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Tomato severe rugose virus: exploring the potential of soybean as source of inoculum and evidence of no horizontal or vertical transmission by *Bemisia tabaci* MEAM1

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Thesis presented to obtain the degree of Doctor in Science. Area. Plant Pathology

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I dedicate with love and gratitude to

My father Savério (in memoriam);

My mother Isabel;

My wife Cintia.

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SUMMARY

RESUMO

Tomato severe rugose virus: explorando o potencial da soja como fonte de inóculo e evidência da não transmissão horizontal ou vertical por *Bemisia tabaci* MEAM1

O tomateiro (*Solanum lycopersicum*) é uma das principais hortaliças do mundo. Infecções por vírus são uma das causas que podem reduzir a produção de tomate. No Brasil, um dos principais vírus que afetam o tomateiro é o tomato severe rugose virus (ToSRV). O ToSRV pertence ao genero *Begomovirus* e é transmitido pela mosca-branca *Bemisia tabaci*, em uma relação vírus-vetor do tipo persistente circulativa. O ToSRV possui uma ampla gama de hospedeiros incluindo plantas cultivadas e daninhas. Recentemente, foi constatado que plantas de soja (*Glycine max*) podem ter importância na epidemiologia do ToSRV ao atuarem como hospedeiro amplificador do vírus e proporcionarem uma quantidade de inóculo suficiente para a ocorrência de epidemias deste begomovírus em tomateiros. Diante da importância deste fato, o potencial de plantas de soja como fontes de inóculo do ToSRV precisa ser mais explorado. Alguns begomovírus podem ser transmitidos verticalmente para a progênie de fêmeas virulíferas de *B. tabaci*. Também já foi constatada a transmissão de begomovírus horizontalmente, durante a reprodução sexuada entre machos e fêmeas de *B. tabaci*. A transmissão vertical e horizontal pode possuir uma grande importância para a epidemiologia da doença causada pelo ToSRV, uma vez que o inóculo viral pode permancer no próprio vetor, sem a necessidade da presença de plantas hospedeiras do vírus no campo. Os objetivos deste trabalho foram explorar o potencial de plantas de soja como fontes de inóculo do ToSRV e verificar se este begomovírus é transmitido de forma vertical ou horizontal por *B. tabaci* MEAM1. Os resultados demonstraram que plantas de soja podem ser consideradas boas fontes de inóculo do ToSRV. Também foi constatado que o ToSRV não foi transmitido verticalmente para a progênie de fêmeas virulíferas de *B. tabaci* MEAM1 e horizontalmente, durante o acasalamento entre machos e fêmeas deste vetor. Os resultados forneceram informações importantes relacionadas a epidemiologia do ToSRV e que devem ser levadas em consideração no manejo deste begomovírus em cultivos de tomateiros.

Palavras-chave: *Solanum lycopersicum*, *Begomovirus*, Mosca-branca, Epidemiologia

ABSTRACT

Tomato severe rugose virus: exploring the potential of soybean as source of inoculum and evidence of no horizontal or vertical transmission by *Bemisia tabaci* MEAM1

Tomato (*Solanum lycopersicum*) is one of the main vegetables worldwide. Virus infections are among the causes that can reduce tomato production. In Brazil, the tomato severe rugose virus (ToSRV) is one of the most critical viruses in tomato crops. ToSRV, a member of the genus *Begomovirus*, is transmitted by the whitefly *Bemisia tabaci* in a persistent circulative manner. ToSRV has a wide host range, including cultivated and weed plants. Recently, it was found that soybean (*Glycine max*) plants can play an essential role in the epidemiology of ToSRV by acting as an amplifier host of the virus and providing sufficient inocula for epidemics of this begomovirus in tomato crops. Because of the importance of this attribute, the potential of soybean plants as a source of ToSRV inoculum needs to be further explored. Some begomoviruses can be transmitted vertically to the progeny of viruliferous females of *B. tabaci*. Horizontal transmission of begomoviruses during sexual reproduction between *B. tabaci* males and females was also described. Vertical and horizontal transmission can be highly important for the epidemiology of the disease caused by ToSRV, since the viral inoculum can remain in the vector without the need for virus host plants in the field. This study explored the potential of soybean plants as sources of ToSRV inoculum and determined if this begomovirus is transmitted vertically or horizontally by *B. tabaci* MEAM1. The results showed that soybean plants could be good sources of ToSRV inoculum. Also, ToSRV was not vertically transmitted to the progeny of viruliferous females of *B. tabaci* MEAM1 and was not horizontally transmitted during mating between males and females of this vector. These results provided important information related to the epidemiology of ToSRV, and should be taken into account in the management of this begomovirus in tomato crops.

Keywords: *Solanum lycopersicum*, *Begomovirus*, Whiteflies, Epidemiology

1. GENERAL INTRODUCTION

Tomato (*Solanum lycopersicum*) is among the most economically and socially essential vegetables globally. Brazil produces about 4 million tons of tomatoes annually, mainly in the Midwest and Southeast regions (IBGE, 2021). Between 5 and 6 jobs are generated directly and indirectly for each hectare planted with this vegetable in the country (Boiteux et al., 2008).

Among the main phytosanitary problems of tomato plants in Brazil are diseases caused by viruses, especially those belonging to the genus *Begomovirus* (Inoue-Nagata et al., 2016a). Begomoviruses have a genome composed of one (monopartite) or two (bipartite) circular, single-stranded DNA molecules encapsulated in twinned particles. They are transmitted in a persistent circulative manner by the whitefly *Bemisia tabaci* complex (Inoue-Nagata et al., 2016b). The genus *Begomovirus* contains the most species in the family *Geminiviridae*, and the International Committee on Taxonomy of Viruses (ICTV) currently recognizes 445 species (ICTV, 2021). Some of these species are responsible for severe economic damage to crops of agricultural importance in several countries (Navas-Castillo et al., 2011).

In Brazil, outbreaks of begomoviruses became more frequent in crops such as tomato after the introduction of the exotic cryptic species *B. tabaci* Middle East-Asia Minor 1 (MEAM1 or biotype B) in the early 1990s (Lourenção and Nagai, 1994; Inoue-Nagata et al., 2016a). Forty-four cryptic species have been described in the *B. tabaci* complex (De Barro et al., 2011; Kanakala and Ghanim, 2019). Four of these cryptic species are present in Brazil: New World I and New World II (biotype A), species considered native to the Americas, and MEAM1 and Mediterranean (MED or biotype Q), which are the most invasive and harmful species of the *B. tabaci* complex in the world (Kanakala and Ghanim, 2019). *B. tabaci* MEAM1 is currently the most important in Brazil, as it predominates in several regions and shows high transmission efficiency of the main species of begomoviruses in the country (Marubayashi et al., 2013; De Marchi et al., 2017; Bello et al., 2020; Rodrigues et al., 2021). *B. tabaci* MED was identified in Brazil in 2013, in the state of Rio Gande do Sul (Barbosa et al., 2015). Since then, *B. tabaci* MED has been spreading and has already been detected in the states of Santa Catarina, Paraná, São Paulo, Minas Gerais, and Goiás (Moraes et al., 2017, 2018; Bello et al., 2020; Rodrigues et al., 2021).

Among the crops affected by begomoviruses in Brazil, tomatoes are infected by a particularly large number of species. The tomato golden mosaic disease, caused by a complex of begomoviruses, can cause up to 100% loss under high whitefly

pressure (Inoue-Nagata et al., 2016b). Tomato severe rugose virus (ToSRV) is one of the most important begomoviruses affecting crops of this vegetable in the states of Minas Gerais, São Paulo, and Goiás (Inoue-Nagata et al., 2016a; Mituti et al., 2019). The first report of ToSRV infecting tomato plants in Brazil was in 1999, in the state of Minas Gerais (Fernandes et al., 2008). This begomovirus has also been found to naturally infect plants of chili pepper (*Capsicum baccatum* var. *pendulum*), sweet pepper (*C. annuum*), potato (*S. tuberosum*), eggplant (*S. melongena*), tamarillo (*S. betaceum*), turkey berry (*S. torvum*), apple-of-Peru (*Nicandra physalodes*), common bean (*Phaseolus vulgaris*), fiddler's spurge (*Euphorbia heterophylla*), *Malva* spp., *Sida* spp., *Crotalaria* spp., ground cherry (*Physalis angulata*), soybean (*Glycine max*), jícama (*Pachyrhizus erosus*), and garden pink-sorrel (*Oxalis latifolia*) (Bezerra-Agasie et al., 2006; Nozaki et al., 2006; Souza-Dias et al., 2008; Barbosa et al., 2009; Barreto et al., 2013; Macedo et al., 2017a, 2017b; Moura et al., 2018; Duarte et al., 2020; Pereira-Silva et al., 2021). Experimentally, ToSRV was also transmitted to *Amaranthus spinosus*, *Emilia sonchifolia*, *Chenopodium album*, *C. ambrosioides*, *Datura stramonium*, *Nicotiana clevelandii*, *N. tabacum*, *N. rustica*, *N. benthamiana*, *N. debney*, *N. glutinosa*, *S. americanum*, *S. stramonifolium*, *S. asperolanatum*, *S. jamaiscense*, and *S. mammosum* (Lima et al., 2008; Barbosa et al., 2011; Michereff-Filho et al., 2012; Macedo et al., 2015). These alternative ToSRV hosts may have epidemiological importance, acting as a reservoir of the virus in the field.

Management of begomoviruses in tomato crops includes the use of resistant cultivars, application of insecticides for vector control, and a tomato-free period of 2 months (officially implemented only in the state of Goiás) to reduce the sources of virus inoculum (Inoue-Nagata et al., 2016b). The genetic resistance of tomato cultivars to begomoviruses is not complete, and under high inoculum pressure, the symptoms can be severe (Boiteux et al., 2007). Application of insecticides does not result in the expected efficiency. Gouvêa et al. (2017) evaluated the efficacy of the insecticides cyantraniliprole, spiromesifen, thiamethoxam, and cartap in simulated primary and secondary transmission of ToSRV. Although some (cyantraniliprole and cartap) were efficient in controlling secondary transmission of ToSRV, none provided efficient control of primary transmission of this begomovirus. Additionally, epidemiological studies indicated that primary infections, due to the continuous influx of viruliferous whiteflies from outside the field, are the most important dispersal mechanism for ToSRV in tomato crops, which makes management of the disease caused by this

begomovirus more difficult (Barbosa et al., 2016; Bergamin Filho et al., 2016; Macedo et al., 2017c, 2019).

A recently proposed hypothesis suggests that soybean plants could serve not only as a ToSRV reservoir, but also as an amplifier host of the virus and provide the inocula for the occurrence of epidemics in tomato crops (Bergamin Filho et al., 2020). This hypothesis was developed after identification of two tomato crops with a high incidence of ToSRV (57% and 70%) near a field of soybeans where approximately 10% of plants were infected with this begomovirus. Of 42 experimentally inoculated soybean cultivars, 14 proved to be susceptible to ToSRV (Bergamin Filho et al., 2020). Given the importance that soybean plants may have in the epidemiology of ToSRV, further studies are needed to assess the potential of this host as a source of virus inoculum.

To be transmitted to plants, begomoviruses need to travel a long way inside *B. tabaci*. Insects ingest viral particles with their stylets; viral particles pass through the alimentary canal and reach the esophagus and mesentery; passing through the filter chamber and mesentery, they enter the hemolymph and translocate to the primary salivary glands, from which they are finally egested with saliva into the phloem of plants (Czosnek et al., 2017). In some situations, although infrequently, begomoviruses can be transmitted vertically, via the transovarian route, to the progeny. Analyzing the progeny of viruliferous *B. tabaci* females carrying the begomovirus tomato yellow leaf curl virus (TYLCV), Ghanim et al. (1998) found the virus in 46/57 eggs, 25/68 nymphs, and 46/81 adults of the first generation of progeny, i.e., F1. The virus was also detected in 26/68 eggs, 35/49 nymphs, and 41/52 adults of the second generation progeny (F2) of viruliferous females. The authors also reported that 5/50 F1 adults and 4/49 F2 adults were able to transmit TYLCV to tomato plants.

B. tabaci may also transmit begomoviruses horizontally, via mating. This type of transmission was observed for tomato yellow leaf curl China virus (TYLCCNV) and TYLCV, which were transmitted from viruliferous females to males and from viruliferous males to females of *B. tabaci* MEAM1 and MED, during mating between individuals of the same species (Wang et al., 2010). Ghanim and Czosnek (2000) reported in studies with *B. tabaci* MEAM1 that TYLCV was transmitted via mating from viruliferous females to 16/49 males and from viruliferous males to 28/54 females. Insects that acquired TYLCV via mating were also able to transmit this begomovirus to tomato plants. Horizontal and vertical transmission of viruses may have consequences

for the epidemiology of the disease, as the inoculum can remain in the insect vector even in the absence of a virus host in the field (Ghanim et al., 2000; Bosco et al., 2004).

