Preliminary findings on biocontrol of bacterial wilt and canker of tomato (*Clavibacter michiganensis* subsp. *michiganensis)* using *Trichoderma harzianum* after biofumigation

Hallazgos preliminares sobre el biocontrol del marchitamiento y cancro bacteriano del tomate (*Clavibacter michiganensis* subsp. *michiganensis*) utilizando *Trichoderma harzianum* luego de una biofumigación

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ABSTRACT

One of the most aggressive bacterial diseases in tomato crops is bacterial wilt and canker caused by *Clavibacter michiganensis* subsp. *michiganensis* (Cmm). Chemical control is questioned due to its negative effects on health and the environment. Within integrated disease management, one alternative is biocontrol with *Trichoderma* species. Another technique is biofumigation, which releases volatile compounds into the soil that inhibit soilborne fungi and stimulate plant health. The aim of this study was to evaluate the potential of biofumigation and the use of *Trichoderma harzianum* for the control of bacterial wilt and tomato canker caused by Cmm *in vitro* and their effect on yields in a commercial tomato crop. The inhibition of phytopathogenic bacteria of the *in vitro* test and the number and weight of fruits per plant in a greenhouse were evaluated. The treatments were: tomato plants inoculated with Cmm with or without two strains of *T. harzianum,* alone and in combination with biofumigation. The *in vitro* test results showed, with both strains, no significant differences between the treatments, although the growth of Cmm was lower in the combination biofumigation and *T. harzianum*. One of the strains of *T. harzianum* (Th118) performed better than the other for yield (weight and number of fruits). However, the results do not show a synergistic effect between *T. harzianum* and biofumigation in the observed yield values.

Key words: biological control, phytopathogenic bacteria, tomato yield, microbial antagonists.

RESUMEN

Una de las enfermedades bacterianas más agresivas en el cultivo de tomate es el marchitamiento y cancro bacteriano ocasionado por *Clavibacter michiganensis* subsp. *michiganensis* (Cmm). Su control químico es cuestionado por sus efectos negativos en la salud y el ambiente. Dentro de un manejo integrado de enfermedades una alternativa es el biocontrol con especies de *Trichoderm*a. Otra técnica es la biofumigación que libera al suelo compuestos volátiles que inhiben fitopatógenos y favorecen la sanidad de las plantas. El objetivo del estudio fue evaluar el potencial de la biofumigación y el uso de *Trichoderma harzianum* para el control de la marchitez bacteriana y cancro del tomate causado por Cmm *in vitro* y observar el efecto sobre los rendimientos en un cultivo comercial. Se evaluó el número y peso de frutos por planta, donde los tratamientos fueron: plantas de tomates inoculadas con Cmm en presencia o ausencia de dos cepas de *T. harzianum* solas y en combinación con biofumigación. Los resultados de los ensayos *in vitro* mostraron que a pesar de que no hubo diferencias significativas entre los tratamientos, el crecimiento de Cmm fue menor en la combinación biofumigación y *T. harzianum*. Una de las cepas de *T. harzianum*, (Th118), tuvo mejor comportamiento que la otra, teniendo en cuenta el efecto sobre el rendimiento (peso y número de frutos). Por otro lado, los resultados no muestran un efecto sinérgico entre *T. harzianum* y la biofumigación en los valores de rendimiento observados.

Palabras clave: control biológico, bacterias fitopatógenas, rendimiento de tomate, antagonistas microbianos.

Introduction

Tomato wilt and bacterial canker is caused by *Clavibacter michiganensis* subsp. *michiganensis* (Davis *et al.,* 1984). It is present in practically all the producing areas of tomato worldwide (Leon *et al*., 2011; Osdaghi, 2015). This disease has caused large losses in tomato crops both in the field and in the greenhouse. The most relevant symptom is the wilting and death of plants, which causes large economic losses (Chalupowicz *et al*., 2016; Osdaghi, 2015; Rolleri & Romero, 2022). When the disease occurs, it can affect all plants in plots or greenhouses in a short time (EPPO,

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2013; Kawaguchi *et al*., 2010). The disease is very difficult to control, and management practices have focused mainly on preventive measures and the use of chemical synthesis products (cuprics and antibiotics). However, these practices have not proven efficient. On the contrary, they not only increase production costs, but also cause harm to humans, animals and the environment. To reduce its incidence, rotation with non-susceptible crops is recommended. However, the effectiveness of this measure depends on the survival of the pathogens on the debris and/or crop supports or on its reintroduction to a new crop (Maeso *et al*., 2012; Malliarakis *et al*., 2023; Vega & Romero, 2015).

