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# Sensitivity of *Colletotrichum* sp. strains from strawberry to systemic fungicides *in vitro*

## Sensibilidad de cepas de *Colletotrichum* sp., en fresa frente a fungicidas sistémicos *in vitro*

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## ABSTRACT

In Pamplona (Colombia), reduced efficacy of anthracnose treatments in strawberries has recently been reported. The objective of this study was to evaluate the sensitivity of *Colletotrichum* sp. strains isolated from strawberry (*Fragaria × ananassa* Duch.) to three systemic fungicides *in vitro*. Two *Colletotrichum* strains were used: Ivan01 (isolated from fruit) and Ivan02 (isolated from leaves); both were collected in the Monteadentro region. Before experimentation, the strains were morphologically characterized. Separate experiments were conducted for each fungicide (difenoconazole, carbendazim, and azoxystrobin) using both strains. Mycelial growth inhibition and conidial production were assessed on each plate. Analysis of variance and regression analyses were performed to estimate the effective concentration ($EC_{50}$) values for each strain-fungicide combination. Strain Ivan01 exhibited dark gray mycelium with a cottony texture, hyaline and melanized hyphae, and chlamydospores. In contrast, strain Ivan02 displayed dense, light gray mycelium, a woolly texture, circular growth, chlamydospores, and cylindrical conidia with rounded, hyaline ends. Difenoconazole demonstrated greater efficacy against both *Colletotrichum* confirming its potential as a key fungicide for managing strawberry anthracnose. In contrast, carbendazim showed markedly reduced effectiveness, with evidence of high resistance and variable impact on sporulation, while azoxystrobin exhibited intermediate levels of resistance. Both strains were sensitive to difenoconazole, moderately sensitive to azoxystrobin, and highly resistant to carbendazim. Integrated disease management strategies are necessary to ensure long-term control of *Colletotrichum* sp. in strawberry cultivation in Pamplona.

**Keywords:** anthracnose; $EC_{50}$; inhibitory sterol demethylation; mycelial growth; quinone outside inhibitors; resistance monitoring

## RESUMEN

En Pamplona (Colombia), se reportó recientemente una baja eficacia de los tratamientos contra la antracnosis en fresa. El objetivo de este estudio fue evaluar la sensibilidad de dos cepas de *Colletotrichum* sp. aisladas de fresa (*Fragaria × ananassa* Duch.) a tres fungicidas sistémicos *in vitro*. Para ello se utilizaron dos cepas de *Colletotrichum*: Ivan01 (aislada de fruto) e Ivan02 (aislada de hojas), obtenidas en la región de Monteadentro. Antes de la experimentación, las cepas se caracterizaron morfológicamente. Se realizaron experimentos con difenoconazol, carbendazim y azoxistrobin, utilizando ambas cepas. La inhibición del crecimiento micelial y la producción de conidios se evaluaron en cada placa. Se realizaron análisis de la varianza y regresión para estimar los valores de la concentración efectiva ($CE_{50}$) para cada combinación cepa-fungicida. La cepa Ivan01 presentó un micelio gris oscuro con textura algodonosa, con hifas hialinas y melanizadas, y clamidosporas. Por el contrario, la cepa Ivan02 presentó un micelio denso, gris claro, textura lanosa, crecimiento circular, clamidosporas y conidios hialinos, con extremos redondeados. Difenoconazol demostró mayor eficacia contra ambas cepas de *Colletotrichum*, confirmando su potencial como fungicida clave para el manejo de la antracnosis de la fresa. En contraste, carbendazim mostró una efectividad marcadamente reducida, con evidencia de alta resistencia e impacto variable en la esporulación, mientras que azoxistrobin exhibe niveles intermedios de resistencia. Ambas cepas fueron sensibles a difenoconazol, moderadamente sensibles a azoxistrobin y altamente resistentes a carbendazim. Se evidencia la necesidad de implementar estrategias integradas de manejo de enfermedades para garantizar el control a largo plazo de *Colletotrichum* sp. en el cultivo de fresas en Pamplona.

**Palabras clave:** antracnosis; $CE_{50}$; crecimiento micelial; inhibición de la desmetilación de esteroles; inhibidor de la quinona externa; monitoreo de resistencia

## INTRODUCTION

The strawberry (*Fragaria* × *ananassa* Duch.) is a fruit consumed worldwide, highly valued for fresh consumption and for making desserts due to its color, aroma, and acidity. It is also rich in vitamins A and C (Qaderi *et al.,* 2022). The strawberry is one of the most cultivated varieties; countries such as the United States, Mexico, Spain, and China are leaders in strawberry production and export (Simpson, 2018). The strawberry industry generates billions of dollars annually and represents an important source of income for farmers and rural economies (Villamizar *et al*., 2022).

The Department of Norte de Santander ranks fourth in strawberry production in Colombia (González & Sanjuán, 2021). Located in the mountains of northeastern Colombia, Pamplona in the Department of Norte de Santander, has established itself as an agricultural epicenter, playing a key role in strawberry production (Castellanos, Céspedes *et al.,* 2020). Favored by a temperate climate and fertile soils, this region has found in strawberries not only a profitable crop but also a fundamental pillar for its economic and social development. Furthermore, the high quality of strawberries produced in Pamplona has facilitated access to both national and international markets, positioning the region as an important competitor in the fruit sector (Minagricultura & Desarrollo Rural, 2023).

