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A reliable begomovirus inoculation method for screening *Lycopersicon esculentum* lines

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ABSTRACT

Due to failures of infection after mechanical inoculation of begomovirus on tomato (*Lycopersicon esculentum* Mill.) plants, an efficient and reliable begomovirus inoculation method was developed using the whitefly vector (*Bemisia tabaci*). Virus acquisition was carried out using a 'Tube-cage', made of a polypropylene tube, containing one to two day-old whiteflies and a tomato plant apex infected with a begomovirus isolate. After 48 h of acquisition access period, inoculation was done using a 'Ring-cage', containing viruliferous whiteflies, attached to the abaxial side of the leaf of young tomato plants. 48 hours after acquisition, the whiteflies were eliminated and the inoculated plants were incubated for 28 days under greenhouse conditions. Three viruliferous whiteflies per plant were enough to cause infection in susceptible tomato cv. Viradoro. This procedure was applied to test the resistance of tomato line 486-1 (resistant line) in comparison with 'Viradoro' (susceptible cultivar). Dot blot hybridization confirmed the susceptibility of 'Viradoro' and the resistance of the line 486-1, showing the efficiency of this method for screening plants for disease resistance. This method is also a useful tool in detecting the presence of virus in inoculated (lower leaves) and in non-inoculated (upper leaves) in a given plant. Using this procedure, it was observed that the resistance of the line 486-1 probably is expressed in the inoculated leaf without virus translocation to the upper leaves.

Keywords: *Lycopersicon esculentum*, inoculation, resistance, screening, geminivirus.

RESUMO

Um método de inoculação de begomovírus confiável para a seleção de linhagens de *Lycopersicon esculentim*

Devido às falhas encontradas no método de inoculação de begomovírus em plantas de tomateiro, um método eficiente e confiável de inoculação de begomovírus foi desenvolvido utilizando como vetor a mosca-branca (*Bemisia tabaci*). A aquisição viral foi realizada em uma armadilha ('Tube-cage') feita a partir de um tubo de polipropileno contendo moscas-brancas de um ou dois dias de idade e o ápice de uma planta de tomate infectada com um begomovírus. Após 48 horas de período de aquisição, procedeu-se à inoculação utilizando-se uma armadilha em forma de anel ('Ring-cage') contendo as moscas-brancas virulíferas. A armadilha foi colocada na parte abaxial das folhas de plântulas de tomateiro. Após o período de inoculação de 48 horas, as moscas-brancas foram eliminadas e as plantas inoculadas foram incubadas por 28 dias em casa-de-vegetação. Utilizando-se este método três moscas-brancas virulíferas por planta foram suficientes para causar infecção em tomateiro susceptível cv. Viradoro. Este procedimento foi aplicado para avaliar a resistência de tomateiro linhagem 486-1 (resistente) em comparação com 'Viradoro'. Hibridização por dot-blot confirmou a susceptibilidade de 'Viradoro' e a resistência da linhagem 486-1, mostrando a utilidade deste método na seleção de plantas resistentes. Este método também permitiu a avaliação da presença do vírus nas folhas inoculadas e em folhas superiores não inoculadas, indicando que o mecanismo de resistência da linhagem 486-1 poderá estar atuando nas folhas inferiores inoculadas, não havendo translocação viral para as folhas superiores.

Palavras-chave: *Lycopersicon esculentum*, inoculação, resistência, screening, geminivírus.

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In tropical and subtropical countries, begomoviruses cause serious problems to tomato. The major symptoms after begomovirus infection are yellow mosaic, mottling, rugosity and leaf distortion, resulting in reduction of the number of flowers, fruits, fruit weight and quality. This disease is known to be transmitted by whiteflies (*Bemisia tabaci*). Transmission of the virus by mechanical inoculation is sporadically successful, hence, in nature the dissemination of this disease agent is supposed to hardly ever occur by mechanical means. The first report on the incidence of begomovirus-like disease in tomatoes in Brazil occurred in the 60's (Flores *et al.*, 1960), and later confirmed as the begomovirus *Tomato golden mosaic virus* (TGMV) (Matyis *et al.*, 1975). Costa *et al.* (1984; 1987) described tomato infected with whitefly-transmitted geminiviruses, but their identification was not carried out and remains unknown. The expansion of begomovirus diseases on tomato was only observed during the last decade (Giordano *et al.*, 1999). The characterization of these begomoviruses and the breeding programs aiming to introgress genes for begomovirus resistance on tomato plants are ongoing in various laboratories.

