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Article

Selenium enrichment alters *Trichoderma* spp. antagonistic activity against phytopathogenic fungi

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A B S T R A C T

Trichoderma harzianum DEMf 1V and *Trichoderma citrinoviride* DEMf: TR3 are confirmed biocontrol agents used in this study to estimate the effects of selenium enrichment on their antifungal activity. Se-enriched cultures were prepared by growing on potato-dextrose broth supplemented with 2.5 and 5 mg Se/L added in the form of Na_2SeO_3. The antifungal activity of Se-enriched and control cultures was estimated by their ability to produce hydrolytic enzymes, which were detected using API ZYM, cellulase and protease tests. The antagonistic potential and impact of volatile compounds were observed in interactions with *Botrytis cinerea*, *Fusarium oxysporum*, and *Fusarium graminearum*. In Se-enriched *T. harzianum* DEMf 1V, the production of esterase, lipase, and N-acetyl-β-glucosaminidase was inhibited, while in Se-enriched *T. citrinoviride* DEMf: TR3, only lipase production was interrupted. All cultures maintained the ability to produce cellulase and protease. Cultures of *T. harzianum* DEMf 1V grown with and without Se enrichment exhibited a high inhibition rate against *B. cinerea* (68–74%) and *F. oxysporum* (62–70%). The antagonistic activity of Se-enriched *T. harzianum* DEMf 1V against *F. graminearum* (65–66%) was higher compared to the control (60%). Se-enriched *T. citrinoviride* DEMf: TR3 expressed lower inhibition against *B. cinerea* and *F. graminearum* compared to the control. Volatile compounds caused a low level of inhibition, indicating that they are among the antifungal mechanisms employed by the isolates, but not the dominant one. Further research should be directed towards the precise determination of metabolic changes induced by Se enrichment and the implications thereof for *Trichoderma*'s antagonistic activities.

Keywords: *Trichoderma harzianum*, *Trichoderma citrinoviride*, selenium, biocontrol, *Botrytis cinerea*, *Fusarium* sp.

И З В О Д

Trichoderma harzianum DEMf 1V и *Trichoderma citrinoviride* DEMf: TR3 су биоконтролни агенси коришћени у овој студији како би се проценили ефекти обогаћивања селеном на њихову антифунгалну активност. Културе обогаћене селеном припремљене су узгајањем у кромпир-декстрозном бујону са 2.5 и 5 mg Se/L додатим у облику Na_2SeO_3. Антифунгална активност култура обогаћених селеном и контролних култура процењивана је кроз способност производње хидролитичких ензима, који су детектовани уз помоћ API ZYM-а, тестовима за утврђивање продукције целулаза и протеаза. Потенцијал за испољавање антагонизма и утицај испарљивих једињења утврђени су у интеракцијама са *Botrytis cinerea, Fusarium oxysporum* и *Fusarium graminearum*. Код Se-обогаћене*T. harzianum* DEMf 1V производња естеразе, липазе и N-ацетил-β-глукозаминидазе је била инхибирана, док је код Se-обогаћене *T. citrinoviride* DEMf: TR3 прекинута производња липазе. Све културе су задржале способност производње целулаза и протеаза. Културе *T. harzianum* DEMf 1V, обогаћене и необогаћене селеном, показале су висок ниво инхибиције *B. cinerea* (68–74%), и *F. oxysporum* (62–70%). Антагонистичка активност Se-обогаћене *T. harzianum* DEMf 1V против *F. graminearum* (65–66%) била је већа у поређењу са контролом (60%). Se-обогаћена *T. citrinoviride* DEMf: TR3 показала је нижу инхибицију *B. cinerea* и *F. graminearum* у поређењу са контролом. Испарљиви метаболити су изазвали низак ниво инхибиције, што указује да су међу антифунгалним механизмима изолата, али не и доминантни. Даља истраживања је потребно усмерити на прецизно утврђивање метаболичких промена изазваних обогаћењем селеном као и на импликације тих промена на антагонистичке активности гљива из рода *Trichoderma*.

Кључне речи: *Trichoderma harzianum*, *Trichoderma citrinoviride*, селен, биоконтрола, *Botrytis cinerea*, *Fusarium* sp.

