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Phytopathological Note

Use of endophytic microorganisms for the management of *Tomato brown rugose fruit virus* in tomato crop (*Solamun lycopersicum*)

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ABSTRACT

Background and objective: The *Tomato brown rugose fruit virus* (ToBRFV) is one of the main pathogens affecting tomato crops in Mexico. Despite efforts to prevent its spread, it is nearly impossible due to its low transmission percentage through seeds and its high susceptibility to being transmitted through cultural practices. Therefore, alternative management strategies are being sought. This research aimed to determine the effect of endophytic microorganisms applied to the soil on tomato plants infected with ToBRFV.

Materials and Methods: A tomato plant was used as an experimental unit, with 13 repetitions per treatment. The treatments on tomato plants infected with ToBRFV were *Beauveria peruviencis*, *Trichoderma longibrachiatum*, *Pseudomonas* sp. and water as a sick witness; a treatment of healthy plants treated with water was also included as an absolute control. The response variables were plant height, fresh weight of the aerial part and root and severity (two evaluations). Measurements were analyzed using Tukey-Kramer HSD tests for each pair.

Results and conclusion: Significant differences were found *Beauveria peruviencis*, *Trichoderma longibrachiatum*, *Pseudomonas* sp. and water as a sick witness. The treatment that most favored the development of infected plants (79% taller and 15% heavier than infected mock) and reduced its severity was *B. peruviensis*, followed by *Pseudomonas* sp. On the other hand, the treatment that resulted in the least plant development (31% smaller than infected mock) and even increased the severity of the infection was *T. longibrachiatum*.

Key words: *Beauveria*, *Pseudomonas*, *Trichoderma*, Severity.

The tomato (*Solanum lycopersicum*) crop stands out as one of the most lucrative and widely consumed agricultural commodities, with a global harvest of 256,770,679.92 tons recorded in 2021 (FAO, 2023). However, its productivity faces a significant threat from the *Tomato brown rugose fruit virus* (ToBRFV), initially identified in 2014 in Israel and subsequently in 2015 in Jordan within tomato plants (Luria *et al*., 2017; Salem *et al*., 2016). Since then, ToBRFV has been identified in 35 countries across Europe, Asia, Africa, and North America, including Mexico (Cambrón-Crisantos *et al*., 2018; Menzel *et al*., 2019; Fidan *et al*., 2019; Skelton *et al*., 2019; Yan *et al*., 2019; Ling *et al*., 2019; Panno *et al*., 2019; Alkowni, 2019; Caruso *et al.,* 2022; EPPO, 2023).

ToBRFV is classified as a Tobamovirus, characterized by rigid rod particles and a single-stranded RNA (+ssRNA) genome consisting of four open reading frames (ORFs) (Luria *et al*., 2017). The virus exhibits high stability, facilitating mechanical transmission, making cultural activities the primary mode of dissemination in greenhouses (Levitzky *et al*., 2019; Panno *et al*., 2020). Additionally, ToBRFV can be transmitted through seeds (Davino *et al*., 2020). Reported transmission percentages include 1.8% in tomato seeds and less than 1% in tobacco (*Nicotiana rustica*) seeds (Davino *et al.*, 2020; Zamora-Macorra *et al*., 2023). Notably, even a transmission percentage as low as 0.001% has the potential to initiate an epidemic (Mohan *et al*., 2020). Given the widespread dissemination of ToBRFV in Mexico, ongoing efforts are directed towards devising preventative strategies. The control of such pathogens is challenging, with limited direct and effective tactics available. However, investigations have explored strategies such as utilizing resistant varieties and inducing natural defenses in plants (Kloepper *et al*., 2004).

Induced systemic resistance in plants is facilitated by endophytic, epiphytic, and rhizospheric microorganisms, establishing mutualistic relationships with plants. These microorganisms contribute to enhanced nutrient assimilation, growth promotion, increased stress tolerance, and the induction of defenses against phytopathogenic microorganisms (Umesha et al., 2018; Yadav et al., 2020). Notably, most bacteria reported as growth promoters belong to the genera *Pseudomonas* and *Bacillus*, among others (Vessey, 2003). Extensive research demonstrates that the inoculation of growth-promoting microorganism strains in crops, including tomatoes, not only improves plant development but also mitigates the incidence and severity of viral diseases (Samaniego, 2017; Kandan et al., 2005; Beris et al., 2018; Murphy et al., 2003; Dashti et al., 2012). Consequently, this study aimed to assess the impact of microorganisms applied to the roots of tomato plants infected with ToBRFV, employing response variables such as aerial and root growth, along with disease severity.