The strategy to control this disease using resistant cultivars focuses mainly on the introgression of resistance genes into tomato commercial cultivars. The introduction of the resistance using transgenic plants is a new approach to develop plants resistant to diseases. However, the current transgenic strategies introducing viral or anti-viral genes into a plant have some disadvantages such as the narrow resistance spectrum to specific virus species, and also some technical and political constraints. Besides, the conventional methods of plant breeding

Table 1. Symptom expression and infection of begomovirus on inoculated tomato plants (Expressão de sintomas e infecção de begomovírus em plantas de tomate inoculadas). Brasília, Embrapa Hortaliças, 2005.

	Viradoro symptom	Dot-Blot	468-1 symptom	Dot-Blot
7-days	1/81	0/4²	0/10	0/3
14-days	6/8	3/4	0/10	0/3
21-days	7/8	3/4	0/10	0/3
28-days	7/8	7/8	1/10	1/10

[1]Number of plants with symptoms (before the slash) out of the total (after the slash); [2]Number of plants positively detected (before the slash) out of the total (after the slash) detected by dot blot hybridization ([1]Número de plantas com sintomas (antes da barra) em relação ao total (após a barra); [2]Número de plantas detectadas positivamente (antes da barra) em relação ao total (após a barra) detectados por hibridização "dot blot").

by inter- and intra-species crosses are viable approaches due to the availability of a large range of resistance genes in the genus *Lycopersicon*. Some level of resistance to *Tomato yellow leaf curl virus* (TYLCV) was already reported in *L. pimpinellifolium*, *L. cheesmani*, *L. hirsutum*, *L. peruvianum* and *L. chilense* (Kasrawi *et al*., 1988; Pilowsky & Cohen, 1974; Picó *et al*., 1996; 1998; Scott *et al*., 1996).

The main limitation for screening resistant plants is related to the inoculation method. Evaluation of plant resistance to a virus is frequently based on mechanical inoculations. However, the systemic infection of tomato begomoviruses by mechanical inoculation is sporadic. Therefore, mechanical inoculation is not a reliable method for screening tomato plants for begomovirus resistance. The particle bombardment method could be a highly efficient option. However, this method requires special equipment and is not suitable for inoculating great numbers of plants. With this method of inoculation it is difficult to control the inoculation pressure in each plant, an important factor to achieve uniformity of infection. Agroinfection is an alternative method, however due to the same reasons mentioned above, it is also not suitable for screening plants for resistance.

Inoculation of begomovirus using whitefly as vectors appears to be the best method at this moment. Hence, it became important to develop a practical and reliable method of begomovirus inoculation using whiteflies. This study introduces a convenient inoculation method, which enables the application of a uniform inoculation pressure to the plants.

MATERIAL AND METHODS

Preparation of viruliferous whiteflies using the Tube-cage

Newly emerged whitefly adults with a maximum age of 48 h were collected using a sucking apparatus from the colonies established on healthy poinsettia plants. A polypropylene tube of 50 ml (Corning) containing a detached infected tomato plant apex (isolate DFM, Tomato mottle leaf curl virus, Inoue-Nagata *et al*., 2006) was attached at the end of the sucking apparatus and the whiteflies were released inside the tube. This new Tube-cage was then covered with a whitefly-proof nylon screen and tightly screw-capped (Figure 1).

Tomato inoculation using the Ring-cage

Three, five or ten viruliferous whiteflies previously prepared using the Tube-cage method (for virus acquisition) were transferred into the newly developed leaf-cages (Figure 2) settled onto the abaxial side of tomato seedlings leaves cv. Viradoro. Tomato plants at four true-leaf staged were used for the inoculation. The whiteflies were placed in contact with the leaves of the plants to be evaluated for disease reaction using a Ring-cage. The inoculation access period was 48 h. The limited space inside the cage forces the insect to feed on the plants to be tested, increasing the efficiency of virus transmission. After 48 h of incubation at room temperature, the whiteflies were eliminated by spraying the insecticide Confidor (imidacloprid, Bayer). The inoculated plants were incubated in the greenhouse up to one month for symptom development. Leaf disks were collected from the inoculated plants to confirm virus infection and tested by dot blot hybridization according to Santos *et al*. (2004).