1. Introduction

The representatives of the genus *Trichoderma* are common soil and plant microbiome inhabitants with a sophisticated package of ecological services expressed in communication with plants' holobiont. The members of *Trichoderma* spp. are confirmed biocontrol agents (BAC), accounting for around 60% of the available biofungicides on the global market (Kubiak et al., 2023). The mechanisms that support their biocontrol activity are versatile, from quick colonisation of the rhizosphere to mycoparasitism, production of highly effective antimicrobial metabolites (cell wall-degrading enzymes and volatile organic compounds), and raising alertness of the host plant. Cell wall-degrading enzymes

(chitinases, glucanases, lipases, N-acetyl-*β*-glucosaminidase, *β*-glucosidase, and protease), produced by *Trichoderma* spp., hydrolyse the components of fungal cell walls, hindering the activity of microbial cells and contributing to lethal effects (Zin and Badaluddi, 2020). They produce versatile volatile organic compounds (VOCs), which work as chemical signals between distant organisms, interfere with the metabolism of pathogens, cause plant growth stimulation, and activate defence systems against a/biotic stressors (Jiménez-Bremont et al., 2024; Li et al., 2024).

Also, representatives of *Trichoderma* spp. act as modulators of microbiome communities, enhance nutrient acquisition, promote plant growth, trigger immune responses, and mediate responses to a/biotic stress (Guzmán-Guzmán et al., 2025). Recent studies have confirmed that the presence of *Trichoderma* members stimulates the growth of other beneficial microorganisms, simultaneously decreasing the presence of detrimental microorganisms in plants' close vicinity (Zhang et al., 2024; Xie et al., 2023). This sort of action brings in a range of effects that impact nutrient availability and improve the physiological condition of the plant.

Selenium (Se) is a micronutrient beneficial for plant growth and development and crucial to humans, animals, and microorganisms owing to its antioxidant capacity and the ability to upgrade the immune system and raise resistance to pests and pathogens (Luo et al., 2025; Li et al., 2023). In interaction with plant pathogens, Se inhibits mycelial growth, causes morphological changes and deformities, and inhibits sclerotial and spore germination (Li et al., 2023). Yuan et al. (2022) reported a decrease in *Botrytis cinerea* mycelium biomass, colony diameter, and conidial germination due to Se presence, while Troni et al. (2021) reported a similar effect on *Fusarium* growth. Moreover, Se treatment strongly influences metabolic processes in microbial cells, causing biochemical changes in phytopathogens, such as reduced acid, dismutase, and catalase production and increased hydrogen peroxide concentration (Luo et al., 2025; Li et al., 2023).

Microorganisms that can grow in media containing Se and accumulate Se in their cells are known as Se-enriched microorganisms. Different representatives of fungi (*Trichoderma* spp., *Aspergillus* spp.), yeasts (*Saccharomyces* spp.), and bacteria (*Bacillus* spp., *Pseudomonas* spp., *Lactobacillus* spp., *Lactococcus* spp., and *Streptococcus* spp.) are capable of metabolising Se species (Luo et al., 2025). Those microorganisms are capable of promoting plant growth and work as biofertilisers, while some of them produce antimicrobial metabolites and can be formulated as biopesticides against plant pathogens (Luo et al., 2025). However, there is a lack of available studies on the possible effects of Se enrichment on the beneficial traits of microorganisms, such as antagonistic activity.

The presented study aimed to determine if Se present in the nutrient medium triggers differences in the antagonistic activity of the tested fungi. For this purpose, *T. harzianum* DEMf 1V, *T.citrinoviride* DEMf: TR3, and their Se-enriched cultures were used to estimate the effects of Se on biocontrol characteristics through:

i) the enzymatic activity; ii) *in vitro* interactions with phytopathogens; and iii) the effects of VOCs produced by enriched and non-enriched cultures on the growth of phytopathogens.

2. Materials and methods

2.1. Fungal material

The study was conducted with *Trichoderma harzianum* DEMf 1V and *Trichoderma citrinoviride* DEMf: TR3 from the collection of the Department of Environmental Microbiology (Faculty of Agriculture, University of Belgrade, Serbia). The fresh cultures were prepared by growing on potato-dextrose agar (PDA, Himedia, India) at 25°C for 48 h. After incubation, three mycelial discs (Ø 10 mm) were transferred into 100 mL of potato-dextrose broth (PDB, Himedia, India) supplemented with 2.5 and 5 mg/L of Se added in the form of Na_2SeO_3 (Alfa Aesar, Thermo Fisher Scientific). Cultures were grown at 25°C/100 rpm for 72 h (KS 260 basic, IKA, Staufen, Germany). The obtained Se-enriched cultures were marked as *T. harzianum* DEMf 1V Se 2.5, *T. harzianum* DEMf 1V Se 5, *T. citrinoviride* DEMf: TR3 Se 2.5, and *T. citrinoviride* DEMf: TR3 Se 5. The cultures grown in PDB without supplementation were used as controls.

