Elicitors in the production of tomato crop infected with Tomato brown rugose fruit virus and Pepino mosaic virus

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Abstract

Elicitors are considered sustainable alternatives for the management of plant viruses. The aim of the present study was to evaluate the effectiveness of nine elicitors on morphology, yield, and fruit quality of tomato plants inoculated with *Tomato brown rugose fruit virus* (ToBRFV), *Pepino mosaic virus* (PepMV), and both (ToBRFV + PepMV). The experiment was set up under a completely randomized design with six replicates. Ten days after transplanting, virus inoculation was done mechanically in all the treatments, except the negative controls. Three morphological, four yield, and seven fruit quality variables were evaluated. Virablock® 3G50 increased yield by more than 44% compared with the positive control in plants inoculated with ToBRFV, PepMV, and the mixed infection, while Supermagro cell extract and enhanced Supermagro increased yield in plants inoculated with ToBRFV and the mixed infection by more than 82% and 34%, respectively. Virablock® 3G50 and an ethanolic extract of *Arracacia bracteata* showed the highest values in fruit quality variables.

Introduction

Plant viruses cause high yield losses, affect the quality of agricultural products and due to disease management, production costs increase (EPPO 2020; Rubio et al. 2020).

Every year, new virus species are reported that have an impact on different crops worldwide. Until 2020, 312 species of viruses, satellite viruses or viroids associated with tomato crops were recorded (Rivarez et al. 2021). 45 out of these 312 species were new ones reported from 2011 to 2020 (Rivarez et al. 2021). In addition, *Tomato brown rugose fruit virus* (ToBRFV) was the only newly reported case in which 13 out of 14 criteria established for a complete biological characterization were met (Hou et al. 2020; Rivarez et al. 2021). This shows the imbalance between the number of new virus species described and the number of biological characterization studies and management strategies. The great diversity of viral species co-infecting a crop makes the pathosystem more complex and it may lead to increased virulence and severity due to a synergistic interaction (Rubio et al. 2020).

To date, there are no viricides available to treat virus-infected plants. The management approaches for viral pathogens include 1) prevention and 2) treatment of diseased plants.

The preventive approach consists of a series of cultural and agronomic practices to avoid infection (Gergerich and Dolja 2006). Preventive actions are associated with the plant itself (use of virus-free certified seed or plant, planting of resistant or tolerant varieties), water (irrigation water quality and treatment), soil (sterilization of substrates, disinfestation of equipment and surfaces prior to crop establishment), and cultural management (establishment of occupational hygiene measures, elimination of alternate hosts of vectors and viruses, vector control, crop rotation, and elimination of inoculum source).

The management of virus infected plants aims to reduce the spread of viruses and infection severity (Rubio et al. 2020). These actions include seed treatment, elimination of infected plants when incidence is low, use of elicitors to reduce infection severity, elimination of alternate hosts of vectors and viruses, the establishment of physical or plant barriers to prevent the appearance of vectors on the crop, installation of traps or attractants for insect vectors, as well as monitoring and controlling insect vectors.

The use of elicitors or defense inducers is an alternative method to reduce infection severity and yield loss in virus infected plants. The effectiveness of each product depends on several factors such as virus species, crop, dosage, timing, and form of application of the product, among other factors. The alternate application of Messenger gold® and MC Cream® on tomato plants infected with *Tobacco mosaic virus* reduced virus concentration and increased total plant dry weight, root length, and average weight of asymptomatic fruits compared to the positive control (Hernández-Santiago et al. 2020). In tomato plants infected with ToBRFV, Virablock 3G50® increased yield per hectare (65%), Optifert® increased the number of fruits per bunch (28.6%), and Silicant® increased the number of bunches per plant (25%) (Ortiz-Martínez and Ochoa-Martínez 2022).

ToBRFV is one of the most important viruses in tomato cultivation worldwide, and its coinfection with PepMV has been reported to increase disease severity (Klap et al. 2020). Therefore, this study aims to evaluate the effectiveness of nine elicitors on morphology, yield, and fruit quality of tomato plants infected with ToBRFV, PepMV, and a mixed infection of ToBRFV + PepMV.

Materials and methods

Plant material

PaiPai tomato seeds® (Enza Zaden, Mexico) were sown in two 200-cavity polystyrene trays with sterile Peat Moss as substrate (PRO-MIX FLEX, Canada) in a controlled environment chamber, with day and night temperature of 27°C ± 1 and 16 hours of light. Seedlings were transplanted 42 days after sowing in 40 x 40 cm black polyethylene bags with volcanic rock as substrate. The spacing between pots was 30 cm, and it was 75 cm between rows. One plant per pot was transplanted at a density of 4.4 plants/m^2.

The crop was grown in a greenhouse, with the average and maximum temperatures of 16°C and 36°C, respectively, and the average and maximum relative humidity of 59%-95%. Plants were kept in a hydroponic system that supplied a nutrient solution with the following concentrations: 127 mg/L N, 77 mg/L P, 231 mg/L K, 244 mg/L Ca, 48 mg/L Mg, 118 mg/L S, 10 mg/L Fe, 1.15 mg/L B, 1 mg/L Mn, 0.5 mg/L Cu and 0.4 mg/L Zn (Juárez-Rodríguez et al. 2021). Lateral shoots were pruned every six days.

