Effect of difenoconazole on populations of *Trichoderma* spp.: a comparative study

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ABSTRACT. Fungi of the genus *Trichoderma* spp. are widely distributed and recognized for their role as biological control agents, as well as for contributing to nutrient uptake, plant growth promotion, organic matter decomposition, tolerance to water and salt stress, and induction of systemic resistance (ISR) in plants. The objective of this study was to determine the lethal dose and sensitivity of *Trichoderma* spp. isolates from different hosts to the fungicide difenoconazole. Sixteen isolates collected from soybean-producing fields in the states of Goiás, Paraná, and the Federal District, Brazil, were evaluated. These isolates are preserved in the Plant Phytopathology Laboratory of the IF Goiano – Urutaí Campus. Monosporic cultures were obtained on potato-dextrose-agar (PDA). The isolates were exposed to difenoconazole concentrations of 0, 0.1, 1, 10, and 100 µL mL^{-1} in a completely randomized design with three replications, totaling 240 experimental units. Colony diameter (mm) was measured from the 1st to the 7th day after inoculation, and the area under the mycelial growth progress curve (AUMGPC), mycelial growth rate (MGR), effective concentration to inhibit 50 % of mycelial growth (EC$_{50}$), and percentage of growth inhibition were calculated. EC$_{50}$ values ranged from 0.26 µL mL^{-1} (IF 299) to 4.40 µL mL^{-1} (IF 310), indicating high sensitivity of most isolates to difenoconazole when compared with populations that show EC$_{50}$ values between 80 and 200 µL mL^{-1}. Only 6.25 % of the isolates showed insensitivity to the fungicide. The isolate IF 316 presented the lowest AUMGPC, indicating greater sensitivity to difenoconazole concentrations.

Key words: EC$_{50}$, sensitivity, bioinput, isolates.

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INTRODUCTION

Brazil stands out as one of the world leaders in the adoption of biological control in agricultural systems, and is even an exporter of technologies related to this type of management. Despite this progress, chemical control through the application of fungicides is still widely used in plant disease management, especially in crops of great economic importance. In this context, the integration of chemical and biological control methods has been considered a promising strategy to make production systems more sustainable (Kubiak et al., 2023).

Species of the genus *Trichoderma* are among the most widely used microorganisms as biocontrol agents in agriculture. These fungi exhibit several mechanisms of action against plant phytopathogens, including competition for space and nutrients, mycoparasitism, production of antifungal metabolites, and induction of systemic resistance in plants. In addition, *Trichoderma* spp. can contribute to plant growth promotion, improvement of nutrient uptake, and modulation of rhizosphere microbiota, enhancing their potential use as bioinputs in sustainable agricultural systems (Kredics et al., 2024; Guzman-Guzman et al., 2023; Martinez et al., 2023).

Alongside the use of biological agents, systemic fungicides remain essential tools for managing several fungal diseases. Among them, difenoconazole stands out as a fungicide belonging to the triazole group,

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widely used in different crops. This compound acts by inhibiting the enzyme lanosterol 14α-demethylase, which is essential for ergosterol biosynthesis, a key structural component of fungal cell membranes (Kong et al., 2023). However, the frequent use of this type of molecule may exert selective pressure on fungal populations, resulting in reduced sensitivity or the development of resistance, as well as possible effects on non-target microorganisms present in the soil and rhizosphere (Mahajan, Deokar & Munde, 2024).

In this context, the compatibility between fungicides and biological control agents becomes a fundamental aspect for the success of integrated disease management. Studies have shown that different isolates of *Trichoderma* spp. may exhibit distinct responses when exposed to fungicides, which can directly influence the feasibility of the simultaneous or sequential use of these management strategies (Manandhar et al., 2020; Budha et al., 2022; Rajesh et al., 2023). Despite the increasing use of bioinputs in agriculture, there are still gaps in knowledge regarding the sensitivity of different *Trichoderma* spp. populations to widely used fungicides such as difenoconazole.

Furthermore, environmental factors and the geographical origin of isolates may significantly influence their tolerance to chemical compounds, as natural microbial populations may be subjected to different selective pressures across agricultural systems. Thus, evaluating isolates from production regions can provide important information about their physiological variability and potential adaptation to environments where fungicides are frequently applied (Massi et al., 2021).