RESULTS AND DISCUSSION

Number of whiteflies used in the inoculation procedure

Forty-eight hours after inoculation the mortality of the whiteflies was low (averaging 10% for both ten and five whitefly inoculation cohorts, and 16.7% for three whitefly inoculation cohort). All the plants inoculated with ten (6/6) and three whiteflies (6/6) were infected within one month. Only one out of six plants was not infected when five viruliferous whiteflies were used. Since all six plants inoculated with three viruliferous whiteflies were infected with begomovirus, the use of three whiteflies per plant for inoculation was demonstrated to be appropriate for most purposes.

The application of the method for the pre-screening test

In order to test the usefulness of this method for screening, two tomato lines were tested, 'Viradoro', the susceptible cultivar, and the tomato line 468-1, previously selected for resistance to the virus (Santana *et al*., 1998; 2001). Ten plants of '468-1' and eight of 'Viradoro' were inoculated using three whiteflies. The development of symptom was observed and recorded weekly. The number of infected plants of 'Viradoro' and '468-1', based on symptom development and dot-blot hybridization, are presented in Table 1. Based upon symptom development, tomato plants ´Viradoro' showed a high infection rate (7/8). On the other hand, tomato line ´468-1' showed only one plant with symptom. To confirm the virus infection, dot blot hybridization was employed using the leaf materials collected 28 days after inoculation. Seven out of eight plants of ´Viradoro'

showed positive signals and two out of ten tomato plants of the line ´468-1' showed positive infection (data not shown). The result indicated that the procedure could differentiate distinct levels of virus susceptibility (resistance) occurring in tomato varieties.

The virus distribution and the development of the infection process in tomato plants can define the mechanism of resistance. One type of resistance, for instance, is related to long distance virus migration in plants. The virus can replicate in the inoculated leaf but is not able to translocate in the whole plant. Since this method allows the differentiation of the inoculated leaf from the non-inoculated ones on a single plant basis, the inoculated leaf and one top leaf were collected separately for both tomato germplasm during a 28-day period. Four plants of each line were separated and the presence of virus was evaluated at 7, 14, 21, and 28 days after inoculation by dot blot hybridization (Figure 3). The virus was detected in the inoculated and in the non-inoculated leaves (top) of ´Viradoro' within 14 days after inoculation (a.i.) (Figure 3). The virus could also be detected in these leaves up to 28 days a.i. No positive signals were obtained from samples of the inoculated and of the top leaves, when tomato ´468-1' was used as test plants. These results suggested that the resistance involved in tomato ´468-1' is not related to the inhibition of long-distance migration of the virus, but most probably with cell-to-cell movement or within even in earlier stages of virus infection. For the *Ty-1* gene (tolerance to *Tomato yellow leaf curl virus* – TYLCV) the resistance, at low titer inoculum, is expressed through a significant reduction of the viral accumulation in the inoculated tissue, and at high titer inoculum through a significant limitation of the long-distance translocation of the virus (Michelson *et al*., 1994). However for the same virus, a different tomato line neither developed disease symptoms nor accumulated high virus titer (Friedmann *et al*., 1998).

The use of polypropylene (PP) tube cage for virus acquisition and leaf cage for inoculation provided important