The objective of this study was to clarify aspects of ToSRV epidemiology. The potential of soybean plants as inoculum sources was explored, and horizontal and vertical transmission of this begomovirus by *B. tabaci* MEAM1 was evaluated. The results obtained will provide critical information for managing the disease caused by ToSRV in tomato crops.

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2. EXPLORING THE POTENTIAL OF SOYBEAN AS SOURCE OF INOCULUM OF TOMATO SEVERE RUGOSE VIRUS

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Abstract

Tomato severe rugose virus (ToSRV) is one of the main begomoviruses that infect tomatoes in Brazil. Recent studies indicate that the soybean crop may have epidemiological importance by acting as an amplifier host for the virus and enabling the dissemination of ToSRV to tomato crops. This study explored the potential of soybean plants as a source of ToSRV inoculum, through experiments carried out under greenhouse and field conditions, where the following epidemiological parameters were evaluated: rate of soybean plant infection with ToSRV; efficiency of acquisition and transmission of ToSRV, using soybean plants as the inoculum source; quantification of ToSRV in soybean plants; and preference of the vector for landing on this host. All these parameters were also evaluated for tomato and *Nicandra physalodes*, species known as good sources of inoculum of this begomovirus and which served as references to compare the potential of soybean plants as an inoculum source for ToSRV. Together, the results showed that soybean plants can be considered good sources of ToSRV inoculum and support the recent theory that the soybean crop, under certain conditions, can play a relevant role as an amplifier host in the epidemiology of this begomovirus.

Keywords: begomovirus, whiteflies, epidemiology, *Glycine max,* reservoir, amplifier host

2.1. Introduction

Tomato severe rugose virus (ToSRV) is a member of the genus *Begomovirus* and the family *Geminiviridae*. The virus has a bipartite genome composed of two single-stranded DNA molecules, and is transmitted in a persistent-circulative manner by whiteflies belonging to the *Bemisia tabaci* complex (Inoue-Nagata et al. 2016). ToSRV is one of the main begomoviruses currently infecting tomatoes (*Solanum lycopersicum*) in Brazil (Mituti et al. 2019; Souza et al. 2020). Symptoms caused by

ToSRV infection in tomato plants include vein clearing, mosaic, leaf wrinkling, and eventually reduced plant size (Barbosa et al. 2011; Inoue-Nagata et al. 2016).

Epidemiological studies have indicated that the spread of ToSRV in tomato crops in Brazil occurs predominantly through primary infections due to the continuous influx of viruliferous whiteflies from outside the field (Barbosa et al. 2016; Bergamin Filho et al. 2016; Macedo et al. 2017c, 2019). This indicates that external sources of inoculum are essential for the occurrence of ToSRV epidemics in tomato crops.

In addition to tomato plants, ToSRV has been found to naturally infect plants of sweet pepper (*Capsicum annuum*), chili pepper (*C. baccatum* var. *pendulum*), potato (*S. tuberosum*), eggplant (*S. melongena*), *N. physalodes*, common bean (*Phaseolus vulgaris*), *Euphorbia heterophylla*, *Malva* spp., *Sida* spp., *Crotalaria* spp., and *Physalis angulata* (Bezerra-Agasie et al. 2006; Nozaki et al. 2006; Souza-Dias et al. 2008; Barbosa et al. 2009; Barreto et al. 2013; Macedo et al. 2017a; Moura et al. 2018; Duarte et al. 2020). Experimentally, the virus has been transmitted to various hosts (Barbosa et al. 2011; Macedo et al. 2015). These alternative ToSRV hosts have epidemiological importance, as they act as a reservoir of the virus in the field.

In 2017, asymptomatic infection of soybean plants (*Glycine max*) with ToSRV was reported in Brazil. The virus was detected in 10 of 301 samples collected during the years 2013–2016, in soybean fields located in the states of São Paulo, Minas Gerais and Goiás, indicating for the first time that asymptomatic ToSRV-infected soybean plants can act as ToSRV reservoirs (Macedo et al. 2017b). As soybean plants are good hosts for whiteflies (Baldin et al. 2017) and cultivation of this species of Fabaceae near tomato crops is relatively common in Brazil, conditions are ideal for these plants to function as a source of ToSRV inoculum for tomato plants.

In 2018, a high incidence of ToSRV was observed in a tomato crop located near a soybean plantation in the municipality of Sumaré, state of São Paulo. At that time, approximately 10% of the soybean plants were asymptomatically infected with ToSRV, while 57–70% of the tomato plants were infected with the virus (Bergamin Filho et al. 2020). The authors pointed out that ToSRV-infected soybean plants can act not only as a virus reservoir but as an amplifier host, capable of providing inocula with sufficient force of infection to cause epidemics in tomato crops, even without the occurrence of secondary infections, due to intensive insecticide spraying (Bergamin Filho et al. 2020).

Soybean is the largest crop in terms of planted area in Brazil, at around 37 million hectares in 2020 (IBGE 2021). The damage caused to soybean plants resulting

from infection with ToSRV is presently unknown, but the presence of virus in this host may represent a phytosanitary threat for tomato crops.

This study explored the potential of soybean plants as sources of ToSRV inoculum, seeking to expand knowledge of the role of this crop in the occurrence of ToSRV epidemics in tomato fields in Brazil.

2.2. Materials and methods

2.2.1. Test plants, ToSRV isolate, and colony of *B. tabaci* MEAM1

Plants of soybean cv. TMG 7262 RR, tomato cv. Compack, *N. physalodes*, and *Solanum americanum* were used for greenhouse and field assays. All plants were obtained from seeds sown in 1.5 liter pots containing a mixture of soil, sand, and autoclaved manure. The plants were kept in a greenhouse inside insect-proof cages until they were used in the experiments.

A ToSRV isolate collected in 2018 from tomato plants in the municipality of Sumaré, state of São Paulo, Brazil, was used. The virus isolate has been maintained in tomato plants in a greenhouse (temperature: 25 ± 10 °C) at the Department of Plant Pathology and Nematology, ESALQ/USP. The partial nucleotide sequence of this isolate has a 96–98% identity with the corresponding nucleotide sequences of other ToSRV isolates (Favara et al. 2020).

For the transmission assays, virus-free adults of *B. tabaci* MEAM1 reared on collard plants (*Brassica oleraceae*) and maintained in a greenhouse were used. To verify the purity of the colony, the identification of whiteflies was performed by PCR, using the primers Bem23F and Bem23R, which makes it possible to identify the cryptic species *B. tabaci* Middle East-Asia Minor 1 (MEAM1) and Mediterranean (MED) (De Barro et al. 2003; Kontsedalov et al. 2012).

2.2.2. Detection of ToSRV by PCR

Total DNA from plant and insect tissues was extracted as Favara et al. (2020) described. DNA extracted from infected plants and viruliferous insects were used as positive controls and DNA extracted from healthy plants and aviruliferous insects as negative controls. The extracted DNA was used to detect the begomovirus, using the universal primers PAR1c496 and PAL1v1978 (Rojas et al. 1993). Reactions were

performed with 3 µl of total DNA, 12.5 µl of GoTaq® Green Master Mix (Promega), 7.5 µl of DNAse-free water, and 1 µl of sense and antisense primers, at a concentration of 20 µM. Amplification conditions were: 95 °C for 2 min, followed by 30 cycles of 94 °C for 40 s, 54 °C for 60 s, 72 °C for 60 s, and a final extension at 72 °C for 10 min. The PCR product was subjected to agarose gel electrophoresis, and the amplicons were visualized on a UV light transilluminator. Whenever necessary, some amplicons were sent for nucleotide sequencing to confirm the virus identity.

2.2.3. ToSRV quantification by qPCR

Initially, a standard curve was constructed using clones previously obtained by Bampi et al. (2019). Plasmids containing a 1.1-kb ToSRV fragment were quantified by spectrophotometer (Nanodrop™ 2000, Thermo Scientific) and used to construct the standard virus concentration curve. Serial dilution was performed by the number of copies (10^2 to 10^8 copies μl^{-1}), following the procedures described by Applied Biosystems (https://tools.thermofisher.com/content/sfs/brochures/cms_042486.pdf). Reactions were performed using 1.25 µl (100 ng/µl) of DNA, 6.25 µl 2X GoTaq qPCR Master Mix (Promega), 0.62 µl of ToSRV-F and ToSRV-R primers (Bampi et al. 2019) at 10 µM, 0.19 µl CXR reference dye, and 3.57 µl of water. DNA samples extracted from healthy soybean, tomato, and *N. physalodes* plants were used as negative controls. Quantified samples were analyzed in triplicate. The presence of non-specific amplifications was determined through analysis of the melting curve. The results obtained were transformed to the log copy number: log 10 = number of virus copies.

2.2.4. Greenhouse experiments

2.2.4.1. ToSRV infection rate in soybean, tomato, and *N. physalodes* plants in no-choice transmission tests with *B. tabaci* MEAM1

With the aid of an entomological aspirator, virus-free *B. tabaci* MEAM1 adults were collected from collard leaves and transferred to glass jars (500 mL) containing tomato leaves infected with the ToSRV isolate. The insects were allowed a virus acquisition access period (AAP) of 24 h. Seven plants of each species, at the 3–4 true-leaf stage, were confined individually in cages made with plastic cups and voile fabric. Thirty-five ToSRV-viruliferous adults of *B. tabaci* MEAM1 were then confined in each

cage for a virus inoculation access period (IAP) of 24 h. After the IAP, the plants were sprayed with the insecticide cyantraniliprole to eliminate the insects. The plants were kept in insect-proof cages inside a greenhouse, and the virus infection was confirmed 30 days after inoculation, through observation of symptoms and PCR. The experiment was performed twice.

2.2.4.2. Efficiency of ToSRV acquisition by *B. tabaci* MEAM1 in infected soybean, tomato, and *N. physalodes* and subsequent transmission to tomato plants

Three ToSRV-infected plants of soybean, tomato, and *N. physalodes* were used as sources of inoculum, 30 days after inoculation. One leaf of each plant was placed in a separate glass jar, and then 60 virus-free adult females of *B. tabaci* MEAM1 were confined in each jar for a virus AAP of 24 h. Thereafter, 10 whitefly adults were collected from each source of inoculum and individually tested for the presence of ToSRV by PCR to determine the efficiency of virus acquisition. To assess transmission efficiency, females from each source of inoculum were transferred to healthy tomato plants for an IAP of 24 h. Six tomato plants were inoculated with 5 adults of *B. tabaci* MEAM1 from each inoculum source. ToSRV-infected leaves of soybean, tomato, and *N. physalodes* used for virus acquisition by whiteflies were analyzed by qPCR to determine the virus titer.

2.2.4.3. Efficiency of ToSRV-infected soybean, tomato, and *N. physalodes* plants as sources of inoculum for *B. tabaci* MEAM1 and subsequent transmission to plants of the same species, in choice tests with the vector

A ToSRV-infected plant each of soybean, tomato, and *N. physalodes* was placed separately in the center of an insect-proof cage. Four healthy plants of soybean, tomato, and *N. physalodes* were randomly placed around the plant source of inoculum, totaling 12 healthy plants per cage. One hundred twenty virus-free adults of *B. tabaci* MEAM1 were released into each cage. The insects had a chance to acquire and transmit the virus to the test plants during a 72-h confinement. After that period, the plants used as sources of inoculum were removed from the cages and the insecticide cyantraniliprole was applied to eliminate any whitefly from the test plants. Virus

infection was confirmed 30 days after inoculation, through observation of symptoms and PCR. The test was performed twice.

2.2.4.4. Preference of viruliferous and aviruliferous adults of *B. tabaci* MEAM1 for landing on ToSRV-infected or mock-inoculated soybean, tomato, and *N. physalodes* plants

Soybean, tomato, and *N. physalodes* plants at the 3–4 true-leaf stage were inoculated with ToSRV, using 35 viruliferous adults of *B. tabaci* MEAM1 per plant. Plants of the same species were mock-inoculated using 35 aviruliferous adults of *B. tabaci* MEAM1 per plant. Twenty-four hours later, all whiteflies were removed from all plants with the aid of an entomological aspirator. Twenty-five days after insect removal, all plants were evaluated to confirm the presence or absence of ToSRV infection by PCR.

Viruliferous adults of *B. tabaci* MEAM1 were obtained after a 24-h virus AAP in ToSRV-infected *S. americanum* leaves. Aviruliferous adults of *B. tabaci* MEAM1 were confined for the same period on healthy *S. americanum* plants. This procedure was adopted to prevent previous acclimatization of the insects to the different species of test plants used for the landing-preference assays.