The evaluated treatments were as follows: 1) *Beauveria peruviencis* on diseased plants, 2) *Trichoderma longibrachiatum* on diseased plants, 3) *Pseudomonas* sp. on diseased plants, 4) Water on healthy plants, and 5) Water on diseased plants. The microorganism strains were sourced from the collection of the biological control laboratory of the Master's program in Plant Protection at Universidad Autónoma Chapingo. Strains, grown for 15 days on Papa Dextrose Agar medium (for *T. longibrachiatum*) and Saboraud Dextrose Agar (for *B. peruviencis*), were incubated at 27 °C. *Pseudomonas* sp. bacteria were cultured on Nutrient Agar at 27 °C for 24 hours, and for higher concentration, they were re-isolated in liquid medium (Nutrient Broth) under agitation at 140 rpm, at room temperature. Subsequently, microorganisms were suspended in liquid for cell counts using the Neubauer chamber, with concentrations set at $1X10^8$ for fungi and $1X10^9$ for bacteria in the treatments.

The experiments were conducted from February to June 2023 in the greenhouse of the Department of Agricultural Parasitology at the Universidad Autónoma Chapingo. Saladette tomato plants were planted in 1 L containers using previously sterilized peat moss as a substrate. When the plants reached two true leaflets, 20 mL of various treatments were applied to the soil. Two days later, the plants were mechanically inoculated with the virus, causing sublethal damage to the leaves with carborundum 400 mesh. Immediately afterward, the leaves were rubbed with a macerate of the inoculum source in phosphate buffer pH 7.5. The inoculum source of ToBRFV was obtained from the biological collection of the laboratory of phytopathogenic viruses at the Colegio de Postgraduados. At 41 days after mechanical inoculation (dami), 20 mL of the microorganism suspension was reapplied to the soil of each plant.

A completely randomized design was employed, with the experimental unit being a tomato plant, and there were 13 replicates per treatment. At 15 dami and at the end of the experiment, the height of each plant and its severity were recorded, utilizing a progressive scale: 1 = no symptoms, 2 = slight chlorosis, 3 = mosaic, 4 = reduction of growth, 5 = deformation of leaves, and 6 = death. Additionally, the fresh weight of the aerial part and roots of each treated plant was recorded at the end of the experiment, coinciding with the onset of flowering. At 30 dami, samples were collected from the treated diseased plants to confirm viral infection by RT-PCR, using primers described by Dovas and collaborators (2004), amplifying a conserved region of 400 base pairs of the replicase viral (RdRp). Composite samples from the control (healthy) plants were also analyzed. The obtained data were subjected to nonparametric tests of means, and once significant differences ($\alpha = 0.05$) were identified, Tukey-Kramer HSD tests were performed for each pair.

All plants inoculated with the virus exhibited systemic symptoms 15 days after inoculation (Figure 1). These symptoms were consistent with those previously reported in tomato plants, characterized by chlorotic mosaic, mottling of dark green