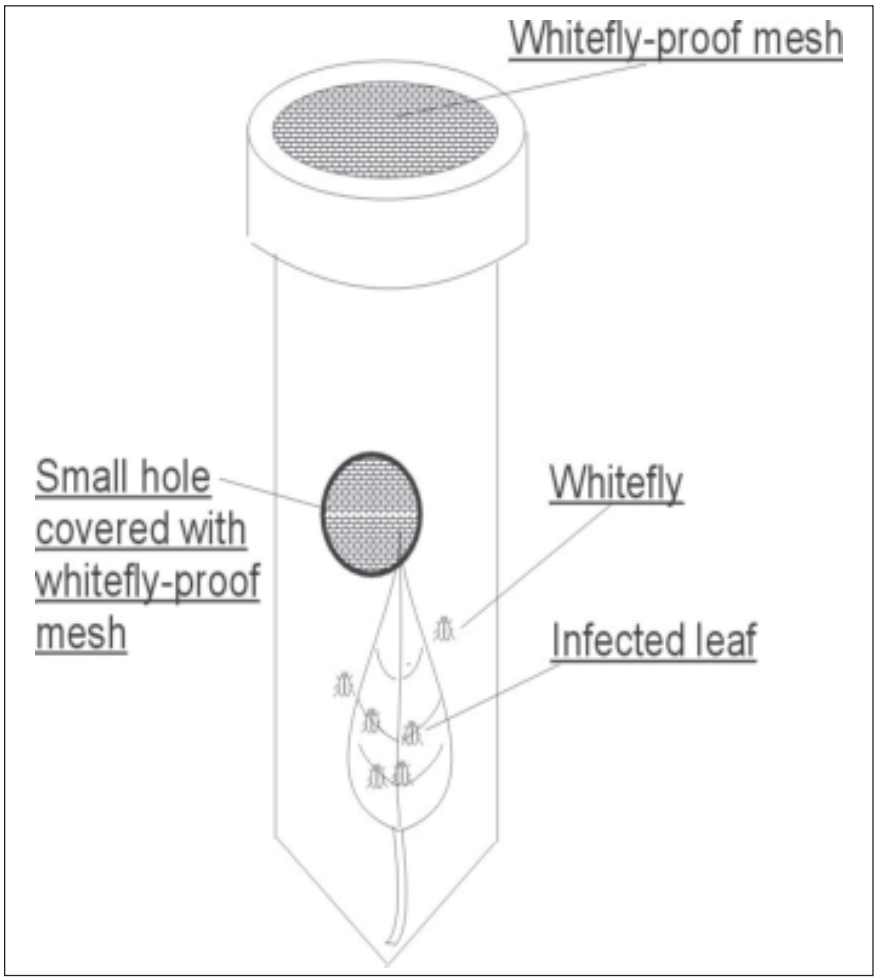

Figure 1. Tube-cage with infected leaf material and whitefly ("Tube-cage" com material foliar infectado e mosca-branca). Brasília, Embrapa Hortaliças, 2005.

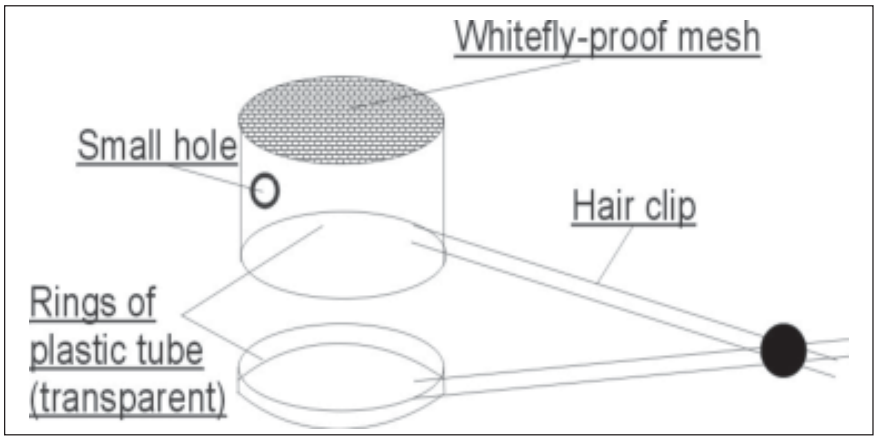

Figure 2. Ring-cage. The leaf is sandwiched between big and small rings of plastic tube and whiteflies were introduced through the small hole by using sucking-blowing apparatus assembled with a narrow silicon tube and yellow tip with wide hole by cutting the top and a piece of screen between them. After the transferrence of the whiteflies, the small hole was sealed with a piece of cotton ("Ring-cage". A folha é colocada entre anéis largos e estreitos de tubos plásticos e moscas-brancas foram introduzidas através de um pequeno orifício usando um aparato sugador, montado com um tubo de silicone estreito com extremidade de ponteira amarela com a ponta alargada por corte e uma barreira de tela entre eles. Após a transferência das moscas-brancas, o pequeno orifício é selado com de algodão). Embrapa Hortaliças, 2005.