To evaluate whitefly preference for landing, the above-described ToSRV-infected and mock-inoculated plants were placed in four insect-proof cages (45 x 45 x 55 cm), and the following treatments were compared: aviruliferous insects and mock-inoculated plants; aviruliferous insects and ToSRV-infected plants; viruliferous insects and mock-inoculated plants, and viruliferous insects and ToSRV-infected plants. Mock-inoculated and ToSRV-infected soybean, tomato, and *N. physalodes* plants were placed equidistantly inside the insect-proof cages, one plant of each species per cage, using two cages with mock-inoculated plants and two with ToSRV-infected plants. According to the respective treatment, 120 aviruliferous or viruliferous adults of *B. tabaci* MEAM1 were released separately in each cage. The insects were initially confined in 1.5-ml microcentrifuge tubes with the aid of an entomological aspirator. The microcentrifuge tubes were then placed on a flight platform positioned on the top (center) of each cage for insect release. The time established for the release of insects was 09:00 h. The number of whiteflies landed on each plant 6 h and 24 h after the insect release was evaluated with the aid of a mirror to facilitate observation of insects on the abaxial side of the leaves. The test was performed three times.

2.2.5. Field experiment

2.2.5.1. Potential of ToSRV-infected soybean, tomato, and *N. physalodes* plants as sources of inoculum in the field

Two experiments were conducted in four areas located on the ESALQ/USP campus, far from each other, to avoid possible interference between the different sources of inoculum and the control. The distance between the experimental areas ranged from 310 to 1,875 m (Supplementary Fig. 1). The first experiment was performed from December 2018 to February 2019 and the second experiment from October to December 2019.

Two hundred healthy tomato plants were transplanted in each experimental area at the 3–4 true-leaf stage. Plants were spaced 1 m x 0.5 m apart. The tomato plants were vertically staked with bamboo poles, and fertilized and irrigated according to their requirements. Ten previously ToSRV-infected tomato, soybean, and *N. physalodes* plants were evenly transplanted outside and around the blocks of tomato plants (one species per block). The control area did not receive a source of ToSRV inoculum. Plants of the different species evaluated as sources of inoculum were produced in pots in a greenhouse. Fifteen days after emergence, they were inoculated with ToSRV by viruliferous adults of *B. tabaci* MEAM1. Virus infection was confirmed by PCR 30 days after inoculation. Plant sources of inoculum and healthy tomato test plants were transplanted to the field on the same day. Four collard plants infested with aviruliferous whiteflies were placed in each experimental area to guarantee the presence of the vector. The plants remained inside the cages, and once a week the cage was opened to release the insects (Supplementary Fig. 2).

To avoid vector colonization on tomato plants and minimize the occurrence of secondary ToSRV infections, the insecticide cyantraniliprole was applied to each plant via the soil at 15-day intervals. For additional prevention of secondary spread, all symptomatic tomato plants identified during the assessments of the incidence of ToSRV were eradicated at each evaluation.

The evaluations of the incidence of ToSRV-infected tomato plants in each experimental area took place at 6-day intervals and were conducted up to 60–70 days after transplanting in the field. Leaf-tissue samples of some symptomatic and asymptomatic plants from each area were collected to confirm the presence and absence of the virus by PCR. Some amplicons obtained were sent to Macrogen Inc (Seoul, South Korea) for nucleotide sequencing. The test was performed twice.

2.2.6. Statistical analysis

The chi-square test was applied to the data for the rate of ToSRV infection, acquisition efficiency, and transmission efficiency in the experiments in the greenhouse and experimental field. For the analyses of ToSRV concentration and landing preference of *B. tabaci* MEAM1, the data were previously analyzed to verify normality by the Shapiro-Wilk test and for homogeneity of variances by the Brown-Forsythe test. Subsequently, the data were analyzed by one-way ANOVA followed by Tukey's test to compare means. Analyses were performed using GraphPad Prism software, version 7.

2.3. Results

2.3.1. Greenhouse experiments

2.3.1.1. ToSRV infection rate in soybean, tomato, and *N. physalodes* plants in no-choice transmission tests with *B. tabaci* MEAM1

There was no difference (X^2 = 1.413, df 2, p = 0.4933) in the rates of infection of soybean, tomato, and *N. physalodes* plants with ToSRV in the transmission tests. Of 14 plants of each species inoculated, 7 soybeans, 10 tomato, and 9 *N. physalodes* plants were infected (Fig. 1a).

2.3.1.2. Efficiency of ToSRV acquisition by *B. tabaci* MEAM1 in infected soybean, tomato, and *N. physalodes* and subsequent transmission to tomato plants

The virus acquisition efficiency of females of *B. tabaci* MEAM1 fed on ToSRV-infected plants of soybean, tomato, and *N. physalodes* was similar (X^2 = 2.2, df 2, p = 0.3297). Of the 60 females allowed a 24-h virus AAP on ToSRV-infected soybean and tomato, 50 and 55 acquired the virus, respectively (Fig. 1b, Supplementary Table 1). For ToSRV-infected *N. physalodes* plants, 45 of 50 females analyzed acquired the begomovirus (Fig. 1b, Supplementary Table 1).

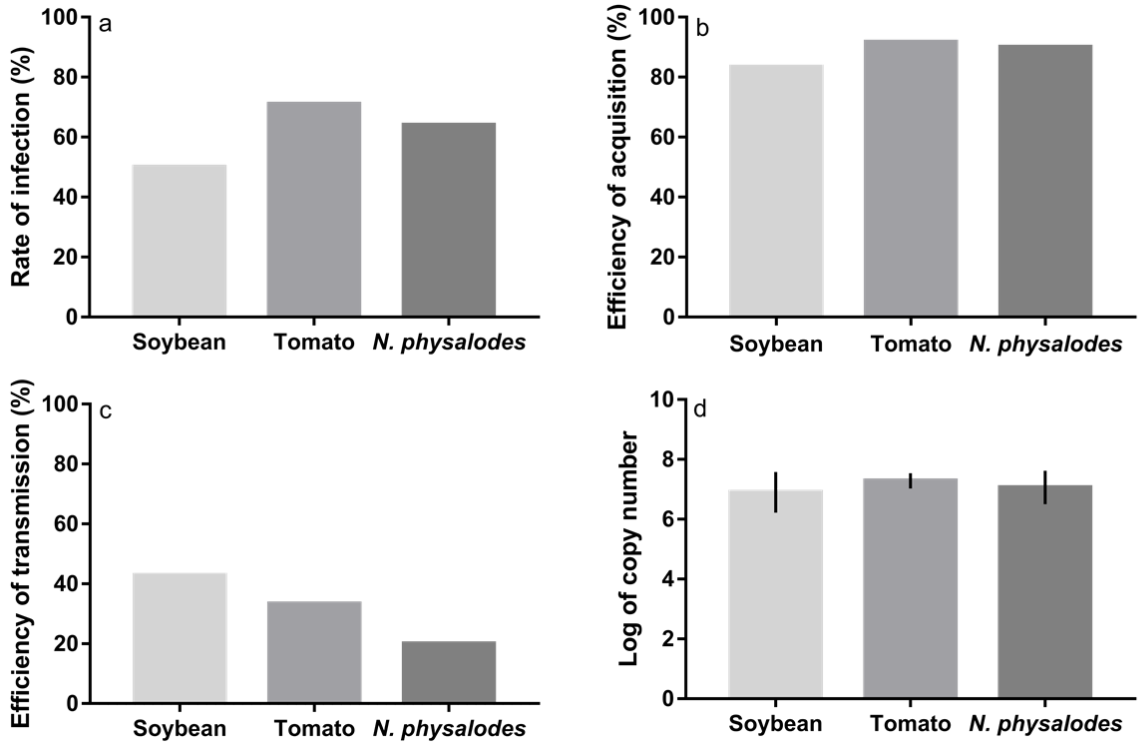

Fig. 1. a) Rate of infection of soybean, tomato, and *N. physalodes* plants with ToSRV in no-choice transmission tests with the vector; b) Efficiency of ToSRV acquisition by individual females of *B. tabaci* MEAM1 fed on infected plants of soybean, tomato, and *N. physalodes*. c) Efficiency of ToSRV transmission to tomato plants, using soybean, tomato, and *N. physalodes* as sources of inoculum. D) Titer of ToSRV in soybean, tomato, and *N. physalodes* infected plants used as sources of inoculum based on standard curve. Slope –3.39, intercept 39.13, R^2 0.99, and efficiency 97%. Means are not significantly different ($p > 0.05$) according to the chi-square (a, b, and c) and Tukey (d) tests.

There was no difference in the transmission efficiency of ToSRV to tomato plants when *B. tabaci* MEAM1 acquired the virus in ToSRV-infected soybean, tomato, and *N. physalodes* plants used as sources of inoculum ($X^2 = 3.848$, df 2, $p = 0.1460$). When ToSRV-infected soybean plants were the sources of inoculum, the transmission efficiency was 43% (15/35). When the virus was acquired in ToSRV-infected tomato and *N. physalodes* plants, the transmission efficiencies were 33% (12/36) and 20% (6/30), respectively (Fig. 1c, Supplementary Table 1).

Leaves of ToSRV-infected soybean, tomato, and *N. physalodes* plants used as sources of inoculum for *B. tabaci* MEAM1 had similar ToSRV titers ($F = 0.7975$, df 2, $p = 0.4699$). The mean log copy numbers of ToSRV in soybean, tomato, and *N. physalodes* leaves used for virus acquisition by *B. tabaci* MEAM1 were 6.9, 7.3, and 7.1, respectively (Fig. 1d, Supplementary Table 1). The cycle threshold (CT) for soybean, tomato, and *N. physalodes* plants used as sources of inoculum varied,

respectively, from 13.67 to 20.22, 13.51 to 15.46, and 12.68 to 17.23 (Supplementary Table 2).

2.3.1.3. Efficiency of ToSRV-infected soybean, tomato, and *N. physalodes* plants as sources of inoculum for *B. tabaci* MEAM1 and subsequent transmission to plants of the same species, in choice tests with the vector

There was a difference (X^2 = 7.664, df 2, p = 0.0217) in the infection rate of soybean, tomato, and *N. physalodes* plants in the transmission tests with a chance of choice by *B. tabaci* MEAM1 that acquired the virus from infected plants of the same species (Table 1). Averaging data from two independent experiments showed that the percentage of tomato plants infected with ToSRV (50%) was higher than that of soybean (17%) and *N. physalodes* (21%) plants infected with this begomovirus (Table 1).

However, ToSRV-infected soybean, tomato, and *N. physalodes* plants were similarly efficient as sources of inoculum for *B. tabaci* MEAM1 and subsequent virus transmission (X^2 = 0.4034, df 2, p = 0.8174) in tests with a chance of choice by the vector (Table 1). Using soybean plants as the source of inoculum, the transmission efficiency of ToSRV was 25% (Table 1). When tomato and *N. physalodes* were used as sources of inoculum, the virus transmission efficiencies were 33% and 29%, respectively (Table 1).

Table 1: Efficiency of ToSRV-infected soybean, tomato, and *N. physalodes* plants as sources of inoculum for *B. tabaci* MEAM1 and rate of infection of the same species, in tests with chance of choice of the vector.

Source of inoculum	no. infected plants/ no. inoculated plants			Efficiency of transmission (%)
	Soybean	Tomato	*N. physalodes*	
Soybean	1/8	4/8	1/8	25 a
Tomato	1/8	5/8	2/8	33 a
N. physalodes	2/8	3/8	2/8	29 a
Rate of infection (%)	17 b	50 a	21 b	

Means followed by different letters within the rows or columns, are significantly different (p<0.05) according to the chi-square test.

2.3.1.4. Preference of viruliferous and aviruliferous adults of *B. tabaci* MEAM1 for landing on mock-inoculated or ToSRV-infected soybean, tomato, and *N. physalodes* plants

The results of three independent experiments indicated a difference in the number of viruliferous adults of *B. tabaci* MEAM1 landing on mock-inoculated soybean, tomato, and *N. physalodes* plants, 6 h ($F = 42.36$, df 8, $p = 0.0003$) and 24 h ($F = 16.06$, df 8, $p = 0.0039$) after insect release. For both periods, more insects landed on tomato plants than on soybean and *N. physalodes* (Fig. 2a, b). There was no difference in the number of aviruliferous whiteflies landed on mock-inoculated soybean, tomato, and *N. physalodes* plants 6 h ($F = 0.2313$, df 8, $p = 0.8003$) and 24 h ($F = 0.2977$, df 8, $P = 0.7529$) after the release (Fig. 2c, d).

