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Article

Biological control of anthracnose using antagonistic microorganisms in *Coffea arabica* in the State of Mexico

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Abstract

Coffee in Mexico is important for the economy, generating jobs and foreign exchange; nevertheless, it faces phytosanitary problems such as anthracnose, caused by the fungus *Colletotrichum* sp., which is managed through chemical products, affecting the environment, so it is necessary to look for friendlier alternatives to control this disease. The research aimed to evaluate the antagonistic effect of three microorganisms (*Trichoderma* sp., *Bacillus* sp., and *Verticillium* sp.) against *Colletotrichum* sp., in samples collected in the municipalities of Temascaltepec and Tejupilco, State of Mexico on different dates in 2024. The experiment was conducted in the Phytopathology Laboratory of the Institute of Agricultural, Aquaculture and Forestry Research and Training of the State of Mexico. Antagonistic strains were confronted *in vitro* against *Colletotrichum* sp., calculating the following with the ImagenJ software: a) degree of mycoparasitism and b) percentage of growth inhibition. To determine the mode of action of the antagonism, an Anova and a Tukey comparison of means ($p > 0.05$) were performed. It was observed that *Trichoderma* sp. presented the highest degree of inhibition on the phytopathogen with 85.15%, *Bacillus* sp. with 59.27%, and *Verticillium* sp. with 47.08%. It was concluded that *Trichoderma* sp. was the microorganism that has the greatest antagonistic effect, which suggested the feasibility of conducting field research to evaluate the impact of this microorganism in the control of the disease under natural conditions.

Keywords:

Bacillus sp., *Trichoderma* sp. *Colletotrichum* sp., *Verticillium* sp.

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Introduction

Currently, coffee is a product of high commercial value. In Mexico, the largest producing states are Chiapas, Veracruz, Oaxaca, and Jalisco. However, the State of Mexico also contributed to national production; in 2021, 530.81 ha were planted, with a production of 639.89 t and an average yield of 1.21 t ha-1 (Bastos-Osorio *et al*., 2019; SIACOM, 2022).

Coffee crops face phytosanitary problems, such as anthracnose caused by the fungus *Colletotrichum* sp., among which *Colletotrichum kahawae* and *Colletotrichum gloeosporioides* have been reported, which affect various parts of the plant (Cannon *et al*., 2012). The development of the disease is influenced by biotic factors, such as fungal sporulation and transmission vectors, as well as abiotic factors, such as temperature, high relative humidity, wind and rain (Rojo-Bi *et al*., 2017).

Traditionally, it is controlled with highly toxic fungicides of restricted use, such as benzimidazole and strobilurins, azoxystrobin, chlorothalonil, mancozeb and thiabendazole, hence the importance of looking for safer crop production alternatives (Rojo-Bí *et al*., 2017; Sood *et al*., 2020). One of the most promising alternatives is the use of microorganisms as biological control agents. *Trichoderma* sp. showed high potential as an antagonist of phytopathogens, including *Fusarium* sp., *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Phytophthora* sp., and *Pythium* sp., among others, in different cultivars of commercial value (Martínez and Coca, 2020; García-Velasco *et al*., 2021).

On the other hand, *Bacillus* sp. produced a wide variety of antibiotics, such as lipopeptides, which inhibit the growth of pathogens such as *Rhizoctonia solani*, *Fusarium* sp., *Xanthomonas* sp., and *Botrytis cinerea* (Astorga-Quirós *et al*., 2014; Pérez and García, 2019; Zuñiga *et al*., 2019). Likewise, *Verticillium* sp. demonstrated great potential as a biocontroller of powdery mildew in cucumbers, producing resistance structures called dictyochlamydospores (Aiavo, 2015; Espinoza *et al*., 2017).

The purpose of the research was to evaluate the antagonistic effect of three microorganisms (*Trichoderma* sp., *Bacillus* sp. and *Verticillium* sp.) against *Colletotrichum* spp. in samples collected in the municipalities of Temascaltepec and Tejupilco, State of Mexico on different dates in 2024.

Materials and methods

Location

The study was carried out in the coffee-producing area of the State of Mexico, in the municipality of Temascaltepec (19° 02’ 35” north latitude, 100° 02’ 29” west longitude, average altitude of 1 719 m) and Tejupilco (18° 48’ 47” north latitude, 100° 08’ 06” west longitude, average altitude of 1 043 m). Samples of coffee leaves and fruits with symptoms of anthracnose (SENASICA, 2014) were collected and transferred to the Phytopathology Laboratory of the Institute of Agricultural, Aquaculture and Forestry Research and Training (ICAMEX, for its acronym in Spanish), where the research was conducted (García-Velasco *et al*., 2021) (Figure 1).