Treatments and experimental design

Nine elicitors (Table 1) were evaluated in three pathosystems: ToBRFV, PepMV, and a mixed infection (ToBRFV + PepMV). Foliar applications were made every 10 days until 130 days after inoculation (dai), for a total of 14 applications. The first application of the elicitors was conducted two hours before the mechanical inoculation of the viruses. The foliar applications were 15 ml/plant in the vegetative stage and 22.5 ml/plant in the flowering and fruiting stages. Each pathosystem was considered as an independent experiment and they were all established under a completely randomized design with six replications in which the experimental unit consisted of one plant.

Table 1
Products evaluated and concentration used

Treatment	Dose
Negative control	N/A[a]
Arracacia bracteata[b]	2.5 ml/L
Plant Extract PLUS 5[b]	2.5 ml/L
Virablock® 3G50	1.8 ml/L
Supermagro[c]	100 ml/L
Enhanced Supermagro[b]	10 ml/L
Supermagro cell extract[b]	10 ml/L
Supermagro cell extract PLUS 5[b]	2.5 ml/L
BacilluZn®	5 g/L
Haifa ProteK Total®	5 g/L
Positive control	N/A

[a]Not applicable; [b]Product developed in this study; [c]Free product and commonly used in organic agriculture; ®Commercial product

Elicitor formulation

Eleven plant species were collected in the municipality of Mixistlán de la Reforma, Oaxaca, Mexico, and evaluated for their elicitor effect on resistance to ToBRFV by the methodology proposed by Madhusudhan et al. (2011).

Ethanolic extracts

Leaves and stems of the five best plants that reduced the number of local lesions were collected, washed, and cut into 5 cm pieces approximately. The pieces were stored in Kraft bags and dried in a 60°C oven for 72 hours. After drying, they were crushed and pulverized. 50 g of the obtained powder was added to a beaker containing 200 ml of 100% ethanol as solvent and placed in a magnetic rack with agitation for 48 hours (Shami et al. 2013; Benítez-Benítez et al. 2019). The extract obtained was filtered using a Wathman No. 1 filter paper, and then the filtrate was placed in a rotary evaporator at 150 rpm at 60°C to remove the solvent and obtain the concentrate extract. A 20% dilution in distilled water was prepared with the *Arracacia bracteate* extract. Likewise, a 50% dilution was prepared by

mixing the concentrated ethanolic extracts of *Arracacia bracteata* (10%), *Litsea glaucescens* (10%), *Calea prunifolia* (10%), *Cleome magnifica* (10%), and *Bouganvillea spectabilis* (10%) in distilled water, which was named Plant Extract Plus 5 (Table 1).

Supermagro is a biofertilizer derived from anaerobic fermentation of bovine manure, a source of microorganisms enriched with minerals. The final product is a liquid that is used in foliar and soil applications to improve crop productivity (Roa 2015). In a previous study, Supermagro was applied to tomato plants infected with ToBRFV (Ortiz-Martínez and Ochoa-Martínez 2022), and elements that were not sufficiently supplemented were identified by the nutritional analysis of the treated plants. Based on this information, these nutrients were added to the original Supermagro (Restrepo 2007) to obtain a preparation known as enhanced Supermagro (Table 1).

A product called Supermagro cell extract (Table 1) was made by centrifuging 4L of enhanced Supermagro at 4000 rpm for 10 min and autoclaving the supernatant at 121°C and 15 lb pressure for 20 min (Ahmed and Baig 2014; Ramirez-Estrada et al. 2016). Another product, named Supermagro cell extract PLUS 5 was formulated by mixing 50% Supermagro cell extract and 50% Vegetable Extract Plus 5. All products were kept at -20°C until use, except for Supermagro cell extract which was kept at room temperature.

ToBRFV and PepMV inoculum

ToBRFV inoculum was obtained from the TBRFV-MX-CP isolate (Ortiz-Martínez and Ochoa-Martínez 2022) by four transfers of a local lesion in *Nicotiana glutinosa*, after which it was increased in *Solanum lycopersicum.* A tomato plant with typical PepMV symptoms (Hanssen and Thomma 2010), positive by RT-PCR for PepMV and negative for tobamovirus, was used as a source of PepMV inoculum.

Ten days after transplanting, mechanical inoculation of the viruses was performed on four apical leaflets per plant dusted with carborundum and swabbed with a swab containing infective sap. For the mixed inoculation, two leaflets were inoculated with ToBRFV and two with PepMV. The macerate consisted of 1 g of infected tissue and 10 mL of 0.01 M phosphate buffer pH 7.0. The apical leaflets of the negative controls were dusted with carborundum and rubbed with phosphate buffer only. All inoculated or rubbed leaflets were rinsed with distilled water.

ToBRFV and PepMV detection

Plants from the 11 treatments of each pathosystem were analyzed by RT-PCR 20 days after inoculation (dai) to confirm the transmission of ToBRFV and/or PepMV. RNA extraction was performed from 100 mg of leaf tissue using the SV Total RNA Isolation System® kit (Promega), following the manufacturer's instructions. RNA quality and concentration were quantified by spectrophotometry on a Nanodrop 2000 (Thermo Scientific™), while RNA integrity was verified on 1% agarose gel. The RT-PCR reaction was performed with the QIAGEN OneStep RT-PCR kit (QIAGEN), following the manufacturer's instructions. The primers used for ToBRFV and PepMV detection were ToBRFV-FMX / ToBRFV-RMX, and KL05-13 / KL05-14, respectively, which amplify 475 base pairs of the viral replicase coding region of ToBRFV and 202 base pairs of the TGB2-3 region of PepMV (Ling et al. 2008; Rodriguez-Mendoza et al. 2019). The amplified products were visualized on 1% agarose gel.