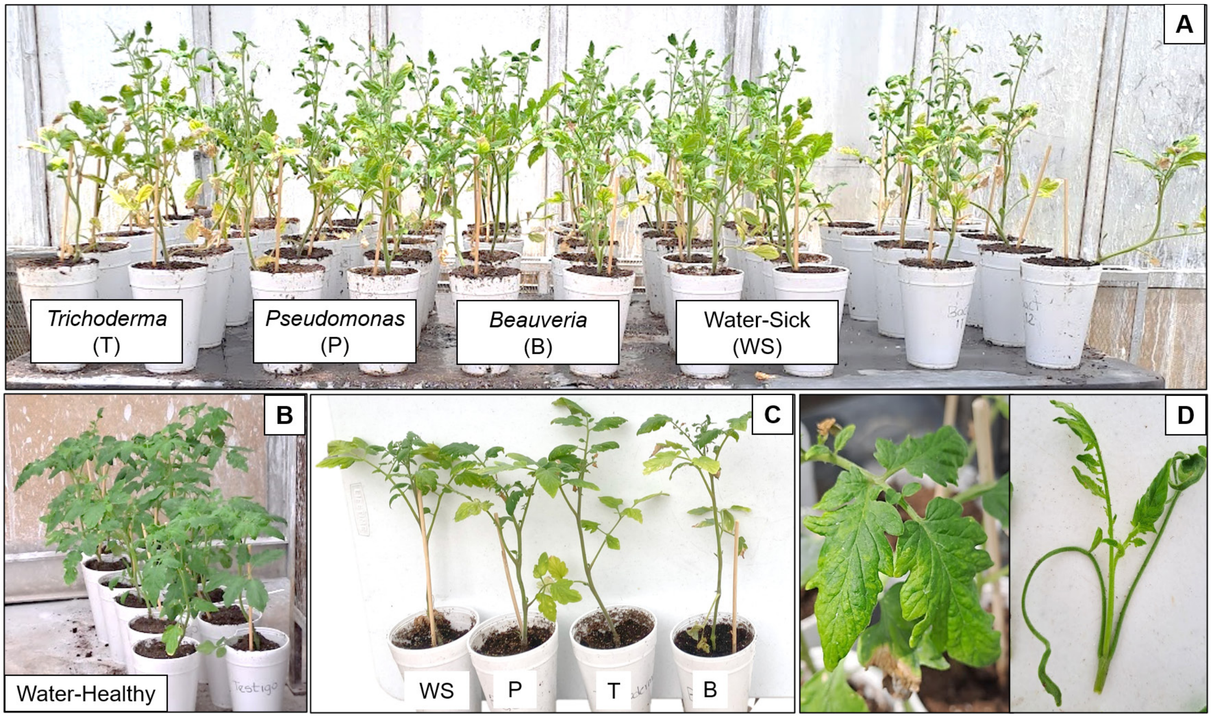

Figure 1. A: Infected plants with *Tomato brown rugose fruit virus* (ToBRFV), treated with various microorganisms, and the diseased mock control. B: Healthy mock plants. C: Comparison among ToBRFV-infected plants treated with different microorganisms. D: Representative symptoms induced by ToBRFV, illustrating leaf mosaic at 20 days after inoculation (dai) and shoot deformation at 35 dai.

areas on leaves, blistering, and even narrowing of the leaf lamina (Fidan *et al*., 2019; Alkowni *et al*., 2019; Menzel *et al.*, 2019). Regarding RT-PCR analysis, only fragments of the expected weight were obtained from the treated diseased plants, confirming the presence of viral infection.

Plants subjected to the Water-Healthy treatment (representing healthy plants) exhibited the most robust root and aerial development, with all response variables displaying statistically significant differences compared to the other treatments ($p < 0.0001$) (Table 1). In comparison to the diseased control (Water-Sick), the treatment that most effectively promoted growth, enhanced development, and reduced severity was *B. peruviencis*, followed by *Pseudomonas* sp. ($p < 0.0001$ and $p= 0.0016$, respectively). Notably, severity levels were statistically similar among these treatments (Table 1). Diseased plants treated with *T. longibrachiatum* exhibited the lowest weight (7.9 g of aerial part and 4.7 g of root) and higher severity (4.6), even surpassing those treated solely with water (control) (Figure 2).

Several growth-promoting rhizobacteria have undergone testing in crops, demonstrating efficacy in reducing the incidence and severity of viral diseases. Notably, species within the *Pseudomonas*, *Bacillus*, and *Azospirillum* genera have

Table 1. Comparison of mean values for response variables (height, severity, and weight of tomato plants) assessed under each treatment, accompanied by Tukey-Kramer HSD test-generated grouping letters.

Response variable	Treatment	Average	Union letters[z]
Height (cm)	Water-Healthy	55.5	A
	Beauveria	35.3	B
	Pseudomonas	32	B C
	Thrichoderma	26.1	C D
	Water-Sick	19.7	D
Severity	*Thrichoderma*	4.6	A
	Water-Sick	4.3	A
	Pseudomonas	4.2	A
	Beauveria	4.1	A
	Water-Healthy	1	B
Shoot fresh weight (g)	Water-Healthy	26.1	A
	Beauveria	11.7	B
	Pseudomonas	11.45	B
	Water-Sick	10.88	B
	Thrichoderma	7.8	B
Root fresh weight (g)	Water-Healthy	11.8	A
	Beauveria	9.4	A B
	Water-Sick	7.4	B C
	Pseudomonas	7	B C
	Thrichoderma	4.6	C

[z]Different letters or sets represent significant differences (P ≤ 0.05, α=0.05).