Anthracnose caused by fungi of the genus *Colletotrichum* is considered one of the most significant diseases affecting strawberry cultivation in different parts of the world, including China (Zhang *et al*., 2020), the United States (Rebello *et al.,* 2022), Iran (Motallebi & Negahban, 2024), Japan (Furuta *et al.,* 2024), and Colombia (Cano, 2013). Anthracnosis are very common diseases that cause a lot of damage to different crops in Colombia, including strawberry (Martínez *et al*., 2020).

Several fungicides are considered systemic because they translocate within the plant and, in many cases, affect specific points in the plant's metabolism. Therefore, fungi develop resistance through selection or variations in their sites of action. Among these are carbendazim, belonging to the benzimidazole group; Sterol Biosynthesis Inhibitors (SBIs), which more specifically belong to the Methyl Benzimidazole Carbamates (MBCs); and difenoconazole, which belongs to the Triazole group and whose mode of action is characterized by inhibitory sterol demethylation (DMI). Azoxystrobin is a Strobilurin, which belongs to the respiration-inhibitor fungicides and specifically to the Quinone-outside Inhibitors (QoIs), and is also systemic (Pérez-Vicente, 2013).

In Mexico, Espinoza-Altamirano *et al.* (2017) determined the loss of sensitivity of *Colletotrichum acutatum*, finding that many isolates were moderately resistant to thiophanate-methyl (benzimidazoles), although they remained sensitive to azoxystrobin (strobitulins). Similarly, in Florida, where strawberry anthracnose fruit rot caused by *Colletotrichum acutatum* is a major disease and frequent applications of quinone-outside inhibitor (QoI) fungicide are required, a collection of 181 *C. acutatum* isolates was evaluated. Different levels of sensitivity were observed to azoxystrobin and pyraclostrobin in strains with or without a history of prolonged treatment with these fungicides (Forcelini *et al.,* 2016).

Similarly, investigations were conducted to determine the sensitivity of *Colletotrichum acutatum* to azoxystrobin, difenoconazole, and thiophanate-methyl in conventional and organic strawberry fields in the states of São Paulo and Espírito Santo in Brazil. The results of the study demonstrated the absence of strains resistant to these fungicides, allowing for the establishment of a baseline for future monitoring with the aim of evaluating the field resistance of *C. acutatum* populations in strawberry fields (Baggio *et al.,* 2018).

In Pamplona, Norte de Santander, strawberry growers face the challenge of managing various foliar diseases caused by phytopathogenic fungi to prevent losses

and deterioration of strawberry quality, including anthracnose. To control this disease, various fungicides are used, including chlorothalonil, difenoconazole, azoxystrobin, and captan (Castellanos, Baldovino *et al.,* 2020; Castellanos, Céspedes *et al.,* 2020).

The literature review did not reveal any research results on the sensitivity or resistance of *Colletotrichum* sp. from strawberry fields in Colombia. However, fungicide resistance is suspected in the case of anthracnose in strawberries due to the indiscriminate use of fungicides in Pamplona (Rodríguez *et al.,* 2024).

Given the above, the objective of this study was to evaluate, under laboratory conditions, the sensitivity of *Colletotrichum* sp. strains collected from strawberries in the Pamplona area to three fungicides from the main systemic groups.

## MATERIALS AND METHODS

### *Study area and experimental approach*

The study was conducted using samples with typical anthracnose symptoms collected from the Los Eucaliptos farm owned by Ivan Portilla, located at 7.346734N and 72.665320W, at an altitude of 2,586.3 m, in the Monteadentro area of Pamplona. The study employed both descriptive and experimental approaches. The descriptive phase enabled the characterization and documentation of *Colletotrichum* sp. strains isolated from strawberry fruits and leaves in Monteadentro, whereas the experimental phase was conducted in vitro at the Microbiology Laboratory of the University of Pamplona.

### *Collection, asolation, and preservation of Colletotrichum sp.*

Samples of strawberry leaves and fruits exhibiting symptoms characteristic of *Colletotrichum* sp. infection were collected. The samples were stored and transported to the microbiology laboratory at the University of Pamplona using established standard methods (Agrios, 2005). Once in the laboratory, they underwent a cleaning, disinfection, and isolation protocol based on the following steps: sterile distilled water, ethanol, and sodium hypochlorite. The disinfected fragments were then inoculated onto Potato Dextrose Agar (PDA) following the standardized protocol of Agrios (2005).

The isolates were preserved on PDA at 25±1 °C, and monoconidial cultures were obtained.

### *Morphological characterization of Colletotrichum sp. Strains*

Morphological characterization was performed on these cultures by evaluating hyphal characteristics, texture, growth pattern, growth rate (Estrada Salazar & Ramírez Galeano, 2019), and colony color (Domínguez Soto *et al.,* 2012). The formation of typical acervuli, the shape of the conidiophores, and the shape and size of the conidia were also considered. For genus classification, the general descriptions provided by Barnett & Hunter (1998) and Agrios (2005) were considered. Finally, two strains deposited in the Microbiology collection of the University of Pamplona, designated as *Colletotrichum* sp. Ivan01 and *Colletotrichum* sp. Ivan02, were used.

### *Experimental design and fungicide treatments*

In the experimental phase, a completely randomized design was implemented in the laboratory to evaluate three fungicides: difenoconazole (triazole group), carbendazim (benzimidazole group), and azoxystrobin (strobilurin group).

A total of six experiments were conducted, corresponding to the evaluation of each fungicide against both strains. Each experiment followed an 8×4 factorial design, consisting

of eight treatments (seven fungicide concentrations, T2–T8, plus a control, T1) and four replicates per treatment. The experimental units consisted of Petri dishes inoculated with the respective phytopathogenic strain, resulting in 32 Petri dishes per trial.