2.2. Biochemical characteristics of Trichoderma harzianum DEMf 1V and Trichoderma citrinoviride DEMf: TR3

The production of hydrolytic enzymes (esterase, esterase-lipase, lipase, and N-acetyl-β-glucosaminidase) by *T. harzianum* DEMf 1V, *T. citrinoviride* DEMf: TR3 (controls), and their Se-enriched cultures was estimated by the API ZYM test (Biomerieux, France) according to the manufacturer's protocol. After incubation for 4.5 h at 37°C, results were read and scored using a colour chart scale: + (5–10 nM), ++ (20–30 nM), and +++ (≥ 40 nM).

Extracellular cellulase activity of the cultures was determined on carboxymethyl cellulose agar (CMC, Sigma Aldrich, USA). The CMC plates were inoculated with fungal cultures and incubated for 48 h at 25°C. Afterwards, a 0.1% Congo red solution in 1M NaCl was poured into Petri dishes and left for 10 min. The final step was rinsing with 1M NaCl, which revealed halo zones around the colonies as indicators of a positive reaction (Angsana et al., 2007). The test was performed in triplicate.

Protease activity was tested on sterile milk agar, prepared by mixing pasteurised skim milk and a 2% agar solution in a 1:1 ratio. The inoculated medium was incubated at 25°C for 48 h. Halo zones around the colonies indicated the production of proteases by the isolates (Chaiharn et al., 2008). The test was performed in triplicate.

2.3. Confrontation test

The interactions with phytopathogens *Botrytis cinerea*, *Fusarium oxysporum*, and *Fusarium graminearum* were assessed using a confrontation test on PDA by placing a 5-mm-diameter plug of *Trichoderma* spp. 3 cm apart from a 5-mm-diameter plug of phytopathogen. The tests were performed at the same time in three replicates. The mycelial growth

inhibition percentage was calculated according to the formula of Mohareb et al. (2017):

$$MGI\ (\%) = (DC - DT)/DC) \times 100$$

where MGI is mycelial growth inhibition, DC is the average diameter of a fungal colony of the control group, and DT is the average diameter of a fungal colony of the treatment group. The degree of growth inhibition was estimated as follows: < 50% low, 51–60% moderate, 61–75% high, and >75% very high inhibition effect (Ruiz-Gómez et al., 2021).

2.4. Effects of Trichoderma harzianum DEMf 1V and Trichoderma citrinoviride DEMf: TR3 VOCs on the mycelial growth of phytopathogens

The effects of *Trichoderma* spp. VOCs on *B. cinerea*, *F. oxysporum*, and *F. graminearum* were detected on two sealed Petri dishes containing 20 mL PDA and inoculated with 5-mm-diameter plugs of *Trichoderma* spp. on one plate and a phytopathogen plug on the other plate. The inoculated plates were placed opposite each other, sealed with Parafilm®, and incubated at 25°C until the control phytopathogen fungi reached the plate's edge. The experiment was performed in triplicate. Using the equation from section 2.3, the mycelial growth inhibition due to the impact of VOCs was calculated.

3. Results and discussion

The determination of the biochemical characteristics of Se-enriched and control *Trichoderma* spp. was conducted through several tests that detected the presence of hydrolytic enzymes involved in biocontrol (Table 1).

The results confirmed changes between the enzymatic profiles of cultures as a result of Se presence in the growth medium. In the case of Se-enriched *T. harzianum* DEMf 1V, esterase-lipase, lipase, and N-acetyl-*β*-glucosaminidase production was inhibited, while esterase production was inhibited due to the presence of a higher Se concentration (5 mg/L) in the medium. Se supplementation did not affect protease and cellulase production for both *T. harzianum* DEMf 1V and *T. citrinoviride* DEMf: TR3, as all tested cultures were positive (Table 1).

Se-enriched *T. citrinoviride* DEMf: TR3 showed the ability to decrease the production of esterase and esterase-lipase, while no production of lipase was noted. There were no effects of Se on N-acetyl-*β*-glucosaminidase activity (Table 1; Figure 1).