Management of diseases in horticulture has traditionally been carried out by soil fumigation, using toxic, volatile compounds (Martin, 2003). Alternative management methods for soil-borne plant pathogens are needed to maintain high agricultural production. In recent years, in the exploration for an ecofriendly disease control, efforts have been directed towards an enhanced understanding of the biological effects of natural products. Biofumigation by means of Brassicaceae green manure incorporation into soil is a promising, ecofriendly alternative to chemical fumigation by methyl bromide for the control of phytopathogens. This biological process is based on the release of glucosinolate-derived toxic compounds, facilitated by endogenous myrosinase (thioglucosidase EC 3.2.1.147) from Brassicaceae plant residues in the presence of water (Brown & Morra, 1997; Hanschen & Winkelman, 2020; Makane *et al.,* 2023; Mitidieri *et al*., 2015; Zhang *et al*., 2020). The beneficial effects observed may not always be related to the activity of glucosinolate-based hydrolysis compounds but may add to other mechanisms that improve plant health. These may play a complimentary or more dominant role in some disease suppression. This is probably due to the incorporation of large quantities of plant residues into the soil. Potentially, this improves soil structure, increases nutrient availability, increases water holding capacity, and stimulates antagonist microbial communities (Kirkegaard & Matthiensen, 2004; Rolleri *et al*., 2021). In addition, biofumigation favors the development of plants, making them more robust. Various authors (Daugovish *et al.,* 2009; Mitidieri *et al.,* 2015) attribute this to the contribution of mineral nutrients, such as nitrogen and phosphorus, and the increase in soil organic matter.

Trichoderma are free-living beneficial fungi commonly found in soil, useful for plant protection purposes in agriculture (Amerio *et al*., 2020; Guzman-Guzman *et al*., 2023). Commercial products based on certain *Trichoderma* isolates are currently utilized in the biocontrol of some soil borne and foliar pathogenic fungi (Sood *et al*., 2020). *Trichoderma* spp. are capable working together both in the plant rhizosphere and in the phyllosphere through several mechanisms, such as antagonism, competition for space and nutrients, mycoparasitism and the discharge of antibiotics and lytic enzymes, which directly inhibit phytopathogen growth (Amerio *et al*., 2020; Harman *et al.,* 2004). In this sense, numerous authors have studied biofumigation and *Trichoderma* spp. *in vitro* (Perniola *et al*., 2014) as well as their incorporation into the soil for management of phytopathogenic fungi (Berlanas *et al*., 2018; Makane *et al*., 2023; Morales-Rodriguez *et al*., 2018; Stocco *et al*., 2016).

This study aimed to explore the effect of biofumigation with *Eruca vesicaria* (L.) Cav. and the incorporation of two strains of *Trichoderma harzianum* on: 1) phytopathogenic bacteria present *in vitro* tests and 2) the control efficacy of bacterial canker and wilt on tomato plants grown under greenhouse conditions. In this regard, this research seeks to determine the synergistic effect of biofumigation and the application of *T. harzianum* on the manifestation of the disease.

Materials and methods

Fungal strains

Two strains of *T. harzianum* (Th118 and Th5cc) were used as antagonists. The Th118 strain was isolated from the tomato leaf phylloplane and previously tested in greenhouse trials. This strain reduced the incidence of the disease caused by *Botrytis cinerea* in tomato plants (Dal Bello *et al*., 2011). The Th5cc strain was isolated from the wheat phylloplane and was previously tested as an antagonist of *Zymoseptoria tritici* (Cordo *et al*., 2007). In addition, in a previous study, the Th5cc strain, when applied as a liquid formulation and as a coating on seeds, was the most effective in maintaining a high population of *T. harzianum* in soil, with a potential biocontrol effect (Stocco *et al.,* 2019). Both strains were molecularly identified following the technique described by Stocco *et al.* (2016) and were deposited in the database of the European Molecular Biology Laboratory (EMBL) under the accession numbers LN869400 (*T. harzianum* Th118) and LN869401 (Th5cc). These strains are also deposited in the fungal collection of the Centro de Investigaciones de Fitopatología (CIDEFI, UNLP, Argentina).