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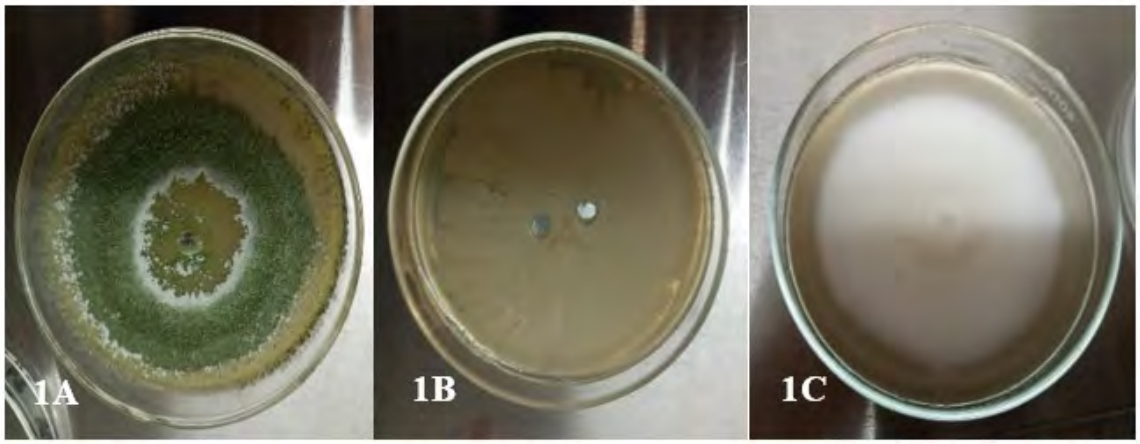
Figure 1. A) *Trichoderma* sp.; B) *Bacillus* sp. and C) *Verticillium* sp., microorganisms identified and provided by the Phytopathology Laboratory of ICAMEX.



To perform the isolation, the methodology used by Rengifo *et al*. (2002); Guzmán and Rivillas (2005), was implemented with some modifications. The leaves with the presence of the pathogen were washed with running water, the leaf blades were immersed in SDW (sterile distilled water) for five minutes and then rinsed with plenty of SDW. They were placed in Petri dishes, simulating a wet chamber in complete darkness at 26 °C and with 100% relative humidity, in a Thermo Scientific Heratherm incubator and checked at 24 and 48 h. A laminar flow chamber and a Labomed stereoscope were used to identify masses of conidia inside the acervuli; the Labomed USA microscope, lenses 10 x and 40 x, was used to identify conidia of *Colletotrichum* sp. on the lesions, these were transferred to 12 Petri dishes containing 20 ml of sterile PDA, and the growth of the pathogen was monitored for six days, selecting the pure colonies to carry out the antagonism (SENASICA, 2014; Ruíz-Campos, 2022).

The antagonism was carried out as proposed by Suárez *et al*. (2008), where potato dextrose agar (PDA) was used in a Petri dish, placing an agar disc of five mm in diameter with the mycelium of the pathogen *Colletotrichum* sp. at one end of the dish and another disc with the mycelium of the antagonists at the opposite end, maintaining an approximate distance of 5 cm between them.

The growth area of the colonies was determined from the images processed by the Imagej software in order to evaluate the antagonistic capacity of the biocontrollers. From these measurements, the percentage of growth inhibition (PGI) was calculated to compare the growth of the pathogen in the presence of the antagonist compared to the control. The formula used, described by Quintana-Obregón *et al*. (2010); Araya *et al*. (2019), was expressed as follows:

$$PGI\%=\frac{R1-R2}{R1}*100$$

Where: R1 growth of the control; R2 growth of the pathogen in antagonism, expressed as a percentage. To assess the degree of mycoparasitism, the scale proposed by Astorga-Quiroz *et al*. (2014) was used (Table 1).

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Table 1. Scale used to assess the degree of mycoparasitism of pathogen biocontrollers.

