seedlings in insect proof muslin chamber (d)


**Plate 2:** Acquisition feeding by pure cultured whiteflies on mungbean seeding (a) & inoculation feeding by viruliferous whiteflies on the healthy mungbean seeding (b)

**Plate 3:** Expression of symptoms on the inoculated mungbean seeding

**Table 2:** Effect of Acquisition Access Period (AAP) on transmission of yellow mosaic virus (YMV) in mungbean by Whitefly

| Acquisition Access Period | Number of Plants | |
|---|---|---|
| | Infected/Inoculated* | Percent Transmission (%) |
| 5 min | 0/10 | 0 |
| 15 min | 0/10 | 0 |
| 30 min | 1/10 | 10 |
| 1 hr | 1/10 | 10 |
| 2 hr | 2/10 | 20 |
| 5 hr | 3/10 | 30 |
| 10 hr | 5/10 | 50 |
| 15 hr | 10/10 | 100 |
| 24 hr | 10/10 | 100 |

*IAP- 24 hr, Group of 13-15 adult whiteflies per plant were used, Variety- LGG-460 (10 days old)

**Table 3:** Influence of latent Period on transmission of yellow mosaic virus (YMV) by Whitefly

| Incubation Period | Number of Plants | |
|---|---|---|
| | Infected/Inoculated* | Percent Transmission (%) |
| 5 min | 0/10 | 0 |
| 15 min | 0/10 | 0 |
| 30 min | 1/10 | 10 |
| 1 hr | 2/10 | 20 |
| 2 hr | 3/10 | 30 |
| 5 hr | 4/10 | 40 |
| 10 hr | 7/10 | 70 |
| 15 hr | 10/10 | 100 |
| 24 hr | 10/10 | 100 |

* AAP & IAP- 24 hr, Group of 13-15 adult whiteflies per plant were used, Variety- LGG-460 (10 days old)

**Table 4:** Effect of inoculation access period (IAP) on transmission of yellow mosaic virus (YMV) in mungbean by Whitefly

| Inoculation Access Period | Number of Plants | |
|---|---|---|
| | Infected/Inoculated* | Percent Transmission (%) |
| 5 min | 0/10 | 0 |
| 15 min | 0/10 | 0 |
| 30 min | 1/10 | 10 |
| 1 hr | 2/10 | 20 |
| 2 hr | 3/10 | 30 |
| 5 hr | 3/10 | 30 |
| 10 hr | 6/10 | 60 |
| 15 hr | 10/10 | 100 |
| 24 hr | 10/10 | 100 |

AAP- 24 hr, Group of 13-15 adult whiteflies per plant were used, Variety- LGG-460 (10 days old)

**Table 5:** Effect of age of the mungbean seedlings on transmission of yellow mosaic virus (YMV) in mungbean by Whitefly

| Age of the seedlings (Days) | Number of Plants | |
|---|---|---|
| | Infected/Inoculated* | Percent Transmission (%) |
| 7 | 9/10 | 90 |
| 10 | 10/10 | 100 |
| 14 | 8/10 | 80 |
| 18 | 6/10 | 60 |
| 21 | 4/10 | 40 |

AAP & IAP- 24 hr, Group of 13-15 adult whiteflies per plant were used, Variety- LGG-460

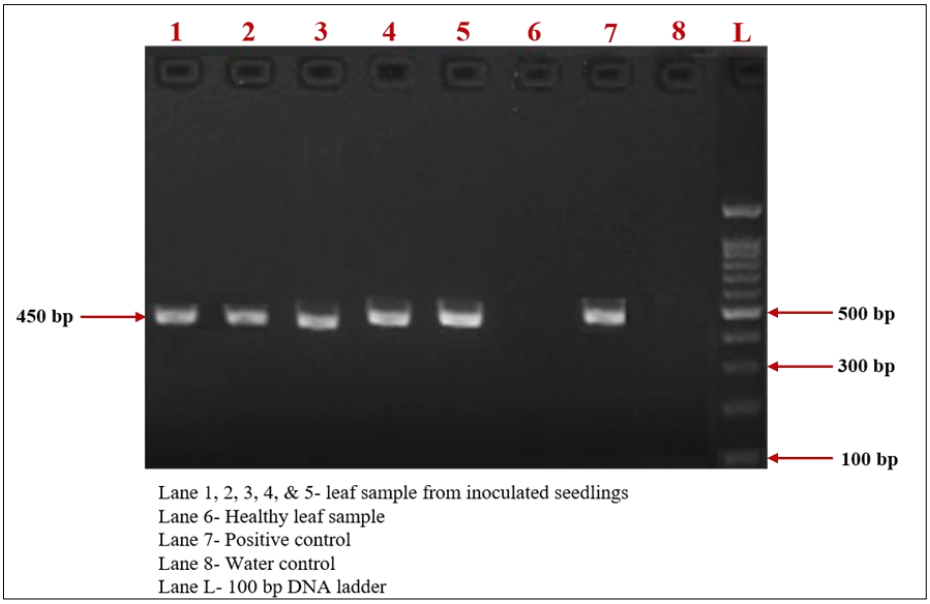


**Plate 4:** Gel image indicating the 450bp amplification of the conserved CP region of virus using RUGEMFI and RUGEMRI Primers

## 4. Conclusion

The results of the virus- whitefly relationship on the basis of% transmission revealed that even single viruliferous whitefly can transmit the YMV successfully. It was also concluded that 100% transmission of the virus by using 13 or more number of whiteflies was achieved with 15 hr and more AAP and IAP. Ten days old seedlings recorded highest level of susceptibility towards the YMV infection. The future research should be based on the genomic level for the virus-vector relationship so that many novel management models to be ultimately be included in integrated disease management (IDM) for yellow mosaic viruses.

## Appendix

**CTAB (Extraction buffer)**

| | |
|---|---|
| CTAB | : 4% |
| NaCl | : 1.4 M |
| EDTA (pH 8.0) | : 20 mM |
| Tris HCl | : 100 mM |

**Stock solution (100 ml)**

372.24 g (molecular weight) in 1000 ml = 1M EDTA
37.224 g in 1000 ml (3.72 g/100 ml) = 0.1 M EDTA
186.12 g in 1000 ml (18.612 g/100 ml) = 0.5 M EDTA
Dissolved the chemical in 50 ml of distilled water and adjusted the pH and the volume made up to 100 ml.

**Loading dye (6X)**

Dissolve the sucrose (40 g) completely in 80 ml of distilled water and add 0.25% of bromophenol blue and finally made volume up to 100 ml and stored at 4 °C.

**50X TAE Buffer (1000 ml)**

| | |
|---|---|
| 0.5 M EDTA | : 100 ml |
| Glacial acetic acid | : 57 ml |
| Tris base | : 242 g |
| Distilled water | : 840 ml |

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