Agroinoculation: a method of screening germplasm resistance to mungbean yellow mosaic geminivirus

KAJAL KUMAR BISWAS* and A. VARMA
Advanced Centre for Plant Virology, Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi 110 012

ABSTRACT: Agroinoculation of dimeric constructs of cloned DNA-A and DNA-B of an isolate of mungbean yellow mosaic virus (MYMV-Bg) to different varieties of six grain legumes, blackgram (Vigna mungo), cowpea (V. unguiculata), Frenchbean (Phaseolus vulgaris), mungbean (V. radiata), pigeonpea (Cajanus cajan) and soybean (Glycine max) was tested. Two methods of agroinoculation i.e., sprouted seed and seedling inoculation were used and both were found equally effective. Agroinfected plants took longer time to develop symptoms as compared to those inoculated by whitefly (Bemisia tabaci) transmitted plants. Symptomatic and symptomless infection of plants was confirmed by nucleic acid spot (NASH) and Southern hybridization. Some symptomless plants showed positive signal by NASH test but not by Southern hybridization. Contrasting results were, however, obtained for some varieties which could be infected by whitefly inoculation, but not by the agroinoculation. Agroinoculation can be a useful tool for screening for resistance in the breeding programme.

Key words: MYMV, agroinoculation, grain legumes, NASH, Southern blot, host resistance

Mungbean yellow mosaic geminivirus (MYMV) belonging to the sub-group Begomovirus causes yellow mosaic disease (YMD) in a variety of grain legumes and adversely affects their production in the Indian subcontinent (Varma et al., 1992). The Indian isolates of MYMV are transmitted by whitefly, Bemisia tabaci, but not by sap inoculation (Nariani, 1960). Sources of resistance to MYMV in mungbean (Vigna radiata) and blackgram (V. mungo) have been identified mostly on the basis of field screening and occasionally under glasshouse conditions using varying number of viruliferous whiteflies for inoculation (Amin et al., 1985-90; Kausal and Singh, 1988; Chhabra and Kooner, 1993; Chhabra et al., 1993). Biological, serological and nucleic acid spot hybridization (NASH) tests have indicated occurrence of natural variants of MYMV which causes rapid breakdown of resistance in different grain legumes (Varma et al., 1992). As in most of the tests resistance is being identified against one or two variants prevailing at a geographical location, so far durable resistance in grain legumes to MYMV has not been developed.

An isolate of MYMV from blackgram (MYMV-Bg) occurring in Delhi has been cloned and was found to contain two split DNAs viz. DNA-A and DNA-B of 2.7 kb each (Varma et al., 1991). The infectivity of the cloned DNAs of MYMV-Bg was demonstrated in blackgram through agroinoculation of the dimeric constructs of the viral DNA; agroinoculation of sprouted seeds resulted in upto 50% infection as compared to 13-20% by agroinoculation of seedlings (Mandal et al., 1997). Agroinoculation of the cloned DNAs has also been reported to be an efficient method of transmission for other Begomoviruses like bean golden mosaic virus (BGMV), tomato golden mosaic virus (TGMV), tomato yellow leaf curl virus (TYLCV), etc. (Morris et al., 1988; Elmer et al., 1988; Kheyr-Pour et al., 1991). Agroinoculation has been mostly utilized to study infectivity, replication and movement of cloned viral genomes (Buraguhain et al., 1994; Czosnek et al., 1993; Mandal et al., 1997; Kheyr-Pour et al., 1991). In this paper we examine the utility of agroinoculation in screening for resistance to MYMV.

MATERIALS AND METHODS

Plant sources

The seeds of different varieties of grain legumes, blackgram (Vigna mungo), cowpea (V. unguiculata), Frenchbean (Phaseolus vulgaris), mungbean (V. radiata), pigeonpea (Cajanus cajan) and soybean (Glycine max) used in the present studies (Table 1) were
obtained from the Division of Genetics, IARI, New Delhi.