Degree	Antagonistic capacity	Biocontrolling potential
0	No invasion of the surface of the pathogenic strain	Very bad
1	1/4 invasion of the surface of the pathogenic strain	Bad
2	1/2 invasion of the surface of the pathogenic strain	Deficient
3	Total invasion of the surface of the pathogenic strain	Good
4	Total invasion of the surface of the pathogenic strain sporulation on it	Very good

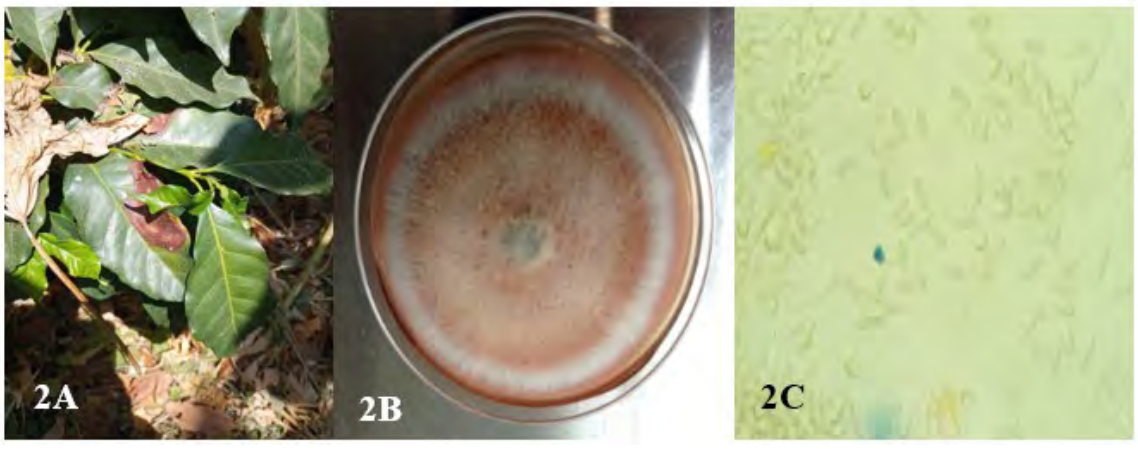
Experimental design and statistical analysis

Antagonism with microorganisms was performed by a completely randomized design with three treatments: a) Antagonism- *Colletotrichum* - *Trichoderma* (ACT); b) Antagonism- *Colletotrichum* - *Bacillus* (ACB) and c) Antagonism- *Colletotrichum* - *Verticillium* (ACV), in addition to a control group, with five replications per treatment, using a Petri dish as an experimental unit; for the comparison between treatments, a Tukey test was performed ($p = 0.05$) and the Shapiro-Wilk and Levene tests were used to validate normality and homoscedasticity, respectively. A Kruskal-Wallis test was also applied. The statistical analysis was performed with the InfoStat 2020 software (Suárez *et al*., 2008; Astorga-Quirós *et al*., 2014).

Results and discussion

The isolation and identification of the pathogen was carried out with samples of coffee leaves with the symptoms of *Colletotrichum* sp. (Figure 2A). Three isolates were obtained, C1, C2, and C3, which grew rapidly in the incubator at a temperature of 26 ±1 °C, covering the entire Petri dish on the sixth day.

Figure 2. A) Collection of leaves with symptoms of anthracnose; B) *Colletotrichum* sp. colony with the presence of masses of acervuli made up of conidia and C) conidia of the fungus *Colletotrichum* sp. in the PDA culture medium.



The colonies had a cottony, grayish-white appearance, with masses of orange acervuli and a coppery-red base (Figure 2B). The morphological description coincides with that reported by Ruíz-

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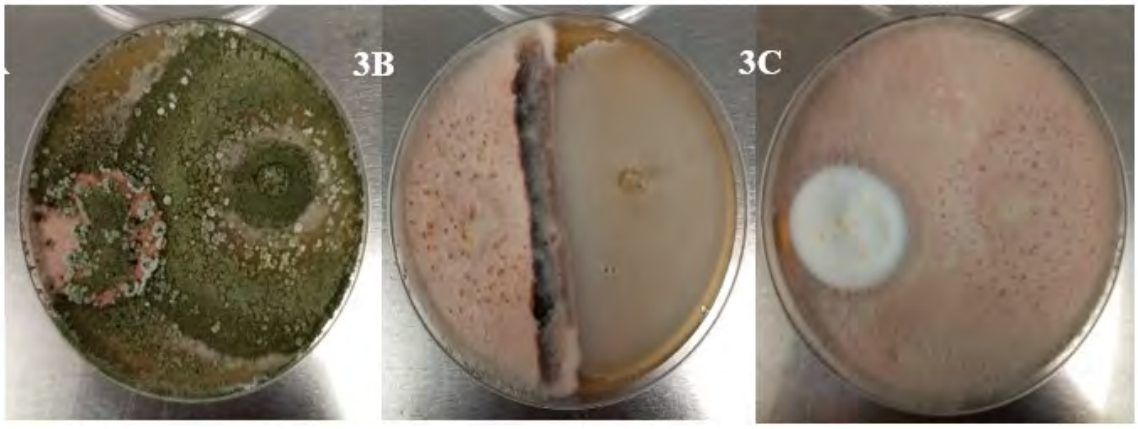
Campos (2022), who identified species of *Colletotrichum* sp. using morphological and molecular techniques. Microscopic observations, with the 10 x and 40 x lenses, showed that the colonies presented: hyaline mycelium, elongated unicellular conidia with rounded ends and a radial and cottony growth (Figure 2C), characteristics also described by Hyde *et al*. (2009). Regarding the growth of the colonies, Domínguez-Guerrero *et al*. (2012) reported a radial or concentric circled, cottony aerial growth, while mushrooms are rarely observed.