Variables evaluated

Fourteen variables classified into three categories, including morphological, yield, and fruit quality variables were evaluated. Among the morphological variables, chlorophyll content (30 dai), plant height (30 and 60 dai), and number of clusters (30, 60, and 113 dai) were evaluated. Yield variables were evaluated 113, 127, and 141 dai, including number of fruits per bunch, number of fruits per plant, weight of fruits per plant, and yield per hectare. The quality variables evaluated were number of small fruits/plant, number and weight of asymptomatic fruits/plant, number and weight of deformed fruits/plant and number and weight of fruits with irregular ripening/plant. The quality variables were evaluated 113, 127, and 141 dai, except for the number of small fruits, which were harvested and evaluated 113 dai.

Chlorophyll content was determined with the portable quantifier atLEAF® CHL Plus (FT Green LLC, USA) on the anterior leaf of the last bunch of the plant (high stratum), while plant height was measured from the base to the apex of the stem with a flexometer. The weight of fruits per plant, the weight of asymptomatic fruits, deformed fruits, and fruits with irregular ripening were measured with a BASE-5EP digital scale; yield per hectare was calculated with the formula:

$$\text{Yield (t/ha)} = \left(\frac{(\text{weightoffruitsperplant(g)xnumberofplantsperm}^2)\text{x}10{,}000\text{m}^2}{1{,}000{,}000\text{(g)}} \right) \text{xnumberofcyclesperyear}$$

Statistical analysis

The normality and homogeneity of variances tests were performed on the data obtained for the variables evaluated. An analysis of variance and a Duncan's Multiple Range test ($P \leq 0.05$) were conducted in those variables that met the assumptions of normality and homogeneity of variance. The statistical analysis was conducted using SAS 9.0 statistical package (SAS Institute Inc. 2002).

Results

Extracts of *Arracacia bracteata, Litsea glaucescens, Calea prunifolia, Cleome magnifica*, and *Bouganvillea spectabilis* reduced the number of local lesions by 72%-90% in *N. glutinosa* plants (unpublished data) and were selected for the formulation of the products described above.

Plants inoculated with PepMV and ToBRFV + PepMV (mixed infection) showed symptoms 11 dai, while plants infected with ToBRFV showed symptoms 15 dai. Mosaic, blistering, and leaf deformation were observed in all infected plants (Fig. 1a-i). Plants infected with both viruses also showed reduced leaf size and general chlorosis (Fig. 1j, k). 60 dai, symptoms were less visible in plants inoculated with PepMV and ToBRFV. However, the expression of symptoms became more evident 113 dai. Plants inoculated with both viruses showed systemic symptoms at all time points. In the BacilluZn treatment®, plants inoculated with ToBRFV ceased to show mosaic and blistering 113 dai. On the other hand, irregular fruit ripening was observed in all three pathosystems, while plants inoculated with PepMV showed a greater reduction in fruit size, and excessive fruit deformation was more frequently observed in plants infected with both viruses (Fig. 1m-o).

Detection of ToBRFV and PepMV

According to the results obtained by RT-PCR analysis, plants infected with either ToBRFV or PepMV were positive only for viruses inoculated (Fig. 2a, b, and Fig. 2e, f, respectively); whereas both viruses were detected in plants inoculated with both viruses (Fig. 2c, d). There was no virus detection in the negative control plants.

Variables evaluated

Morphological variables

Chlorophyll content increased by 35%, 29%, and 26% in plants inoculated with ToBRFV and treated with ethanolic extract of *Arracacia bracteata*, Supermagro cell extract, and enhanced Supermagro, respectively, compared to the positive control. Plant Extract Plus 5 increased chlorophyll concentration by 52% (Fig. 3a) in plants inoculated with ToBRFV + PepMV. No significant differences were obtained in this variable in plants inoculated with PepMV.

The height of those plants that were inoculated with ToBRFV and treated with Supermagro cell extract PLUS 5, and Supermagro increased by 15% and 20%, respectively, when compared to the positive control (Fig. 3b). In PepMV-inoculated plants with applications of Supermagro cell extract, enhanced Supermagro, Virablock® 3G50, Supermagro, and ethanolic extract of *Arracacia bracteate*, height increased by more than 10% compared to the positive control. In plants inoculated with both viruses, the plant height in the positive control was statistically similar to that of the best treatments.

The ToBRFV positive control and the plants inoculated by the mixed infection showed the lowest chlorophyll concentration, while the positive control plants inoculated with ToBRFV or PepMV showed the lowest height values.

Performance variables

The number of fruits per bunch was increased by more than 165% compared to the positive control in plants infected with ToBRFV + PepMV and treated with Supermagro, enhanced Supermagro, Supermagro cell extract, BacilluZn®, and Haifa ProteK Total® (Fig. 4a). Virablock® 3G50 increased the number of fruits per bunch in PepMV-infected plants by 89%. No significant differences between treatments were observed in plants inoculated with ToBRFV.

Virablock® 3G50 increased more than 117% of fruit number per plant with respect to the positive control in PepMV-infected and mixed-infected plants (Fig. 4b). On the other hand, treatments with ethanolic extract of *Arracacia bracteata*, PLUS 5 plant extract, Supermagro, enhanced Supermagro, Supermagro cell extract, Supermagro cell extract PLUS 5, and BacilluZn® were significantly different with respect to the positive control in plants inoculated with both viruses.