The treatments for each fungicide versus pathogen confrontation to be evaluated were as follows (Table 1):

**Table 1.** *Concentrations of each of the fungicides (difenoconazole, carbendazim, and azoxystrobin) that were used as treatments.*

| Treatments of each fungicide | Concentration of active ingredient (mg/L) | Quantity of standard solution of each fungicide | Quantity of agar - PDA (ml) |
|---|---|---|---|
| T1 (Control) | 0 | - | 100 |
| T2 | 0.001 | 1 mL de S4 | 99.0 |
| T3 | 0.01 | 5 mL de S3 | 95.0 |
| T4 | 0.1 | 2 mL de S3 | 98.0 |
| T5 | 0.5 | 1 mL de S2 | 99.0 |
| T6 | 5 | 1 mL de S1 | 99.0 |
| T7 | 10 | 2 mL de S1 | 98.0 |
| T8 | 100 | 20 mL de S1 | 80.0 |

***Preparation of fungicide solutions and culture media***

Three commercial fungicide formulations officially registered in Colombia (ICA, 2024) were used in this study: Conazole 250 EC (active ingredient: difenoconazole), Bélico 500 SC (carbendazim), and Chakal 250 SC (azoxystrobin). Prior to establishing the treatment concentrations, four stock solutions were prepared: S1 = 500 mg/L, S2 = 50 mg/L, S3 = 5 mg/L, and S4 = 0.1 mg/L of active ingredient for each fungicide. These stock solutions were used to adjust the proportions in the culture media to achieve the desired final concentrations. The volume of each stock solution prepared was determined based on the number of experimental units required. Sterile distilled water (SDW) was used as the solvent, following the protocol described by Pérez-Vicente (2013).

Fungicide solutions were added to liquefied agar in Erlenmeyer flasks once it had cooled to ~40 °C. The mixture was gently agitated to ensure homogenization while avoiding bubble formation. Each concentration was poured into four Petri dishes (replicates) and allowed to solidify at room temperature. A 1-cm mycelial disc of the *Colletotrichum* strain was placed at the center of each dish. Fungicide concentration served as the independent variable, while mycelial growth inhibition and sporulation reduction were considered dependent variables.

***Evaluation of mycelial growth inhibition***

Colony diameter (cm) was measured twice perpendicularly, and the percentage of inhibition was calculated using the Abbott formula (Püntener & Zahner, 1981), based on the mean of both measurements. Assessments were conducted daily for 12 days following inoculation with the mycelial disc.

***Assessment of sporulation capacity***

To evaluate sporulation capacity, one colony per treatment was selected. Ten mL of sterile distilled water (SDW) was added, and the mycelial surface was gently scraped

with a sterile scalpel. The suspension was homogenized by shaking, and a 1 mL aliquot was transferred to a tube containing 9 mL of SDW (1:10 dilution). Four subsamples of this dilution were used for conidia quantification with a Neubauer chamber, so four repetitions were performed.

***Statistical analysis and $EC_{50}$ determination***

Data on colony diameter, sporulation reduction, and germination were analyzed using one-way Analysis of variance after verifying the assumptions of normality and uniformity of variances. The conidia data were transformed into √X+1 due to the existence of zeros. When significant differences were detected ($p < 0.05$), Tukey's HSD test was applied at a 95% confidence level. Statistical analyses were performed using SPSS v.23.

$EC_{50}$ values were estimated from dose–response curves relating fungicide concentration to percentage inhibition of pathogen growth over time, using a 5% error probability. Inhibition data were transformed into probit units, and concentrations into logarithmic values. Regression analyses were conducted using Probit software developed by the Cuban Plant Health Research Institute (INISAV) (Pérez-Vicente, 2013). Susceptibility classification followed the criteria proposed by Torres-Calzada *et al.* (2015).

## RESULTS

***Morphological characteristics of Colletotrichum sp. Strains***

Strain Ivan01 exhibited dark gray mycelium with light gray to whitish margins and a typical concentric growth pattern. Strain Ivan02 formed dense grayish-white colonies with well-defined concentric rings. Both *Colletotrichum* sp. strains showed progressive and uniform mycelial growth over 12 days of incubation. The average radial growth rate was 0.45 cm/day for Ivan01 and 0.43 cm/day for Ivan02, indicating moderate development under optimal laboratory conditions.

Both strains, Ivan01 and Ivan02, exhibited septate, thin-walled, branched, hyaline hyphae with an interlaced growth pattern. Melanized hyphae—brown to black with thickened cell walls—were also observed, consistent with the protective structures typical of the *Colletotrichum* genus. Strain Ivan02 showed thick hyphae, though melanization was less frequent.

Strains Ivan01 (Figure 1 A and B) and Ivan02 (Figure 1 D and E) presented a well-defined convex elevation, with entire and regular margins, adopting a symmetrical circular morphology.

