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The invasive tomato pest *Tuta absoluta* can transmit the emergent tomato brown rugose fruit virus

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With 2 figures and 1 table

Abstract: The tomato brown rugose fruit virus (ToBRFV) currently represents one of the most significant threats to tomato crop worldwide. Its transmission occurs mainly through contact between plants and infected surfaces, and only one case of mechanical transmission by arthropods has been reported. Here, we aim to assess the role of an invasive tomato pest, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), in ToBRFV transmission. Laboratory tests demonstrated the presence of the ToBRFV in adult moths obtained from larvae developed on ToBRFV-infected plants. Infected adults of *T. absoluta* were able to infect healthy tomato plants. In order to evaluate whether the occurrence of ToBRFV on *T. absoluta* adults was internal or external to the pupae, as results of larvae feeding on infected plants, pupae obtained from larvae fed on infected plants were externally disinfected and then analyzed for the presence of ToBRFV by RT-qPCR. Adults obtained from disinfected and not-disinfected pupae were also analyzed. Both adults and pupae were positive for the virus, suggesting its presence in the inner surface of the pupal exuvia. Electron microscopy, western blot analyses and hemolymph bioassay showed the absence of ToBRFV virions and viral coat protein in the hemolymph collected from disinfected pupae, demonstrating that the ToBRFV is not circulative in the progeny of *T. absoluta* adults obtained from larvae fed in infected plants, but probably adults got contaminated during their emergence, due to virus presence in the inner surface of pupal exuvia. This study demonstrates for the first time that *T. absoluta* can carry an infectious primary *inoculum* of ToBRFV, highlighting a potential epidemiological role of *T. absoluta* in spreading ToBRFV in the field.

Keywords: South American tomato pinworm; insect vector; ToBRFV; tobamovirus; plant virus transmission

1 Introduction

Tomato (*Solanum lycopersicum* L., family Solanaceae) is one of the most important cultivated vegetable crops worldwide, with over 5 million hectares and a total production over 186 million tons, according to the latest data available (FAO 2022). In Europe, Italy is one of the most relevant tomato producers, with 38% of the total production (Eurostat 2021). Tomato cultivation, for both fresh and processed products, is constantly threatened by numerous arthropod pests. Among insect pests, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), the South American tomato pinworm, is one of the key pests but other insects, such as whiteflies, mainly the *Bemisia tabaci* complex (Homoptera: Aleyrodidae), aphids and thrips (Biondi et al. 2018) are also relevant. Homoptera and Thysanoptera can damage host plants not only for their direct feeding activity but also for their ability to transmit plant pathogens (Gilbertson et al. 2015). Tomato is also attacked by several pathogens, mostly viruses (Panno et al. 2021a), leading to yield losses.

Currently, among the viral entities recently identified in the Mediterranean basin, tomato brown rugose fruit virus (ToBRFV, family *Virgaviridae*), represents one of the most significant threats to this crop. After its initial finding in 2014 in few countries (Luria et al. 2017; Salem et al. 2016; Panno et al. 2019a; Alfaro-Fernández et al. 2021), ToBRFV is now widely distributed, and different outbreaks have been reported in European, Asiatic and American countries

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(EPPO 2022a, Fig. 1). In Italy, ToBRFV was first detected in 2018 in tomato greenhouses in the Ragusa province (Sicily) (Panno et al. 2019b). In nature, ToBRFV host range seems to be limited to tomato, pepper, and other solanaceous plants, but various grasses and weeds occurring in tomato fields, belonging to several botanical families are also infected (Panno et al. 2020a; Zhang et al. 2022). Therefore weeds, although in most cases asymptomatic, may represent a virus reservoir across tomato growing cycles (Salem et al. 2022). Noteworthy, ToBRFV can also infect tomato cultivars carrying the *Tm-2²* gene, inducing resistance to the tobamoviruses tomato mosaic virus (ToMV) and tomato mottle mosaic virus (ToMMV) (Chanda et al. 2021).

Symptoms caused by ToBRFV on tomato plants consist of interveinal yellowing and leaf deformation, mild to severe chlorotic mosaic, young leaf deformation and necrosis, sepal necrosis and deformation. On fruits, discoloration of young fruits, marbling, deformation, and necrosis are visible; these symptoms and the reduction in the number of berries, contribute to lower both yield and commercial value of the product. Moreover, severe attacks can occur, bringing to whole plant collapse and death within a short time (Caruso et al. 2022).