Bacterial strain

For this study, the strain of Cmm LPAb158 was used, which is deposited in the collection of microbial cultures

of the Centro de Investigaciones de Fitopatología (CIDEFI, Argentina). It was identified by microbiological and molecular techniques using specific primers that amplify the intergenic region 16S-23S of rRNA (EPPO, 2013; Schaad *et al.,* 2001), as described by Rolleri (2015).

In vitro test

To evaluate the effect of biofumigation and the two strains of *Trichoderma harzianum* (Th5cc and Th118) on the pathogen, fresh plant tissue of arugula (*Eruca vesicaria*) was collected from the greenhouse experiments. The plants were uprooted at the 50% flowering stage and taken immediately to the laboratory in autoclaved polypropylene bags. The plants were washed with sterilized water, cut into small pieces, and then 5 g of the moistened plant tissue was placed at the bottom of a 9 cm Petri dish. For the preparation of the assay, a 4 cm long guideline was drawn on the bases of the Petri dishes where the bacteria were seeded with a bacteriological loop on Nutrient Agar. Then, Cmm was seeded 3 cm from the edge of the dish and 3 cm from the location where the antagonist was placed simultaneously. Five mm diameter discs of actively growing mycelium of *Trichoderma* strains were taken from the margins of 7-d-old cultures and transferred to Petri dishes, maintaining a distance of 4 cm between Cmm and *Trichoderma* (dual culture). One plug of each *Trichoderma* strain was seeded in each dual culture. The lid of the Petri dishes containing the pieces of arugula was replaced with the bottom of the Petri dishes with the fungal plug and bacteria. The pieces of arugula were not in contact with the bacteria or with *Trichoderma* sp. The plates were immediately sealed with parafilm and incubated in an inverted position at 27±2°C until the *Trichoderma* almost covered the medium surface. Petri dishes without biofumigation or *Trichoderma* were used as controls.

To evaluate the area of the Cmm colony, the growth in length was measured on the marked line of the bacterial colony and three perpendicular measurements of width were taken (at the center and 2 cm from it to the right and left). Two days after sowing, the three width measurements were averaged and multiplied by the growth length, thus obtaining the growth value of the bacteria (Peñalba, 2022). The treatments were: 1) Cmm, 2) Cmm + biofumigation, 3) Cmm + *T. harzianum* (Th5cc) or *T. harzianum* (Th118), 4) Cmm + *T. harzianum* (Th5cc) or *T. harzianum* (Th118).

To evaluate the growth of *T. harzianum*, the growth in length was measured 2 d after sowing.

Application of *Trichoderma harzianum* to tomato seedlings

The strains of *T. harzianum* were incorporated in liquid form to the substrate of tomato seedlings var. Elpida. For the incorporation, a suspension of spores of each strain of *T. harzianum* developed in PDA medium (potato dextrose agar at 2%) was prepared from a culture 8 d-old. The suspension was made by adding sterile water over the colonized Petri dish and scraping with a sterile ansa. Then it was adjusted to 1 x 10^8 spores ml^{-1} and Tween 20 (0.01%) was added. The fungal inoculum was applied only once, at the time of sowing, in the form of irrigation using 10 ml of suspension for each cell of the planting tray. Finally, tomato seeds were sown, one for each cell in the planting tray. The control treatment consisted of sowing tomato seeds on substrate without inoculum of *T. harzianum*. This methodology was used according to the results obtained by Rolleri *et al.* (2021), who tested two techniques for infesting the substrate with *Trichoderma* sp.

Greenhouse assays

The assays were carried out in a greenhouse (6 m x 20 m, with wooden masonry and 180 µm thick polyethylene) during September 2021 – January 2022 and September 2022 – January 2023. The greenhouse was located at the Chacra Experimental Gorina of the Ministry of Agrarian Development of the province of Buenos Aires, Argentina (34°54'56.4" S, 58°02'21.5" W) belonging to the Platense Horticultural Belt.