In plants inoculated with ToBRFV, PepMV, and mixed infection treated with Virablock® 3G50, fruit weight per plant and yield per hectare increased by 44%, 131%, and 134%, respectively (Fig. 4c, d). Supermagro cell extract increased fruit weight per plant and yield per hectare in plants inoculated with ToBRFV and the mixed infection by 92% and 83%, respectively. Enhanced Supermagro showed an increase of 35% and 74% in the ToBRFV and mixed infection pathosystems. The positive control had the lowest value in all three pathosystems.

Quality variables

Virablock® 3G50, ethanolic extract of *Arracacia bracteata*, and Supermagro cell extract increased the number of asymptomatic fruits by more than 100% with respect to the positive control in PepMV-inoculated plants (Fig. 5a). There were no significant differences between treatments in the ToBRFV pathosystems and mixed infection.

Virablock® 3G50, ethanolic extract of *Arracacia bracteata*, Supermagro cell extract, and Supermagro reduced the number of small fruits by more than 31% with respect to the positive control in PepMV-infected plants (Fig. 5b). No significant differences were observed for the ToBRFV pathosystems and mixed infection.

Virablock® 3G50 and ethanolic extract of *Arracacia bracteata* increased asymptomatic fruit weight by more than 100% with respect to the positive control in PepMV-infected plants, while BacilluZn® increased asymptomatic fruit weight by 240% in plants inoculated with ToBRFV (Fig. 5c). No significant differences were obtained between treatments in plants with the mixed infection.

Positive controls on fruit yield and quality

The ToBRFV positive control showed higher yield compared to the PepMV positive control and mixed infection 127 and 141 dai (Fig. 6a), while the mixed infection showed the lowest yield113 dai.

The PepMV positive control had higher weight of asymptomatic fruit relative to the ToBRFV + PepMV positive control 113, 127, and 141 dai (Fig. 6b). The ToBRFV positive control was statistically similar to the mixed infection in all three evaluations.

Discussion

The severity and expression of symptoms in virus infected plants depend on environmental conditions, virus isolates, and plant variety (Hanssen and Thomma 2010). In general, as reported by different authors, PaiPai tomato plants® inoculated with ToBRFV, PepMV, and both viruses showed the typical symptoms of mosaic, blistering, leaf deformation, and irregular fruit ripening (Hanssen and Thomma 2010; Menzel et al. 2019; Zhang et al. 2022). Additionally, plants infected with ToBRFV + PepMV showed reduced leaf size, general chlorosis, and severe fruit deformation suggesting a phenomenon of synergism.

Virablock® 3G50 had the highest values in yield variables in plants inoculated individually or with both viruses, the same results were observed previously in plants infected with ToBRFV (Ortiz-Martínez and Ochoa-Martínez 2022). It is known that some elicitors can be specific and useful in case of a certain virus but they can prove to be ineffective in case of others (Zellner et al. 2011). This characteristic of elicitors was not observed in the case of Virablock® 3G50, possibly because it is formulated with more than 50 active ingredients of natural origin (GreenCorp 2022) that can activate or potentiate a greater number of plant defense mechanisms.

Supermagro cell extract and enhanced Supermagro increased fruit weight per plant and yield per hectare with respect to the positive control in the ToBRFV pathosystem and mixed infection, while Supermagro was only superior to the positive control in the ToBRFV pathosystem. These results demonstrate that the sterilization process and the addition of certain nutrients in the original formulation (Supermagro) (Ramírez-Estrada et al. 2016; Ortiz-Martínez and Ochoa-Martínez 2022) improved the response of infected plants, despite the fact that the modified formulas (Supermagro cell extract and enhanced Supermagro) had a reduction in application rates from 10–1%.

The ethanolic extract of *Arracacia bracteata* increased the number and weight of fruits per plant, yield per hectare, plant height, chlorophyll content, number and weight of asymptomatic fruits, and reduced the number of small fruits relative to the positive control in single or combined infections of ToBRFV and PepMV. It has also been reported that *Solanum nigrum* ethanolic extract has the ability to reduce the incidence and severity of *Tomato mosaic virus* (ToMV) in *Capsicum annum* plants by 20% and 15%, respectively (Elhelaly and El-shennawy 2022). Some plant extracts such as *Bougainvillea spectabilis, Azadirachta indica*, and *Pongamia glabra* reduced viral concentration in ToMV-infected tomato plants (Madhusudhan et al. 2011). Likewise, the ability of methanolic extracts of *Combretum micranthum* and *Allium sativum* to disintegrate ToBRFV viral particles has recently been reported (Iobbi et al. 2022; Iobbi et al. 2023).

These results show the need to obtain methanolic extracts of the plants used in the present investigation and to evaluate their behavior individually or combined with ethanolic extracts as elicitors.

The results of the present study demonstrate that the use of elicitors or defense inducers is a sustainable alternative to reduce yield loss in plants infected with ToBRFV and PepMV.

Declarations

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Conflict of interest. The authors declare that they have no conflicts of interest.