The isolates used in this study were obtained from agricultural areas located in the states of Goiás, Paraná, and the Federal District, regions of great relevance for soybean production in Brazil and characterized by intensive agricultural systems in which the use of fungicides is a common practice in disease management. Evaluating the sensitivity of *Trichoderma* spp. isolates from these regions may contribute to the development of more efficient strategies for integrating biological and chemical control.

MATERIALS AND METHODS

The experiments were conducted at the Plant Pathology Laboratory of the Federal Institute Goiano – Urutaí Campus. Sixteen *Trichoderma* spp. isolates were used, obtained from soybean plants, volunteer soybean, abiu, and *Gomphrena* sp., collected from different locations in the states of Goiás, Paraná, and the Federal District (Table 1). The isolates were obtained from internal fragments of plant collar tissue, which were subjected to surface disinfection in 50 % alcohol solution for 1 min, followed by sodium hypochlorite (NaClO) for 1 min. Subsequently, the fragments were dried on autoclaved paper and transferred to Petri dishes containing water agar (WA). The plates were incubated at 25 °C for five days. After mycelial growth, the isolates were subcultured onto potato dextrose agar (PDA) to obtain pure cultures.

Table 1. List of *Trichoderma* spp. isolate codes, host of origin, and collection sites in the states of Goiás, Paraná, and the Federal District (Brazil).

Ord.	Isolate code	Host of origin	Identification of the isolate	Site and State of the collection
1	IF 295	volunteer soybean st 2.4	*Trichoderma* sp.	Luziânia, GO
2	IF 298	soybean	*Trichoderma* sp.	Urutaí, GO
3	IF 299	volunteer soybean 3.1.	*Trichoderma* sp.	Luziânia, GO
4	IF 300	volunteer soybean 3.2.	*Trichoderma* sp.	Luziânia, GO
5	IF 303	unknown	*Trichoderma asperellum*	Edéia, GO
6	IF 304	abiu	*Trichoderma* sp.	Paranaguá, PR
7	IF 305	volunteer soybean C6A1	*Trichoderma* sp.	Silvânia, GO
8	IF 306	*Gomphrena* sp.	*Trichoderma* sp.	Brasília, DF
9	IF 307	volunteer soybean c6a3	*Trichoderma* sp.	Silvânia, GO
10	IF 309	volunteer soybean c5a3	*Trichoderma* sp.	Catalão, GO
11	IF 310	soybean st 3.5	*Trichoderma* sp.	Luziânia, GO
12	IF 316	volunteer soybean f03	*Trichoderma* sp.	Orizona, GO
13	IF 454	soybean	*Trichoderma* sp.	Urutaí, GO
14	IF 455	soil	*Trichoderma* sp.	Urutaí, GO
15	IF 473	soybean am 9.4	*Trichoderma* sp.	Vianópolis, GO
16	IF 526	volunteer soybean c6a2	*Trichoderma* sp.	Silvânia, GO

The cultures were maintained as stock cultures of the same age and used in sensitivity assays to the commercial fungicide Score® (Syngenta®), containing the active ingredient difenoconazole (250 g L^{-1}). The fungicide was incorporated into PDA medium to obtain concentrations of 0.1, 1, 10, and 100 µL mL^{-1}, in addition to the control (0 µL mL^{-1}). Stock solutions were prepared in 250 mL Erlenmeyer flasks and stored under freezing conditions until use. For treatment preparation, 3 mL of the stock solution were added to

500 mL of molten PDA medium (50 °C), and approximately 20 mL were then poured into each Petri dish.

The experiment was conducted in a completely randomized design, with three replicates per treatment. Considering 16 isolates and five fungicide concentrations, a total of 240 experimental units (Petri dishes) were used.

Mycelial discs of 9 mm in diameter, obtained from monosporic cultures, were transferred to the center of the plates containing the different treatments. The plates were sealed with PVC plastic film and incubated in a growth chamber at 27 °C. Colony diameter was measured with a digital caliper every 24 hours for seven days, obtaining measurements at 1, 2, 3, 4, 5, 6, and 7 days after inoculation (DAI).