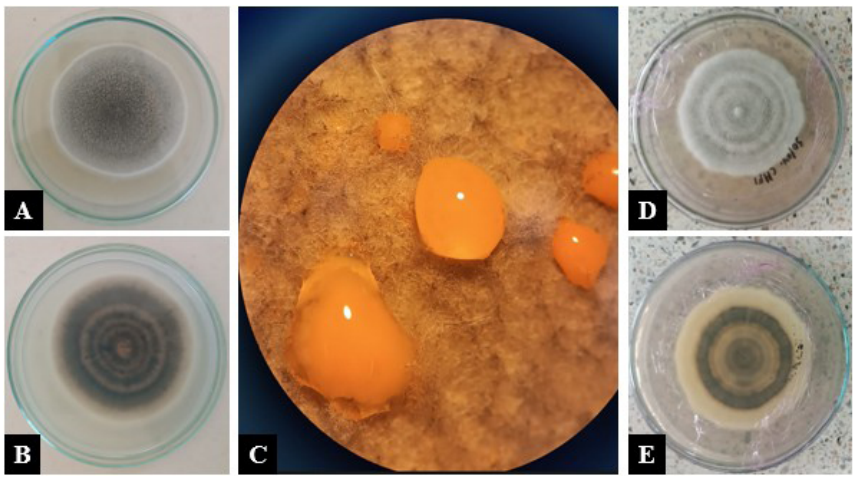



**Figure 1.** *Colletotrichum spp. A. Ivan01 strain, top of the colony on PDA. B. Ivan01 strain, reverse of the colony on PDA. C. Ivan01 strain, conidiomata seen under a stereomicroscope. D. Ivan02 strain, top of the colony on PDA. E. Ivan02 strain, reverse of the colony on PDA.*

Stained preparations revealed chlamydospores in both strains, Ivan01 and Ivan02, appearing as thick-walled, spherical to subglobose cells with more intense coloration than the surrounding hyphae. In Ivan01, conidia were abundant, cylindrical to slightly fusiform, with one angled and one rounded end, showing visible constrictions ("waists"), and hyaline in color. Ivan02 conidia were also abundant, cylindrical with rounded ends, lacking constrictions, and similarly hyaline.

***Sensitivity of the strain Ivan01 of the fungus Colletotrichum sp. to different fungicides***

Analysis of variance (ANOVA) revealed highly significant differences ($p < 0.01$) in the radial growth of *Colletotrichum* sp. (strain Ivan01) colonies in response to varying concentrations of difenoconazole, azoxystrobin, and carbendazim, based on evaluations conducted at four, eight, and twelve days post-inoculation (Table 2). Tukey's means comparison test ($p<0.05$) for difenoconazole, at all-time points, the treatment without fungicide (T1: 0 mg/L) and the lowest concentration (T2: 0.001 mg/L) exhibited the largest mycelial diameters, with no statistically significant differences between them. From 0.01 mg/L (T3) onward, a significant reduction in mycelial growth was observed, with the effect becoming more pronounced as the concentration increased. After 12 days, treatments T6, T7, and T8 (5, 10, and 100 mg/L, respectively) showed the lowest radial growth, statistically different from the other treatments. These results indicate a strong fungicidal effect at concentrations ≥ 0.01 mg/L, with complete growth inhibition beginning at 5 mg/L.

**Table 2.** *Results of the statistical analysis comparing the radial growth of Colletotrichum sp. strain Ivan01 colonies at different fungicide concentrations*

| | **Difenoconazole** | | | **Azoxystrobin** | | | **Carbendazim** | | |
|---|---|---|---|---|---|---|---|---|---|
| | Results of ANOVA | | | | | | | | |
| | 3 d | 8 d | 12 d | 3 d | 8 d | 12 d | 3 d | 8 d | 12 |
| **F** | 109.87 | 180.68 | 155.36 | 19.69 | 10.15 | 4.33 | 14.73 | 2.97 | 4.36 |
| ***P* valor** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.021 | 0.003 |
| | **Comparison of media** | | | | | | | | |
| mg/L | 3 d | 8 d | 12 d | 3 d | 8 d | 12 d | 3 d | 8 d | 12 d |
| 0 | 2.90 a | 5.28 a | 7.13 a | 2.70 a | 4.96 a | 6.60 a | 3.59 a | 5.09 a | 6.68 a |
| 0.001 | 2.80 a | 5.25 a | 7.11 a | 2.43 ab | 5.11 ab | 6.38 a | 3.42 a | 4.08 a | 6.22 ab |
| 0.01 | 1.71 b | 2.52 b | 3.40 b | 2.15 ab | 3.59 abc | 5.09 ab | 3.28 a | 4.80 a | 6.24 ab |
| 0.1 | 1.55 b | 2.27 b | 3.03 b | 2.00 b | 3.68 bc | 5.15 ab | 3.35 a | 5.12 a | 5.68 ab |
| 0.5 | 1 c | 1.47 c | 1.81 c | 1.88 bc | 3.48 bc | 4.63 ab | 3.23 a | 4.05 a | 4.84 ab |
| 5 | 1.00 c | 1.00 c | 1.00 c | 1.35 cd | 3.15 c | 4.20 ab | 2.55 b | 3.38 a | 4.75 ab |
| 10 | 1.00 c | 1.00 c | 1.00 c | 1.30 cd | 2.23 c | 3.43 b | 2.41b | 3.59 a | 4.63 b |
| 100 | 1.00 c | 1.00 c | 1.00 c | 1.00 **d** | 2.63 c | 3.38 b | 2.31 b | 3.30 a | 4.20 b |
| ET | 0.077 | 0.135 | 0.208 | 0.133 | 0.318 | 0.580 | 0.13 | 0.43 | 0.48 |
| CV (%) | 9.56 | 10.93 | 13.11 | 14.40 | 17.70 | 18.90 | 8.72 | 19.71 | 18.28 |

*Note.* Means with unequal letters in the columns differ for $p<0.05$, according to Tukey's test.