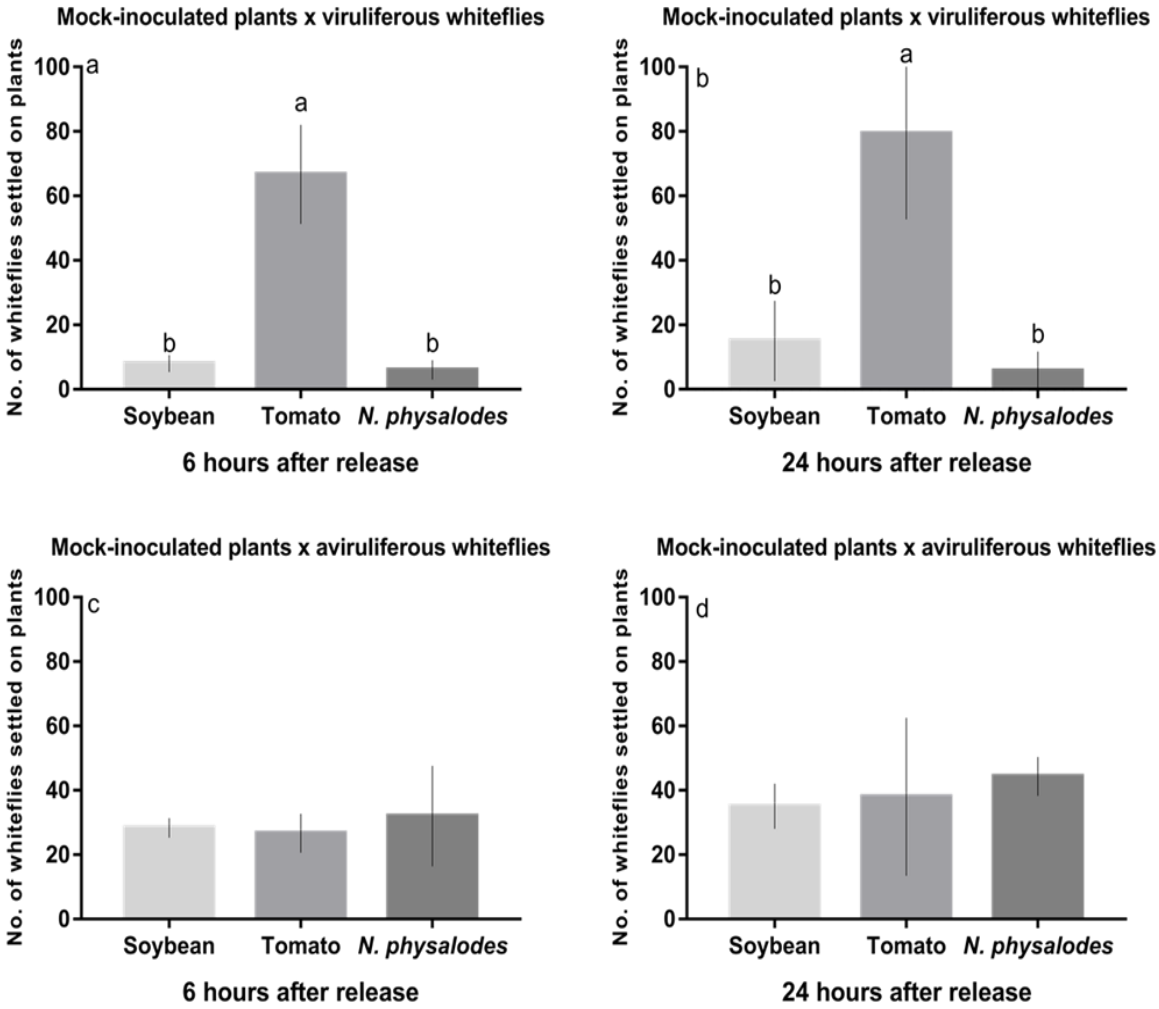

Fig. 2. Number of viruliferous (a/b) and aviruliferous (c/d) adults of *B. tabaci* MEAM1 settled on soybean, tomato, and *N. physalodes* mock-inoculated plants, 6 (a/c) and 24 (b/d) h after the insects were released in the cage. Means with different letters are significantly different ($p < 0.05$) according to the Tukey test.

There was a difference in the preference of viruliferous *B. tabaci* MEAM1 for landing on ToSRV-infected plants of soybean, tomato, and *N. physalodes* 6 h ($F = 14.82$, df 8, $p = 0.0048$) and 24 h ($F = 6.51$, df 8, $p = 0.0314$) after insect release. Tomato plants were the most preferred for landing by adults of *B. tabaci* at 6 h (Fig. 3a). After 24 h, tomato and soybean were the most preferred for landing (Fig. 3b). There was no difference in the number of aviruliferous whiteflies landed on ToSRV-infected soybean, tomato, and *N. physalodes* plants 6 h ($F = 0.3194$, df 8, $p = 0.7382$) and 24 h ($F = 1.112$, df 8, $p = 0.3882$) after insect release (Fig. 3c, d). Insect host preference studies are by nature highly variables among replications and this fact was observed in the present study (see error standard [vertical lines] on Figs 2 and 3).

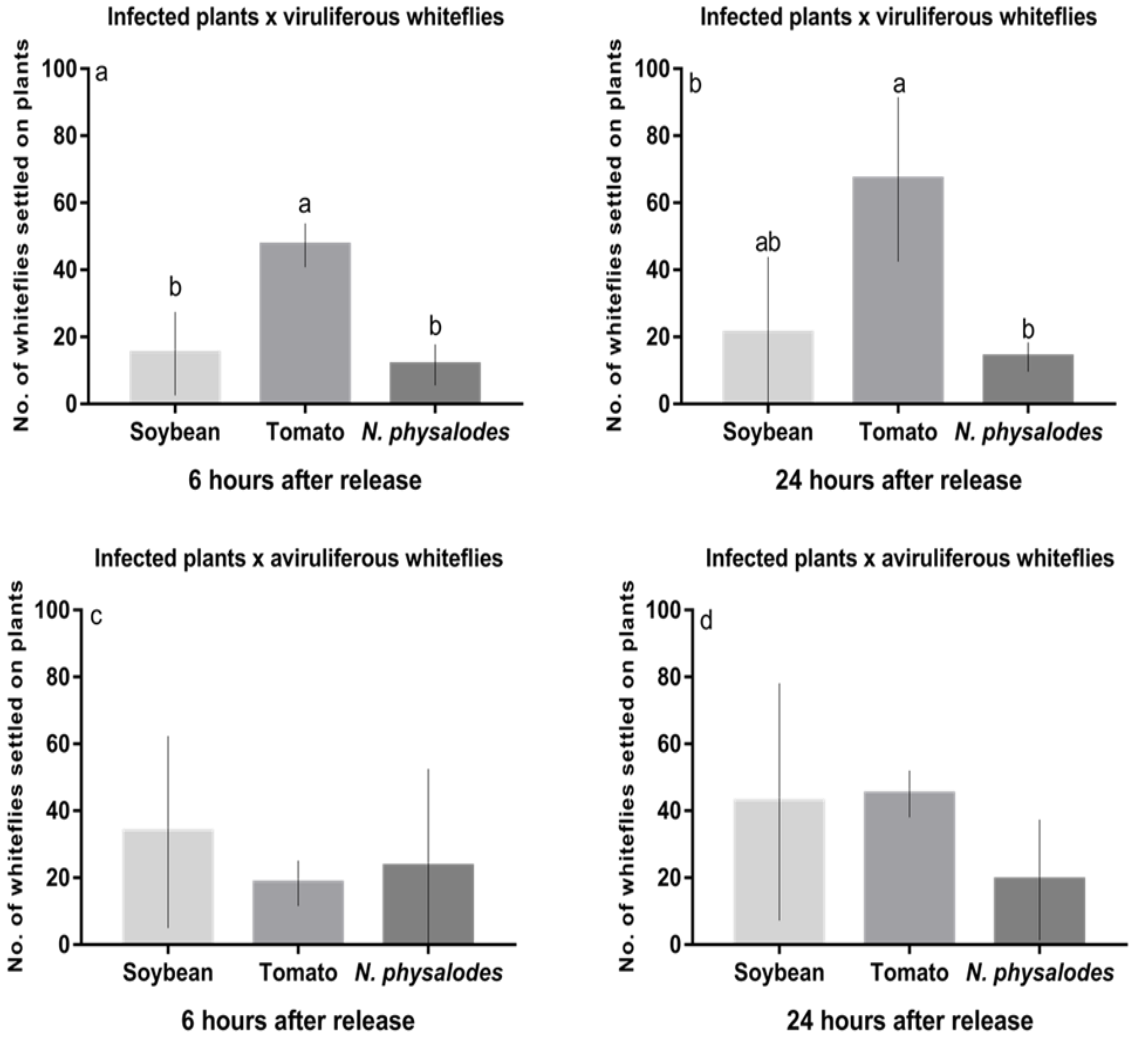

Fig. 3. Number of viruliferous (a/b) and aviruliferous (c/d) adults of *B. tabaci* MEAM1 whiteflies settled on ToSRV-infected soybean, tomato, and *N. physalodes* plants, 6 (a/c) and 24 (b/d) h after the insects were released in the cage. Means with different letters are significantly different ($p < 0.05$) according to the Tukey test.

2.3.2. Field experiment

2.3.2.1. Potential of ToSRV-infected soybean, tomato, and *N. physalodes* plants as sources of inoculum for tomato plants in the field

In the first experiment, the highest incidence of ToSRV-infected tomato plants occurred in the area where infected *N. physalodes* plants were used as sources of inoculum. In this area, the onset of symptoms occurred 28 days after transplanting (DAT), and at 64 DAT the incidence of infected plants reached 50% (100/200) (Fig. 4a). In the area where ToSRV-infected tomato plants were used as the source of inoculum, the first symptomatic plants were observed at 28 DAT. The maximum incidence of infected tomato plants in this area, 38.5% (77/200), was obtained at 64 DAT (Fig. 4a). In the experimental area where ToSRV-infected soybean plants were evaluated as the source of inoculum, 8% (16/200) of tomato plants were infected with the virus 64 DAT (Fig. 4a). In this field, the first symptomatic plants were observed at 35 DAT (Fig. 4a). No tomato plants showed symptoms of infection in the area without a ToSRV source of inoculum (control) at 60 DAT (Fig. 4a).

In the second experiment, the highest incidence of ToSRV-infected tomato plants was in the area where infected tomato plants were the sources of inoculum. In this area, the onset of symptoms occurred at 27 DAT, and at 45 DAT the incidence of infected plants reached 100% (186/186) (Fig. 4b). In the area where ToSRV-infected *N. physalodes* plants were used as sources of inoculum, the first symptomatic tomato plants were observed at 29 DAT. The maximum incidence of infected tomato plants (65%, 113/175) was obtained at 59 DAT (Fig. 4b). In the experimental area where ToSRV-infected soybean plants were evaluated as sources of inoculum, 28% of tomato plants were infected (49/177) (Fig. 4b). No ToSRV-symptomatic tomato plants were observed in the area without sources of ToSRV inoculum (control) at 59 DAT (Fig. 4b).

The means of data from the two experiments indicated that the percentage of tomato plants infected with ToSRV in the field was influenced by the plant used as the source of inoculum (X^2 = 497, df 3, $p < 0.0001$). The percentage of ToSRV-infected tomato plants was higher in the area where ToSRV-infected tomato plants were used as sources of inoculum, followed by areas where ToSRV-infected *N. physalodes* and soybean plants, respectively, were used as virus sources of inoculum (Fig. 4c).

PCR detected ToSRV in all 10 samples of symptomatic tomato plants collected from each experimental area where ToSRV-infected soybean, tomato, and *N. physalodes* plants were evaluated as sources of inoculum in the first experiment. In the second experiment, all 20 tomato samples from each area tested positive for ToSRV by PCR. Three amplicons obtained from tomato plants of each experimental area, in both experiments, were sequenced. The analysis of the nucleotide sequences confirmed the infection with ToSRV. The begomovirus was not detected in 10 and 20 asymptomatic tomato samples collected from the control area, in the first and second experiments, respectively.