Based on the mycelial growth data, the following variables were calculated: area under the mycelial growth progress curve (AUMGPC), calculated based on the integration of colony diameter values over time, according to the methodology described by Shaner and Finney (1977), and mycelial growth rate (MGR), estimated by linear regression between colony diameter (mm) and evaluation time (days), with the slope of the equation corresponding to the growth rate (mm day^{-1}).

The effective concentration capable of inhibiting 50 % of mycelial growth (EC_{50}) was determined by linear regression between the logarithm of fungicide concentrations and the percentage of mycelial growth inhibition. The model equation was expressed as $y = a + bx$, with the EC_{50} value obtained by substituting $y = 50$ into the fitted equation (Edgington et al., 1971). The isolates were classified according to their sensitivity to the fungicide based on EC_{50} values, being considered sensitive when presenting values between 0–100 ppm, intermediate between 100–800 ppm, and insensitive when above 800 ppm.

Data on AUMGPC, MGR, and percentage of mycelial growth inhibition were subjected to analysis of variance (ANOVA). When significant, the Scott-Knott mean comparison test at 5% probability was applied. For variables that did not meet normality assumptions, a non-parametric test was used followed by mean comparison using the Tukey test ($p \leq 0.05$). All analyses were performed using the R statistical software.

RESULTS AND DISCUSSION

Information regarding the *Trichoderma* spp. isolates, including their hosts of origin and collection sites (Table 1), highlights the diversity of environments in which these fungi can be found. This ecological variability is relevant for the selection of strains with desirable characteristics, such as biocontrol potential, enzyme production, or other biotechnological applications. The exploration of this diversity may contribute to the development of more sustainable technologies for agriculture and for the production of bioproducts, since *Trichoderma* species exhibit broad ecological adaptability and multiple mechanisms of action against plant pathogens (Harman et al., 2004; Mukherjee et al., 2012).

The results of the experiment demonstrated variability in the sensitivity of different *Trichoderma* spp. isolates to the fungicide difenoconazole (Table 2). The EC_{50} values, which represent the concentration required to inhibit 50 % of mycelial growth, indicate differences among the isolates regarding their level of tolerance to the fungicide. This heterogeneity suggests that the evaluated population presents different levels of response to the compound, with some isolates showing higher sensitivity and others greater relative tolerance. Variability in sensitivity to fungicides within fungal populations is frequently reported in the literature and may be associated with genetic or physiological differences among isolates (Brent & Hollomon, 2007).

Table 2. Difenoconazole doses required to inhibit 50 % of mycelial growth (EC_{50}) and standard errors of *Trichoderma* spp. Isolates

Order	Isolates	EC_{50}	Standard error
1	IF 295	1.3387	1.1727
2	IF 298	1.4867	1.1089
3	IF 299	0.2622	1.1872
4	IF 300	1.9427	1.5115
5	IF 303	0.3764	0.0771
6	IF 304	1.3015	0.6586
7	IF 305	0.9398	1.7404
8	IF 306	2.3105	0.6124
9	IF 307	2.1193	1.0270
10	IF 309	1.8563	1.1331
11	IF 310	4.4033	7.6336
12	IF 316	1.0917	1.4739
13	IF 454	1.7613	0.4062
14	IF 455	1.7704	0.3315
15	IF 473	1.7096	0.0720
16	IF 526	1.1244	0.2402

Studies conducted with different plant phytopathogen fungi also demonstrate a wide variation in EC_{50} values for difenoconazole. In populations of *Lasiodiplodia theobromae*, for example, EC_{50} values varied widely among isolates, indicating a continuous distribution of sensitivity within the population (Wang et al., 2021). This pattern reinforces that natural differences in tolerance to fungicides may occur even among isolates belonging to the same species or genus.

The growth of the *Trichoderma* spp. isolate IF 299 under different concentrations of difenoconazole (Figure 1). A progressive reduction in mycelial growth is observed as the fungicide concentration increases. In the absence of the fungicide, the isolate exhibited vigorous growth and colonization of the culture medium, whereas higher concentrations resulted in a strong reduction in mycelial growth. This behavior demonstrates the sensitivity of the isolate to difenoconazole and confirms the inhibitory effect of the fungicide on mycelial development.

