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Article

Antagonistic microorganisms as management of *Fusarium oxysporum* wilt of blackberry

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Abstract

In the states of Michoacán, Mexico and California, USA, blackberry (*Rubus* sp.) production is affected by wilt, which causes the loss of thousands of hectares. In the present work, the causative agent of the disease was identified and control alternatives with antagonistic microorganisms were proposed. In 2022, the causative agent of the disease was isolated and morphologically identified from an orchard in Tocumbo, Michoacán. A preliminary laboratory bioassay was performed with strains of *B. subtilis*, *B. amyloliquefaciens*, *P. fluorescens*, and *Trichoderma* spp., native to Tacámbaro and Taretan, Mich. The outstanding strains were tested in a greenhouse on plants of blackberry cv Tupy as a preventive measure and as a control, under an experimental design of randomized blocks with six repetitions. In the preliminary tests, all the strains used showed an inhibition of the mycelial growth of the pathogen greater than 50%, with *Trichoderma* sp. from Tacámbaro, *B. subtilis* and *P. fluorescens* standing out. In the tests carried out *in situ*, preventively applied treatments decreased the incidence of wilt by 57% to 66%. In terms of severity and plant height, *Trichoderma* sp. stood out, while the percentage of root necrosis decreased by 56% to 70% with all treatments. Control treatments also showed antagonistic efficacy, although to a lesser extent, they decreased the incidence of wilt by 2 to 58% and the percentages of root necrosis by 19 to 62%. Antagonistic microorganisms can be incorporated as a comprehensive management strategy with high efficiency.

Keywords:

Rubus, *Fusarium*, biocontrol, comprehensive management, wilt.

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Introduction

Mexico stands out as a global producer of berries, with blackberry (*Rubus* sp.) being the most prominent (Ricárdez-Luna *et al*., 2016). Michoacán contributes significantly 93.3% of blackberry production (SIAP, 2021). Although this fruit adapts well to temperate conditions, it suffers from production and marketing restrictions due to *Fusarium oxysporum*, which causes wilt in blackberry plantations in California and Mexico (Gordon *et al*., 2017).

A new lineage of *F. oxysporum*, recently identified, has emerged as responsible for the disease, becoming a devastating threat in Mexico, especially with severe epidemic expression in the Tupy variety in commercial plantations (Hernández-Cruz *et al*., 2020).

Due to the environmental and health risks posed by the extensive use of synthetic fungicides and fumigants (Guédez *et al*., 2009; Vásquez-Ramírez, 2017), it is imperative to reduce the use of chemical pesticides that affect the ecosystem (Villa *et al*., 2005). The application of antagonistic microorganisms emerges as an indispensable alternative, not only for phytopathological control but also to improve stress tolerance in plants (Guédez *et al*., 2008; Tian *et al*., 2018).

Given the urgency of approaches with lower environmental impact, this work aims to evaluate the efficacy of antagonistic microorganisms in the management of blackberry wilt disease.

Materials and methods

Collection, isolation, and identification In 2022, in the municipality of Tocumbo, a targeted sampling of wilt symptomatic plants was carried out in an orchard of 'Tupy' blackberry (19° 38' 21.5" north latitude and 102° 29' 02.9" west longitude) at 1 300 masl. The second-year and conventionally managed plant was grown in the open field. Phytopathological techniques from Agrios (2005) and Trigiano *et al*. (2004) were followed. Symptomatic roots were sown in ½X potato dextrose agar (PDA) culture medium and incubated at room temperature in darkness, hyphae tips. Monosporic cultures were obtained in Spezieller Nührstoffarmer agar (SNA) culture medium and stored at -70 °C in sterile 25% glycerol.

Barnett and Hunter (1998); Leslie and Summerell (2006) keys were used for identification, comparing morphologically with fixed preparations. A cultural characterization was performed in 1XPDA medium and 20 µl of the conidia suspension was inoculated with 25% glycerol. Morphological and cultural characteristics were recorded, including mycelium type and thickness, colony color, agar pigmentation, sporodochia presence and color, presence of chlamydospores, and possible presence of rolled hyphae at 15, 30, and 60 days.