Tukey's mean comparison test ($p < 0.05$) for azoxystrobin treatments showed that the control (T1: 0 mg/L) had the largest mycelial diameters at all three evaluation times, although several treatments did not differ significantly from it. In contrast, the highest concentrations (T7: 10 mg/L and T8: 100 mg/L) exhibited significantly reduced growth. From day four onward, concentrations ≥ 0.5 mg/L consistently produced significant inhibition of mycelial development. After eight days, no statistically significant differences were observed among T1 (0 mg/L), T2 (0.001 mg/L), and T3 (0.01 mg/L), indicating that these concentrations were ineffective in suppressing growth. Conversely, T4 (0.1 mg/L) and T5 (0.5 mg/L) showed significant differences compared to the control, reflecting reduced fungal development. Finally, T6, T7, and T8 (5, 10, and 100 mg/L, respectively) recorded the lowest mycelial diameters and were statistically distinct from all other treatments.

Tukey's test ($p < 0.05$) for carbendazim treatments indicated that the control (T1: 0 mg/L) showed significant differences beginning on day four at a concentration of 0.5 mg/L (T5), with this trend persisting in subsequent evaluations. After eight days, no statistical differences were observed between T1 (0 mg/L) and T8 (100 mg/L). By day 12, only treatments T7 and T8 maintained significantly lower mycelial diameters compared to the control.

***Estimation of $EC_{50}$ of strain Ivan01 of the fungus Colletotrichum sp. to fungicides***

Once inhibition values were transformed into logits and concentrations into logarithmic units, regression analysis yielded the linear equation Y = 0.939X + 5.719 for difenoconazole, showing a significant fit to the data. This resulted in an estimated $EC_{50}$ of 0.17 mg/L for *Colletotrichum* sp., strain Ivan01. For azoxystrobin, the regression equation was Y = 0.347X + 4.678, also with a significant fit, yielding an $EC_{50}$ of 8.88 mg/L. For carbendazim, the equation Y = 0.283X + 4.365 was obtained, with a significant fit, resulting in an $EC_{50}$ of 174.35 mg/L for the pathogen (Table 3).

**Table 3.** *Determination of $EC_{50}$ values for the strain Ivan01 of the fungus Colletotrichum sp. according to the fungicide dose/percentage inhibition of fungal growth curve*

| Fungicide | Equation | $X^{2*}$ | R | $R^2$ | $EC_{50}$ (mg/L) | | |
|---|---|---|---|---|---|---|---|
| | | | | | Cal. | L inf. | L. sup. |
| **Difenoconazole** | Y= 0.939X + 5.719 | 4.02 | 0.82 | 0.68 | 0.17 | 0.03 | 0.31 |
| **Azoxystrobin** | Y= 0.347 X + 4.678 | 3.05 | 0.82 | 0.68 | 8.88 | 8.28 | 9.48 |
| **Carbendazim** | Y= 0.347 X + 4.678 | 0.13 | 0.95 | 0.92 | 174.35 | 167.0 | 181.05 |

*Note.* No statistical difference by the $X^2$ test.

These results indicate that relatively high concentrations of carbendazim are necessary to achieve a 50% inhibition of fungal growth, suggesting reduced sensitivity of the tested *Colletotrichum* strain to this fungicide.

***Sporulating capacity of the Ivan01 strain against fungicides***

ANOVA results for conidia production across treatments with the three fungicides against *Colletotrichum* sp., strain Ivan01, revealed a significant effect among concentrations (Table 4). Mean comparison for difenoconazole showed a significant

reduction in conidia /mL starting at 0.001 mg/L, with no conidia production observed from 5 to 100 mg/L due to the absence of mycelial growth. For azoxystrobin, a significant reduction in CFU/mL was recorded from 0.1 mg/L onward compared to the control. In the case of carbendazim, a significant decrease in conidia production was observed only at 100 mg/L relative to the control.

**Table 4.** *Results of the statistical analysis of the effect of the fungicides on conidia production of Colletotrichum sp. strain Ivan01*

| | **Difenoconazole** | | **Azoxystrobin** | | **Carbendazim** | |
|---|---|---|---|---|---|---|
| mg/L | Conidia/ 10 mL | Data transformed | Conidia/ 10 mL | Data transformed | Conidia/ 10 mL | Data transformed |
| | Conidia X $10^4$ | √ Conidia + 1 | Conidia X $10^4$ | √ Conidia + 1 | Conidia X $10^4$ | √ Conidia + 1 |
| 0 | 102.00 | 11.07 a | 103.5 | 11.14 a | 71.25 | 9.39 a |
| 0.001 | 42.25 | 7.48 b | 46.75 | 7.83 ab | 66.75 | 9.16 a |
| 0.01 | 47.25 | 7.82 b | 51.0 | 7.94 ab | 62 | 8.45 a |
| 0.1 | 33.25 | 6.73 b | 46.5 | 7.32 b | 61.75 | 8.80 a |
| 0.5 | 17.75 | 5.13 c | 34.5 | 6.80 b | 54.75 | 8.28 ab |
| 5 | 0 | 1.00 d | 22.5 | 6.00 b | 55 | 7.98 ab |
| 10 | 0 | 1.00 d | 25.5 | 5.67 b | 47.5 | 7.83 ab |
| 100 | 0 | 1.00 d | 18.5 | 5.24 b | 12 | 4.45 b |
| ET | | 0.31 | | 0.71 | | 0.83 |
| CV (%) | | 12 | | 19.7 | | 20.0 |

*Note.* Means with unequal letters in the columns differ for $p<0.05$, according to Tukey's test.