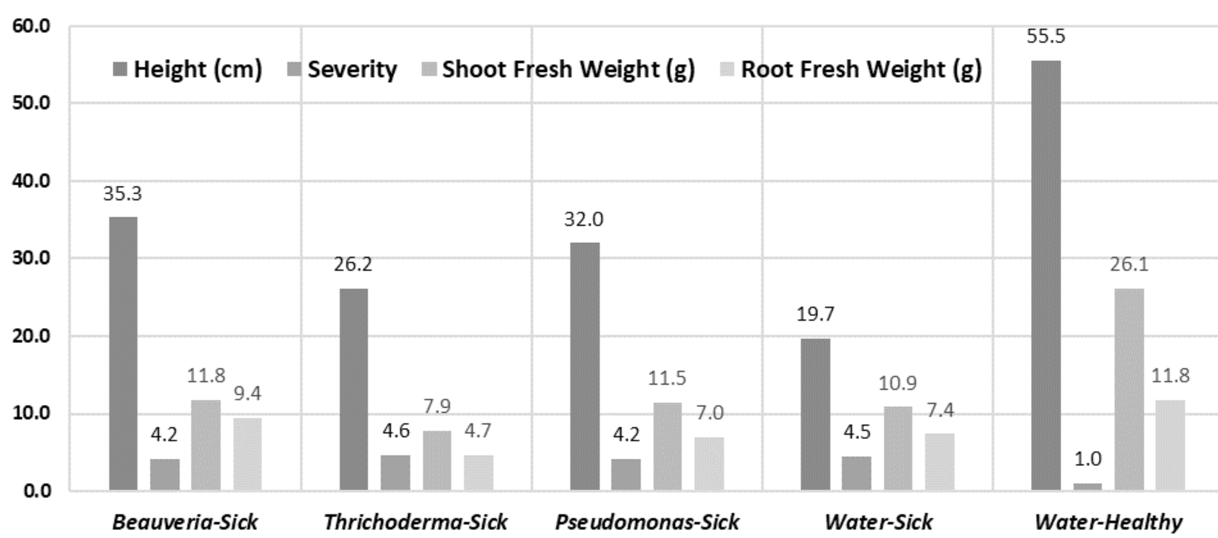

Figure 2. Mean of the response variables, obtained at the end of the experiment, for each treatment applied to ToBRFV-infected (diseased) and healthy tomato plants.

proven effective against various viruses, including *Cucumber mosaic virus* (CMV), *Tobacco mosaic virus*, *Tomato chlorotic spot virus*, *Tomato mottle virus*, and *Tomato yellow leaf curl virus* (TYLCV) (Dashti *et al*., 2007; Wang *et al*., 2009; Choi *et al*., 2014; Abdalla *et al*., 2017; Li *et al*., 2016; Beris *et al*., 2018). For instance, the application of *Pseudomonas fluorescens* on seeds, seedlings, leaves, and soil has been shown to enhance peroxidase and phenylalanine-ammonia-lyase activity. This, in turn, promotes the accumulation of phenolic compounds and stimulates phenylpropanoids, resulting in a significant reduction in the severity and incidence of *Tomato spotted wilt virus* (Kandan *et al*., 2005).

Similarly, fungi have been noted to exert a positive impact on virus-infected plants. Investigations into cucumber plants infected with CMV revealed that *Trichoderma asperellum* heightened transcript levels of resistance-related genes (pr1, pal1, etr1, sod, rip, and lox1) and enzymes (SOD, LOX1, POX, CAT) known to induce systemic resistance in the plant (Tamandegani *et al*., 2021). While *T. longibrachiatum* has been identified as a pathogen in the cultivation of various fungi, such as mushrooms and *Ganoderma* (Zhang *et al*., 2018), and has demonstrated efficacy as a biological control agent against *Sclerotinia cepivorum* in onions (*Allium cepa*) (Camacho-Luna *et al*., 2023), as well as *Thielaviopsis paradoxa* in agave (*Agave tequilana*) (Sánchez and Rebolledo, 2010), its role as an endophyte was previously unknown. In the present experiment, it was observed that *T. longibrachiatum* increased the severity of virus-infected plants and reduced plant growth by 31% compared to the Water-Sick treatment. Further research is required to determine the likely mechanisms involved and its overall effect.

Certain entomopathogenic fungi are recognized as endophytes in plants. For instance, *B*. *bassiana* has been documented to colonize various plant species, including wheat (*Triticum*), soybean (*Glycine max*), rice (*Oryza sativa*), bean (*Phaseolus vulgaris*), onion (*Allium cepa*), tomato (S. lycopersicum), palm (*Elaeis guineensis*), grape (*Vitis vinifera*), potato (*Solanum tuberosum*), cotton (*Gossypium hirsutum*), and maize (*Zea mays*) (Vega, 2018; Liu *et al*., 2022; Jaber and Ownley, 2018). Upon inoculation on seeds, foliage, or soil, these fungi infiltrate plant tissue, fostering plant growth (Jaber and Enkerli, 2017). In the realm of virus management, El-Deeb and colleagues (2021) explored the application of *B. bassiana* to assess resistance against *Tomato yellow leaf curl virus* (TYLCV) and *Bemisia tabaci* populations. Through injection applications to plant tissue for colonization, they observed an increase in phenol content, enhanced plant height and fruit yield, reduced TYLCV incidence, and a decline in whitefly (*B. tabaci*) population. The *B. peruviencis* isolate utilized in this study, derived from an anonaceae weevil (*Optatus palmaris*) (Hernández, 2023), was previously unknown for its potential as an endophyte in plants. Notably, in the present investigation, it emerged as the treatment most conducive to the development of infected plants (79% taller and

15% heavier than the Water-Sick treatment), while concurrently reducing severity. This outcome underscores its promising potential for future research.

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