Additional morphological characterization was performed in carnation leaf agar (CLA) medium, 5 µl of the suspension was inoculated, and the length and width of 50 macroconidia and 50 microconidia of each isolate were measured. The characteristics determined were shape, foot type and apical cell in macroconidia and shape, arrangement, and type of phialide in microconidia, along with the presence and arrangement of chlamydospores and rolled hyphae at 10 days.

For growth rate, 5 µl of the suspension was inoculated in paperless SNA medium, incubating for 7 days at 25 °C in darkness. A 6 mm diameter disc was transferred to the center of a Petri dish with PDA medium, incubated for 72 h, and the growth (mm) rate was recorded. The analysis of variance was performed with the R program, version 3.5.1 (R Core Team, 2020) and the R Studio interface, version 1.1.463 (Rstudio Team, 2020), with six repetitions and two repetitions of the experiment.

Pathogenicity tests

According to Dhingra and Sinclair (1985), healthy blackberry seedlings from a nursery in Zirimícuaro, Michoacán, grown in granzón as a substrate, were inoculated with 100 ml in a 1 x 10^c conidia ml^{-1} suspension. The plants were evaluated every 15 days, observing from the first symptoms to the total wilting characteristic of the disease. Koch's postulates were corroborated.

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In vitro bioassay

In vitro confrontation tests were performed between antagonists (*Trichoderma* native to Tacámbaro and Taretan, *Bacillus subtilis*, *Bacillus amyloliquefaciens* and *Pseudomonas fluorescens*) and the pathogen using the quadrant confrontation method, in Petri dishes with 20 ml of PDA culture medium. The dish was divided into quadrants, placing discs (10 mm in diameter) of the pathogen and antagonist at opposite ends, the control treatment consisted only of the pathogen without the antagonist.

A completely randomized experimental design with six repetitions was used, measuring radial growth every 24 h. Measurements of the radial growth of the pathogen (RG mm) in interaction with the antagonists and of the isolated control (pathogen) determined the *in vitro* antagonistic effect. An analysis of variance of daily growth was performed for eight days, with Tukey's mean separation (0.05). The analyses were performed using the R program, version 3.5.1 (R Core Team, 2020) and the R Studio interface, version 1.1.463 (Rstudio Team, 2020). Based on the results of the *in vitro* assay, the antagonists were confronted under greenhouse conditions to evaluate their *in vivo* efficiency in controlling the pathogen.

Greenhouse bioassay

In healthy four-month-old Tupy blackberry plants from a commercial nursery in Zirimícuaro, Michoacán, 100 ml of a solution of 1 x 10# CFU ml^{-1} of *Bacillus subtilis* and *Pseudomonas fluorescens*, and 100 ml of a solution of 1 x 10^{c} conidia ml^{-1} of *Trichoderma* sp., a native to Tacámbaro, were applied as a preventive measure and as a control, individually and in combination, generating 14 treatments under a randomized block design. Each experimental unit consisted of 6 repetitions, the plants were placed in sterile granzón as a substrate.

Preventive treatments were applied two weeks prior to inoculating the plants with a conidia solution of 1 x 10# conidia ml^{-1} of pathogen. In control treatments, inoculation of the pathogen preceded the antagonists by two weeks, using the same concentration of spores. Controls were established for each case.

The variables of incidence of yellowing and wilting of foliage were evaluated, determining the percentage of foliage with symptoms of the disease, and it was evaluated according to the following formula:

$$\%\ \text{of yellowing} = \left(\frac{\text{Number of leaves with symptoms}}{\text{Total number of leaves}} \right) 100$$

Disease severity was measured on a scale of 1 to 100% based on foliar damage. Plant height was compared to healthy plants. Weight, length, and necrosis of the root were recorded, establishing percentages of rot on a scale of 1 to 100%. The data were transformed with the arcsine function for analysis.