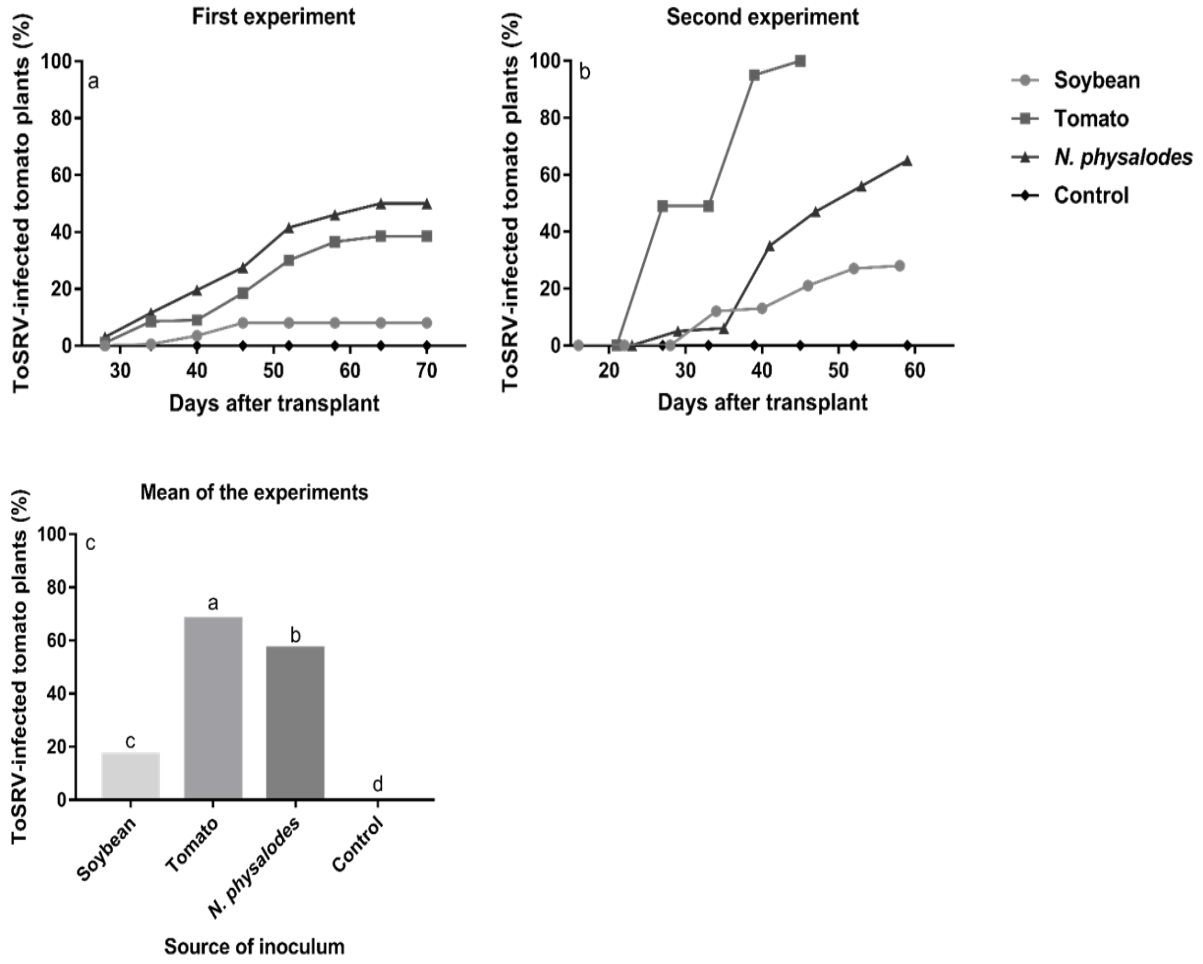

Fig. 4. Progress curves of ToSRV-infected tomato plants, in the first (a) and second (b) experiment, in the areas where ToSRV-infected soybean, tomato, and *N. physalodes* plants were used as sources of inoculum, and in the control area with no source of inoculum. C) Means of the percentage of ToSRV-infected tomato plants in the respective areas. Means with different letters are significantly different ($p < 0.05$) according to the chi-square test.

2.4. Discussion

An essential piece of information for the proper management of diseases caused by viruses is the potential that alternative hosts have in the epidemiology of the disease in the field. The following parameters can affect the potential of a host as a source of virus inoculum: infection rate, virus concentration in the plant tissue, viral isolate, host population density near the crop, length of stay of the host in the field (annual or perennial), and the vector's preference for the host (Kil et al. 2015; Esquivel-Fariña et al. 2021). Some of these parameters were evaluated in the present study, to better understand the potential of soybean plants as a source of ToSRV inoculum for tomato crops. Tomato (primary host) and *N. physalodes* (weed) plants were chosen as references for evaluating the potential of ToSRV-infected soybean plants as a source of inoculum for this begomovirus, as previous studies reported plants of both species as good sources of inoculum of ToSRV under experimental conditions (Barbosa et al. 2011; Macedo et al. 2015; Gorayeb et al. 2020).

In the experimental transmission assays conducted with no chance of choice by *B. tabaci* MEAM1, the rates of ToSRV infection of soybean, tomato, and *N. physalodes* plants were not significantly different (Fig. 1a). Soybean plants had an infection rate of 50%. As shown by Bergamin Filho et al. (2020), the cultivar influences the infection rate of soybean plants with ToSRV. In a trial with 42 soybean cultivars, 14 were susceptible to ToSRV, with infection rates of 10–40%.

When ToSRV-infected soybean, tomato, and *N. physalodes* plants were used as sources of inoculum for tests of transmission by *B. tabaci* MEAM1 to healthy plants of soybean, tomato, and *N. physalodes*, with a chance of choice by the vector, the infection rates of tomato plants were 50%, 62.5%, and 37.5%, respectively. The ToSRV infection rates of plants of soybean and *N. physalodes*, when the same plants were used as sources of inoculum, ranged from 12.5% to 25% (Table 1). The difference in the rates of ToSRV infection in soybean, tomato, and *N. physalodes* obtained in the transmission experiments performed with and without a chance of choice by *B. tabaci* MEAM1 could be explained by the fact that in the choice tests, some plants may not have been properly inoculated, due to the presence of other, more attractive hosts for the vector (Macedo et al. 2015). The results indicated that tomato plants were probably more attractive to whiteflies than were soybean and *N. physalodes* plants.

The virus concentration in the host source of inoculum can significantly affect its acquisition and transmission efficiencies by the vector. A study conducted with alternative hosts of the cucurbit yellow stunting disorder virus (CYSDV) found that *Cucurbita foetadissima* plants had the highest concentration of the virus among the evaluated host species. Consequently, the transmission rate of CYSDV to zucchini (*Cucurbita pepo*) plants was highest when *C. foetadissima* was used as a source of virus inoculum (Wintermantel et al. 2016). Esquivel-Fariña et al. (2021) reported a positive correlation between the concentration of tomato chlorosis virus (ToCV) in the plant source of inoculum and the amount of virus acquired by *B. tabaci* MEAM1, and subsequently, there was also a positive correlation between the concentration of the virus in the vector and the transmission rate for tomato plants. The lack of differences in ToSRV transmission rates by *B. tabaci* MEAM1 from infected soybean, tomato, and *N. physalodes* plants to healthy tomato plants found in the present study (Fig. 1c) may be associated with the similarity in virus concentration in the leaf tissue of the sources of inoculum (Fig. 1d).

The preference of the vector for the virus host may also influence its potential as a source of inoculum in the field (Wintermantel et al. 2016; Esquivel-Fariña et al. 2021). Viruses can cause morphological and physiological changes in infected plants, altering the vector's preference for the host. Fereres et al. (2016) reported that adults of *B. tabaci* MEAM1 preferred to land on leaves of tomato plants infected with ToSRV or ToCV over leaves of mock-inoculated tomato plants. Furthermore, the presence of the virus in the vector itself can directly affect its behavior. Adults of aviruliferous *B. tabaci* MEAM1 and MED preferred to land on healthy soybean plants compared to soybean plants infected with cowpea mild mottle virus (CPMMV). Contrariwise, viruliferous adults of *B. tabaci* MEAM1 and MED preferred to land on infected soybean plants rather than on healthy ones (Bello et al. 2021). In the present study, aviruliferous adults of *B. tabaci* MEAM1 landed in the same proportion on mock-inoculated (Fig. 3c, d) or ToSRV-infected (Fig. 4c, d) plants of soybean, tomato, and *N. physalodes.* However, the presence of ToSRV in adults of *B. tabaci* MEAM1 caused an alteration in their behavior. The viruliferous insects landed preferentially, in most of the cases analyzed, on tomato plants rather than on soybean or *N. physalodes* plants, regardless of whether the plants were mock-inoculated (Fig. 3a, b) or infected with ToSRV (Fig. 4a). These results help to understand why the tomato plants were more infected than the soybean and *N. physalodes* plants in the transmission experiments carried out with a chance of choice by *B. tabaci* MEAM1 (Table 1). The preference of viruliferous

insects for tomato plants is a characteristic that may favor the spread of ToSRV in the field, since tomato is the main host for the virus.

Together, these experimental results, under greenhouse conditions, indicate that ToSRV-infected soybean plants are good sources of inoculum for virus acquisition by adults of *B. tabaci* MEAM1 and subsequent transmission to tomato plants. To explore this further, the potential of ToSRV-infected soybean plants as a source of inoculum was compared with that of ToSRV-infected tomato and *N. physalodes* plants under experimental field conditions. Under the same proportions, ToSRV-infected tomato and *N. physalodes* plants acted more efficiently as sources of inoculum than ToSRV-infected soybean plants (Fig. 4). However, this experiment demonstrated that 10 infected soybean plants were able to provide ToSRV transmission for, on average, 18% of the tomato plants evaluated.

As pointed out by Bergamin Filho et al. (2020), the potential of an alternative host to favor the occurrence of a ToSRV epidemic in tomato fields, where secondary infection is absent or incipient due to efficient control of *B. tabaci* MEAM1, is highly dependent on the population of infected plants in the field. Although ToSRV-infected *N. physalodes* plants were more efficient experimental sources of inoculum than ToSRV-infected soybean plants, the population of *N. physalodes* plants near tomato crops is unknown. Observations during the last ten years in the tomato-producing region of Sumaré, state of São Paulo, indicate that *N. physalodes* populations are consistently small, especially plants infected with ToSRV (unpublished data). The high frequency of insecticide application (2 to 3 per week) and the efficient control of *B. tabaci* MEAM1 makes transmission of ToSRV between tomato crops unlikely. Due to these factors, tomato and *N. physalodes* plants might have only a weak force of infection to cause epidemics in tomato crops in a scenario where only primary infections occur (Bergamin Filho et al. 2020). In turn, the application of insecticides in soybean crops is much less frequent, approximately 2 to 6 per crop season (Bortolotto et al., 2015). The difference in the number of insecticide applications allows the more significant growth of the whitefly population in soybeans than in tomato crops. Furthermore, the population of soybean plants is around 240,000 to 330,000 plants per ha (Faria et al. 2019). Because of this, the number of soybean plants infected with ToSRV in the field can be very large. Macedo et al. (2017b) and Bergamin Filho et al. (2020) reported, respectively, incidences of 3.3% and 10% of ToSRV-infected soybean plants in Brazil. These incidences represent, respectively, 7,920 to 10,890 and 24,000 to 33,000 ToSRV-infected soybean plants per ha acting as sources of inoculum in the

field. The results obtained in this study under experimental field conditions support the suggestion of Bergamin Filho et al. (2020) that the soybean crop has the potential to act as an amplifier host of ToSRV and be an important factor in the occurrence of epidemics of this begomovirus in tomato crops. However, noted by these authors, the epidemiological importance of the soybean crop as a source of ToSRV inoculum will depend on the cultivar, its development stage at the time of infection, the ToSRV isolate, and the synchrony between the planting of tomatoes near soybean fields infected with ToSRV.