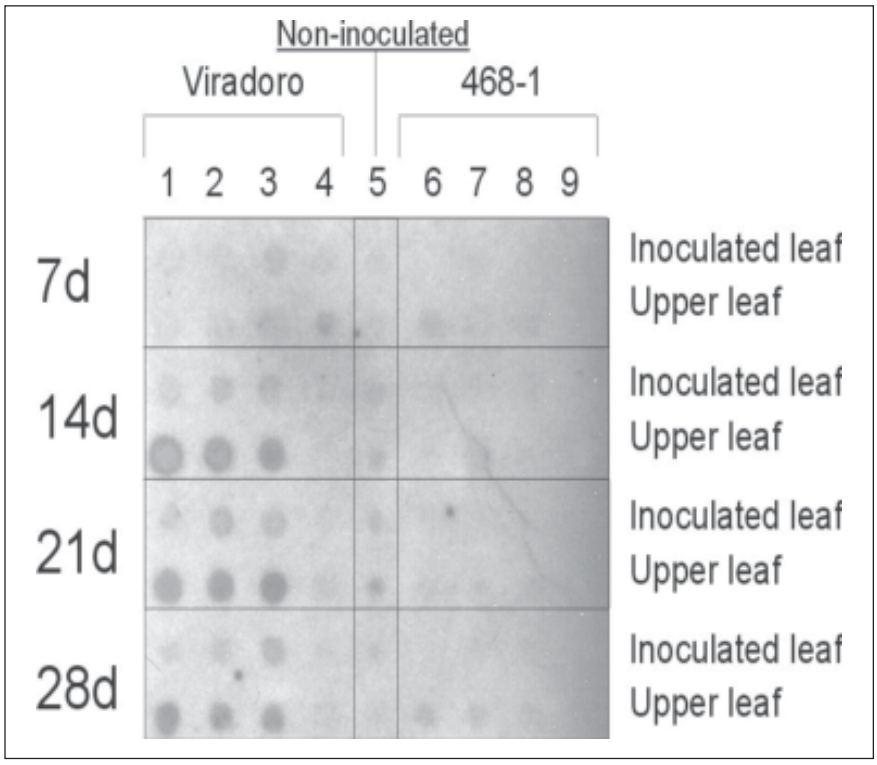

Figure 3. Comparison of susceptibility between ´Viradoro' (lanes 1-4) and ´468-1' (lanes 6-9) to begomovirus infection by dot blot hybridization. Infection on tomato ´Viradoro' (lanes 1-3) became clear from 14 days after inoculation. No positive signals were found in tomato ´468-1' and one tomato plant ´Viradoro' (lane 4). Weak reactions were considered as background reaction. Lane 5 = non-inoculated tomato ´Viradoro'. 7d, 14d, 21d and 28d = 7, 14, 21, 28 days after inoculation (Comparação de susceptibilidade entre 'Viradoro' (colunas 1-4) e '468-1' (colunas 6-9) a infecção por begomovírus por hibridização "dot blot". Infecção de tomate 'Viradoro' (colunas 1-3) tornou-se clara a partir de 14 dias após a inoculação. Nenhum sinal positivo foi encontrado nos tomates '468-1' e em um tomate 'Viradoro' (coluna 4). Reações fracas foram consideradas como negativas. Coluna 5 = tomate 'Viradoro' não inoculado. 7d, 14d, 21d e 28d = 7, 14, 21, 28 dias após a inoculação). Embrapa Hortaliças, 2005.

alternative methods for the improvement of whitefly mediated inoculation during the screening procedure. The PP tube cage with infected leaves was a very effective method for obtaining viruliferous whiteflies. The leaf cage allows the utilization of only three viruliferous whiteflies per plant for proceeding with the inoculation process. It is also worthwhile to observe that the protocol takes a very short time to prepare the viruliferous whiteflies. In addition, this procedure makes inoculation pressure much more uniform and the results more reliable. This inoculation procedure proved to be an efficient and easy method, and demanding a small numbers of whiteflies individuals. Furthermore, it turned out to be a good tool to study the mechanism of begomovirus resistance on tomato.

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