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Figures

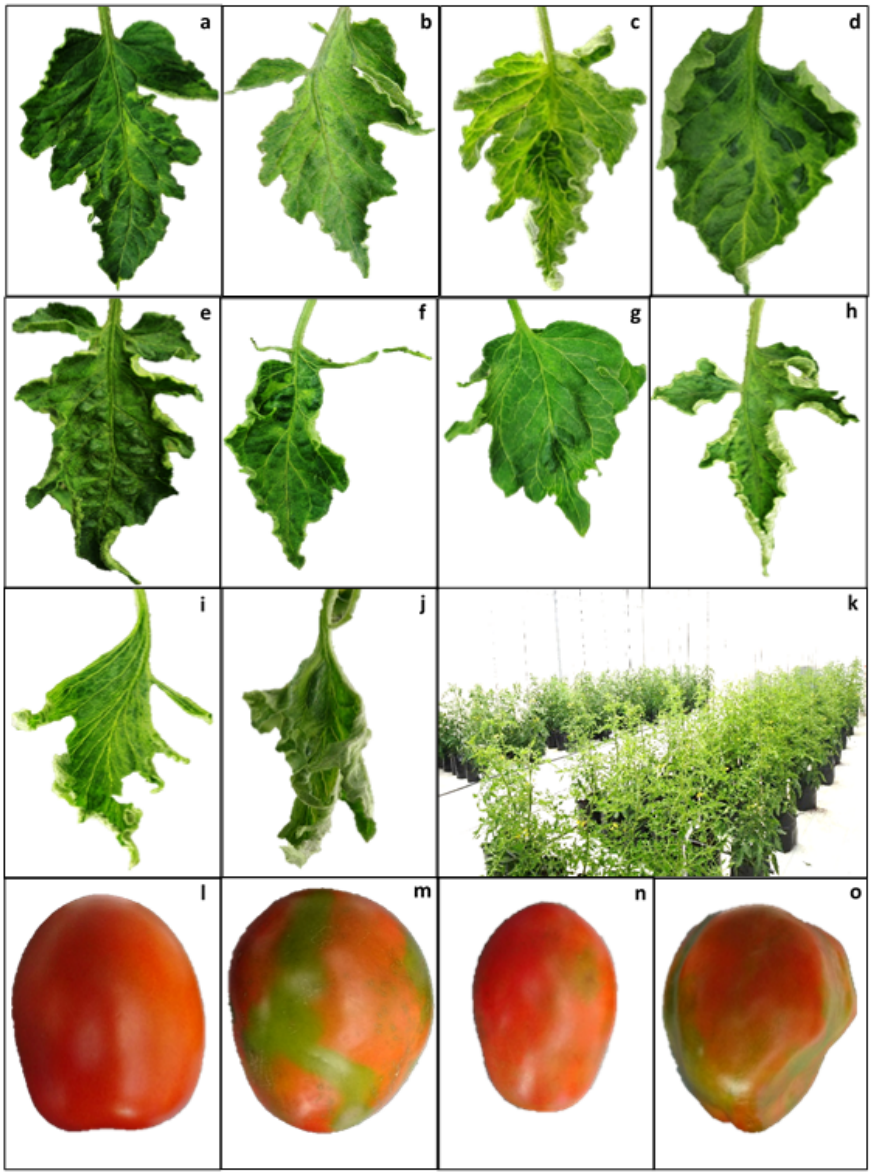

Figure 1

Viral symptoms in saladette tomato var. Pai pai®. **a**, **b,** and **c** mosaic; **d**, **e,** and **f** blistering; **g**, **h.** and **i** leaf deformation induced by ToBRFV, PepMV and mixed infection, respectively; **j** leaf size reduction induced by mixed infection; **k** general chlorosis in plants with mixed infection; **l** healthy fruit; **m** irregular ripening by ToBRFV; **n** irregular ripening and fruit size reduction induced by PepMV; **o** irregular ripening and excessive fruit deformation in plants with mixed infection