ToBRFV is extremely infectious and, once established in fields or nurseries, it spreads very quickly. It is classified as a seed-borne virus; viral particles are localized in the external teguments but were never found in the embryo (Davino et al. 2020). Its transmission occurs through direct contact between plants, including propagation material, through wounds inflicted on leaves or roots of seedlings during transplant (Salem et al. 2016; Panno et al. 2020b), through infected sap contaminating different surfaces (human body, clothes, work tools, soil, etc.), and even though irrigation/drainage water (Li et al. 2016) or nutrient solutions (Wilstermann & Ziebell 2019).

To the best of our knowledge, no scientific evidence of ToBRFV transmission by arthropods is available, except for a recent study demonstrating that ToBRFV can be mechanically transmitted by *Bombus terrestris* L. (Hymenoptera: Apidae) during its pollination activity; transmission occurred without ingestion and incubation of viral particles, through transfer of crude sap with insect mandibles or vibrating bodies (Levitzky et al. 2019). In this context, we sought to investigate the potential role of other insects to act as vector of this pathogen, focusing on *T. absoluta*. It is one of the most invasive pests of tomato, accidentally introduced in Spain in 2006 from South America, and currently widespread in Europe, Asia and Africa (Biondi et al. 2018; EPPO 2022b, Fig. 1). Its host range mainly includes solanaceous species, but eggs can be laid on a wide range of wild and cultivated plants of other families, although in many cases larvae fail to complete their development up to the adult stage (Cherif & Verheggen 2019).

Females of *T. absoluta* lay eggs mainly on apical leaves. Young larvae (1st–2nd instar) bore into the leaf mesophyll, whereas 3rd–4th instar larvae leave their mines and start to feed on all the epigeal parts of the plant, including stems, twigs, and fruits. In the Mediterranean basin, *T. absoluta* can complete up to ten generations per year (Biondi et al. 2018). The larval feeding activity results in significant yield losses both in the open field and in greenhouses (Campos et al. 2017; Mansour et al. 2018; Desneux et al. 2022). To the best of our knowledge, there is no evidence of this pest acting as a vector of plant diseases.

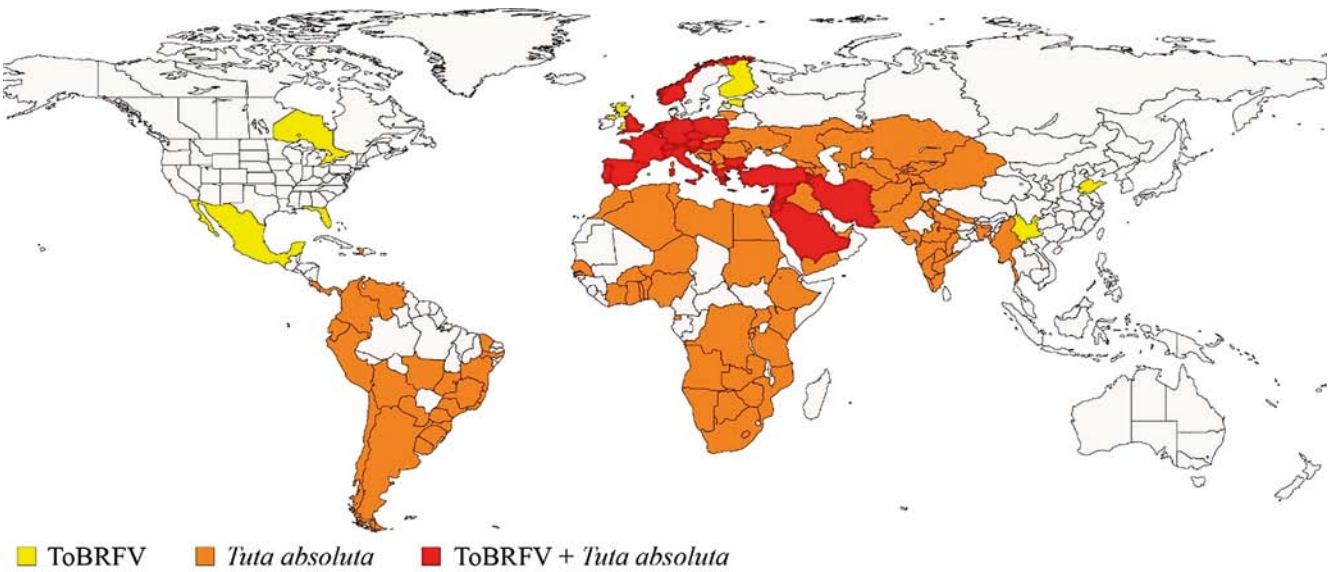

Fig. 1. Current worldwide tomato brown rugose fruit virus and *Tuta absoluta* distribution. The red area in which both *Tuta absoluta* and the ToBRFV are present, refer to the years after 2014, when the virus was firstly detected and characterized (distribution data from EPPO 2022a and EPPO 2022b).

