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RS Marabi
Department of Entomology
College of Agriculture, JNKVV,
Jabalpur, Madhya Pradesh, India

SB Das
Department of Entomology
College of Agriculture, JNKVV,
Jabalpur, Madhya Pradesh, India

N Tripathi
Directorate of Research Services
JNKVV, Jabalpur, Madhya
Pradesh, India

AK Bhowmick
Department of Entomology
College of Agriculture, JNKVV,
Jabalpur, Madhya Pradesh, India

R Pachori
Department of Entomology
College of Agriculture, JNKVV,
Jabalpur, Madhya Pradesh, India

Vibha
Department of Plant Pathology
College of Agriculture, JNKVV,
Jabalpur, Madhya Pradesh, India

Correspondence
RS Marabi
Department of Entomology
College of Agriculture, JNKVV,
Jabalpur, Madhya Pradesh, India

Molecular identification of Mungbean yellow mosaic India virus (MYMIV) from whitefly and soybean in Jabalpur district of Madhya Pradesh, Central India

RS Marabi, SB Das, N Tripathi, AK Bhowmick, R Pachori and Vibha

Abstract
Yellow mosaic disease caused by genus Begomovirus which is transmitted through whitefly, *Bemisia tabaci* has become an important constraint for legume production particularly in soybean, urdbean, mungbean and other bean crops of India. The present investigation was performed with the aim to identify Mungbean yellow mosaic India virus (MYMIV) infection in soybean as well as whitefly with the use of coat protein (CP) primer. The genomic DNA templates from both were extracted and amplified with CP primer. DNA templates from yellow mosaic symptomatic plants and carrier whiteflies were amplified with a band size of ~750bp. The present study confirms the utility of CP primers for the detection of MYMIV which is found to be most prevalent yellow mosaic disease in soybean crop at Jabalpur district of Madhya Pradesh, Central India.

Keywords: Soybean, Coat protein, MYMIV, Whitefly, *Bemisia tabaci*, Begomovirus

1. Introduction
Yellow mosaic diseases (YMD) are major biotic constraints on the productivity of legume crops in India. YMD in soybean was first reported from the northern region of the country, had spread to different parts of Central India where large acreage is under soybean (*Glycine max*) cultivation with yield losses of 21–70% (Dasgupta *et al*., 2003) [1].YMD caused by Mungbean yellow mosaic India virus (MYMIV) is one of the important constraints to soybean, urdbean (*Vigna mungo*) and mungbean (*Vigna radiata*) production in Central India (Ramesh *et al.* 2016) [2]. MYMIV is transmitted by the whitefly, *Bemisia tabaci* Gennadius infect the legumes such as soybean, urdbean and mungbean reported by Govindan *et al.*[3] and Marabi *et al.*[4] In India, the annual monetary losses in legumes (soybean, urdbean and mungbean) caused by YMD have been estimated to be approximately US $300 million per year (Varma *et al.* 2003) [5]. Since last few decades it has been experienced that the total cultivated area of soybean is declined continuously due to severe incidence of yellow mosaic disease. Information on the alternative weed hosts of MYMIV is limited which need to be addressed to unveil the reasons. Although a single whitefly is able to acquire virus and transmit to plants. Female *B. tabaci* is more active and efficient to transmit virus than male. Many weeds in and around the agricultural field throughout the year are often seen with YMD symptoms and occurrence of whiteflies are also observed on many weed species. Whiteflies take shelters on alternative hosts (weeds) which are found to be major and sometime act as transient reservoir of MYMIV after harvesting the main crops and carry over to the next season. Therefore, the current study was taken up to confirm the identity of the virus causing YMD in soybean as well as its vector at molecular level.

2. Material and Methods
2.1 Culturing of insect vector and virus source
Culturing of vector (*B. tabaci*) and virus sources were maintained in insect proof net house (Size: 50 mesh) following the methodology proposed by Aidawati *et al*. (2002) [6]. Healthy non-viruliferous colonies of whiteflies were maintained on healthy soybean plants (cv. JS 335) which were used for MYMIV transmission studies. For inoculation study, single healthy seedling of soybean was grown in each earthen pot.