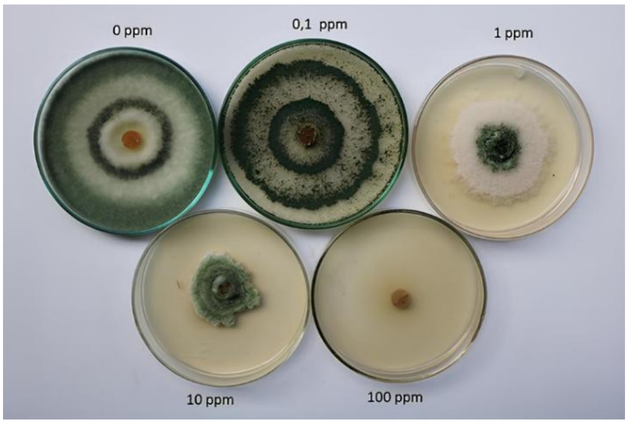

Figure 1. Growth of the *Trichoderma* spp. isolate IF 299 at different doses (ppm = μL mL⁻¹) of difenoconazole on PDA medium.

Similar results have been reported for other fungi. In studies with *Penicillium expansum*, it was observed that increasing difenoconazole concentrations resulted in greater inhibition of mycelial growth, with complete inhibition occurring at higher fungicide concentrations (Khadiri et al., 2024). These results demonstrate that fungicides from the triazole group exhibit high efficiency in suppressing the growth of various fungi.

The classification of *Trichoderma* spp. isolates into different sensitivity classes (Table 3) indicates a predominance of moderately sensitive isolates to difenoconazole. The absence of moderately insensitive or completely insensitive isolates suggests that the evaluated population generally exhibits high susceptibility to fungicide. This information is relevant for integrated disease management strategies, as compatibility between fungicides and biological control agents must be carefully considered (Brent & Hollomon, 2007). Recent studies have shown that fungicides containing difenoconazole, either alone or in combination with other active ingredients, can significantly reduce the mycelial growth of *Trichoderma* isolates, although variation exists among different strains (Limdolthamand et al., 2023). Thus, the selection of more tolerant isolates becomes an important factor to ensure the effectiveness of biological control in agricultural systems where fungicides are used.

The analysis of mycelial growth parameters, including AUMGPC and MGR (Table 4), also revealed differences among the evaluated isolates. Some isolates showed greater growth over the experimental period, suggesting higher tolerance to the fungicide, while others exhibited reduced growth, indicating greater sensitivity to the tested concentrations.

The observed variability reinforces the importance of proper selection of *Trichoderma* isolates for use in biological control programs, especially in agricultural systems where fungicides from the triazole group are widely used. More tolerant isolates may exhibit greater compatibility with chemical management, whereas more sensitive isolates may have their performance compromised when exposed to the fungicide (Harman et al., 2004; Mukherjee et al., 2012).

CONCLUSION

The results obtained in this study show a wide variation in EC_{50} among *Trichoderma* spp. isolates, ranging from 0.26 ± 1.18 μL mL⁻¹ (IF 299) to 4.40 ± 7.63 μL mL⁻¹ (IF 310) (EC_{50} ± standard error), highlighting the diversity of responses of *Trichoderma* spp. isolates to difenoconazole. This heterogeneity among isolates may be attributed to different mechanisms of resistance or tolerance to the fungicide. The evaluation of isolate sensitivity revealed a heterogeneous distribution, with 12.5 % showing high sensitivity to the fungicide. However, most isolates (50 %) exhibited moderate sensitivity, while 31.25 % were classified as intermediate and 6.25 % as insensitive. It is important to highlight the absence of isolates classified as moderately insensitive, indicating a general trend of sensitivity among the tested isolates.

Table 3. Number of *Trichoderma* spp. isolates, names of sensitivity classes, and percentage of isolates in each EC_{50} class range of difenoconazole required to inhibit 50 % of mycelial growth.