Here, we investigated the potential role of *T. absoluta* as vector of ToBRFV, evaluating in laboratory conditions the potential for this arthropod to acquire ToBRFV from infected plants and to transmit the primary *inoculum* to healthy tomato plants.

2 Materials and methods

2.1 Plant growth and insect rearing

Plant growth and insect rearing were carried out at the Research Centre for Plant Protection and Certification (CREA-DC, Bagheria, Italy) under laboratory conditions (25 ± 2 °C; 50 ± 10% RH; 14:10 L:D). Tomato plants (cv 'San Marzano nano') obtained from tobamovirus-free seeds were grown in screened cages (45 × 45 × 90 cm) to avoid any accidental pest infestations. Seedlings were grown in one-liter pots (10 × 10 × 15 cm) with peat and topsoil sterilized at 121 °C for 20 min. Plants were fertilized once a week with Foliar Gold® (Hydro Fert, Italy); no pesticides were used. Plants used for rearing and experiments were about 50 cm in height and with at least ten true leaves.

A healthy *T. absoluta* colony was started in February 2021 from plants infested by a laboratory colony. Insects were reared in screened cages (45 × 45 × 90 cm), providing adults with a solution of 10% honey in water. Twice a year, field collected adults were added to the rearing group to avoid inbreeding problems (Campolo et al. 2017).

To obtain coetaneous insects for the bioassays, about one hundred unsexed newly emerged adults were collected using a mechanical aspirator and released into rearing cages with four healthy tomato plants. Moths were left to lay eggs for 72 hours and then removed. Infested plant material containing mainly 4th instar larvae was collected and maintained into five-liter screened plastic containers until adult emergence.

All cages, plastic containers, and mechanical aspirators used in the experiments were cleaned each time before use with a solution of distilled water and NaClO (90:10 v/v). Moreover, before each bioassay, a sample of at least 3 leaves and/or 30 insects was preliminarily tested for the presence of ToBRFV.

3 Plant inoculation and molecular analyses by RT-qPCR assay

In order to obtain plants suitable for virus acquisition experiments, tomato plants were mechanically inoculated with the Sicilian ToBRFV isolate (ToB-SIC01/19) (Panno et al. 2019b) using an homogenate prepared by grinding in a mortar 100 mg of ToBRFV-infected leaf tissue with 3 mL phosphate buffer (0.2 M NaH_2PO_4, 0.2 M $Na_2HPO_4 \times 7H_2O$, pH 7.0). The homogenate was applied to leaves previously rubbed with Carborundum (320 mesh).

Thirty days post-inoculation, to confirm the successful ToBRFV transmission, visual inspection of the symptoms on leaves was conducted, followed by RT-qPCR analysis on two young leaves per inoculated plant. Total RNA was extracted from young leaves using the NucleoSpin® RNA Plant Kit (Macherey-Nagel GmbH & Co., Dueren, Germany) following manufacturer's instructions. Total RNA was re-suspended in 50 µL of RNase-free water and its concentration adjusted to 10 ng/µL. RT-qPCR was carried out using specific primer pairs (CaTa28 Fw 5′-GGTGGTGTCAGTGTCTGTTT-3′ and CaTa28 Rv 5′-GCGTCCTTGGTAGTGATGTT-3′) and probe (CaTa28 Pr 5′-6FAM-AGAGAATGGAGAGAGCGGACGAGG-BHQ1-3′) described in the ISHI-Veg protocol (EPPO 2021), with the following cycling conditions: reverse transcription at 50 °C for 10 min, incubation at 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min, with fluorescence acquisition at the end of each cycle, using a Rotor-Gene Q2plex HRM Platform Thermal Cycler (Qiagen, Hilden, Germany). According to the EPPO protocol, samples were considered positive when the Ct value is < 32. The Ct value is directly correlated to viral titer; specifically, a low Ct value corresponds to a high viral titer, and vice versa. To determine the copy numbers of the viral RNA molecules present in each sample, an external standard curve was generated, using 10-fold serial dilutions of in vitro-synthesized RNA transcripts from the ToB-SIC01/19 isolate; RT-qPCR curves were generated by linear regression analysis, plotting the Ct value *vs* the logarithm of the starting RNA dilutions (data not shown).