**Agroinoculation**

Tandem dimeric constructs of MYMV-Bg DNA-A (pBMA2) and DNA-B (pBMB2) in T-DNA derivative vector pBin 19 (Fig. 1; Mandal et al., 1997) were used for inoculating different varieties of six grain legumes which are commonly grown in India. Tandem dimeric pBMA2 and pBMB2 were transferred from *E. coli* strain NM 522 by tri-parental mating using pRK 2013 as a helper plasmid vector into *Agrobacterium tumefaciens* strain C 58 which contains the nopaline type Ti-plasmid pTiC 58 (Hamilton and Fall, 1971). *A. tumefaciens* C 58 was grown in LB (Luria Broth) containing kanamycin (50 mg/ml) and nalidixic acid (100 mg/ml) at 28°C for 24 h with continuous shaking at 200 rpm in an incubator. *E. coli* strain JM 83 containing helper plasmid pRK 2013 and *E. coli* strain NM 522 containing dimeric constructs of MYMV-Bg DNA-A and DNA-B were also grown for 24 h in LB containing kanamycin (50 mg/ml) and nalidixic acid (10 mg/ml) at 37°C with continuous shaking at 200 rpm. Two ml of *Agrobacterium*, 1 ml each of helper *E. coli* JM 83 and of *E. coli* NM 522 cells were transferred into a culture tube and mixed well. An aliquot of 50-100 ml of the mixture was layered on 1 sq. cm. sterile nitrocellulose membrane (NCM) piece on Luria Agar (LA) plate. The plates were air dried and sealed with parafilm and incubated at 28°C for 24 h. The bacterial cells which grew on NCM were collected, diluted in 5 ml of sterile double distilled water (ddw) and mixed with gentle shaking. These were further diluted ten times in sterile ddw and 10 ml suspension spread on LA plate supplemented with kanamycin (50 mg/ml); the plates were incubated at 28°C for 36 h. Twenty bacterial colonies from each construct were transferred to 5 ml LB supplemented with kanamycin (10 mg/ml) and nalidixic acid (10 mg/ml) and incubated at 28°C for 24 h.

For inoculation, both the constructs i.e. DNA-A and DNA-B were mixed in equal proportion. Bacterial cells were collected by low speed centrifugation, resuspended in 200 ml sterile water and used for inoculation immediately. Plants were inoculated by two methods. In the first method, four to five days old seedlings of test plants were inoculated by pricking the tender stem near the cotyledon with fine entomological needles after placing 2 ml of bacterial suspension containing the MYMV constructs. In the second method sprouted seeds were used for inoculation. Seeds of test plants were soaked in sterile water overnight at 37°C, the seed coats were removed gently and then the collar region of the sprouted seeds were pricked with fine entomological needle and immediately submerged in the overnight grown culture of *A. tumefaciens* C58 mixture containing DNA-A and -B constructs. The seeds were then incubated at 28°C overnight, followed by washing of individual seeds and sowing in earthen pots containing natural field soil. Agroinoculated plants were maintained in a limited access chamber following appropriate bio-safety measures (Elmer et al., 1988).

**Nucleic acid spot and Southern hybridization**

Agroinfection in various plants was confirmed by nucleic acid spot (NASH) and Southern hybridization tests. For each variety pooled samples from all the plants or 10 randomly selected plants (where the number of plants inoculated was more than 10) consisting of 0.1 g leaf from each plant were tested six weeks after inoculation. In the case of varieties developing symptoms, only the plants showing symptoms were tested. Total DNA was isolated from the pooled samples by the modified method of Maule et al., 1983. The leaf tissues were ground in extraction buffer (0.1M Tris-HCl pH 8.0, 0.01M NaCl, 0.01M EDTA, 10% SDS) using pestle and mortar. The extract was centrifuged at 10,000 rpm for 10 min. The supernatant was mixed well with equal volume of phenol, chloroform, isooamylalcohol (25:25:1), and centrifuged to separate the aqueous phase which was collected and DNA precipitated using 0.8 volume of isopropanol and 0.1 volume of 3M sodium acetate, pH 4.8 at -20°C for 30 min. The DNA precipitate, separated by centrifugation, was dried and dissolved in 100 ml of sterile ddw. Four microlitre of the isolated DNA was dotted on NCM and 10 ml was loaded for Southern hybridization (Southern, 1975).
Whitefly inoculation

Healthy non-viruliferous adult whiteflies were given acquisition feeding of 24 h on trifoliate leaves of infected plants and transferred to the healthy 2-4 days old test plant seedlings for inoculation feeding of 24 h at the rate of 8-10 whiteflies per seedling. After inoculation the plants were kept in an insect-proof growth chamber. Each pot containing 4-5 seedlings, was individually covered with cylindrical macrocage made up of transparent cellulose acetate sheet to provide complete insect-proof condition.