Table 1.
Biochemical characteristics of *T. harzianum* DEMf 1V, *T. citrinoviride* DEMf: TR3, and Se-enriched *T. harzianum* DEMf 1V and *T. citrinoviride* DEMf: TR3

Metabolites	*T. harzianum* DEMf 1V -control-	*T. harzianum* DEMf 1V Se 2.5	*T. harzianum* DEMf 1V Se 5	*T. citrinoviride* DEMf: TR3 -control-	*T. citrinoviride* DEMf: TR3 Se 2.5	*T. citrinoviride* DEMf: TR3 Se 5
Esterase – C4	+*	+	-	++	+	+
Esterase-lipase C8	+*	-	-	++	+	+
Lipase (C14)	+*	-	-	++	-	-
N-acetil-*β*-	+*	-	-	+	+	+
Protease	+	+	+	+	+	+
Celullase	+	+	+	+	+	+

(-) negative reaction; (+) positive reaction, 5-10 nM; (++) 20-30 nM; (+++) ≥ 40 nM;
*Karličić et al. (2022).

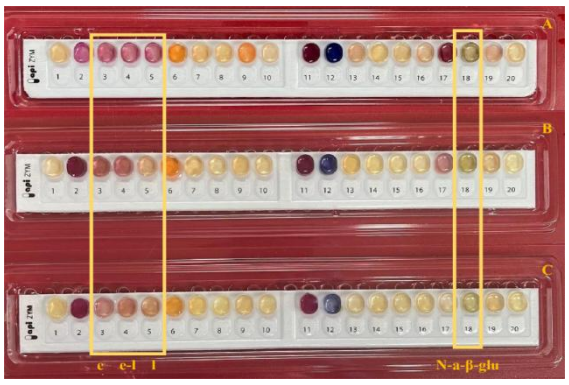

Figure 1. Enzymatic profiles of *T. citrinoviride* DEMf TR3 (control, A); *T. citrinoviride* Se 2.5 (B); *T. citrinoviride* Se 5 (C); e: esterase – C4; e-l: esterase-lipase C8; l – lipase (C14); N-a-β-glu: N-acetyl-*β*-glucosaminidase.

The confrontation tests performed between *B. cinerea*, *F. oxysporum,* and *F. graminearum,* on one side, and *Trichoderma* spp. as the BCA, on the other, showed significant levels of antagonism (Table 2). The confrontation test revealed high inhibition caused by *T. harzianum* DEMf 1V against *B. cinerea* according to the Ruiz-Gómez et al. (2021) classification. The Se-enriched cultures showed no difference in the level of antagonistic activity compared to the control. Similarly, Suyal and Tripathi (2025) reported several *T. harzianum* strains with the ability to cause radial growth inhibition of *B. cinerea* ranging from 68 to 74%. Geng et al. (2022) also reported 62% inhibition of *B. cinerea* caused by *T. harzianum*.

As reported by Karličić et al. (2022), *T. harzianum* DEMf 1V showed a high level of inhibition towards *F. oxysporum*, and Se-enriched cultures maintained the same level of inhibition according to the Ruiz-Gómez et al. (2021) classification. Similarly, Hugar and Nayaka (2025) reported over 62% inhibition of *F. oxysporum* f. sp. *udum* growth by *T. harzianum* AKH-5. *T. harzianum* DEMf 1V showed a moderate level of inhibition towards *F. graminearum* (Karličić et al., 2022), while Se-enriched cultures expressed high inhibition. Pedrero-Méndez et al. (2025) reported around 60%

inhibition of *F. graminearum* by *T. harzianum* T136, which is similar to the inhibition percentage obtained in our study (60–66%). The antagonistic effect of *T. harzianum* against several-soil borne pathogens showed the presence of inhibition zone indicating production of antibiotics (Pešaković et al., 2022).

T. citrinoviride DEMf: TR3 showed very high antagonistic activity toward *B. cinerea.* However, the antagonistic effect has dropped to a high level in the case of both Se-enriched *T. citrinoviride* DEMf: TR3 according to the Ruiz-Gómez et al. (2021) classification. Park et al. (2019) reported *T. citrinoviride*'s ability to inhibit *B. cinerea* growth in a dual culture test and to reduce the disease symptoms in ginseng plants. *T. citrinoviride* DEMf: TR3 expressed high inhibition toward *F. oxysporum*, reaching from 61 to 70% of inhibition, and those results are in accordance with Chen et al. (2022), who reported 71.85% of *F. oxysporum* growth inhibition by *T. citrinoviride* HT-1. Also, Gezgin et al. (2019) reported *T. citrinoviride* antagonistic activity toward *F. oxysporum*, indicating mycoparasitism as a mechanism of action. *T. citrinoviride* DEMf: TR3 vs *F. graminearum* resulted in very high antagonistic activity, while Karličić et al. (2022) reported 63% of *F. graminearum* inhibition by *T. citrinoviride* TR1. Interestingly, Se-enriched *T. citrinoviride* DEMf: TR3 exhibited a decrease in mycelial growth inhibition to a moderate level, and this is the most prominent difference in antagonistic activity noted in this study.