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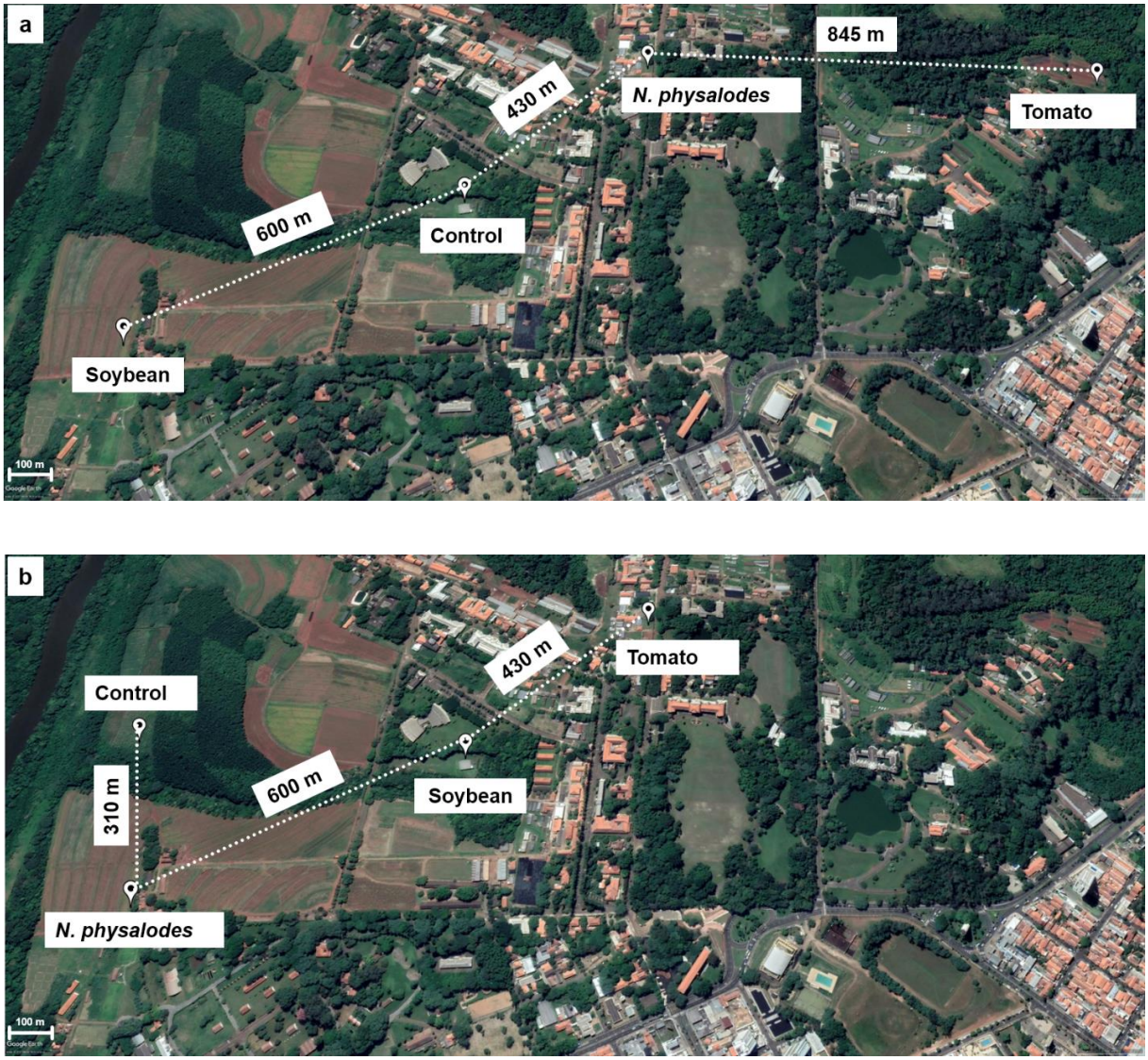
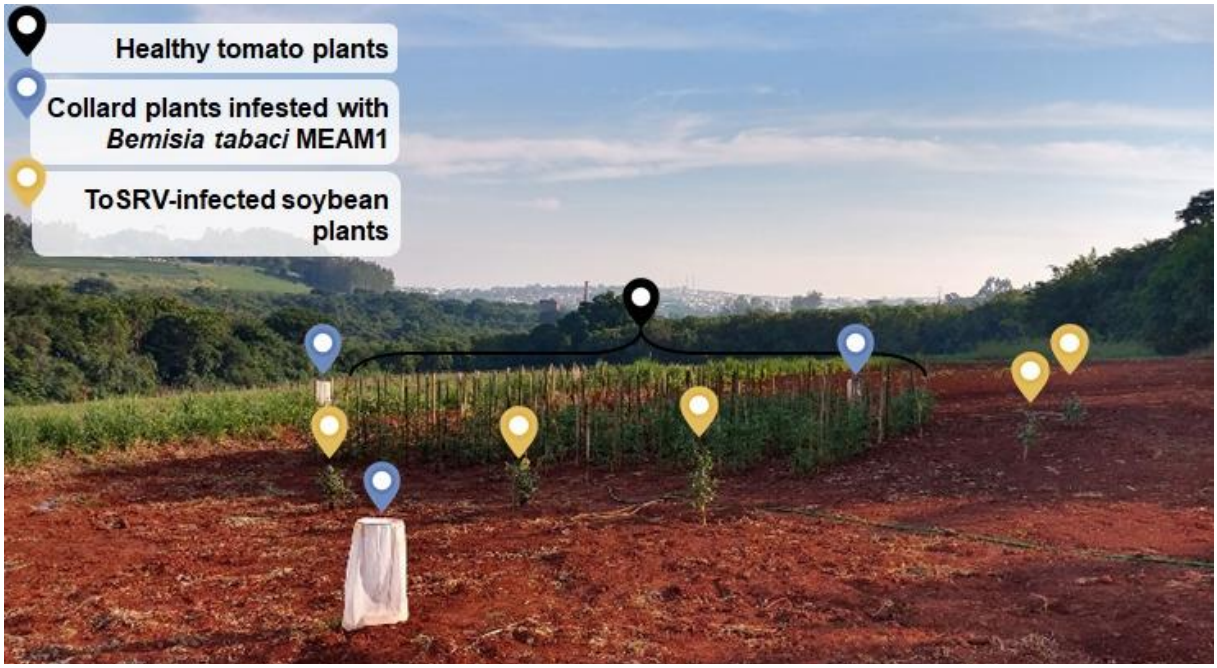
SUPPORTING INFORMATION



Supplementary Fig. 1. Aerial view of part of the ESALQ/USP campus with the locations and distances of the areas where ToSRV-infected soybean, tomato, and *N. physalodes* plants were used as sources of inoculum, and the control area with no source of inoculum, in the first (a) and second (b) experiment. Photo: Google Earth.



Supplementary Fig. 2. Detail of the area where ToSRV-infected soybean plants were used as sources of inoculum in the first experiment.

Supplementary Table 1: Efficiency of ToSRV acquisition and transmission using soybean, tomato and *N. physalodes* as sources of inoculum and titer of the virus in the source plants.

Source of inoculum	Efficiency of acquisition (%) (no. of viruliferous insects/ no. of insects analyzed)	Efficiency of transmission (%) (no. of infected tomato plants/ no. of inoculated tomato plants)	Titer of ToSRV (log of copy number)
		First experiment	
Soybean 1	90 (9/10)	0 (0/6)	7.2
Soybean 2	90 (9/10)	40 (2/5)	7
Soybean 3	90 (9/10)	50 (3/6)	5.6
Tomato 1	90 (9/10)	50 (3/6)	7.6
Tomato 2	90 (9/10)	17 (1/6)	7
Tomato 3	100 (10/10)	50 (3/6)	7.6
N. physalodes 1	80 (8/10)	33 (2/6)	6.8
N. physalodes 2	100 (10/10)	50 (3/6)	6.5
N. physalodes 3	90 (9/10)	0 (0/6)	6.7
		Second experiment	
Soybean 1	70 (7/10)	33 (2/6)	6.8
Soybean 2	80 (8/10)	67 (4/6)	7.3
Soybean 3	80 (8/10)	67 (4/6)	7.5
Tomato 1	100 (10/10)	33 (2/6)	7.1
Tomato 2	90 (9/10)	17 (1/6)	7.2
Tomato 3	80 (8/10)	33 (2/6)	7.2
N. physalodes 1	90 (9/10)	0 (0/6)	7.8
N. physalodes 2	90 (9/10)	17 (1/6)	7.5

Supplementary Table 2. Cycle threshold (CT) values of qPCR (mean triplicate + standard deviation) of tomato severe rugose virus in soybean, tomato and *N. physalodes* plants used as sources of inoculum in two independent experiments.

Experiment	Source of inoculum	Plant 1	Plant 2	Plant 3	Mean
1	Soybean	14.62 ± 0.10	15.46 ± 0.16	20.22 ± 0.14	16.77
	Tomato	13.53 ± 0.10	15.46 ± 0.11	13.51 ± 0.03	14.17
	N. physalodes	16.09 ± 0.17	17.23 ± 0.22	16.30 ± 0.07	16.54
2	Soybean	15.97 ± 0.05	14.46 ± 0.26	13.67 ± 0.36	14.70
	Tomato	15.24 ± 0.41	14.72 ± 0.26	14.67 ± 0.13	14.88
	N. physalodes	12.68 ± 0.10	13.87 ± 0.28	-	13.26

-: not tested

3. EVIDENCE OF NO HORIZONTAL OR VERTICAL TRANSMISSION OF TOMATO SEVERE RUGOSE VIRUS BY *BEMISIA TABACI* MEAM1

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Abstract

Bemisia tabaci is important in agriculture worldwide, mainly because it is a vector of numerous plant viruses, probably the most important of which are members of the genus *Begomovirus*. Dozens of begomoviruses have been reported to infect tomato plants in Brazil, although tomato severe rugose virus (ToSRV) predominates in tomato crops. ToSRV, found so far only in Brazil, is efficiently transmitted by *B. tabaci* MEAM1. However, no studies have assessed the occurrence of vertical and horizontal transmission of the virus in the insect, which may have epidemiological consequences affecting disease management. This study evaluated the possibility of transmission of ToSRV between whiteflies during copulation and transovarial transmission from viruliferous females of *B. tabaci* MEAM1 to their progeny. Transmission of ToSRV did not occur during mating between males and females of *B. tabaci* MEAM1. Aviruliferous males and females confined with viruliferous insects of the opposite sex were also unable to transmit the virus to tomato plants. ToSRV was detected, by PCR, in the ovaries of viruliferous females of *B. tabaci* MEAM1 but not in eggs, nymphs, or adults of the progeny of viruliferous females. Adult progeny of viruliferous females also did not transmit ToSRV to tomato plants. Together, the results indicate that vertical and horizontal transmission of ToSRV by *B. tabaci* MEAM1 is unlikely. Sustainable management of the tomato golden mosaic disease caused by ToSRV should continue to focus on using resistant varieties, managing sources of inoculum around tomato fields, and rational chemical control of the vector.

Keywords: begomovirus, horizontal transmission, transovarial transmission, whitefly

3.1. Introduction

Bemisia tabaci (Hemiptera: Aleyrodidae) is a highly polyphagous whitefly and one of the most important pests in agriculture worldwide (De Barro *et al.*, 2011; Navas-Castillo *et al.*, 2011). *B. tabaci* is a complex of 44 cryptic species, of which *B. tabaci*

Middle East-Asia Minor 1 (MEAM1, formerly biotype B) and *B. tabaci* Mediterranean (MED, formerly biotype Q) are the most important in many regions of the world (De Barro *et al.*, 2011; Kanakala & Ghanim, 2019). In Brazil, *B. tabaci* MEAM1 is the predominant species, although *B. tabaci* MED is becoming more widely distributed since its first detection in 2013 (Marubayashi *et al.*, 2013; Barbosa *et al.*, 2015; Moraes *et al.*, 2018; Bello *et al.*, 2020).

Adults of *B. tabaci* transmit viruses belonging to the genera *Begomovirus*, *Crinivirus*, *Torradovirus*, *Carlavirus*, and *Ipomovirus* (Navas-Castillo *et al.*, 2011). Recently, transmission of two poleroviruses by *B. tabaci* (Ghosh *et al.*, 2019; Costa *et al.*, 2020) has also been reported. Because of its high reproduction rate, dispersal ability, polyphagy, manipulation of plant defense mechanisms, and ability to transmit hundreds of viruses, *B. tabaci* is recognized as a “super vector of plant viruses” (Gilbertson *et al.*, 2015).

In Brazil, *B. tabaci* is responsible for the transmission of begomoviruses and a crinivirus in solanaceous crops, particularly tomato (Inoue-Nagata *et al.*, 2016). Several species of begomovirus have been reported to infect tomato plants in Brazil, however, a survey between 2013 and 2017 in tomato fields in the states of São Paulo, Minas Gerais, Goiás, Rio de Janeiro, Bahia and Espírito Santo indicated that tomato severe rugose virus (ToSRV) was prevalent, being detected in 105/136 (77.2%) of plants analyzed (Mituti *et al.*, 2019). So far, this begomovirus has been found only in Brazil.

In nature, begomoviruses are perpetuated mainly in susceptible host plants, through transmission between plants by adults of *B. tabaci*, in a persistent circulative manner (Navas-Castillo *et al.*, 2011). A few begomoviruses can be perpetuated in the vector itself, through copulation and transovarial transmission from viruliferous females to their progeny (Ghanim *et al.*, 1998; Ghanim & Czosnek, 2000; Bosco *et al.*, 2004; Wang *et al.*, 2010). Some reports indicate that insects that acquire a begomovirus through these mechanisms can transmit it to healthy plants (Ghanim *et al.*, 1998; Ghanim & Czosnek, 2000; Wei *et al.*, 2017). The perpetuation of the virus in the vector has epidemiological importance, as the virus can remain in the field even without the presence of host plants (Accotto & Sardo, 2009; Wang *et al.*, 2010), as occurs with rice dwarf virus (RDV), family *Reoviridae*, transmitted by leafhoppers (Hibino, 1996). Due to the importance of this information for the management of tomato golden mosaic disease caused by ToSRV in the field, this study evaluated the possibility of vertical and horizontal transmission of this begomovirus by *B. tabaci* MEAM1.