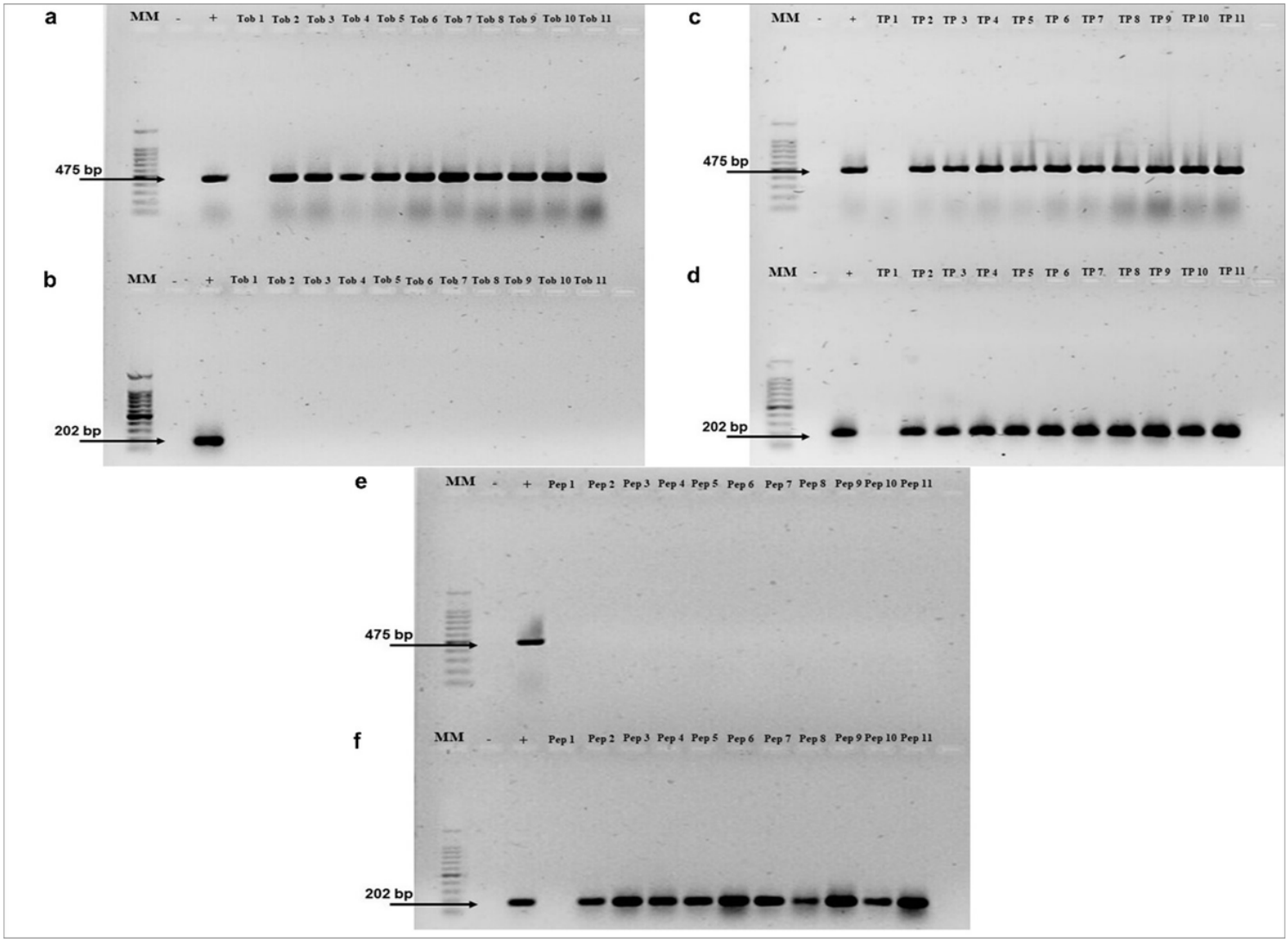

Figure 2

RT-PCR products obtained with primers ToBRFV-FMX/ToBRFV-RMX and KL05-13/KL05-14. MM (Molecular marker 100 bp); - (Negative control with water). **a** + ToBRFV; lanes Tob 1-Tob 11 ToBRFV pathosystem. **b** + PepMV. **c** + ToBRFV; lanes TP1-TP11 ToBRFV pathosystem + PepMV. **d** + PepMV. **e** + ToBRFV; lanes Pep 1-Pep 11 PepMV pathosystem. **f** + PepMV

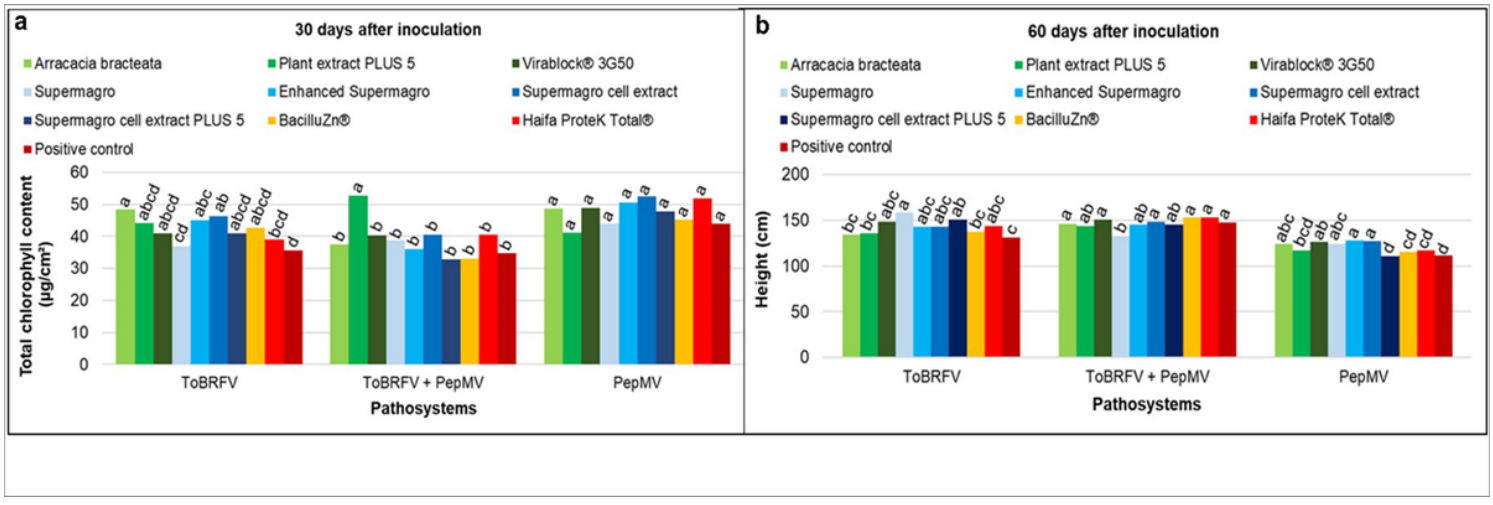

Figure 3

Morphological variables evaluated. **a** total chlorophyll content, and **b** height. Bars with different letters in each pathosystem are statistically different ($P \leq 0.05$)