Table 2.
The mycelia growth inhibition (%) of phytopathogens by *T. harzianum* DEMf 1V, *T. citrinoviride* DEMf: TR3, and Se-enriched *T. harzianum* DEMf 1V, and *T. citrinoviride* DEMf: TR3

Isolates	MGI (%)		
	B. cinerea	*F. oxysporum*	*F. graminearum*
T. harzianum DEMf 1V	74 ± 3.1	62 ± 1.8*	60 ± 2.5*
T. harzianum DEMf 1V Se 2.5	72 ± 2.7	70 ± 3.4	66 ± 1.7
T. harzianum DEMf 1V Se 5	68 ± 2.5	70 ± 2.1	65 ± 3.2
T. citrinoviride DEMf: TR3	79 ± 4.2	61 ± 3.3	78 ± 1.1
T. citrinoviride DEMf: TR3 Se 2.5	73 ± 3.5	70 ± 3.6	58 ± 3.5
T. citrinoviride DEMf: TR3 Se 5	64 ± 2.6	63 ± 2.1	52 ± 3.4

MGI: mycelial growth inhibition;
*Karličić et al. (2022).

The effects of VOCs produced by Se-enriched and control *Trichoderma* spp. on selected phytopathogens expressed a low level of mycelial growth inhibition according to the Ruiz-Gómez et al. (2021) classification (Table 3). The highest growth inhibition of *B. cinerea* was noted in treatments with *T. harzianum* DEMf 1V Se 5 and *T. citrinoviride* DEMf: TR3 Se 5, reaching 15%. On the contrary, You et al. (2022) reported over 60% inhibition of *B. cinerea* growth caused by *T. harzianum* VOCs, proving that those compounds are powerful factors in pathogen control. As reported by Karličić et al. (2022), *F. oxysporum* growth was decreased by 36% by VOCs produced by *T. harzianum*, and Se-enriched cultures maintained a similar level of inhibition. *F. oxysporum* growth decreased by 25% during interaction with VOCs produced by *T. citrinoviride* DEMf: TR3 Se 5. Karličić et al. (2022) reported a lower percentage of *F. oxysporum* inhibition (13%) caused by *T. citrinoviride* TR1 VOCs. *F. graminearum* was decreased by 21–23% during interaction with VOCs produced by Se-enriched *T. harzianum* DEMf 1V while Karličić et al. (2022) reported 26% inhibition of *F. graminearum* by *T. harzianum* volatile metabolites. Ren et al. (2025) reported 10–20% inhibition of *F. graminearum* growth caused by *T. harzianum* VOCs and 8–18% inhibition caused by VOCs produced by *T. citrinoviride*. *T. citrinoviride* DEMf: TR3 Se 5 decreased the growth of *F. graminearum* by 23% (Figure 2). The low levels of inhibition caused by VOCs indicate that they play only a partial role in fungal growth inhibition, suggesting that other mechanisms are likely dominant.

Table 3.
The effects of volatile compounds emitted by *T. harzianum* spp. and Se-enriched *Trichoderma* spp. on mycelial growth of phytopathogens

Isolates	MGI (%)		
	B. cinerea	*F. oxysporum*	*F. graminearum*
T. harzianum DEMf 1V	8 ± 0.5	36 ± 1.4*	26 ± 2.1*
T. harzianum DEMf 1V Se 2.5	12 ± 1.2	29 ± 1.3	21 ± 1.3
T. harzianum DEMf 1V Se 5	15 ± 1.1	31 ± 2.2	23 ± 2.4
T. citrinoviride DEMf: TR3	13 ± 2.3	17 ± 1.8	10 ± 1.6
T. citrinoviride DEMf: TR3 Se 2.5	13 ± 1.5	21 ± 1.9	15 ± 1.7
T. citrinoviride DEMf: TR3 Se 5	15 ± 0.7	25 ± 1.3	23 ± 2.0

MGI: mycelial growth inhibition;
*Karličić et al. (2022).