Simultaneously soybean plants showing typical yellow mosaic disease symptoms were collected from the soybean field which was confirmed as MYMIV by molecular studies through PCR technique and were maintained as virus source.

2.2 Virus-vector relationship

A known number of healthy non-viruliferous adult female whiteflies were released on MYMIV-infected soybean plants and was given 24 hrs to acquire virus *i.e.* acquisition access period (AAP). After the AAP, the whiteflies were re-collected individually with the help of aspirator and transferred them on 7-14 days old healthy soybean plants for 24 hrs *i.e.* inoculation access period (IAP) for transmitting the virus. Ten adult female whiteflies per plant were used and replicated it ten times. After inoculation the whiteflies were completely removed and plants were maintained under insect-free condition for development of disease symptoms. Percentage of virus infection (*i.e.* percent disease incidence) was computed from inoculated test plants which were expressed disease symptoms.

2.3 Isolation of DNA from whitefly sample

Whiteflies were collected by using the aspirator from experimental field and preserved in 100 percent acetone at 4°C until use. A total of 30µl of STE buffer [100mM NaCl, 1mM EDTA(pH8.0), 10mM Tris-HCl(pH8.0)] was taken in a microcentrifuge tube and a single whitefly was introduced in it using fine pointed paint brush (Zero number brush- Camel). The whitefly was crushed using micro pestle to make homogenate solution and 2 µl of proteinase-K (10mg/1ml) was added to the homogenate and mixed thoroughly. Homogenate containing microcentrifuge tubes were incubated at 55^0C for 30 min in heating block. Microcentrifuge tubes were then incubated at 90 ^{0}C for 5 min using another heating block. Microcentrifuge tubes were centrifuged slightly to collect the liquid on the bottom. Resultant DNA solution was stored in refrigerator until further activities.

2.4 Isolation of DNA from soybean leaf sample

Leaf sample of soybean (cv. JS 335) was collected from the field and individually kept in sterilized polythene bag containing zip. After bringing the samples in laboratory 100mg leaf sample was marked and wrapped in aluminum foil and then frozen in liquid nitrogen before storing in -80 ^{0}C. DNA from soybean leaf samples was isolated using DNeasy Plant Mini Kit (Qiagen) and stored in refrigerator until its use.

2.4 PCR amplification

Molecular markers were designed for DNA-A (CP) genomes of mungbean yellow mosaic India virus (MYMIV): DNA-A (CP) forward primer – 5'ACACGGATCCGTTGCATACACAGGATTTG3'; reverse primer – 5'ACACGAGCTCCTCTACCCCGATATCGAATG3'. PCR was carried out with genomic DNA using molecular markers in Bio-Rad Thermal cycler. The reaction was carried out in 25 µl volumes, which contains 1.0µl (25ng) of soybean genomic DNA, 1.0µl (2.5pmole) of forward and reverse primers each, 1.0µl (2.0mM) of dNTPs, 1.0µl of Taq buffer (10X), 1.0µl of $MgCl_2$ (25mM) and 1 units of *Taq* polymerase. All the chemicals and plasticwares used were obtained from Genei and Tarsons Company, respectively. PCR Programme was standardized to carry out amplification with DNA-A genome specific primer as mentioned in the Table 1. The amplified products were resolved on 1.0% agarose gel and visualized under Syngene gel documentation system.

Table 1: PCR programme

Steps followed in Thermal cycler	Temperature in ºC for one cycle	Time for one cycle
Marker	CP	
Step 1	94 ºC	1 min.
Step 2	94 ºC	20 sec.
Step 3	56 ºC	20 sec.
Step 4	72 ºC	1 min.
Step 2 – Step 4 are repeated for 30 cycles		
Step 5	72 ºC	3 min.
Step 6	Hold at 4 ºC	

3. Results and Discussion

Improved frequency of whitefly outbreaks due to the expansion of insecticide resistance in whitefly has increased the incidence of MYMIV and therefore needs for resistant cultivars (Ahmad *et al*., 2010) [7]. Identification of MYMIV and whitefly resistant soybean cultivars is an environmentally compatible and effective control method (Martin and Fereres, 2003) [8]. In the present study, an evaluation of MYMIV infection on soybean genotypes and its vector whitefly (*B. tabaci*) was carried out under natural infection in field and in the net-house using whiteflies inoculations.