4 Experiment 1 – *Tuta absoluta* contamination by primary *inoculum* of ToBRFV

To assess whether *T. absoluta* adults obtained from larvae developed feeding on infected plants can be contaminated by ToBRFV, a first experiment was carried out placing in each experimental cage (45 × 45 × 90 cm) three ToBRFV-infected plants and sixty virus-free *T. absoluta* adults (sex ratio 1:1); insects were removed after 48 hours. The experiment was conducted in four replicates.

After 20–25 days, the infested plant material from each cage was collected, placed in five-liter screened plastic boxes (one box per cage) and daily checked for adult emergence. Adults were analyzed for the presence of ToBRFV RNA by RT-qPCR, using 20 adults from each replicate (cage) (Supplementary Fig. 1). Total RNA was extracted from each sample (20 adults/replicate) using the NucleoSpin RNA – Mini kit for RNA purification (Macherey-Nagel GmbH & Co., Dueren, Germany) following manufacturer's instructions. Viral titer was assessed following the same procedure adopted for plant material (see above).

5 Experiment 2 – occurrence of ToBRFV within the body of *Tuta absoluta*

To evaluate whether ToBRFV was inside or outside the *T. absoluta* body, further specific bioassays were performed. Adults emerged from pupae externally disinfected and not-disinfected were analyzed for the ToBRFV occurrence. All pupae used in this experiment were obtained from larvae reared on infected plants. In this case, 20/25 days after placing infected plants and healthy adults in rearing cages, the infested plant material was collected, placed inside five-liter screened plastic boxes (one box for each cage) and daily checked for pupae occurrence. Pupae were collected and placed in sterile Petri dishes. Pupae were disinfected with a solution of sterile H_2O and NaClO (90:10 v/v) for 4 minutes and then cleaned in sterile H_2O for 2 min by immersion. Disinfection procedure was chosen after a specific test (see Supplementary material S1) in order to obtain at the same time the disinfection effectiveness and the highest percentage of emerged adults.

Four pupae groups (3 replicates per group) were handled as follow (Supplementary Fig. 1):

- *Group 1*: 60 not-disinfected pupae (20 pupae/replicate);
- *Group 2*: 90 not-disinfected pupae (30 pupae/replicate) reared in a sterile Petri dish until adult emergence, analyzing at least 20 adults from each replicate;
- *Group 3*: 60 pupae (20 pupae/replicate) disinfected with a solution of sterile H_2O and NaClO (90:10 v/v) for 4 minutes, cleaned by immersion in sterile H_2O for 2 min and dried;
- *Group 4*: 90 pupae (30 pupae/replicate) disinfected following the same procedure adopted in Group 3, and then reared in a Petri dish until adult emergence, analyzing at least 20 adults from each replicate.

Pupae and adults obtained at the end of each experiment were kept at −20 °C and subsequently analyzed following the procedure described above.

6 Experiment 3 – movement of ToBRFV primary *inoculum* by contaminated *Tuta absoluta* adults to healthy tomato plants

To evaluate the ability of contaminated adults to transmit primary *inoculum* of ToBRFV to healthy tomato plants, adults of *T. absoluta* emerged from both disinfected and not-disinfected pupae, obtained from larvae reared on ToBRFV-infected plants, were released in experimental cages containing healthy tomato plants. Three healthy plants and sixty *T. absoluta* adults from not-disinfected pupae (sex ratio 1:1) were placed in each cage. Thirty days later, three leaves from each plant were collected and analyzed for ToBRFV, as described above. A total of four replicates were performed. The same procedure was applied using *T. absoluta* adults emerged from disinfected pupae.

