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Colony Age of *Trichoderma azevedoi* Alters the Profile of Volatile Organic Compounds and Ability to Suppress *Sclerotinia sclerotiorum* in Bean Plants

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Common bean (*Phaseolus vulgaris* L.) is one of the most important crops in human food production. The occurrence of diseases, such as white mold, caused by *Sclerotinia sclerotiorum* can limit the production of this legume. The use of *Trichoderma* has become an important strategy in the suppression of this disease. The aim of the present study was to evaluate the effect of volatile organic compounds (VOCs) emitted by *Trichoderma azevedoi* CEN1241 in five different growth periods on the severity of white mold in common bean. The *in vitro* assays were carried out in double-plate and split-plate, and the *in vivo* assays, through the exposure of the mycelia of *S. sclerotiorum* to the VOCs of *T. azevedoi* CEN1241 and subsequent inoculation in bean plants. Chemical analysis by gas chromatography coupled to mass spectrometry detected 37 VOCs produced by *T. azevedoi* CEN1241, covering six major chemical classes. The profile of VOCs produced by *T. azevedoi* CEN1241 varied according to colony age and was shown to be related to the ability of the biocontrol agent to suppress *S. sclerotiorum*. *T. azevedoi* CEN1241 VOCs reduced the size of *S. sclerotiorum* lesions on bean fragments *in vitro* and reduced disease severity in a greenhouse. This study demonstrated in a more applied way that the mechanism of antibiosis through the production of volatile compounds exerted by *Trichoderma* can complement other mechanisms, such as parasitism and competition, thus contributing to a better efficiency in the control of white mold in bean plants.

Keywords **:** biological control, gas chromatography, mycelial inhibition, soil pathogen, volatile metabolites

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Common bean (*Phaseolus vulgaris* L.) is one of the most important legumes for human consumption (Mayo-Prieto et al., 2021). On a global scale, Brazil currently occupies fourth position in the production of this legume, behind China, India and Myammar. According to the Brazilian Institute of Beans and Pulses (IBRAFE, 2021), in 2021, the country exported more than 200,000 tons of the product and earned approximately US$ 1 billion. This legume contributes approximately 25% of the protein in the diet of the poorest Brazilian population and its production is related to food security, besides representing an important source of income and employment for the rural population (Cardoso and Ferreira, 2021; Ganascini et al., 2019).

Bean cultivation can be affected by several pathogens that limit commercial production, including the soil fungus *Sclerotinia sclerotiorum* (Lib.) de Bary (Carvalho et al., 2015; de Figueirêdo et al., 2010). The disease caused

by this phytopathogen is known as white mold and has, as a characteristic sign, the presence of white and cottony mycelia on stems, leaves, flowers, pods and grains of the common bean (Miklas et al., 2013). After colonization of the host, *S. sclerotiorum* produces survival structures called sclerotia, which are constituted by a cluster of rigid, black and irregular hyphae, which can be formed in the inner and outer layers of the host (Ordóñez-Valencia et al., 2015; Smolińska and Kowalska, 2018).

Crop rotation with non-host species, use of the no-tillage system, change in plant density and application of synthetic fungicides for seed treatment, as well as aerial spraying, are measures that can reduce the incidence and severity of the disease (Mahoney et al., 2014). The use of synthetic fungicides is influenced by the need for quick results to prevent the disease from advancing in the field. However, growing concern about the effects of such products on the environment and their residues in food has increased society's interest in the biological control of plant diseases (O'Brien, 2017; Smolińska and Kowalska, 2018).

Fungi of the genus *Trichoderma* have potential for the control of phytopathogens due to their different mechanisms of action, such as parasitism, competition, induction of resistance and antibiosis. The latter stems from the production of volatile (VOCs) and non-volatile organic compounds (Castillo et al., 2011; Nawrocka et al., 2018; Srivastava et al., 2016). Several studies have demonstrated the ability of *Trichoderma* spp. to parasitize sclerotia and, consequently, reduce the density of apothecia, and to inhibit the mycelial growth of *S. sclerotiorum*, thus reducing the incidence of white mold (Carvalho et al., 2011, 2015; Geraldine et al., 2013; Kumar et al., 2021). However, the number of reports dealing with antagonistic activity against *S. sclerotiorum* due to *Trichoderma* VOCs exclusively, under applied conditions, is quite limited. This is mainly because the available techniques have limitations in obtaining results that effectively demonstrate the role of VOCs in disease control.