Once the pathogen was isolated and identified, antagonistic tests were performed, through which the percentage of growth inhibition was analyzed. This analysis was carried out on the second, fourth, and sixth day after establishing the antagonism experiment in the laboratory. The ACT treatment, identified as *Trichoderma* sp., was the most effective in controlling the mycelial growth of *Colletotrichum* sp. with a PGI of 17.09% ±8.25 on the second day, 79.03% ±8.55 on the fourth day and 85.15% ±1.34 on the sixth day (Figure 3A).

Figure 3. Antagonism of microorganisms. A) *Trichoderma* sp.; B) *Bacillus* sp. and C) *Verticillium* sp. against *Colletotrichum* sp. on the sixth day, the last day of evaluation .



This effect was attributed to the fact that the mode of action of *Trichoderma* sp. is competition for space and nutrients, mycoparasitism, and antibiosis is the main mechanism of action (Martínez and Coca, 2020). For their part, Sanmartín *et al*. (2012) obtained percentages of growth inhibition, with strains of *T. asperellum* against *C. gloeosporioides* ranging from 42.4% to 66.8%, being evaluated with a high degree of antagonism.

On the other hand, Zuñiga *et al*. (2019) obtained a PGI of 31% with the PDA culture medium with a concentration of 450 µg ml-1 of secondary metabolites extracted with *Trichoderma* sp. spores; changes were observed in the phenotype of *C. gloeosporioides*, such as modifications in texture, color, a thinning of the mycelium, and a lower biomass production of the phytopathogen after treatment.

The antagonist *Bacillus* sp. showed a PGI of 27.08% ±8.25 on the second day, 59.27% ±8.55 on the fourth day, and 47.02% ±1.34 on the sixth day (Figure 3B). This effect can be attributed to the mode of action of *Bacillus* sp., which included the production of antimicrobial compounds, lytic enzymes, competition for nutrients and space, as well as the induction of defenses in the host plant (Pérez and García, 2019). These values are within the ranges reported by Amaro *et al*. (2018), who obtained a reduction in mycelial growth of the pathogen between 48.75% and 72.01%; likewise, Zúñiga *et al*. (2019) reported a growth inhibition of 52% when using secondary metabolites of *Bacillus subtilis*, obtained by hexanic extracts and ethyl acetate.

Regarding *Verticillium* sp., the PGI was 36.89% ±8.25 on the second day, 47.08% ±8.55 on the fourth day, and 39.48% ±1.34 on the sixth day (Figure 3C). This antagonistic behavior was due to their ability to produce antimicrobial metabolites, competition for nutrients and space, and

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direct parasitism (Aiavo, 2015). These values coincide with those described by Bello *et al*. (2018), who, in their analysis, observed up to 82.6% inhibition of rust pustules 65 days after spraying *Verticillium* sp., in the field, this being the highest percentage recorded in coffee crops. Sharma *et al*. (2023) mentioned that recent hybrids of *Verticillium* sp. generated by protoplast fusion have greater virulence and a wider range of hosts.

Regarding Anova, on the second day, there is no statistically significant effect on the percentage of growth inhibition of *Colletotrichum* sp. between treatments (Table 2); on the fourth day, given that F is 7.31 and $p < 0.05$, there is a significant difference between treatments, confirming the data in the analysis of day six, where the F-value is 376.75 and the *p*-value is 0.0001, the variations in the PGI are much clearer and more pronounced.

Table 2. Analysis of variance (Anova) for the comparison of the PGI on days D2, D4 and D6.