7 Experiment 4 – localization of ToBRFV viral particles in *Tuta absoluta* disinfected pupae

Based on results obtained in Experiment 2, to ascertain the localization of ToBRFV viral particles within the body of *T. absoluta* pupae, the hemolymph was extracted from pupae developed on infected tomato plants and externally disinfected following the protocol described in Supplementary material S1. Each *T. absoluta* pupa was inserted into a sterile tip (0–100 µL) to block the body, as suggested by MacMillan & Hughson (2014). With a sterile micropipette (0–200 µL), a small lesion was created in the cuticle at the pupae's head. Subsequently, the hemolymph was sampled with the micropipette, collected in Eppendorf tube (0.2 mL) and stored at −20 °C for further analyses. The obtained hemolymph (about 0.5 ± 1 µL per pupa) was used for the following tests.

7.1 Electron microscopy

The hemolymph samples were analyzed at appropriate dilutions by negative staining with 0.5% aqueous uranyl acetate following adsorption onto Pelco® formvar and carbon-coated grids for 5 min. Grids were observed under a CM 10 electron microscope (Philips, Eindhoven, Netherlands) operating at 60 kV.

7.2 Western blot analysis with specific antibodies for coat protein-ToBRFV

Thirty-µL aliquots of hemolymph sample (extracted from about 60 pupae) were homogenized in 10 µL of 3X Laemmli sample buffer and incubated for 3 min at 95 °C. Following centrifugation (12,000 rpm, 5 min), 1 µL of this extract was loaded on 4–20% gradient Mini-PROTEAN-TGX gel (Bio-Rad, Richmond, CA) in Tris-glycine SDS-PAGE buffer and either stained with Coomassie brilliant blue and electroblotted to polyvinylidene difluoride membranes (Millipore, Billerica, MA) in transfer buffer (25 mM Tris, 192 mM glycine, 20% ethanol). Following blocking in 5% nonfat dry milk in PBS containing 0.05% Tween-20 for 1 h, membranes were incubated for 1 h with an anti-CP (coat protein) ToBRFV antibodies (A128IVPS, 1:2,000, kindly provided by Dr. Marina Ciuffo, IPSP-CNR) in PBS with 0.05% Tween-20. After three washings with PBS-0.05% Tween-20, membranes were further incubated for 1 h with the anti-rabbit horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich), diluted 1:20,000 in PBS-0.05% Tween-20, containing 1% bovine serum albumin. After three washes, the reaction was detected using the Supersignal West Pico Chemiluminescent substrate (Thermo Fisher Scientific, Rockford, IL). All incubations were performed at room temperature. Controls consisted of total protein extracts from a ToBRFV-infected tomato plant (positive control) and from a healthy tomato plant.

7.3 Hemolymph bioassay

Five µL of hemolymph were mixed in 15 µL phosphate buffer (pH 7.0); subsequently this suspension (5 µL/leaf) was mechanically inoculated in four leaves of healthy tomato plants, previously dusted with Carborundum (320 mesh). A total of 4 healthy tomato plants were inoculated, while two tomato plants inoculated with healthy sap were used as negative control. Thirty days after inoculation, plants were scored for symptoms and tested for the ToBRFV presence by RT-qPCR (Supplementary Fig. 1).

8 Results

All the tomato plants inoculated with the ToB-SIC01/19 isolate, showed typical ToBRFV symptoms as mosaic, bubbling and deformation on leaves 30 days-post-inoculation; molecular analyses by RT-qPCR confirmed the infection status. In Experiment 1, all *T. absoluta* adults obtained from larvae from non-contaminated eggs and developed on ToBRFV-infected tomato plants resulted positive to the virus in the RT-qPCR assay, with a mean Ct value (± SD) of 17.01 ± 2.59 (1.47×10^6 RNA copies).

The results obtained in Experiment 2 show that both not-disinfected pupae (group 1) and adults of *T. absoluta* obtained from not-disinfected pupae (group 2) resulted positive to ToBRFV, with mean Ct values of 15.21 ± 0.70 (4.28×10^6 RNA copies) and 13.25 ± 0.84 (1.37×10^7 RNA copies), respectively (Table 1). Disinfected pupae (group 3) and adults of *T. absoluta* obtained from disinfected pupae (group 4) were also found positive to ToBRFV, with mean Ct values of 20.11 ± 0.73 (2.33×10^5 RNA copies), and 14.62 ± 0.66 (6.08×10^6 RNA copies), respectively (Table 1). The highest and the lowest Ct values were found in disinfected pupae and in adults from not-disinfected pupae, respectively.