In the greenhouse, *E. vesicaria* seeds were sown at the rate of 10 g m^2 and after flowering the plants were cut and integrated into soil using a common rotary cultivator at a rate of 5 kg fresh biomass m^{-2}. After integration, the plot was sheltered with linear low-density polyethylene sheets and left for one month (biofumigation). At the time of tomato planting, the polyethylene sheet was removed and the soil was thoroughly mixed. In this greenhouse, the traditional management carried out by producers without the use of agrochemicals was followed. Biofumigation was carried out in half of the greenhouse, while the other half did not receive it. The tomato plants were transplanted at the state of three fully expanded leaves, at a distance of 0.35 m between plants and 0.60 m between rows. The treatments were the following: tomato seedlings inoculated with Cmm and biofumigation; tomato seedlings treated with *T. harzianum* (Th5cc), inoculated with Cmm and biofumigation; tomato seedlings treated with *T. harzianum* (Th118), inoculated with Cmm and biofumigation; tomato seedlings without

Trichoderma spp. inoculated with Cmm (control); seedlings treated with *T. harzianum* (Th5cc) and inoculated with Cmm, and tomato seedlings treated with *T. harzianum* (Th118) and inoculated with Cmm.

A single inoculation with the pathogenic bacteria was carried out at the time the first shoot was cut, when the plants had between 12 and 14 leaves. For this, the bacterial suspension was placed in the shoot wound. To prepare the bacterial suspension, the bacteria were streaked in Petri dishes, in Nutrient Agar (NA) medium, and incubated at 27°C (+/- 2°C) for 48 to 72 h. Subsequently, sterile distilled water was added, adjusting to a spectrophotometer reading of OD600 = 0.3 (~5.5 x 10^8 CFU ml^{-1}) diluted 1:10 to reach the final used concentration of 10^7 CFU ml^{-1}. The experimental design was a randomized block design with 6 treatments and 10 replicates.

The evaluation consisted of determining yield parameters, number and weight of fruits per plant. Three evaluations were carried out on a weekly basis.

Statistical analysis

To ensure reproducibility and reliability, the experiments were conducted twice. Data from the *in vitro* tests and the greenhouse trials were analyzed using analysis of variance (ANOVA) with the Infostat® program (Di Rienzo *et al*., 2020). Treatment means for all variables were compared using the Tukey test at a significance level of 5% ($P \leq 0.05$). If the data did not meet the assumptions of normality, homoscedasticity and randomness, non-parametric statistics were applied using the Kruskal-Wallis test.

Results

In vitro test

The results of the *in vitro* assays showed that the diameter of the *Trichoderma* colonies (Th118 and Th5cc) did not present significant differences among the different treatments ($P \leq 0.05$) (Tab. 1).

According to the results, although significant differences were observed between treatments, the combination of Th5cc and biofumigation showed the lowest values in the growth of Cmm but did not differ significantly from the control (Cmm) (Fig. 1).

With respect to Th118, the bacterial growth was only slightly affected by biofumigation and the presence of *T. harzianum*. However, although no significant differences were observed between the treatments, the growth of Cmm was lower in the combination biofumigation and *T. harzianum*. These results can be observed in Figure 2.

TABLE 1. Growth (cm^2) of *Trichoderma harzianum* (Th118 and Th5cc) with and without biofumigation (Bio) in the presence or absence of *Clavibacter michiganensis* subsp. *michiganensis* (Cmm).

Treatment	Mean	Standard deviation	Treatment	Mean	Standard deviation
Th118+Bio+Cmm	3.07	0.62	Th5cc+Bio+Cmm	5.57	0.53
Th118+Bio	4.64	1.20	Th5cc+Bio	5.57	0.53
Th118+ Cmm	3.39	1.26	Th5cc+ Cmm	5.05	0.27
Th118	3.37	1.00	Th5cc	5.09	0.35

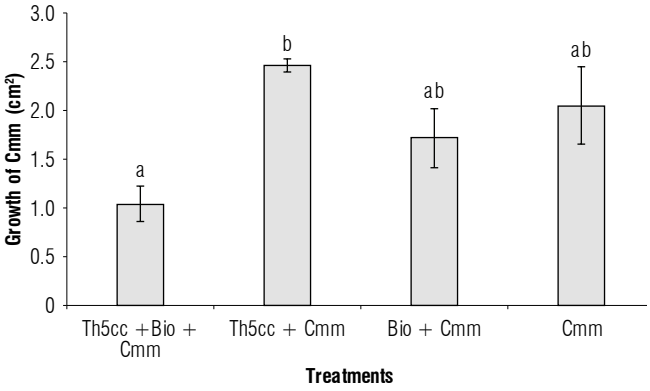

FIGURE 1. *In vitro* growth (cm^2) of *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) with and without biofumigation (Bio) and in the presence or absence *Trichoderma harzianum* (Th5cc). Averages with the same letter do not differ significantly according to the Tukey's test ($P \leq 0.05$). Vertical bars represent standard error (n=5).