EC_{50} class ranges (μL mL⁻¹)	Number of isolates	Sensitivity class names	% of isolates
0 - 0.88	2	Sensitive	12.5
0.81 - 1.76	8	Moderately sensitive	50
1.77 - 2.64	5	Intermediate	31.25
2.65 - 3.52	0	Moderately insensitive	0
3.53 - 4.40	1	Insensitive	6.25

Table 4. Mean colony diameter (mm) at difenoconazole doses at different days after inoculation (DAI), area under the mycelial growth progress curve (AUMGPC), and mycelial growth rate (MGR, mm day^{-1}) of different *Trichoderma* spp. isolates subjected to difenoconazole doses.

Isolates	1	2	3	4	5	6	7	AUMGPC	MGR
IF 295	16.00 cde	34.10 cde	52.40 bcde	68.26 bcde	81.72 abcd	93.18 abcd	106.70 abcd	375.69 abcd	14.97 abcd
IF 298	17.60 abcd	36.20 bcd	49.40 bcdef	61.83 def	68.85 def	80.28 cde	86.80 de	338.14 cde	11.26 de
IF 299	11.40 fg	26.70 f	43.00 f	54.23 ef	60.84 ef	71.27 e	86.55 de	301.40 fg	11.88 de
IF 300	19.70 a	43.10 a	56.60 ab	71.98 ab	85.49 abc	98.60 ab	111.51 abcd	402.09 ab	14.82 abcd
IF 303	14.80 defg	32.90 def	44.80 ef	53.71 f	62.89 f	71.36 de	80.69 e	309.15 ef	10.46 e
IF 304	18.00 abc	37.30 bcd	51.80 bcdef	66.48 bcde	79.12 cdef	89.80 abcd	103.60 bcde	371.15 abcd	13.90 bcde
IF 305	14.30 efg	31.00 ef	44.10 ef	57.94 def	66.48 bcdef	80.08 de	95.62 cde	328.55 def	13.02 de
IF 306	18.00 abc	39.50 ab	55.60 ab	70.87 abc	86.83 abc	103.39 a	117.95 ab	405.92 ab	16.38 ab
IF 307	18.80 ab	42.50 a	59.40 a	75.30 a	87.93 a	100.48 ab	119.57 ab	412.46 a	15.96 abc
IF 309	17.20 bcde	38.30 b	53.60 abc	70.47 abcd	84.74 abc	97.51 ab	112.86 abc	392.63 abc	15.58 abc
IF 310	11.30 gh	28.70 ef	45.50 def	59.01 def	72.03 abcd	88.36 bcde	137.11 a	360.56 cde	18.69 a
IF 316	0.00 h	2.00 g	4.90 g	6.70 g	7.20 g	9.30 f	10.50 f	74.55 g	1.73 f
IF 454	16.20 bcde	37.40 bc	52.85 bcde	71.15 ab	83.65 ab	94.66 abc	115.09 ab	388.36 abc	15.79 abc
IF 455	17.80 abc	35.70 bcd	53.00 abcd	65.63 bcde	77.59 abcde	90.15 abcd	106.20 abcd	369.77 bcd	14.24 abcde
IF 473	17.40 bcd	36.80 bcd	52.30 bcde	62.82 cdef	73.57 def	87.07 cde	102.00 cde	359.71 cde	13.42 cde
IF 526	16.00 cdef	35.50 bcd	49.00 cdef	63.53 bcde	77.20 abcd	90.46 abcd	104.60 abcd	364.70 cde	14.41 abcd
Shapiro–Wilk test (normality)	0.95888***	0.9271***	0.9856***	0.9908***	0.9920***	0.9921***	0.7758***	0.9872***	0.91622***
Bartlett's test (homogeneity of variances)	4.0000ns	4.0000ns	4.0000ns	4.0000ns	4.0000ns	4.0000ns	4.0000ns	4.0000ns	4.0000ns
Coefficient of variation (%)	26.0857	21.1458	19.6256	19.1810	21.0429	22.8073	32.4110	17.4352	31.7252
F test	14.0140***	17.9260***	17.3570***	18.3120***	16.1630***	13.2470***	7.3161***	17.3440***	7.6643***
Friedman test	70.3386***	81.4247***	60.4021***	57.3480***	56.1835***	55.8673***	55.8673***	65.9827***	52.2159***

CONFLICT OF INTEREST

The authors declare that this work presents no conflict of interest.

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