RESULTS

Of the sixteen varieties of different grain legumes inoculated with MYMV-Bg by whitefly inoculation, one variety of pigeonpea and two varieties of cowpea were not infected and the other varieties developed varied symptoms (Table 1). Of the 14 varieties tested by agroinoculation of sprouted seeds, three varieties of Frenchbean and two varieties of mungbean developed typical symptoms and contained good amount of viral DNA (Table 1; Fig. 2). Whereas, in two varieties each of pigeonpea and soybean very faint signals were obtained in NASH (Fig. 2; Lanes 8, 9, 16 and 17), but the replicative forms of viral DNA were not detected in Southern hybridization (Fig. 3). Therefore, infection in these varieties was not considered positive; moreover, these plants also did not develop any symptoms. In all, 16 varieties of different species were tested by agroinoculation of seedlings. Of these, six varieties were infected by the virus, and developed characteristics symptoms and the remaining ten varieties were not infected (Table 1), although Frenchbean var. PDR 14 developed doubtful symptoms and faint signals were obtained in NASH in soybean var. Pusa 16.

Notably, some contrasting results were obtained. Frenchbean var. VL 67 could be infected by agroinoculation of sprouted seeds but not by agroinoculation of seedlings. Converse was the case with blackgram var. Pusa 2. Differences in infection by whitefly inoculation and agroinoculation were also obtained. Pigeonpea var. ICPL 84023, soybean vars Pusa 16 and Pusa 24, and blackgram var. KU 91 could be infected by whitefly inoculation but not by agroinoculation.

Table 1. Infection of leguminous hosts with cloned DNA components of MYMV-Bg using A. tumefaciens strain C 58 and virus through whitefly transmission

<table>
<thead>
<tr>
<th>Host</th>
<th>Variety</th>
<th>Agroinoculation of sprouted seed</th>
<th>Agroinoculation of seedling</th>
<th>Whitefly inoculation</th>
<th>Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Disease reaction</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frenchbean</td>
<td>VL 67</td>
<td>4/6* (22)</td>
<td>0/4</td>
<td>4/9(8)</td>
<td>LCr,IC,SL</td>
</tr>
<tr>
<td></td>
<td>Top Crop</td>
<td>3/6(22)</td>
<td>6/6(15)</td>
<td>7/10(8)</td>
<td>LCr,IC,SL</td>
</tr>
<tr>
<td></td>
<td>Contender</td>
<td>2/6(22)</td>
<td>6/11(15)</td>
<td>8/10(10)</td>
<td>IC</td>
</tr>
<tr>
<td></td>
<td>Jwala</td>
<td>0/5</td>
<td>0/4</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HUR 137</td>
<td>-</td>
<td>2/4(15)</td>
<td>7/10(10)</td>
<td>IC</td>
</tr>
<tr>
<td></td>
<td>K.Wonder</td>
<td>-</td>
<td>5/8(15)</td>
<td>3/7(9)</td>
<td>IC</td>
</tr>
<tr>
<td></td>
<td>PDR 14</td>
<td>-</td>
<td>1*8 (15)</td>
<td>5/8(8)</td>
<td>LCr</td>
</tr>
<tr>
<td>Pigeonpea</td>
<td>ICPL 84023</td>
<td>?+21</td>
<td>0/3</td>
<td>2/9(17)</td>
<td>Y speck</td>
</tr>
<tr>
<td></td>
<td>TAT 14</td>
<td>?+25</td>
<td>0/8</td>
<td>0/10</td>
<td></td>
</tr>
<tr>
<td>Cowpea</td>
<td>V 130</td>
<td>-</td>
<td>0/32</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>V 322</td>
<td>0.8</td>
<td>0/24</td>
<td>0/10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>V 218-1</td>
<td>-</td>
<td>0/10</td>
<td>0/10</td>
<td></td>
</tr>
<tr>
<td>Soybean</td>
<td>Ds 75-12-1</td>
<td>?+30</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pusa 16</td>
<td>?+38</td>
<td>?+42</td>
<td>2/5(20)</td>
<td>Y speck</td>
</tr>
<tr>
<td></td>
<td>Pusa 24</td>
<td>0/32</td>
<td>0/13</td>
<td>4/6(15)</td>
<td>Mild YM</td>
</tr>
<tr>
<td>Mungbean</td>
<td>PS 16</td>
<td>18/148(25)</td>
<td>18/50(17)</td>
<td>4/6(8)</td>
<td>Y speck</td>
</tr>
<tr>
<td></td>
<td>P. Baisakhi</td>
<td>4/29(23)</td>
<td>-</td>
<td>4/5(9)</td>
<td>Mild YM</td>
</tr>
<tr>
<td>Blackgram</td>
<td>Pusa 2</td>
<td>0/77</td>
<td>20/100(16)</td>
<td>5/5(9)</td>
<td>Severe YM</td>
</tr>
<tr>
<td></td>
<td>KU 91</td>
<td>0/32</td>
<td>0/32</td>
<td>4/5(9)</td>
<td>Mild YM</td>
</tr>
</tbody>
</table>