SV	DF	SS	F	P	Kruskal Wallis
D2	2	261.89	0.38	0.6886	0.6771
Error	12	4 082.44			
Total	14	4 344.33			
D4	2	5 338.89	7.31	0.0084	0.0271
Error	12	4 382.43			
Total	14	9 721.33			
D6	2	6 750.09	376.75	0.0001	0.0019
Error	12	107.5			
Total	14	6 857.59			

S.V.= source of variation; DF= degrees of freedom; SS= sum of squares; F= F-value; *p* = *p*-value; Kruskal Wallis (nonparametric test).

These data were confirmed with Tukey's comparison of means ($p > 0.05$) (Table 3) and the Kruskal-Wallis test, where it was observed that treatments begin to show different effects from the fourth day (Table 2); these values coincide with Sanmartín *et al*. (2012); Sharma *et al*. (2023).

Table 3. Comparison of PGI means on days D2, D4 and D6 for the ACB, ACT and ACV treatments, Tukey ($p > 0.05$).

Variable	Treatment	Means	n	Letter
PGI D2	ACV	13.96 (8.25)	5	A
	ACT	6.64 (8.25)	5	a
	ACB	4.1 (8.25)	5	a
PGI D4	ACT	58.24 (8.55)	5	a
	ACB	24.46 (8.55)	5	b
	ACV	14.04 (8.55)	5	b
PGI D6	ACT	82.51 (1.34)	5	a
	ACB	45.19 (1.34)	5	b
	ACV	32.54 (1.34)	5	c

n= number of replications.

Regarding the degree of mycoparasitism, growth tests in dual culture of *Trichoderma* sp. against *Colletotrichum* sp. (Table 4) show a progressive inhibition of growth from the second day of incubation; *Trichoderma* sp. obtained level 4, which rated it with very good biocontrolling potential.

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Table 4. Antagonistic capacity of *Trichoderma* sp., *Bacillus* sp. and *Verticillium* sp. against *Colletotrichum*.

Antagonist	Contact antagonist/ pathogen (days)	Degree	Antagonistic capacity
Trichoderma sp.	2	4	Very good
Bacillus sp.	3	2	Deficient
Verticillium sp.	5	1	Bad

According to Suárez *et al*. (2008), this antagonistic fungus acts through a combination of nutrient competition, production of antifungal metabolites, hydrolytic enzymes, mycoparasitism and production of plant growth-promoting substances; this was also observed by Sanmartín *et al*. (2012), where they rated *T. asperellum* at an antagonistic level four, completely covering *C. gloeosporioides* at 10 days, according to the scale used in the work by Ezziyyani *et al*. (2004); Astorga-Quirós *et al*. (2014).

On the other hand, *Bacillus* sp. against *Colletotrichum* sp. (Table 4) showed that the antagonist came into contact with the pathogen on the third day, showing a progressive inhibition of its growth, according to the scale used by Ezziyyani *et al*. (2004); Astorga-Quirós *et al*. (2014), *Bacillus* sp., it obtained a level 2, which rated it with a deficient antagonistic capacity; nevertheless, Amaro *et al*. (2018) point out that once the antagonist comes into with the pathogen, it produces a wide variety of antibiotics that inhibit the growth of the pathogen due to competition for space and nutrients, not allowing the growth of *Colletotrichum* sp.

As for the antagonistic fungus *Verticillium* sp., according to the scale used by Ezziyyani *et al*. (2004); Astorga-Quirós *et al*. (2014), it obtained a level 1 (Table 4), which was considered bad. This is because the antagonistic fungus came into contact with *Colletotrichum* sp. until the fifth day, invading approximately a quarter of the surface of the experimental unit. This indicates that the ability of the antagonistic fungus to inhibit the growth of the pathogen was limited; according to Sood *et al*. (2020), the partial invasion suggests that the action of the antagonist is not sufficient to prevent the growth and development of the pathogen.

Conclusions

The treatments showed a high significant effect on PGI, the ACT treatment was the one with the highest value, reaching up to 85.15% ±1.34, followed by ACB with values of up to 59.27% ±8.55 and ACV sp. with values of 47.08% ±1.34. These results showed that ACT is the most effective treatment in terms of inhibiting the growth of the pathogen's colony. On the other hand, *Trichoderma* sp. possibly acted by mycoparasitism, competition for space and nutrients and antibiosis, on *Colletotrichum* sp., it obtained a level four, because it invaded and destroyed the colonies of the phytopathogen, which indicated that *Trichoderma*

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