VOCs can easily evaporate at room temperature, and cross cell membranes to be released into the air or soil (Siddiquee et al., 2012; Spiteller, 2015). The antifungal capacity of these metabolites emitted by *Trichoderma* is interesting due to the possible applicability of such products as biofumigants in the control of plant diseases or as a complement to other mechanisms exerted by the biocontrol agent (Ruangwong et al., 2021). The chemical classes of these compounds include alcohols, ketones, terpenes, aromatic compounds, lactones, esters, and aldehydes, among others (Estrada-Rivera et al., 2019; González-Pérez et al., 2018; Nieto Jacobo et al., 2017).

The biosynthesis, diversity and amount of fungal VOCs is species or even strain-specific and depends on growth conditions such as nutrient availability, medium pH, light and temperature (Guo et al., 2019; Lee et al., 2016). Recently, it was also reported that the age of the *Trichoderma* colony is a key factor for more effective indices of *in vitro* mycelial inhibition of *S. sclerotiorum* (da Silva et al., 2021a). In this context, the objective of this study was to identify the diversity of VOCs emitted by *Trichoderma azevedoi* CEN1241 in five different growth periods, to evaluate the ability of these VOCs to suppress *S. sclerotiorum in vitro* and to control white mold in bean plants.

Materials and Methods

Origin and maintenance of strains. *Trichoderma azevedoi* strain CEN1241 and pathogen *S. sclerotiorum* strain CEN1147 were used, both belonging to the Collection of Biological Control Agents of Embrapa Genetic Resources and Biotechnology. The cultures, conserved in liquid nitrogen (N_2), were reactivated in potato dextrose agar (PDA) Merck medium and then grown in Petri dishes (90 × 15 mm), containing 20 ml of the same medium, at a temperature of 6ºC. For each experiment, samples taken from these plates were cultured for five days in new Petri dishes containing 20 ml of PDA medium. This made it possible to standardize the colonies in terms of age, generating greater reliability in the data.

VOCs collected from *T. azevedoi* CEN1241 in different growth periods. The VOC collections were performed using 2 l glass chambers (internal volume). Each chamber received a Petri dish (90 × 15 mm), containing 20 ml of PDA medium and one mycelium disk (5 mm Ø) colonized by *T. azevedoi* in different growth periods: 0 h, 24 h, 48 h, 72 h, and 96 h. In this VOC collection system, the air enters through an activated carbon filter, thus ensuring the passage of purified air. The air escapes through a flow rate of 0.5 l/min using a vacuum pump connected to a glass tube containing 100 mg of Porapak Q adsorbent polymer (60-80 mesh, Supelco, Bellefonte, PA, USA), via polytetrafluoroethylene connections. In order to quantify and identify which VOCs are part of the mycelial inhibition of *S. sclerotiorum* at the exact moment when the shared atmosphere is set up, we chose to collect them at each growth period within a 4 h interval. After this period, the collected VOCs were eluted from the adsorbents with 500 µl of the organic solvent *n*-hexane and concentrated to 20 µl, with a gentle stream of N_2 flow. The obtained samples were stored at –20ºC for further analysis by a gas chromatograph

(GC) connected to a flame ionization detector (GC-FID) and GC coupled to a mass spectrometer detector (GC-MS). The experiment was conducted with four replications per treatment.

Chemical analysis of collected VOCs. For the quantitative analysis of VOCs, each sample received 1 µl of 16-hexadecanolide (97%, Sigma-Aldrich, St. Louis, MO, USA), as an internal standard (IS), prepared at a 1 mg/ml concentration in distilled *n*-hexane. Subsequently, 2 µl of each sample was injected into the CG-FID (Agilent 7890-A, Agilent Technologies, Santa Clara, CA, USA) using a nonpolar DB-5MS column (0.25 mm internal diameter × 30 m long with 0.25 µm film thickness; J&W Scientific, Folsom, CA, USA), using splitless injection mode, with helium as carrier gas with a flow rate of 1.5 ml/min. The initial ramp temperature was 50°C for 2 min, gradually increasing 5°C per minute until reaching 180°C, and then a second gradient of 10°C per minute until reaching 250°C, in which it was maintained for 20 min. The detector temperature was 270°C and the injector temperature was 250°C. The quantification of released compounds was performed by comparing the areas of each compound in relation to the IS area. Thus, the detector response factor for all compounds was considered equal to 1. Data were collected and analyzed using the GC Open Lab CDS ChemStation software (Agilent Technologies, Santa Clara, CA, USA).