3.2. Materials and Methods

3.2.1. Colony of *B. tabaci* MEAM1 and ToSRV isolate

A colony of virus-free *B. tabaci* MEAM1 was reared in collard plants (*Brassica oleraceae*) kept in an insect-proof cage in a greenhouse. The purity of the colony was checked periodically by PCR from total DNA extracted from adult insects, with the primers Bem23F and Bem23R, whose generated amplicons allow differentiation of the cryptic species *B. tabaci* MEAM1 and MED (De Barro *et al.*, 2003).

The ToSRV isolate was collected in 2018 from field-infected tomato plants in the municipality of Sumaré, state of São Paulo. Partial nucleotide sequencing of its genome revealed a 96–98% identity with corresponding nucleotide sequences of other ToSRV isolates (GenBank access nos. EU086591.2 and JX415196.1). Since then, the ToSRV isolate was maintained in tomato plants cv. Compack in insect-proof cages, and frequently checked for virus identity by nucleotide sequencing of PCR amplicons. The inoculum was frequently renewed by transmitting the virus to young tomato plants with *B. tabaci* MEAM1.

3.2.2. Detection of ToSRV by PCR

Total DNA was extracted from groups of ovaries, eggs, nymphs, and adults of *B. tabaci* MEAM1 and from leaf tissues of tomato plants, using an adaptation of the protocol described by Doyle (1991). Ovaries, eggs, nymphs, and adult insects, placed in 1.5-ml microcentrifuge tubes, were ground with 150 µl of CTAB buffer (2% CTAB, 1.4 M NaCl, 0.2% 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCI, pH 8.0). Leaf tissues, approximately 100 mg per sample, were ground with 650 µl of CTAB buffer. The samples were incubated at 65 °C for 5 min and equal volumes (150 µl and 650 µl) of chloroform-isoamyl alcohol (24:1) were added to samples of *B. tabaci* and leaf tissue, respectively. The samples were centrifuged at 12,000 *g* for 10 min. One hundred µl of supernatant from samples of ovaries, eggs, nymphs, and adults and 300 µl of leaf-tissue samples were transferred to new tubes and mixed with 100 µl and 300 µl of isopropanol, respectively. The samples were kept at –20 °C for 1 h and then centrifuged at 12,000 *g* for 10 min. The supernatants were discarded, and 250 µl and 500 µl of 70% ethanol were added, respectively, to samples of *B. tabaci* MEAM1 (ovaries, eggs, nymphs, and adults) and tomato leaf tissue. The samples were centrifuged at 12,000 *g* for 5 min and the supernatant was discarded. The pellets were

vacuum-dried for 5 min and the DNA was eluted in 50 µl of nuclease-free water. The extracted DNAs were stored at –20 °C until used for polymerase chain reaction (PCR).

For detection of ToSRV by PCR, the begomovirus universal primers PAR1c496 and PAL1v1978, developed by Rojas (1993), were used at a concentration of 20 µM. PCR was performed using the PCR Master Mix kit (Promega). For a volume of 25 µl, were added 12.5 µl of 2× GoTaq Colorless Master Mix, 7.5 µl of DNA-free water, 3 µl of total DNA and 1 µl of the specific primers. The thermocycler conditions were: 92 °C for 2 min, followed by 30 cycles of 94 °C for 40 s, 54 °C for 60 s, 72 °C for 60 s, and a final extension at 72 °C for 10 min. The amplicons obtained were analyzed by electrophoresis on 1% agarose gel, stained with SYBR Safe DNA gel stain (Invitrogen), and visualized on a UV light transilluminator.

DNA extracted from viruliferous adults of *B. tabaci* MEAM1 and ToSRV-infected tomato leaves, as well as virus-free adults of *B. tabaci* MEAM1 and healthy tomato leaves, was used as positive and negative controls, respectively. As an internal control, DNA extracted from ovaries, eggs, nymphs, and adults of *B. tabaci* MEAM1 was analyzed by PCR with a pair of primers specific for the *B. tabaci* elongation factor (EF_1) gene, as described by Ghanim *et al.* (2007) and Wang *et al.* (2010). Only samples that had the EF_1 gene amplified, which guarantees the quality of the extracted DNA, were subsequently analyzed for detection of ToSRV by PCR.

3.2.3. Test of vertical transmission of ToSRV by *B. tabaci* MEAM1

Trials to assess transovarial (vertical) transmission of ToSRV by *B. tabaci* MEAM1 started with virus-free females (termed F_0) 1–4 days after emergence (DAE), which started oviposition 3–6 DAE. Transovarial transmission of this begomovirus by females of unknown age at the time of virus acquisition and oviposition was also evaluated. Virus-free F_0 females of *B. tabaci* MEAM1 were confined on ToSRV-infected tomato leaves in glass jars for a virus acquisition access period (AAP) of 48 h. Subsequently, the F_0 females were individually confined in clip-cages placed on the abaxial surface of cotton leaves (*Gossypium hirsutum*), a non-host of ToSRV, and maintained for 5 days for oviposition. After that period, the adult F_0 females were collected with the aid of an entomological aspirator and stored in groups of three adults at –20 °C, for PCR analysis to confirm the presence of ToSRV. Only the offspring of the F_0 females present in the groups that tested positive for ToSRV were analyzed subsequently.

Detection of ToSRV in eggs, nymphs, and first-generation adults (termed F_1) was performed separately, as follows. Soon after the removal of the F_0 females, all eggs deposited on some plants were immediately collected for analysis by PCR. In other plants, the eggs were left on the leaves for hatching. Ten days after the end of the oviposition period of the F_0 females, all the nymphs were collected for PCR detection of the virus. Finally, in a third group of plants, the eggs deposited on the leaves were left until the first-generation adults (F_1) developed, which occurred 25–30 days after the end of the oviposition period of the F_0 females. All adults of the offspring were then collected for PCR detection of the virus. The presence of ToSRV was assessed through PCR performed with DNA extracted from groups of up to 20 eggs, 10 nymphs, and 5 adults. The experiment was performed twice.

3.2.4. Transmission test of ToSRV by F_1 adults of *B. tabaci* MEAM1

About 200 virus-free adults of *B. tabaci* MEAM1 were confined on ToSRV-infected tomato leaves for an AAP of 48 h. Subsequently, the insects were transferred to cotton plants in an insect-proof cage for 5 days, for oviposition. After that period, all insects were collected using an entomological aspirator and stored at –20 °C to later confirm the presence of ToSRV by PCR, in groups of five females. After adults emerged in the cotton plants, 12 healthy tomato plants cv. Compack, at the stage of 3–4 true leaves, were placed in the cage to allow virus transmission by potentially ToSRV-viruliferous F_1 adults of *B. tabaci* MEAM1. After 30 days of confinement, all tomato plants were analyzed based on the expression of symptoms and detection of ToSRV by PCR. The experiment was performed twice.

3.2.5. ToSRV detection in the ovaries and eggs of *B. tabaci* MEAM1

The presence of the ToSRV was examined in the ovaries of viruliferous females after a virus AAP of 48 h on infected tomato leaves. The age of the females at the beginning of the acquisition period was 1-4 DAE and 11 DAE. After virus acquisition, the females were collected using an entomological aspirator and were placed at –20 °C until they died. The ovaries were dissected under a stereoscopic microscope in 1× phosphate-buffered saline (PBS). The head and thorax of dissected females were separately analyzed by PCR to confirm virus acquisition. The presence of ToSRV was assessed through PCR performed with DNA extracted from groups of

up to 5 ovaries and 5 heads+thoraxes. Eggs oviposited by females of 1-4 DAE and 11 DAE were PCR analyzed for the presence of ToSRV, to verify whether virus transovarial transmission by *B. tabaci* MEAM1 is dependent on the developmental stage of the whiteflies, as pointed out by Wei et al (2017). The F_0 females were maintained confined on ToSRV-infected tomato leaves for a virus AAP of 48 h. After the virus acquisition, 10 and 8 viruliferous F_0 females of 1-4 DAE and 11 DAE, respectively, were transferred, separately, to oviposition in cotton plants, kept in insect-proof cages for five days. After that, the F_0 females and the eggs deposited on the leaves were collected to detect the virus. The presence of ToSRV was assessed through PCR performed with DNA extracted from individual females and groups of up to 10 eggs.

3.2.6. Tests of horizontal transmission of ToSRV through *B. tabaci* MEAM1

Horizontal transmission of ToSRV was evaluated among females and males of ToSRV-viruliferous adults of *B. tabaci* MEAM1, which acquired the virus 1–4 DAE. Copulation occurred when potentially ToSRV-viruliferous and aviruliferous adults were 3–6 DAE. ToSRV transmission was also evaluated between males and females of unknown age at the time of virus acquisition and copulation. Males and females were differentiated by abdomen morphology (Souza & Vendramim, 2000), using a stereoscopic microscope. *B. tabaci* MEAM1 adults of both sexes together were allowed a 48-h virus AAP on ToSRV-infected tomato leaves. After that, 15 viruliferous females were confined with 15 virus-free males on detached cotton leaves in glass jars covered with voile fabric. The same procedure was performed by confining 15 viruliferous males with 15 virus-free females in other glass jars. Confinement for copulation lasted for 48 h, after which the insects were collected with an entomological aspirator and transferred to 1.5-ml microcentrifuge tubes. The insects were stored at –20 °C and were later analyzed for ToSRV by PCR. DNA extracted from viruliferous and aviruliferous adults of *B. tabaci* MEAM1, was used as positive and negative controls, respectively. The experiment was performed twice.

3.2.7. Transmission test of ToSRV by adults of *B. tabaci* MEAM1 after copulation

The procedure for copulation of potential-ToSRV viruliferous and aviruliferous insects was conducted as described above. After copulation, potentially viruliferous insects of both sexes were collected with an entomological aspirator and stored at –20 °C to later confirm the presence of ToSRV by PCR. Previously virus-free males and females, which copulated with potentially ToSRV-viruliferous females and males, respectively, were then transferred to healthy tomato plants cv. Compack at the stage of 3–4 true leaves, confined in cages made with acetate sheets covered with voile fabric. The insects were kept in the cages for an IAP of 48 h. Ten insects per plant were used for inoculation. After inoculation, tomato plants were sprayed with the insecticide flupiradifurone and kept in insect-proof cages in the greenhouse. Transmission of ToSRV to tomato plants was evaluated by monitoring the appearance of symptoms and by PCR for virus detection, 30 days after inoculation.

3.3. Results

3.3.1. Vertical transmission of ToSRV through *B. tabaci* MEAM1

All data on virus detection in eggs, nymphs, and F_1 adults of *B. tabaci* MEAM1 refer to samples that tested positive in PCR using elongation factor (EF_1)-specific primers, thus proving that the insect preparations contained DNA appropriate for PCR (data not shown). ToSRV was not detected in 127 eggs, 63 nymphs, or 71 adults of the first generation (F_1) from F_0 females of *B. tabaci* MEAM1 that acquired the virus at 1–4 DAE. Similarly, the presence of ToSRV was not detected in 80 eggs, 110 nymphs, or 75 adults of the F_1 generation from F_0 females of unknown age at the time of virus acquisition and oviposition. PCR analysis with total DNA extracted from adult F_0 females of *B. tabaci*, after virus acquisition, confirmed that they were viruliferous for ToSRV (Table 1).

None of the 24 tomato plants inoculated with adults of the first generation of ToSRV-viruliferous females developed symptoms, nor was the virus detected by PCR in the DNA extracted from newly emerged leaves, 30 days after inoculation.

TABLE 1: Detection of tomato severe rugose virus in eggs, nymphs, and adults of the first-generation progeny of viruliferous females of *Bemisia tabaci* MEAM1 of known and unknown ages, at the beginning of the virus acquisition access period on ToSRV-infected tomato leaves

Developmental stage	Number of positive groups/number of groups analyzed (total insects evaluated)[a]	
	Females 1–4 days old	Females of unknown age
Eggs (F_1)	0/7 (127)	0/4 (80)
Nymphs (F_1)	0/9 (63)	0/11 (110)
Adults (F_1)	0/20 (71)	0/15 (75)
[b]Viruliferous females (F_0)[b]	4/4 (12)	6/6 (18)

[a]PCR performed with DNA extracted from groups of up to 20 eggs, 10 nymphs, and 5 adults from the first generation of viruliferous females (F_1).
[b]Groups of up to three females (F0) submitted to an acquisition access period (AAP) of 48 hr on ToSRV-infected tomato leaves and whose progeny was evaluated for the presence of ToSRV.