Statistical analyses of each variable for a completely randomized design and Tukey's mean separation (0.05) were performed with the R program, version 3.5.1 (R Core Team, 2020) and the R Studio interface, version 1.1.463 (Rstudio Team, 2020).

Results and discussion

An isolate was obtained from the isolations carried out, which by comparison of its morphological characteristics belongs to the genus *Fusarium*.

Cultural characteristics

The colony in PDA had radial growth, abundant, cottony, floppy, aerial, entire-margin mycelium. White on the obverse and white to pink, light violet, and dark violet on the reverse as the colony aged (Figure 1).

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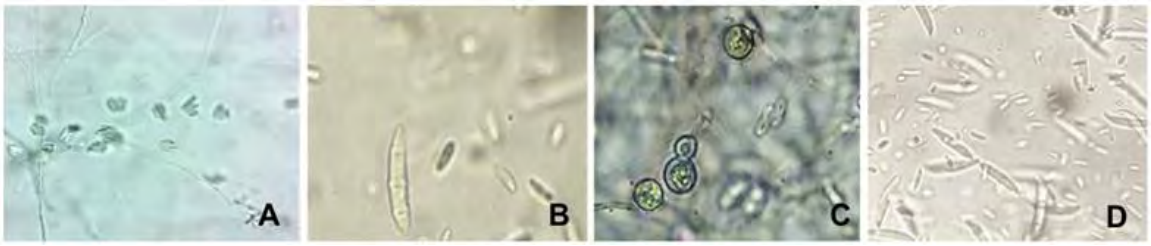
Figure 1. Cultural characterization of *Fusarium* isolates in the culture media: PDA, SNA, and CLA.

Temperature, darkness, and culture medium are important factors for the formation of pigments in this genus. PDA medium, due to its carbohydrate content, allows ideal development and expression of the culture such as shape, color, pigmentation of the medium and growth (Leslie and Summerell, 2006).

Morphological characteristics

Elliptic microconidia in false heads, without septa, rarely with 1 septum, intercalated chlamydospores were observed in PDA medium. In SNA medium, uniform microconidia, in short monophialides, with aerial mycelium. The formation of macroconidia was only observed in the CLA culture medium, almost straight in shape, with three septa, curved only at the tips, thin, with a curved apical cell and basal cell in the shape of a foot with 3 septa (Figure 2).

Figure 2. *Fusarium* structures observed in the culture media PDA, SNA, and CLA. A) microconidia in false heads in SNA; B) macroconidia in CLA; C) chlamydospores in PDA and D) micro and macroconidia in CLA.



Growth rate

The rate ranged from 28 to 30 mm at 72 h at 25 °C and although it is a secondary trait, traditionally in PDA medium and allowed to grow for three days at 25 or 30 °C, it allows to distinguish fast and slow growing species (Leslie and Summerell *et al*., 2006). The characteristics of the colony in the PDA, SNA and CLA culture media, as well as the growth rate, coincide with those reported by Leslie and Summerell (2006) for the species *Fusarium oxysporum*.

Pathogenicity tests

The symptoms appeared two months after inoculation, they began with yellowing and flaccidity in the leaves at the base of the plant, wilting the plant five months after inoculation. The roots showed necrosis and brown discoloration of the xylem tissues. Root re-isolation confirmed the presence of the inoculated *Fusarium oxysporum*. Uninoculated plants continued without the presence of symptoms (Figure 3).

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Figure 3. Symptoms of yellowing, wilting, and necrosis present in blackberry plants. A) healthy plant and B) plant inoculated with the pathogen.

In vitro bioassay

The results showed significant variation between treatments. From the fourth day, the effects of *Trichoderma* sp., Tacámbaro, *Trichoderma* sp., Taretan, and *B. subtilis* were statistically similar to and superior to *B. amyloliquefaciens* and *P. fluorescens* in inhibiting the radial growth (RG) of *Fusarium*. On the eighth day, *P. fluorescens* showed a 78% decrease in RG, while *Trichoderma* sp., Tacámbaro, *Trichoderma* sp., Taretan, and *B. subtilis* recorded a decrease of 71%, 68%, and 65%, respectively (Figures 4 and 5).