***Sensitivity of the Colletotrichum sp. strain Ivan02 to different fungicides***

Analysis of variance (ANOVA) revealed highly significant differences ($p < 0.01$) in the radial growth of *Colletotrichum* sp. (strain Ivan02) colonies in response to varying fungicide concentrations, based on evaluations conducted at four, eight, and twelve days post-inoculation (Table 5). For difenoconazole, mean comparisons showed that at all time points, the control (T1: 0 mg/L) and the lowest concentration (T2: 0.001 mg/L) exhibited the largest mycelial diameters, with no statistically significant differences between them. From 0.01 mg/L (T3) onward, a significant reduction in mycelial growth was observed, with the effect becoming more pronounced as the concentration increased. After 12 days, treatments T6, T7, and T8 (5, 10, and 100 mg/L, respectively) showed the smallest diameters (1.00 cm), statistically distinct from the other treatments, indicating complete inhibition of mycelial growth.

**Table 5.** *Results of the statistical analysis comparing the radial growth of colonies of strain Ivan02 at different fungicide concentrations*

| | **Difenoconazole** | | | **Azoxystrobin** | | | **Carbendazim** | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Results of ANOVA | | | | | |
| | 3 d | 8 d | 12 d | 3 d | 8 d | 12 d | 3 d | 8 d | 12 |
| F | 89.19 | 186.76 | 173.36 | 85.45 | 9.45 | 3.03 | 12.07 | 9.51 | 18.80 |
| P valor | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.020 | 0.000 | 0.021 | 0.003 |
| | | | | **Comparison of media** | | | | | |
| mg/L | 3 d | 8 d | 12 d | 3 d | 8 d | 12 d | 3 d | 8 d | 12 d |
| 0 | 2.70 a | 4.12 a | 5.27 a | 2.50 a | 3.62 a | 4.62 a | 2.27 a | 4.06 a | 4.8 a |
| 0.001 | 2.52 a | 1.97 a | 5.02 a | 2.27 a | 3.12 ab | 3.61 ab | 2.56 a | 4.03 a | 4.68 a |
| 0.01 | 1.00 b | 1.97 a | 2.32 b | 1.00 b | 3.06 abc | 3.31 ab | 2.18 ab | 2.72 b | 4.55 a |
| 0.1 | 1.00 b | 1.72 bc | 1.77 b | 1.00 b | 3.00 abc | 3.20 ab | 2.00 bc | 2.58 b | 4.40 a |
| 0.5 | 1.00 b | 1.52 bc | 1.75 b | 1.00 b | 2.02 bcd | 2.46 ab | 1.97 bc | 2.45 b | 4.27 a |
| 5 | 1.00 b | 1.00 c | 1.00 c | 1.00 b | 1.97 cd | 2.41 b | 1.95 bc | 2.38 b | 4.20 a |
| 10 | 1.00 b | 1.00 c | 1.00 c | 1.00 b | 1.85 cd | 2.32 b | 1.81 bc | 2.32 b | 3.37 b |
| 100 | 1.00 b | 1.00 c | 1.00 c | 1.00 b | 1.72 cd | 2.30 b | 1.75 bc | 2.25 b | 2.91 b |
| ET | 0.079 | 0.090 | 0.135 | 0.068 | 0.237 | 0.384 | 0.090 | 0.075 | 0.155 |
| CV (%) | 9.56 | 10.93 | 13.11 | 10.28 | 18.87 | 15.35 | 8.65 | 5.20 | 7.48 |

*Note.* Means with unequal letters in the columns differ for $p<0.05$, according to Tukey's test.

Among the different concentrations of azoxystrobin, evaluated at four, eight, and twelve days post-inoculation, the control treatment (T1: 0 mg/L) consistently showed the largest mycelial diameters, followed by T2 (0.001 mg/L), with no statistically significant differences between them. Beginning at 0.01 mg/L (T3), a progressive reduction in fungal growth was observed, with statistically significant differences compared to the control. This reduction became more pronounced from T5 (0.5 mg/L) onward. After 12 days, treatments T7 (10 mg/L) and T8 (100 mg/L) recorded the smallest diameters (2.01 and 1.80 cm, respectively), significantly different from treatments with lower concentrations.

For carbendazim, across all three evaluation times; four, eight, and twelve days post-inoculation, the control (T1: 0 mg/L) and the lowest concentration (T2: 0.001 mg/L) consistently showed the largest mycelial diameters, with no statistical differences between them, indicating low or no efficacy at these concentrations. From 0.01 mg/L (T3) onward, a progressive reduction in fungal growth was observed, with statistically significant differences compared to the control, especially from day four. This decrease became more marked as the fungicide dose increased. After 12 days, treatments T6 (5 mg/L), T7 (10 mg/L), and T8 (100 mg/L) recorded the smallest diameters (4.20, 3.73, and 2.91 cm, respectively), significantly different from the control and from treatments with lower concentrations.

***Estimation of $EC_{50}$ of Strain Ivan02 of the Fungus Colletotrichum sp. to Fungicides***

Once inhibition values were transformed into logits and concentrations into logarithmic units, regression analysis yielded the linear equation Y = 0.4418X + 5.3495 for difenoconazole, with a significant fit to the data. Using this method, the $EC_{50}$ of *Colletotrichum* sp., strain Ivan02, was estimated at 0.1 mg/L. For azoxystrobin, the regression equation was Y = 0.203X + 4.6582, also showing a significant fit, resulting in an $EC_{50}$ of 2.66 mg/L. In the case of carbendazim, the regression analysis produced the equation Y = 0.3233X + 3.958, with a significant fit, from which an $EC_{50}$ of 1665.26 mg/L was estimated (Table 6).