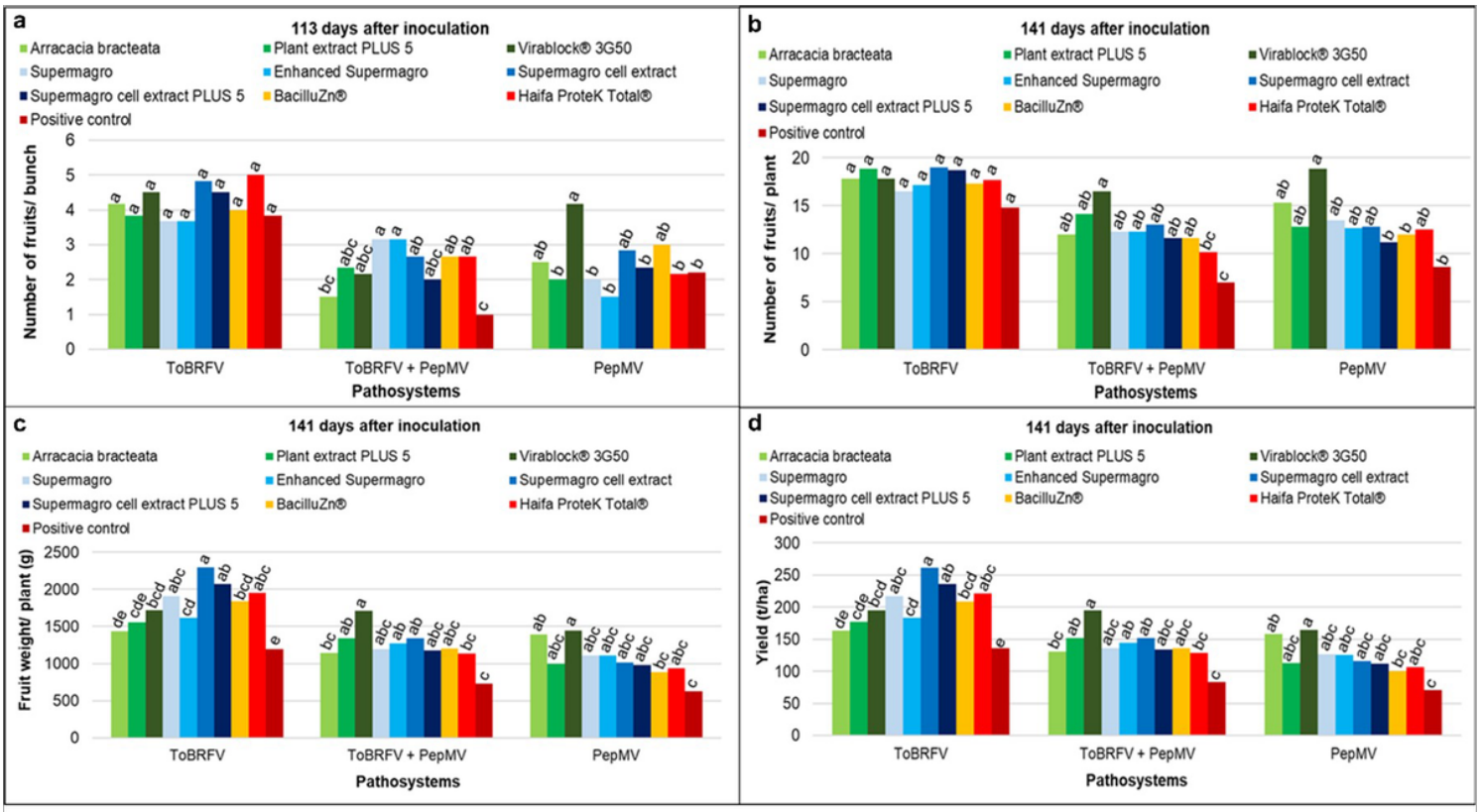

Figure 4

Yield variables evaluated. **a** number of fruits per bunch, **b** number of fruits per plant, **c** fruit weight per plant, and **d** yield per hectare. Bars with different letters in each pathosystem are statistically different (Duncan, $P \leq 0.05$)