In Experiment 3, at 30 days post inoculation plants showed mosaic, bubbling and leaves deformation. Analyses conducted on plant material collected from plants infested by adults, obtained from disinfected and not-disinfected pupae, confirmed the virus presence, with a mean Ct values of 26.21 ± 3.41 (6.23×10^3 RNA copies) and 28.74 ± 2.09 (1.38×10^3 RNA copies), respectively.

Experiment 4 was carried out to verify if the viral particles were located or not into hemolymph. Electron microscopy revealed no rod-shaped structures typical of tobamoviruses in the hemolymph. In addition, the Western blot analysis, carried out in hemolymph extract, using specific antibodies for ToBRFV coat protein, did not show bands with a molecular weight (ca. 17.5 kDa), corresponding to coat protein of ToBRFV (Fig. 2). These results were further confirmed by hemolymph bioassay, as none of the four hemolymph-inoculated plants displayed typical ToBRFV symptoms and none of them resulted positive in the subsequently RT-qPCR assay.

Table 1. Results of the four groups of *Tuta absoluta* pupae and adults analyzed by RT-qPCR.

Tested material (group)	Replicate	Ct values	RNA copies
Not-disinfected pupae (1)	A	15.84	2.94×10^6
	B	15.37	3.89×10^6
	C	14.42	6.84×10^6
	Mean	15.21	4.28×10^6
Adults from not-disinfected pupae (2)	A	14.08	8.38×10^6
	B	13.27	1.35×10^7
	C	12.40	2.27×10^7
	Mean	13.25	1.37×10^7
Disinfected pupae (3)	A	20.84	1.51×10^5
	B	19.37	3.62×10^5
	C	20.13	2.30×10^5
	Mean	20.11	2.33×10^5
Adults from disinfected pupae (4)	A	14.87	5.24×10^6
	B	13.87	9.49×10^6
	C	15.13	4.49×10^6
	Mean	14.62	6.08×10^6

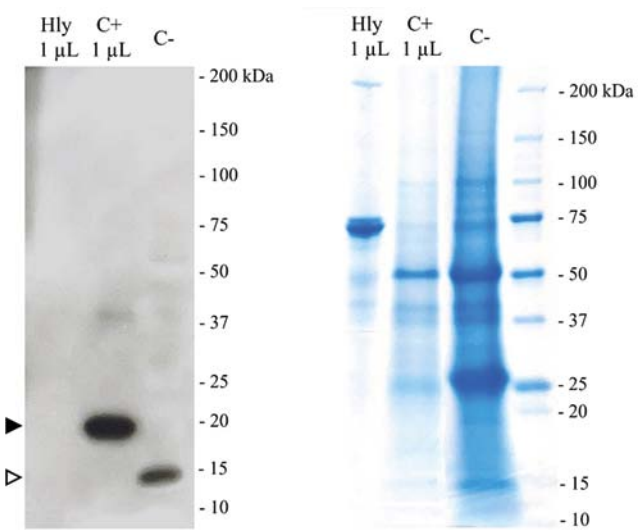

Fig. 2. Western blot analysis of ToBRFV in the hemolymph of disinfected *Tuta absoluta* pupae. Total proteins were extracted from the hemolymph (Hly) collected from disinfected pupae (group 3). C+ and C- represented total protein extracts from ToBRFV-infected or healthy tomato plants, respectively. Samples were loaded on a 4–20% gradient SDS-PAGE and the gels stained with Coomassie (right) or electroblotted onto PVDF membranes (left). The western blot was developed with an anti-CP (coat protein) ToBRFV polyclonal antibodies (1:2000) from IPSP-CNR, preabsorbed with soluble protein extracts from a healthy tomato plant. The black arrow indicates the coat protein of ToBRFV, while the empty arrow denotes an aspecific signal of unknown origin.

9 Discussion

Plant virus transmission by insects is well known for sap sucking insects, such as whiteflies or aphids (De Lillo et al. 2021; Mu et al. 2022). Few cases are reported in the literature regarding virus transmission by leaf-feeders which are responsible for the mechanical transmission of viruses, as a result of feeding damage with biting mouthparts. Some Coleoptera (Chrysomelidae and Coccinellidae) and Orthoptera (Tettigoniidae, Acrididae, Tetrigidae, Pyrgomorphidae and Gryllidae), are considered vectors of the Sobemovirus rice yellow mottle virus (RYMV) in Africa (Koudamiloro et al. 2015).