**Table 6.** *Determination of $EC_{50}$ values for the strain Ivan02 of the fungus Colletotrichum sp. according to the fungicide dose/percentage inhibition of fungal growth curve*

| Fungicide | Equation | $X^{2*}$ | R | $R^2$ | $EC_{50}$ (mg/L) | | |
|---|---|---|---|---|---|---|---|
| | | | | | Cal. | L inf. | L. sup. |
| **Difenoconazole** | Y=0.4418X + 5.3495 | 1.368* | 0.852 | | 0.1 | 0 | 0.3 |
| **Azoxystrobin** | Y= 0.203 X + 4.6582 | 9.93 | 0.75 | 0.63 | 2.66 | 1.53 | 2.70 |
| **Carbendazim** | Y= 0.3233 X + 3.958 | 0.158 | 0.96 | 0.92 | 1665.26 | 1596.5 | 17341.0 |

*Note.* No statistical difference by the X2 test.

***Spore-forming capacity of strain Ivan02 against fungicides***

For all three fungicides, ANOVA revealed statistically significant differences in conidia production across concentrations. In the case of difenoconazole, mean comparisons showed a significant reduction in conidia /mL relative to the control starting at 0.001 mg/L, with complete inhibition of sporulation observed from 0.1 to 100 mg/L. Azoxystrobin did not fully inhibit conidia production; although a decrease was noted from 0.1 mg/L onward, statistical differences between treatments were not pronounced. The control (T1) maintained high spore production ($40 \times 10^4$ conidia / mL), comparable to several treatments, with a statistically significant difference only at 100 mg/L. Regarding conidia production of *Colletotrichum* sp., strain Ivan02, under carbendazim treatments, a significant effect was observed among concentrations. The control (T1), without fungicide, produced the highest number of conidia ($36 \times 10^4$ conidia /mL), but from 0.001 mg/L onward, a reduction in sporulation was evident, and from 0.01 mg/L onward, conidia production was completely inhibited. Treatments T2 to T8 did not differ statistically among themselves, but all showed significant differences compared to the control (Table 7).

**Table 7.** *Determination of $EC_{50}$ values for the strain Ivan02 of the fungus Colletotrichum sp. according to the fungicide dose/percentage inhibition of fungal growth curve*

| | Difenoconazole | | Azoxystrobin | | Carbendazim | |
|---|---|---|---|---|---|---|
| (mg/L) | Conidia / 10 mL | Data transformed | Conidia / 10 mL | Data transformed | Conidia / 10 mL | Data transformed |
| | Conidia X $10^4$ | √Conidia + 1 | Conidia X $10^4$ | √ Conidia + 1 | Conidia X $10^4$ | √ Conidia + 1 |
| 0 | 37 | 3.18 a | 40 | 3.30 ab | 36 | 3.15 a |
| 0.001 | 25 | 2.69 b | 45 | 3.447 a | 2 | 1.20 ab |
| 0.01 | 4 | 1.38 c | 20 | 2.43 abc | 1 | 1.102 b |
| 0.1 | 0 | 1.00 c | 23 | 2.60 abc | 0 | 1.00 b |
| 0.5 | 0 | 1.00 c | 22 | 2.51 abc | 0 | 1.00 b |
| 5 | 0 | 1.00 c | 24 | 2.57 abc | 0 | 1.00 b |
| 10 | 0 | 1.00 c | 17 | 2.24 bc | 0 | 1.00 b |
| 100 | 0 | 1.00 c | 8 | 1.71 c | 0 | 1.00 b |
| ET | | 0.098 | | 0.238 | | 0.075 |
| CV (%) | | 12.90 | | 18.36 | | 11.66 |

*Note.* Means with unequal letters in the columns differ for $p<0.05$, according to Tukey's test.

## DISCUSSION

The results revealed that difenoconazole was the most effective fungicide against both *Colletotrichum* strains, with $EC_{50}$ values ranging between 0.10 and 0.17 mg/L, indicating high sensitivity and efficacy even at very low doses. In contrast, azoxystrobin recorded $EC_{50}$ values of 8.88 mg/L and 2.66 mg/L for strains Ivan01 and Ivan02, respectively, remaining within the sensitivity range, although close to the upper limit for strain Ivan01, suggesting a potential risk of reduced efficacy if not properly managed.

Conversely, carbendazim exhibited $EC_{50}$ values of 174.35 mg/L and 1665.26 mg/L for strains Ivan01 and Ivan02, respectively. These results indicate that both strains are highly resistant to carbendazim, demonstrating a significant loss of sensitivity to this active ingredient. Overall, the findings confirm that carbendazim has limited efficacy in inhibiting the radial growth of *Colletotrichum* spp. strains. This low efficacy is consistent with the results reported by Chung *et al*. (2006).

Despite the above, the data obtained on the sporulation capacity of strain Ivan01 indicate that carbendazim only achieves significant inhibition of conidia production at high doses (100 mg/L). In contrast, for strain Ivan02, sporulation was drastically reduced compared to the control at just 0.001 mg/L, and conidia production was virtually eliminated at 0.01 mg/L.

These findings suggest that the effect of carbendazim on conidia production could differ between the two strains. For strain Ivan01, sensitivity appears to be lost with respect to both mycelial growth inhibition and conidia production. In contrast, strain Ivan02 continues to exhibit some degree of sensitivity to carbendazim with respect to conidia production.

Based on the $EC_{50}$ values obtained for the fungicides, and following the classification criteria proposed by Chung *et al.* (2006) and Torres-Calzada *et al.* (2015), the sensitivity of C*olletotrichum* sp. strains Ivan01 and Ivan02 can be categorized as follows: sensitive to difenoconazole ($EC_{50}$ < 10 mg/L), intermediately resistant to azoxystrobin ($EC_{50}$ = 10–100 mg/L), and highly resistant to carbendazim ($EC_{50}$ > 100 mg/L).