3.1 Transmission

Yellow mosaic symptom was first recorded after 15 days of inoculation. The expression of disease symptoms was produced in the form of typical yellow specks and golden mosaic on the leaves of soybean test plant (Figure 1B). After inoculation, all soybean test plants were found to be 100 per cent yellow mosaic disease symptoms within 15-21 days similar to those seen in the field indicating that the causal agent of virus was transmitted by the whiteflies in same manner. Present finding is accordance with the result of Usharani *et al*. (2004) [9] who studied whitefly inoculation in the glass house on soybean (cv. Bragg). Gazala *et al*. (2013) [10] also reported that at 18 hrs of AAP and 24 hrs of IAP the MYMIV symptoms was developed after 20 days of inoculation in the form of mild scattered yellow specks in the leaves soybean (cv. JS 335) plants.

3.2 Amplification and data analysis

Because of its high degree of conservation, the coat protein ORF (CP) is the only begomovirus sequence approved by the International Committee on Taxonomy of Viruses for ascertaining the identity of a begomovirus (Mayo and Pringle, 1998) [11] it was used to amplify template DNA isolated from whitefly and soybean plants both.

PCR tests yielded amplified DNA fragments of the expected size ~750 bp, of MYMIV in the symptomatic leaf samples of soybean collected from the field as well as in the whitefly. Gel photographs of PCR amplified products of all the samples

are shown in Figures 2 and 3, respectively. The findings indicate that the primer CP specific to coat protein gene is important for detection of MYMIV infection in plants and from viruliferous whiteflies.

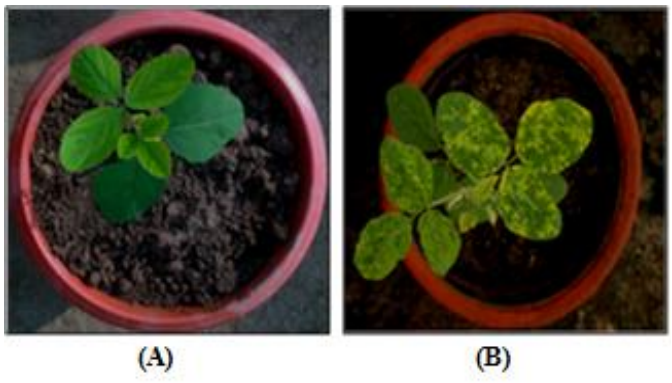

Fig 1: (A) Healthy soybean plant (B) MYMIV symptoms on soybean plant after inoculation through viruliferous whiteflies

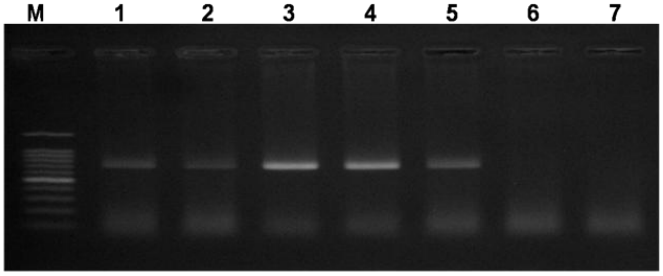

Fig 2: PCR amplifications of whitefly DNA using CP primer. 1-5 whiteflies collected form MYMIV infected soybean plants and 6-7 whiteflies collected form healthy soybean plants, M=100bp DNA ladder

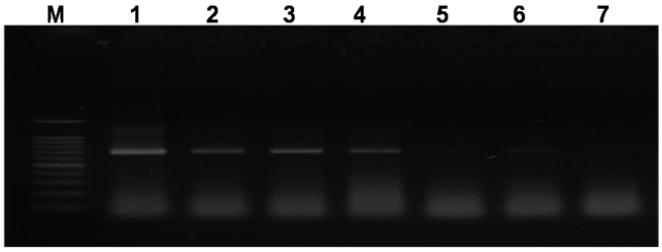

Fig 3: PCR amplifications of soybean leaf DNA using CP Primer. 1-6 MYMIV infected soybean leaves and 7 healthy soybean leaf, M=100bp DNA ladder

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