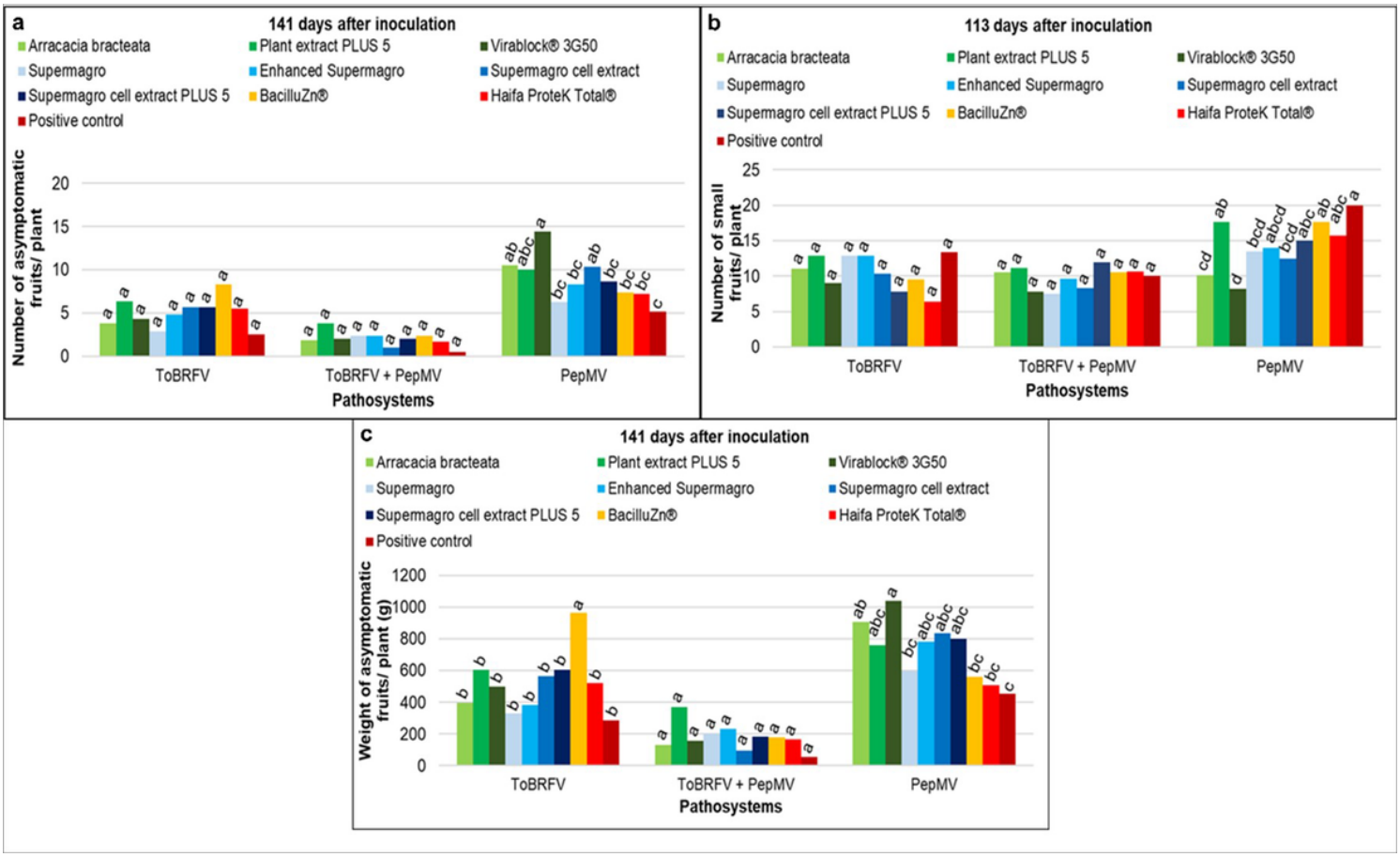

Figure 5

Fruit quality variables evaluated. **a** number of asymptomatic fruits per plant, **b** number of small fruits per plant, and **c** weight of asymptomatic fruits per plant. Bars with different letters in each pathosystem are statistically different (Duncan, $P \leq 0.05$)

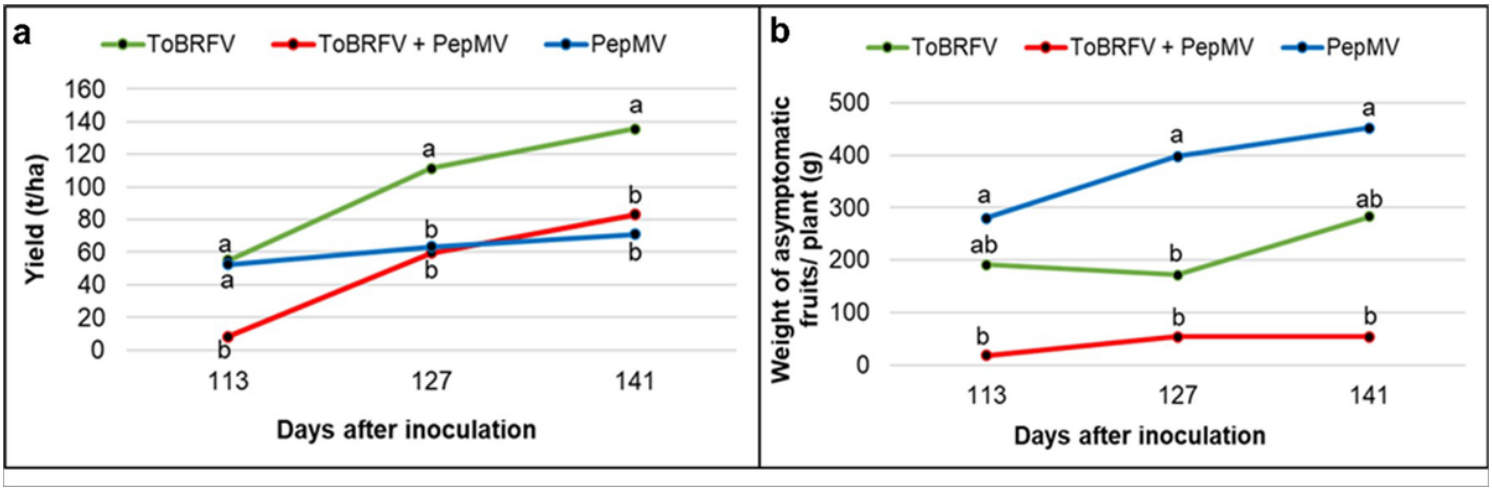

Figure 6

Performance of positive controls in three evaluations. **a** yield per hectare and **b** weight of asymptomatic fruit per plant. Points with different letters in each evaluation stage are statistically different (Duncan, $P \leq 0.05$)