a: Number of symptomatic plants/Number of inoculated plants; ( ): Days required for symptoms appearance; *: Symptom was doubtful; not considered as positive infection; LCr: Leaf crinkle; IC: Inward curling; SL: Small leaf; Y: Yellow; YM: Yellow mosaic; +: Small amount of viral DNA detected by NASH in pooled samples of each varieties; these, however, were not considered positive for infection as the replicative forms of viral DNA were not detected in southern hybridization.
In general, the plants infected by agroinoculation took a longer time for symptom development as compared to the plants infected by whitefly inoculation.

**DISCUSSION**

The results presented here clearly demonstrate that the dimeric constructs of MYMV-Bg DNAs are infectious to not only the original host blackgram but also to the other hosts, as has also been found for the cloned DNAs of other Begomovirus like cassava latent virus (CLV) (Morris et al., 1988), BGMV (Morinaga et al., 1988), potato yellow mosaic mosaic virus (PYMV) (Buraguhain et al., 1984), squash leaf curl virus (SLCV) (Lazarowitz and Lazdins, 1991), TGMV (Elmer et al., 1988; Rochester et al., 1990) and TYLCV (Kheyr-Pour et al., 1991; Stenger et al., 1992). Some hosts, however, could be infected by whitefly inoculation but not by agroinoculation (Table 1). This could be due to the possibility of the presence of more than one natural variant of MYMV in the source plants in which the original virus isolate (MYMV-Bg) was maintained. Therefore, for screening for resistance, it would be desirable to use clones of all the commonly occurring variants of MYMV (Varma, 1993).

In all the hosts the time taken for symptoms development after agroinoculation was considerably longer than after inoculation through whiteflies. This difference could be due to the delay in the initial excisions and release of normal length of infectious DNAs from the dimeric constructs used for agroinoculation as described by Mandal et al. (1997). Differences in the development of symptoms in *Nicotiana clevelandii* infected by different constructs of CLV through agroinoculation have also been noticed by earlier workers (Elmer et al., 1988; Morris et al., 1988).

Efficiency of agroinoculation varied from host to host. The Frenchbean varieties appear to be more amenable to agroinfection by MYMV than the other hosts tested. Frenchbean have also been shown to be highly susceptible to agroinoculation of BGMV (Morinaga et al., 1988). Standardization of the method...
of agroinoculation is important as is obvious from the contrasting results obtained in infecting Frenchbean var. VL 67 and blackgram var. Pusa 2 by the two methods tested in the present investigation (Table 1). Agroinoculation can be a useful method for screening for resistance in the breeding programmes but it requires standardization and testing of plants for infection by NASH to avoid inadvertent development and use of susceptible genotypes. As a procedure it would be useful to first screen for resistance by the standardized agroinoculation method and further test the identified resistant genotypes by whitefly inoculation.

ACKNOWLEDGEMENT

The senior author is thankful to the Director, IARI, New Delhi for the award of Senior Research Fellowship.

REFERENCES


Morris, B. A. M., Richardson, K. A., Anderson, M. T.
infections mediated by the Ti plasmid of Agrobacterium
*ti anfaciens* either monomeric or dimeric viral DNA.
*Plant Mol. Biol.* 11: 795-803.

Nariani, T. K. (1960). Yellow mosaic of mung (*Phaseolus

Systemic movement and symptom production of
following agroinoculation with a single DNA of tomato
yellow leaf curl geminivirus (Thiland). *Virology* 178:
520-526.

Southern, E. M. (1975). Detection of specific sequences
among DNA fragments separated by gel electrophoresis.

Limited replication of tomato golden mosaic virus DNA
Interactions* 5: 525-527.

and restriction analysis of mungbean yellow mosaic virus.
In: *International conference on Virology in the Tropics,*
Lucknow, India, pp. 114.

transmission and control in India. In: *Mungbean Yellow
Mosaic Disease* (S. K. Green and D. Kim eds) Asian
vegetable Research and Development Centre, Taipei, pp.
8-27.

disease diseases. In: *Crop protection and sustainable
agriculture*, Wiley Chichester (Ciba Foundation

Received for publication May 3, 2000