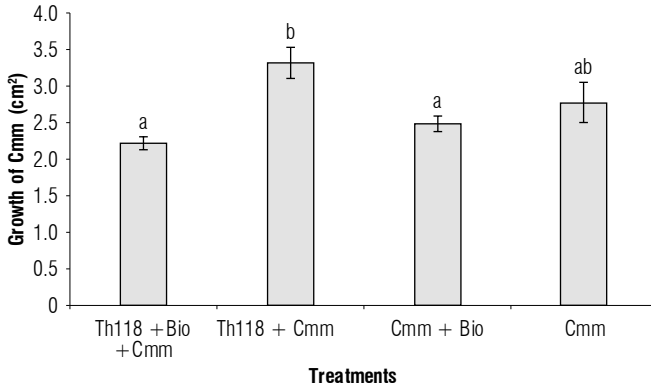

FIGURE 2. *In vitro* growth (cm^2) of *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) with and without biofumigation (Bio) and in the presence or absence of *Trichoderma harzianum* (Th118). Averages with the same letter do not differ significantly according to the Tukey's test ($P \leq 0.05$). Vertical bars represent standard error (n=5).

Greenhouse assays

Related to the assays in the greenhouse, in 2021-2022, the inoculated control with biofumigation presented the lowest yield in terms of the weight of the harvested fruits. The Th118 treatment produced the highest weight of tomato fruits, but it did not differ significantly from the control (Fig. 3).

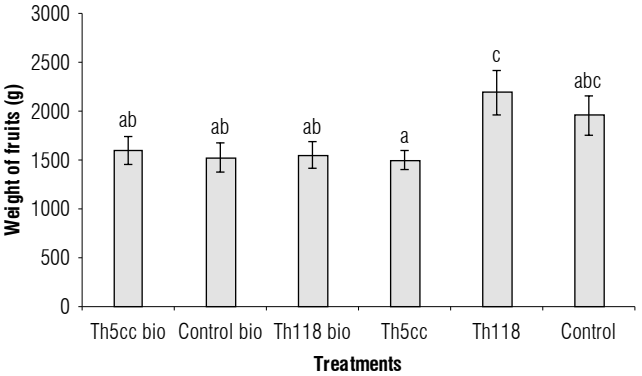

FIGURE 3. Effect of the treatments on the weight of tomato fruits during 2021-2022. Averages with the same letter do not differ significantly according to the Tukey's test ($P \leq 0.05$). Th5cc bio: with *Trichoderma harzianum* 5cc and biofumigation; Th118 bio: with *T. harzianum* 118 and biofumigation; Control bio: with biofumigation without *T. harzianum*. The error bars correspond to standard error (n=27).

The effect of the treatments on the number of fruits is shown in Figure 4. The Th118 treatment had the greatest number of fruits, and it differed significantly from the control treatment with biofumigation. The same observation was made in relation to fruit weight.

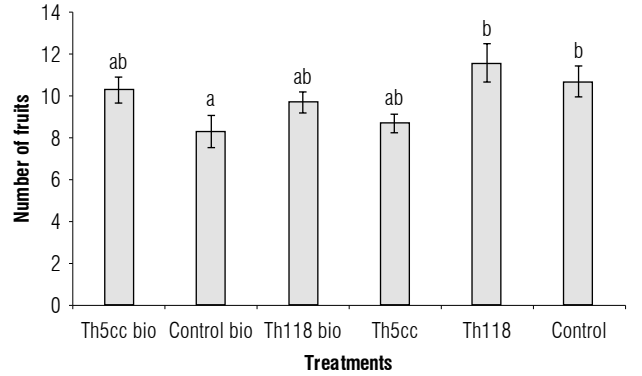

FIGURE 4. Effect of the treatments on the number of tomato fruits harvested during 2021-2022. Averages with the same letter do not differ significantly according to the Tukey's test ($P \leq 0.05$). Th5cc bio: with *Trichoderma harzianum* 5cc and biofumigation; Th118 bio: with *T. harzianum* 118 and biofumigation; Control bio: with biofumigation without *T. harzianum*. The error bars correspond to standard error (n=27).