Tobamoviruses, including ToBRFV, are efficiently transmitted by mechanical contact, a frequent scenario occurring during agronomic practices. To date, the only reported case of tobamoviruses transmission by insect regards bumblebees, which are capable to transfer tobacco mosaic virus (TMV) and ToBRFV from contaminated to healthy tomato plants (Okada et al. 2000; Levitzky et al. 2019). Moreover, bumblebees seem to be involved in the transmission of the potexvirus pepino mosaic virus (PepMV) to tomato (Shipp et al. 2008). ToBRFV transmission by bumblebees possibly occurs during pollination, as bumblebee buzzing may contribute to mechanical transmission of the virus, because no evidence of the presence of ToBRFV within the insect body has been reported (Levitzky et al. 2019). As ToBRFV spreads very rapidly, reaching up to 100% plant infection in protected tomato crops (Panno et al. 2020b), the potential epidemiological role of the main herbivore arthropods needs to be clarified.

In this context, the main aim of this work was to ascertain whether *T. absoluta* have the ability to transmit ToBRFV from infected to healthy tomato plants. Our experiments demonstrated the transmission of ToBRFV primary *inoculum* by *T. absoluta* adults, obtained from larvae developed on infected tomato plants in laboratory conditions. All adults reared from eggs laid by uninfected females on ToBRFV-infected plants, resulted positive to ToBRFV and were able to transfer the virus to healthy tomato plants. In addition, the results of Experiment 3 showed that adults of *T. absoluta* obtained from not-disinfected and disinfected pupae were positive to ToBRFV, and were able to infect healthy tomato plants, indicating that the virus presence is not limited to the pupae external surface. Moreover, the virus acquired during larval stage survives across the pupal and resulting adult stages.

Furthermore, the Experiment 4, in which electron microscopy and western blot analyses using hemolymph were carried out, showed the absence of ToBRFV virions and viral coat protein in the hemolymph collected from disinfected pupae, demonstrating that the ToBRFV is not circulative in the progeny of *T. absoluta* adults obtained from larvae fed in infected plants. These results were further confirmed by bioassay, in fact none of the inoculated plants with the hemolymph collected from disinfected pupae resulted positive to ToBRFV at 30 days post inoculation.

These results allow to speculate that the virus contamination of adults obtained from disinfected pupae (Experiment 3) could occur during their emergence, when touching the inner surface of the pupal exuvia. The transmission of ToBRFV primary *inoculum* by infected adults to healthy plants could be explained by direct mechanical transmission, possibly due to small wounds on the upper tomato leaf surface, caused by infected adults tapping with the uncoiled proboscis (Baetan et al. 2015), or through wounds produced by larvae during their penetration or during feeding activity inside their mines.

The high mobility of this pest, together with the different and efficient transmission modes of the virus (Biondi et al. 2018; Chanda et al. 2021), suggest that *T. absoluta* could be responsible for virus spread among crops and persistence across cropping seasons. This is particularly important in the cropping systems, such as protected tomato crop in the Mediterranean basin, where arthropod and disease movement occurs not only between inner and outer areas of the greenhouses, but also among greenhouses (Gabarra et al. 2004). Currently, the management of this pest is mainly based on the adoption of Integrated Pest Management (IPM) programs (Desneux et al. 2022) involving non-chemical approaches and tools, with biological control being the main component (Passos et al. 2022; Ricupero et al. 2022; Yao et al. 2022). However, biological control of plant virus vectors has rarely been implemented as its efficiency in keeping the incidence of the virus under the economic threshold has not been clearly demonstrated (Roudine et al. 2023). Therefore, in this scenario, it will be important to specifically assess whether key natural enemies of *T. absoluta* can prevent virus spread in the short-distance in the case of biocontrol agents of moth juveniles (Desneux et al. 2022), and potentially in the long-distance in the case of natural enemies of *T. absoluta* adults, that are mainly birds, bats and reptiles (Díaz-Siefer et al. 2022; Janssen et al. 2023). Moreover, it could be important clarifying how biocontrol agents could be directly be involved in ToBRFV plant-to-plant transmission. This could be more important for omnivorous predators, such as mirid bugs (Heteroptera: Miridae), which can feed on both the pest and the plants (Biondi et al. 2016).

In conclusion, new integrated pest management strategies for *T. absoluta* control in the countries in which ToBRFV occurs will have to be adapted to the concurrent presence of both the insect pest and the virus.

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