Figure 4. Obverse and reverse of the quadrant confrontation of *Fusarium oxysporum* with each of the antagonistic treatments on day eight.

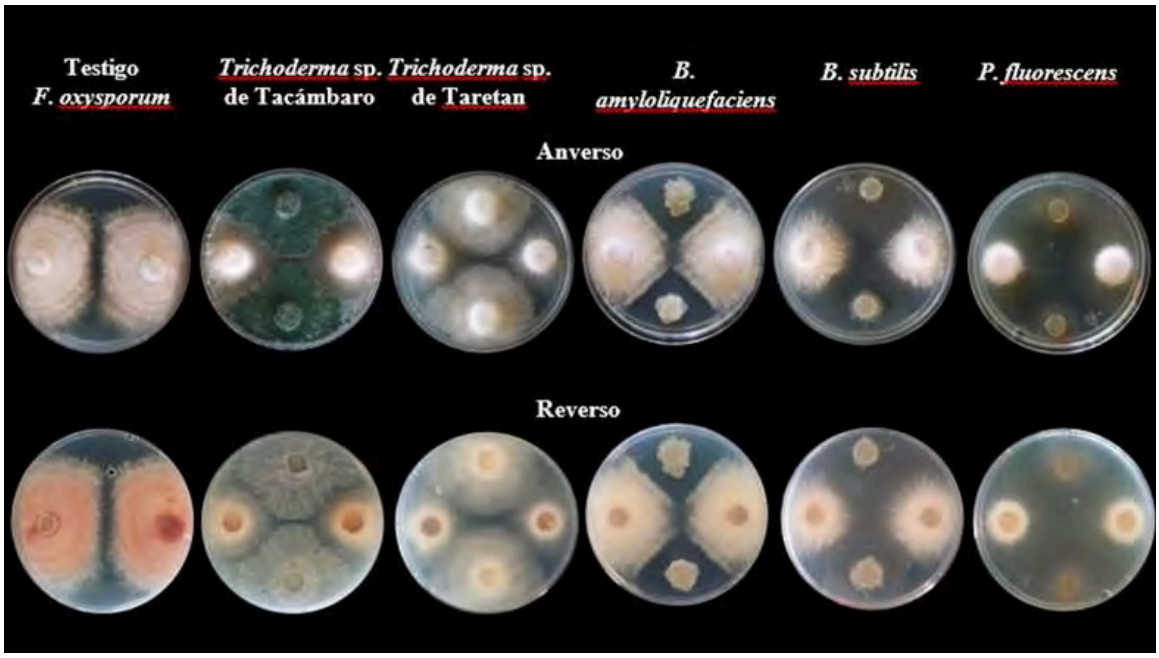

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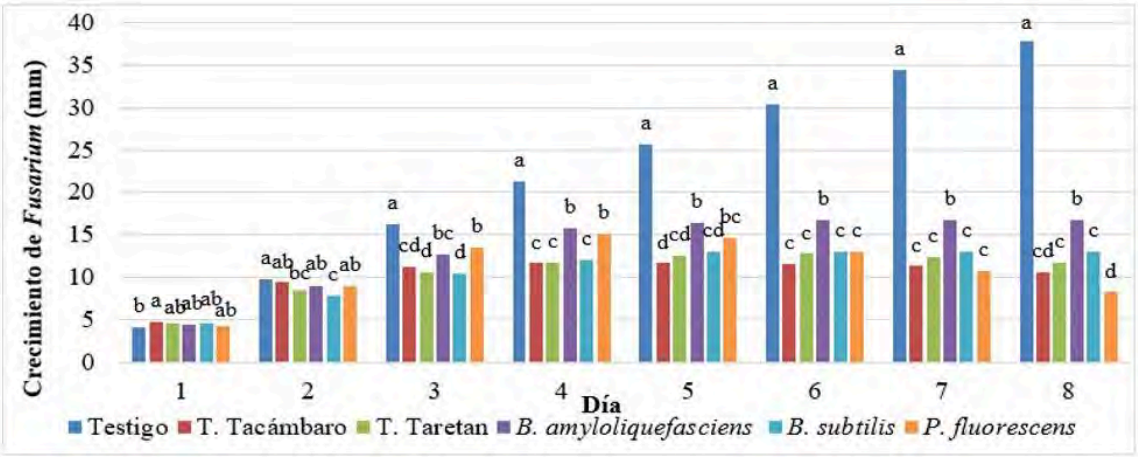
Figure 5. Comparison of means (Tukey, 0.05) of radial growth of *Fusarium oxysporum* over eight days.