In addition, the effects of VOCs of enriched and non-enriched *Trichoderma* spp. on the growth of phytopathogens (Table 3) lead to the assumption that the content of volatile compounds may be affected by Se addition and its concentration. Xu et al. (2021) noted the production of Se volatiles by Se-enriched *Ganoderma lucidum,* while no production was reported in control conditions. The analysis of volatile components produced by *Cordyceps cicadae* confirmed the presence of numerous compounds in the Se-enriched samples (alcohols, organic acids, formamide, N-methylthio, and N-formylglycine) which were absent in the control (Dou et al., 2025). While literature confirms changes in volatile spectra of macrophytes grown in Se-enriched media, similar data covering metabolic changes in filamentous fungi are missing. Considering the importance of VOCs in establishing microbial interactions, those changes may impact and modulate the communication between the BCA and the phytopathogen, but profound research is needed to precisely determine the influence of Se enrichment on VOCs profiles, and the consequences for pathogens.

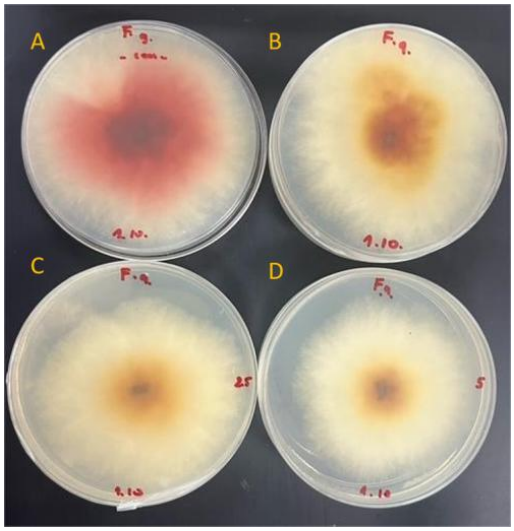

Figure 2. Effects of *T. citrinoviride* DEMf: TR3 volatile compounds on growth of *F. graminearum* (control; A); *T. citrinoviride* DEMf: TR3 vs F. *graminearum* (B); *T. citrinoviride* DEMf: TR3 Se 2.5 vs *F. graminearum* (C); *T. citrinoviride* DEMf: TR3 Se 5 vs *F. graminearum* (D).

The results confirmed that the presence of Se in the growth medium resulted in changes in fungal cultures' characteristics. The confrontation test revealed different responses to the presence of phytopathogens. Se-enriched *T. harzianum* DEMf 1V showed an increase in *F. graminearum* growth inhibition compared to the control. In the case of Se-enriched *T. citrinoviride* DEMf: TR3, antagonistic activity towards *B. cinerea* and *F. graminearum* was decreased.

Numerous mechanisms are involved in phytopathogen–biocontrol agent interactions, and hydrolytic enzymes are among them (Guzmán-Guzmán et al., 2025). Se-enriched *T. harzianum* DEMf 1V ceased the production of several enzymes, suggesting that different mechanisms are crucial for the interaction with *F. graminearum*. The decrease in antagonistic activity towards *B. cinerea* and *F. graminearum* in the case of Se-enriched *T. citrinoviride* DEMf: TR3 may be caused by changes in the production of esterase, esterase-lipase, and lipase, but that has to be determined in further study. The differences observed in the phytopathogens' responses to the VOCs produced by Se-enriched *Trichoderma* spp. indicate potential changes in the spectra, but further quantitative research should be devoted to the determination of the precise degree of influence.

Conclusion

The presence of selenium in the nutrient medium caused changes in the biochemical and ecological characteristics of *T. harzianum* DEMf 1V and *T. citrinoviride* DEMf: TR3. The Se-enrichment of *T. harzianum* DEMf 1V inhibited the production of the hydrolytic enzymes involved in biocontrol activity (esterase-lipase, lipase, and N-acetyl-β-glucosaminidase). Se-enriched *T. citrinoviride* DEMf: TR3 ceased the production of lipase, while the production of esterase and esterase-lipase was decreased. Both cultures maintained their ability to produce proteases and cellulases. While the antagonistic activity of Se-enriched *T. harzianum* DEMf 1V against *F. graminearum* was increased, the opposite occurred in Se-enriched *T. citrinoviride* DEMf: TR3 against *B. cinerea* and *F. graminearum*. The low level of inhibition caused by the volatile metabolites confirmed that they are only partially involved in the mechanisms of the antifungal activity of the tested isolates. Further research should be directed towards the precise determination of metabolic changes induced by Se-enrichment and the implications thereof for *Trichoderma*'s antagonistic activities.

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Declaration of competing interests

Authors declare no competing interests.

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