3.3.2. ToSRV detection in the ovaries and eggs of *B. tabaci* MEAM1

PCR analysis detected ToSRV in 80% (16/20) of the groups of 5 ovaries from *B. tabaci* MEAM1 females that acquired the virus at 1-4 DAE. The virus was detected in all groups of 5 heads+thoraxes (20/20) from these females (Table 2). PCR detected ToSRV in 40% (8/20) of the groups of ovaries from females that acquired the virus at 11 DAE. The presence of the virus was also confirmed in 100% (20/20) of the groups of 5 heads+thoraxes from these females (Table 2). These results indicated that the ToSRV can invade the reproductive organs of viruliferous females of *B. tabaci* MEAM1**.** PCR did not detect the begomovirus in 100 and 170 eggs oviposited by females of 1-4 DAE and 11 DAE, respectively (Table 2), confirming that ToSRV was not transmitted vertically to the offspring. PCR detected the begomovirus in all the F_0 females that were placed for oviposition on the cotton leaves.

TABLE 2: Detection of tomato severe rugose virus in ovaries, heads + thoraxes, and eggs of viruliferous females of *Bemisia tabaci* MEAM1, 1–4 and 11 days after emergence, at the beginning of the virus acquisition access period on ToSRV-infected tomato leaves

Part of the body analyzed[a]	No. of positive groups/number of groups analysed (total evaluated)	
	Females 1–4 days old	Females 11 days old
Ovaries	16/20 (100)	8/20 (100)
Heads+thoraxes	20/20 (100)	20/20 (100)
Eggs	0/10 (100)	0/17 (170)

[a]PCR performed with DNA extracted from groups of up to 5 ovaries, five heads + thoraxes, and 10 eggs.

3.3.3. Analysis of ToSRV transmission by *B. tabaci* MEAM1 via mating

Regardless of the age of adults of *B. tabaci* MEAM1 at the time of copulation, ToSRV-viruliferous males did not transmit the begomovirus to virus-free females and vice-versa (Table 3). All 39 males and 48 females of known ages at the time of copulation with ToSRV-viruliferous partners tested negative for the presence of the virus by PCR. Negative results were also obtained for 79 males and 108 females of unknown age at the time of copulation with ToSRV-viruliferous partners. All the females and males tested positive in PCR using EF_1-specific primers, thus proving that the insect preparations contained DNA appropriate for PCR (data not shown).

None of the eight tomato plants inoculated with males or females of *B. tabaci* MEAM1 previously copulated with ToSRV-viruliferous insects of the opposite sex developed symptoms of infection, nor is the virus detected by PCR 30 days after inoculation.

TABLE 3: Transmission of tomato severe rugose virus between males and females of *Bemisia tabaci* MEAM1 of known and unknown ages, at the beginning of the mating period

Treatment[a]	Insects evaluated	No. of positive groups/number of groups analysed (total insects evaluated)[b]	
		Insects 3–6 days old	Insects of unknown age
F^+ X M°	M°	0/8 (39)	0/18 (79)
F^+ X M°	F^+	6/10 (47)	20/23 (86)
M^+ X F°	F°	0/10 (48)	0/24 (108)
M^+ X F°	M^+	3/8 (31)	15/21 (84)

[a]F^+, viruliferous female; F°, initially aviruliferous female; M^+, viruliferous male; M°, initially aviruliferous male.
[b]PCR performed with total DNA extracted from groups of up to five insects.

3.4. Discussion

The results of the present study indicate that the begomovirus ToSRV was not transmitted to the offspring of viruliferous females of *B. tabaci* MEAM1. The virus was not detected by PCR in total DNA extracted from eggs, nymphs, or adults of the first-generation progeny (Table 1). Similarly, papaya leaf curl China virus (PaLCuCNV) and TYLCV were not detected at any stage of the first-generation progeny of viruliferous females of *B. tabaci* MEAM1 and MED (Wei *et al.*, 2017) and Asia II 7 o (Guo *et al.*, 2019), respectively.

In some cases, begomoviruses can be transovarial-transmitted to the progeny of *B. tabaci*. The first study that reported infectivity in adults of the first-generation progeny of *B. tabaci* was conducted with TYLCV by Ghanim *et al.* (1998). The authors detected the presence of TYLCV in 80.7% (46/57) of eggs, 36.7% (25/68) of nymphs, and 56.7% (46/81) of adults of the first-generation progeny of viruliferous females of *B. tabaci* MEAM1. The virus was also detected in 38.6% (26/68) of eggs, 71.4% (35/49) of nymphs, and 78.8% (41/52) of adults of the second-generation progeny of viruliferous females. Even more importantly, Ghanim and co-workers found that 10% (5/50) and 8.1% (4/49) of the adults of the first- and second-generation progeny, respectively, were able to transmit TYLCV to tomato plants. A later study also demonstrated the ability of adults of the first-generation progeny of *B. tabaci* MEAM1 and MED to transmit TYLCV to 20.8% (5/24) and 33.3% (8/24) of healthy tomato plants, respectively. Second-generation adults were able to transmit the begomovirus

to 13% (4/30) and 3.3% (1/30) of tomato plants, respectively (Wei *et al.*, 2017). Contrasting with these results, Bosco *et al.* (2004) did not detect TYLCV when analyzing 100 eggs, 100 nymphs, and 125 adults of the first-generation progeny of viruliferous females of *B. tabaci* MEAM1. These contrasting results may have occurred due to variations between TYLCV isolates, *B. tabaci* MEAM1 colonies, and/or the age of the whiteflies used in the experiments (Bosco *et al.*, 2004; Wang *et al.*, 2010).

The present results also indicated that the adult insects of the first generation of *B. tabaci* MEAM1 did not transmit ToSRV to tomato plants, as confirmed by the absence of symptoms in the inoculated plants and by negative PCRs. As commented by Accotto & Sardo (2009), transmission of begomovirus DNA to the progeny of *B. tabaci* seems to be relatively common. Different studies have demonstrated the presence of begomovirus, to a greater or lesser extent, in eggs, nymphs, and adults of the progeny of *B. tabaci* (Santos *et al.*, 2003; Bosco *et al.*, 2004; Wang *et al.*, 2010; Pan *et al.*, 2012; Guo *et al.*, 2019; Gadhave et al., 2020). However, infectivity, i.e., the ability of these adults to transmit begomoviruses to plants, seems to be a rare event, and so far has been found only for TYLCV (Ghanim *et al.*, 1998; Accotto & Sardo, 2009; Wei *et al.*, 2017).

Studies with TYLCV have shown that the entry of the virus into the reproductive organs was dependent on the developmental stage of females of *B. tabaci*. Transovarial transmission of this begomovirus was greater for the progeny of females of *B. tabaci* MEAM1 at 11 DAE, compared to the progeny of females at 1 DAE (Wei *et al.*, 2017). To verify if the developmental stage was also determinant for the occurrence of the transovarial transmission of ToSRV by *B. tabaci* MEAM1, the presence of the begomovirus in eggs oviposited by females at 1-4 DAE and 11 DAE was evaluated. ToSRV was not detected in 100 and 170 eggs from viruliferous females at 1-4 DAE and 11 DAE, respectively, suggesting that the age of the whitefly did not influence the transmission of ToSRV to the progeny of viruliferous females. However, PCR detected ToSRV in 16/20 and 8/20 groups of five dissected ovaries from females that acquired the virus at 1-4 DAE and 11 DAE, respectively, indicating that the begomovirus invaded the reproductive organs of the whiteflies, but did not reach the eggs.

A determining factor for the entry of the begomovirus into the ovary of *B. tabaci* is the interaction between the virus-coat protein (CP) and the vitellogenin (Vg) of the insect. The interaction of the CP of TYLCV with the whitefly Vg was interrupted when a 423-bp fragment (nt 241–663) of the virus CP was exchanged with a 420-bp fragment (nt 241–660) of the coat-protein gene of PaLCuCNV. Consequently, the virus lost the

ability to entry into the ovaries and be transmitted to the progeny of *B. tabaci*. On the other hand, the CP of PaLCuCNV, containing the fragment of the CP gene of TYLCV, interacted with Vg and allowed the virus to invade the ovaries and be transmitted transovarially (Wei *et al.*, 2017). The interaction of the major CP pc3 of the tenuivirus rice stripe virus (RSV) and the Vg of the small brown planthopper vector (*Laodelphax striatellus*) also appears to be a critical step in RSV transovarial transmission (Huo *et al*., 2014).

Another via that plant viruses can use to invade the reproductive organs of the vector is through the interaction with bacterial symbionts. Jia *et. al* (2017) showed that the entrance of RDV into the oocytes of the leafhopper vector *Nephotettix cincticeps* occurs through the binding of the minor outer viral CP P2 and an outer membrane protein of the bacterial symbiont *Sulcia.* Whether the entry of ToSRV in the ovaries of *B. tabaci* MEAM1 is mediated by the interaction with Vg, or a whitefly bacterial symbiont protein or any other strategy needs investigation.

The reason why ToSRV can invade the ovaries of viruliferous whiteflies but cannot enter the eggs and be transmitted to the progeny is not clear. Liao *et al*. (2017) characterized the rice gall dwarf virus (RGDV) entrance into the oocyte for transovarial transmission by its leafhopper vector *Recilia dorsalis*. They showed that RGDV initially entered the germ cells in the germarium, moved between follicular cells, then translocated across the microvilli to enter the oocyte. Virus transport occurs through tubules formed by the non-structural protein Pns11, which interact with actin-based junctions between follicular cells or actin-based microvilli from follicular cells into the oocyte to overcome transovarial transmission barriers. Studies are needed to identify possible barriers that prevent the transovarial transmission of ToSRV in *B. tabaci* MEAM1.

The present study verified that viruliferous males of *B. tabaci* MEAM1 did not transmit ToSRV to females during copulation, and vice-versa (Table 3). Virus-free males or females of *B. tabaci* MEAM1 that copulated with ToSRV-viruliferous insects of the opposite sex were unable to transmit the virus to tomato plants. Ghanim & Czosnek (2000) reported that TYLCV was transmitted, via copulation of *B. tabaci* MEAM1, to 32.6% (16/49) of males and 51.8% (28/54) of females. The insects that acquired TYLCV via copulation were able to inoculate the begomovirus into tomato plants. TYLCV was also transmitted during sexual reproduction between males and females of *B. tabaci* MED (Ghanim *et al.*, 2007). In addition to TYLCV, the tomato yellow leaf curl China virus (TYLCCNV) was transmitted during copulation between

males and females of *B. tabaci* MEAM1 and MED, and the bipartite squash leaf curl virus (SLCV) and the watermelon chlorotic stunt virus (WmCSV) were transmitted during sexual reproduction among adults of *B. tabaci* MEAM1 (Ghanim *et al.*, 2007; Wang *et al.*, 2010). Gadhave et. al (2020) reported a low frequency of horizontal transmission of cucurbit leaf crumple virus (CuLCrV) in *B. tabaci* MEAM1. Viruliferous males transmitted the begomovirus to 4% (1/25) of aviruliferous females, but viruliferous females did not transmit the begomovirus to aviruliferous males (0/26). The way in which begomoviruses are transmitted horizontally between adults of *B. tabaci* is not yet fully understood. Some studies suggest that transmission occurs through the exchange of hemolymph between males and females during copulation (Ghanim & Czosnek, 2000; Czosnek *et al.*, 2002; Ghanim *et al.*, 2007). The results obtained here indicated that there must be one or more other determining factors for the occurrence of horizontal transmission of begomoviruses through *B. tabaci* MEAM1, because mating between males and females of *B. tabaci* MEAM1 is a natural process and the efficient transmission of ToSRV to plants by this vector indicates that the virus reaches the hemolymph, which is a prerequisite for the transmission of begomoviruses by whiteflies (Czosnek *et al.*, 2017; Toloy *et al.*, 2017). Further studies are needed for a better understanding of the mechanisms involved in the transmission of begomoviruses during mating between males and females of *B. tabaci*.

The results of the present study strongly indicate that ToSRV does not perpetuate in the field in adults of *B. tabaci* MEAM1 through vertical or horizontal transmission, which therefore do not play a role in the epidemiology of the disease. The perpetuation of ToSRV in the field between growing seasons must depend entirely on the host plants of the virus. Epidemiological studies have shown that ToSRV spreads in tomato crops predominantly by primary infections, through the continuous influx of viruliferous whiteflies from external areas (Barbosa *et al.*, 2016; Macedo *et al.*, 2019). Because many plant species are susceptible to ToSRV (Barbosa *et al.*, 2011; Macedo *et al.*, 2015), the most abundant species that are potentially most efficient as sources of inoculum for *B. tabaci* MEAM1 in an area must be properly identified, in order to formulate management strategies. The establishment of a virus-free period, i.e., with no tomato or other ToSRV-susceptible solanaceous species present, together with elimination of alternative hosts will certainly help to reduce the sources of ToSRV inoculum in the field and consequently the incidence of the virus and the damage to tomato crops.

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