Microorganisms such as *P. fluorescens*, *B. subtilis* and both *Trichoderma* species, which stand out for their efficacy in controlling *Fusarium* spp. and *F. oxysporum* in controlled environments, have been supported by previous studies (Guerra *et al*., 2011; Tejera-Hernández *et al*., 2011; Guédez *et al*., 2012; Mejía-Bautista *et al*., 2016; Boughalleb-M'Hamdi *et al*., 2018).

Biocontrol with *Trichoderma* is attributed to the production of enzymes with antifungal activity, affecting the mycelium of *F. oxysporum* by penetration and lysis (Martínez *et al*., 2013). Competition for space and nutrients also play a role (Infante *et al*., 2009). In the treatment with *Trichoderma* sp. from Tacámbaro, an initial parasitism of the pathogen was observed, corroborating previous findings with *T. viride* against *P. infestans* (Bouziane *et al*., 2016).

On the other hand, the antagonism of *P. fluorescens* and *B. subtilis* could be attributed to antibiosis, competition for resources, and induction of systemic resistance. In addition, the production of enzymes that degrade the cell wall and volatile organic compounds, phytoalexins and siderophores (Cornelis *et al*., 2008; Bhattacharyya and Jha, 2012; Singh and Singh, 2013).

Greenhouse bioassay

Yellowing and wilting in blackberry plants inoculated with *F. oxysporum* and with the antagonists showed significant variation. Preventive treatments with *B. subtilis* (37.65%), *Trichoderma* sp. (33.64%), *P. fluorescens* (42.32%) and the consortium of antagonistic agents (37.46), as well as *P. fluorescens* (41.97%) as a control, showed to be statistically equal, decreasing the incidence compared to the control inoculated with *Fusarium*, which had 100%; the preventive treatment with *Trichoderma* sp. reduced the incidence by 66.36% (Figure 6).

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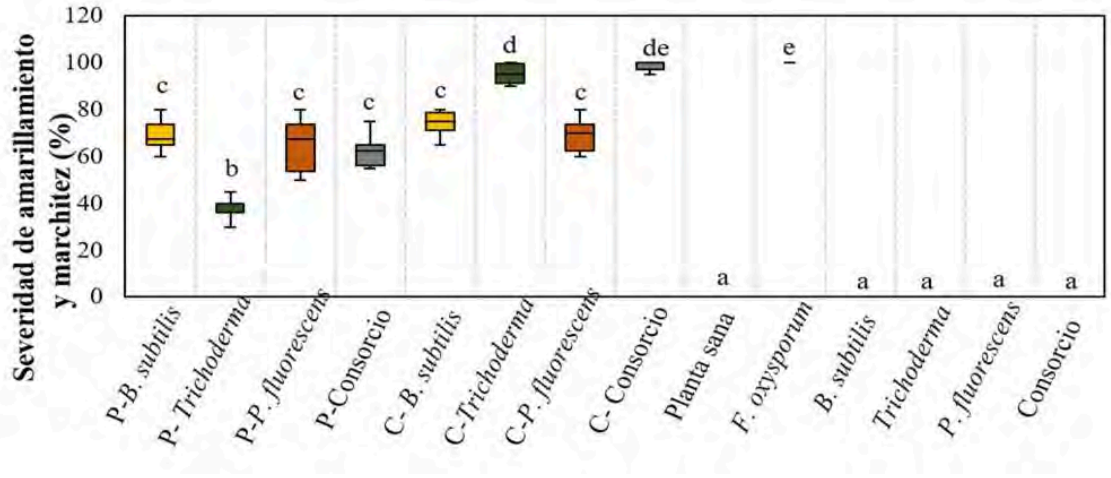
Figure 6. Comparison of means and percentage of severity of foliar symptoms in blackberry plants inoculated with *F. oxysporum*. P= preventive and C= control.