For tomato yield and weight of fruits, Th118 without biofumigation had the highest values and differed statistically from the treatments Th5cc with and without biofumigation and the treatment control without biofumigation (Fig. 5). For the numbers of fruits, the treatment Th118 without biofumigation had the highest value and differed statistically from the other *T. harzianum* strain, both with and without biofumigation (Fig. 6).

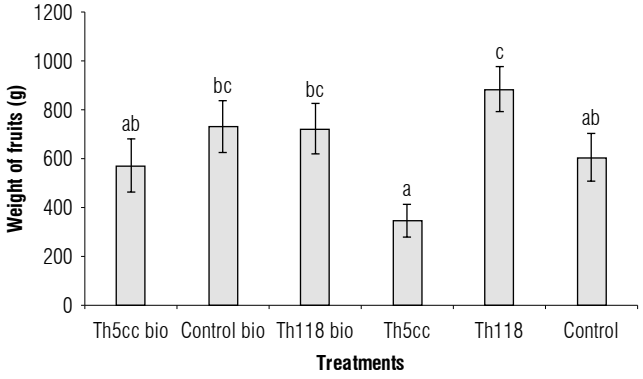

FIGURE 5. Effect of the treatments on the weight of tomato fruits during 2022-2023. Averages with the same letter do not differ significantly according to the Tukey's test ($P \leq 0.05$). Th5cc bio: with *Trichoderma harzianum* 5cc and biofumigation; Th118 bio: with *T. harzianum* 118 and biofumigation; Control bio: with biofumigation without *T. harzianum*. The error bars correspond to standard error (n=27).

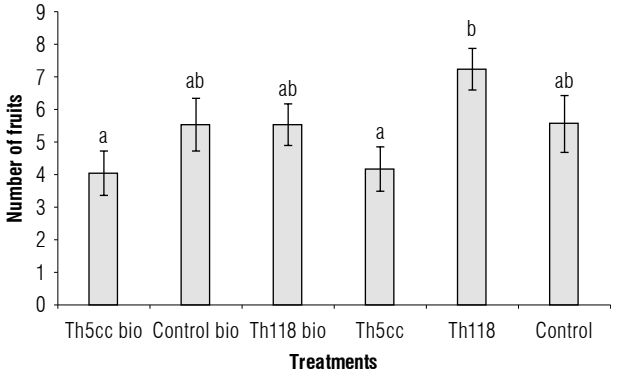

FIGURE 6. Effect of the treatments on the number of tomato fruits harvested during 2022-2023. Averages with the same letter do not differ significantly according to the Tukey's test ($P \leq 0.05$). Th5cc bio: with *Trichoderma harzianum* 5cc and biofumigation; Th118 bio: with *T. harzianum* 118 and biofumigation; Control bio: with biofumigation without *T. harzianum*. The error bars correspond to standard error (n=27).

Discussion

In recent years, there has been increased interest in the biological control of plant pathogens, particularly phytopathogenic bacteria (Abo-Elyonsr *et al*., 2019; Amerio *et al*., 2020; Berlanas *et al*., 2018; Sarandon & Flores, 2014; Zahir *et al*., 2018). Within this context, and based on the results of the previous studies, the use of *T. harzianum* is a good alternative to control wilt and bacterial canker in tomato

(Rolleri *et al*., 2021). Additionally, the use of Cruciferous species for biofumigation, which can be carried out alone or combined with biocontrol microorganisms as control mechanisms for crop diseases, has gained significance (Dugassa *et al*., 2021; Mitidieri *et al*., 2015).