It is important to highlight that these three fungicides evaluated are systemic, single-site inhibitors. This mode of action is associated with a high risk of resistance development, particularly when fungicides are not applied in accordance with principles of mode-of-action rotation and integrated disease management.

These results are particularly important, even with the limitation that strains of the pathogen were studied, since those three fungicide molecules are frequently used in Pamplona and are currently registered and in use in Colombia for strawberry cultivation, according to the National Registry of Chemical Pesticides for Agricultural Use of the Colombian Agricultural Institute (ICA, 2024).

In the case of carbendazim, despite its compromised efficacy, it remains available in several formulations, the commercialization of which may be contributing to the maintenance or even intensification of selection pressure on resistant populations. The use of other benomyl- and thiabendazole-based fungicides is also questionable, as recent studies have confirmed cross-resistance among benzimidazole fungicides in *Colletotrichum* spp. isolates. For instance, Kongtragoul *et al.* (2022) reported that 25 out of 34 *Colletotrichum* isolates exhibited resistance to both benomyl and carbendazim, with $EC_{50}$ values exceeding 100 $mg \cdot L^{-1}$ for each compound. Conversely, benomyl was reported as the most effective fungicide for controlling *Colletotrichum gloeosporioides* causing anthracnose in the Red Globe grape variety in Caldas, Colombia (López-Zapata & Castaño-Zapata, 2020).

Several products containing azoxystrobin as the active ingredient are registered for strawberry cultivation (ICA, 2024), which still represents a viable option, although its use should be carefully monitored. Zhang *et al*. (2020) confirmed the sensitivity of strains from the *C. gloeosporioides* complex isolated from strawberries to azoxystrobin; however, they concluded that this fungicide was no longer suitable for managing anthracnose in this crop.

Meanwhile, multiple difenoconazole formulations are registered (ICA, 2024), and this molecule stands out as the most effective for controlling *Colletotrichum* sp. in strawberry, according to the present results, which align with findings reported in eastern China by Zhang *et al.* (2020).

These results, in the case of carbendazim, correspond to those of Alonso *et al.* (2003), who evaluated resistance to benomyl and thiabendazole (benzimidazoles) in *Colletotrichum gloesporioides* isolates obtained from mango fruits. Most isolates presented an $EC_{50}$ > 20 mg/L for both fungicides, and were considered resistant, although others proved to be sensitive.

Similarly, Gaviria-Hernández *et al.* (2013) evaluated the in vitro efficacy of chemically synthesized fungicides, including difenoconazole and azoxystrobin, against *Colletotrichum gloeosporioides* and *C. acutatum,* on blackberry in Colombia. The best results in mycelial growth inhibition were observed with difenoconazole, with 100% inhibition.

For other fungicides also registered in Colombia, such as fludioxonil, resistance in *Colletotrichum* species affecting strawberry crops has been reported in Japan (Ishii *et al*., 2022; Furuta *et al.,* 2024) and in China (Ren *et al*., 2025). These findings underscore the need to continue studies on the sensitivity behavior of *Colletotrichum* strains to the studied fungicides, and to establish baseline sensitivity data for new molecules intended

for use by farmers in Pamplona. This is essential to ensure their long-term efficacy and to guide sustainable disease management strategies.

Other strategies that need further development to reduce the risks of fungicide resistance include the use of antagonistic microorganisms. For instance, *Bacillus atrophaeus* significantly reduced disease severity under both in vitro and greenhouse conditions (Alijani *et al.,* 2022), while *Trichoderma harzianum* and *Bacillus* spp. showed effectiveness under field conditions in Pamplona (Rodríguez *et al.,* 2024). Additionally, plant extracts from neem seeds formulated for managing strawberry anthracnose have been suggested as a viable alternative (Motallebi & Negahban, 2024).

## CONCLUSIONS

Difenoconazole demonstrated the highest efficacy against *Colletotrichum* spp. strains Ivan01 and Ivan02, confirming its potential as a key fungicide for strawberry anthracnose management in Pamplona, Colombia. In contrast, carbendazim showed markedly reduced effectiveness, with evidence of high resistance and variable impact on sporulation, underscoring the need to reassess its continued use. Azoxystrobin remains a viable option but requires careful monitoring due to intermediate resistance levels.

The results emphasize the urgency of implementing integrated disease management strategies, including fungicide rotation and the exploration of biological alternatives such as *Bacillus* spp., *Trichoderma harzianum*, and vegetable-based extracts. Establishing baseline sensitivity data for emerging fungicides and promoting sustainable practices will be essential to mitigate resistance development and ensure long-term control of *Colletotrichum* spp. in strawberry cultivation.

## AUTHOR CONTRIBUTIONS

Conceptualization, L.C.G. and C.O.Z.G.; Methodology, L.C.G. and A.G.S.; Validation, L.C.G., A.G.S. and C.O.Z.G.; Formal Analysis, L.C.G., A.G.S. and C.O.Z.G; Investigation, L.C.G., A.G.S. and C.O.Z.G.; Resources, L.C.G. and C.O.Z.G..; Data Curation, L.C.G. and A.G.S.; Writing—Original Draft Preparation, A.G.S.; Writing—Review & Editing, L.C.G., A.G.S. and C.O.Z.G.; Visualization, L.C.G., A.G.S. and C.O.Z.G..; Supervision, L.C.G. and C.O.Z.G.; Project Administration L.C.G.; Funding Acquisition, L.C.G.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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