The control treatments with the consortium of antagonistic agents and with *Trichoderma* sp. behaved statistically the same as the control, with 98.97% and 96.69% yellowing and wilting. The treatment without inoculation and those inoculated with the antagonists *B. subtilis*, *Trichoderma* sp., *P. fluorescens* alone or in consortium did not present any incidence.

As a preventive measure, each antagonist decreased the percentage of incidence, agreeing with Acosta-González *et al*. (2018), who tested commercial and native biological control agents on the incidence of blackberry wilt due to *Fusarium* and reported that strains of *Trichoderma*, *Pseudomonas fluorescens*, and bacterial complexes showed positive results as they decreased the percentage of incidence. As a preventive measure, *Trichoderma* decreased the severity of yellowing by 61.73%, as reported by Rebollar and Silva (2019) under greenhouse conditions for blackberry wilt.

The high incidence of wilt in the antagonist consortium as a control could be attributed to the complex interrelationships between microorganisms at the soil-plant-microorganism-environment interface. *Trichoderma* and *Pseudomonas* often rely on these factors to manifest their beneficial effects. Nevertheless, in the interaction of these three types of microorganisms, synergistic effects may arise that amplify the benefits or conversely, that do not generate any appreciable effect (Cano, 2011).

The treatment inoculated with *Trichoderma* sp. and the preventive treatment, both statistically equal, exhibited the greatest increase in plant height, resembling previous findings by Ruiz-Cisneros *et al*. (2018) (Figure 7). *Trichoderma* generates secondary metabolites that influence plant growth and development (Contreras-Cornejo *et al*., 2016). In addition, it can activate genes related to plant defense, increasing the level of ergosterol, and promoting faster growth in the soil, thus explaining its positive effects on plant growth and defense (Mayo *et al*., 2015).

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Figure 7. Appearance of blackberry plants at the end of the tests. *A) healthy plant; B) control P-B. sub lis; C) control Trichoderma sp.; D) P-B. sub lis; E) P-Trichoderma sp.; F) P-P. fluorescens; G) P-consor um; H) control Fusarium oxysporum*; I) control *P. fluorescens*; J) consortium control; K) C-*B. subtilis*; L) C-*Trichoderma* sp.; M) C-*P. fluorescens*; and N) C-consortium.

The roots of the blackberry plants of the treatment without inoculation and the controls inoculated with *B. subtilis*, *Trichoderma* sp., *P. fluorescens* and the consortium of antagonists showed a slight percentage of root necrosis, isolates of these were made and the presence of pathogens was ruled out. This necrosis could be caused by excess water in the substrate, oxygen becomes scarce, causing asphyxiation and subsequent root rot.

In the preventive or control treatments inoculated with *Fusarium*, *B. subtilis* and *Trichoderma* sp. were statistically the same, with mean percentage of necrosis of 29.84%, as in the case of the preventive treatment with *P. fluorescens*. In comparison, the control treatment with the antagonist consortium and the control exhibited similar statistics and the highest percentages of necrosis (Figure 8).