In this research, we studied the influence of the incorporation of *Eruca vesicaria* material in combination with *T. harzianum* biocontrol agent *in vitro* to control the pathogenic bacteria *Clavibacter michiganensis* subsp. *michiganensis* in tomato. In this assay, *Trichoderma* strains were not inhibited by the volatiles released by cruciferous species; similar results have been reported by Perniola *et al*. (2014). Kirkegaard and Matthiessen (2004) found that to stop the growth of certain pathogens, such as *Bipolaris* spp., *Sclerotinia* spp. or *Phytophthora* spp., low concentrations of isothiocyanates are necessary; however, to affect *Trichoderma* spp., high doses of these compounds are required. Furthermore, in our *in vitro* assay results, we observed that *T. harzianum* strains behaved differently. In this sense, the Th5cc and Th118 strains did not cause significant differences in the growth of the bacteria; similar results were observed with biofumigation. On the other hand, the Th118 strain, although it did not differ statistically from the control, caused a decrease in bacterial growth in combination with biofumigation, demonstrating a synergistic effect, as suggested by Perniola *et al*. (2014).

Additionally, the increase in microorganisms is undoubtedly due to the incorporation of cruciferous plant residues, which have an important role in the suppression of plant pathogens and improving plant health (Bakker *et al*., 2010; Bonanomi *et al*., 2010). In this sense, Mitidieri *et al.* (2015) mention that some fungi, such as *Trichoderma*, are tolerant to isothiocyanates. The management of bacterial diseases is difficult when epidemics develop during favorable weather. One of the ways to partially solve this problem may be the incorporation of Cruciferae residues in soil. Organic amendments improve soil contents of mineral nutrients, increase biodiversity, prevent degradation, and contribute to general suppressiveness through enhanced soil microbial biomass. Regarding the greenhouse results, one of the strains of *T. harzianum* (Th118) had a better performance than the other strain, considering the effect on the yield (with an average fruit weight between 900 and 2200 g). In this sense, *T. harzianum* Th118 strain exhibited a better performance than Th5cc, both in the in vitro and greenhouse assays. Rolleri *et al.* (2021) obtained similar results when they applied Th118 in the form of irrigation at the time of sowing in tomato plants from La Plata. This makes sense, considering that strain Th118 was isolated from the phylloplane of tomato plants (Dal Bello *et al*., 2011) and was better adapted to the agroecosystem studied. One concern about the use of *Trichoderma* spp. in greenhouses is the introduction of new species in the area, which is why native species are used (Dugassa *et al*., 2021; Guzman-Guzman *et al.,* 2023; Zhang *et al*., 2020). And if, in addition to this effect, a biocontrol agent such as *T. harzianum* is incorporated, the effect on the development of the disease and the growth of the plants is enhanced.

Some authors, such as Galletti *et al.* (2008), found a synergistic effect of the two biological control methods carried out in soil under controlled conditions, applying separately and together *Trichoderma* spp. and biofumigation with seeds of *Brassica carinata*. However, in our study and according to Berlanas *et al*. (2018), we did not observe a synergistic effect between biofumigation and the incorporation of *T. harzianum* to the tray of tomato seedlings on the yield parameters (weight and number of the fruits). Regarding tomato yield and fruit weight, the Th118 treatment without biofumigation resulted in the highest values; the same treatment had the highest number of tomato fruits. This *Trichoderma* strain, applied as irrigation to the seedlings, could be a good alternative within an integrated disease management plan in tomato cultivation. In this sense, the proposed hypothesis could not be demonstrated, since the plants transplanted in a biofumigated soil did not present higher yield values when infested with *T. harzianum* and inoculated with Cmm.

Further research is required to analyze the effect of this integrated approach with different species of *Brassica* or non-*Brassica* genera on other phytopathogens and in field conditions to study the effect of biofumigation and *T. harzianum* on the incidence of bacterial wilt and canker of tomato.

Conclusions

This work highlighted the effect of *T. harzianum* (Th118) on the severity of bacterial canker and yield in tomato plants. We also demonstrated that no synergistic effect on yield was observed between Th118 and biofumigation. Further complementary studies are required to evaluate the integrated effect between biofumigation and other species of *Trichoderma*.

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Conflict of interest statement

The authors declare that there is no conflict of interests regarding the publication of this article.

Author's contributions

MS, JR and CM designed the experiment, developed the methodology and analyzed the evaluated data. PM and JP carried out the experimentation in the greenhouse and collected data. MS performed the statistical analysis of the data.CM wrote the initial draft of the manuscrip. All authors reviewed the final version of the manuscript.

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