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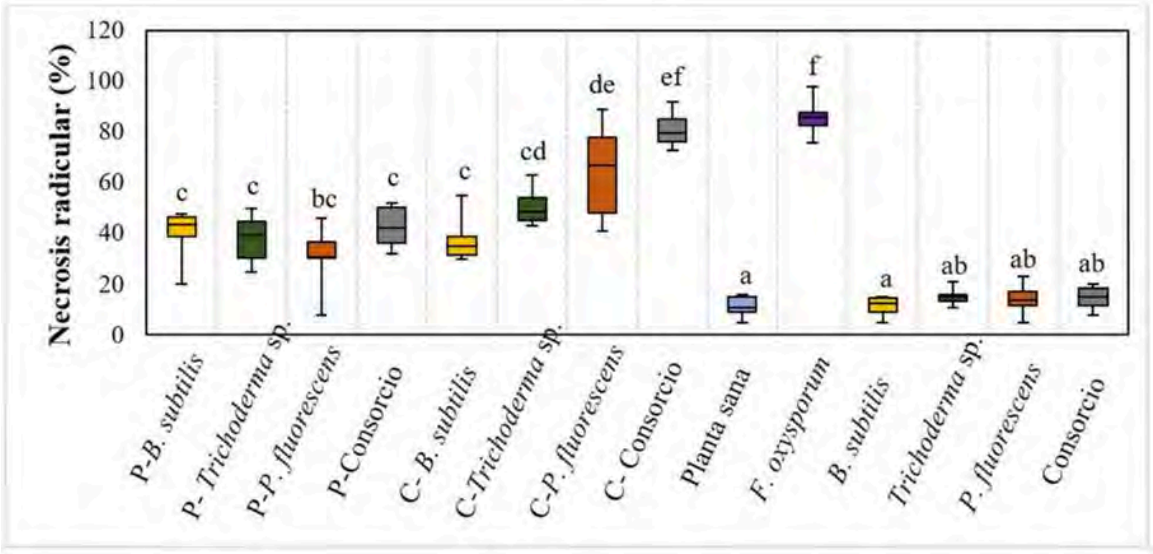
Figure 8. Comparison of means (Tukey, 0.05) of the percentage of necrosis present in the root of blackberry plants. P= preventive; C= control.



The efficiency in reducing the percentage of necrosis with the preventive treatment of *P. fluorescens* could be due to its ability to colonize the rhizosphere of plants and its antagonistic activity towards various pathogens (Perotti *et al*., 2005) as it is able to chelate iron, making it less available to pathogens or other microorganisms (Susan and Castiel, 2005). This coincides with Guerra *et al*. (2011), who reported efficient *P. fluorescens* strains for the control of *F. oxysporum* under controlled and greenhouse conditions.

Control and preventive treatments with *Trichoderma* sp. decreased the percentage of root necrosis since when colonizing the rhizosphere, any pathogen that crosses this protection is destroyed by the hyperparasitism it has, in addition to acting as a barrier to prevent the entry of pathogens into the roots (Gómez *et al*., 2013).

Conclusions

Fusarium oxysporum, the causative agent of yellowing and wilting in blackberry plants, was identified by its morphological and cultural characteristics. The strains of *Trichoderma*, *Bacillus* and *P. fluorescens* showed *in vitro* antagonistic capacity greater than 50% against *F. oxysporum*, *P. fluorescens* stood out with a remarkable 78% inhibition in radial growth.

In the greenhouse, preventive treatments exhibited remarkable antagonistic capacity, reducing the incidence of wilt by more than 57%. *Trichoderma* sp. stood out with a decrease of more than 66% and an increase in height of more than 50%. *B. subtilis* and *P. fluorescens* as controls also showed reductions of more than 45%.

In the preventive and control treatments, *B. subtilis* and *Trichoderma* sp. reduced root necrosis by more than 49%, the preventive treatment with *Trichoderma* sp. stood out, with a decrease of more than 62%. The preventive application of the antagonistic microorganisms showed greater efficacy in the prevention of blackberry yellowing and wilting compared to their application as a control.

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