In the qualitative analysis, the previously selected samples were injected into the GC-MS (Agilent 5975 MSD, Beijing, China) equipped with a quadrupole analyzer, in a nonpolar DB-5MS column (0.25 mm internal diameter × 30 m long with 0.25 µm film thickness; J&W Scientific), electron impact ionization (70 eV, at 200ºC) and injector in splitless mode. Helium gas was used as carrier gas, with a flow rate of 1 ml/min. The same temperature program was used. Data were collected and analyzed using MassHunter Workstation 10.1.49 software (Agilent Technologies, Santa Clara, CA, USA). The identification of VOCs was performed by comparing the fragmentation pattern of the sample components with the data cataloged in spectral libraries (National Institute of Standards and Technology, 2020) and also by calculating the retention index (RI). To calculate the RI, a mixture of linear alkane hydrocarbons (C_8-C_{26}) was injected under the same conditions described above. For final confirmation, the fragmentation pattern and the RI of the compounds were compared with the data obtained from the injection of authentic standards with the samples, when they were available.

Chemicals. Authentic chemical standards of 16-hexadecanolide (97%), benzaldehyde (99%), 3-octanone (98%), 3-octanol (99%), limonene (97%), decanal (98%), undecanal (97%), undecane (99%), dodecane (99%), tridecane (99%), tetradecane (99%), pentadecane (98%), hexadecane (98.5%), heptadecane (98.5%), β-caryophyllene (98%), β-cedrene (95%), geranylacetone (97%), and the solvent *n*-hexane (97% redistilled) were purchased from Sigma-Aldrich. (*E*)-2-Octen-1-ol (98%) was purchased from Alfa Aesar. 1-Octen-3-ol, octanal, nonanal, 2-ethylhexan-1-ol, phenylacetaldehyde, phenylethanol, and β-bisabolene were donated by Dr. Jeffrey Aldrich (Agricultural Research Service – USDA). β-Sesquiphellandrene was purified from ginger oil and α-himachalene was purified from *Cedrus atlantica* oil.

Double-plate mycelial inhibition. Petri dishes (90 × 15 mm) containing 20 ml of PDA medium, received at the center disks of PDA (5 mm Ø) colonized by *T. azevedoi* CEN1241, in different growth periods: 0, 24, 48, 72 and 96 h. In the same way, in another Petri dish, a PDA disc (5 mm Ø) colonized by *S. sclerotiorum* was placed. The plates containing antagonist and pathogen were placed facing each other, forming an atmosphere sharing set. The set was sealed with plastic paraffin film and wrapped with clear plastic, so that the plates containing the pathogen were in the upper position (Dennis and Webster, 1971). The control treatment was represented by plate bases with *S. sclerotiorum*, but without the antagonist at the bottom. The system was maintained at a temperature between 23 and 25°C and a photoperiod of 12 h. After the total colonization of the PDA medium in the control treatments, the measurements of the pathogen colonies were taken with the aid of a digital caliper (Digimess, São Paulo, SP, Brazil). Mean inhibition values were calculated using the formula: $I = (C - T)/C \times 100$, where "I" represents the inhibition percentage, "C" is the control diameter and "T" is the treatment diameter value in the presence of the antagonist, in millimeters. The experiment was carried out in a completely randomized design with four replications per treatment and performed twice.

Inhibition of mycelogenic germination in double-plate. For the production of sclerotia, discs of mycelium (5 mm Ø) colonized by *S. sclerotiorum* were placed in the center of Petri dishes (90 × 15 mm) containing 20 ml of PDA medium, and the colonies were maintained at a temperature between 23ºC and 25ºC and photoperiod of 12 h. After 14 days, the mature sclerotia were collected and then disinfected with ethanol (70%) for 1 min, followed by sodium hypochlorite (50%) for 3 min and then washed three times

in sterilized water for 30 s. Subsequently, Petri dishes (90 × 15 mm) containing 20 ml of PDA received 12 of these sclerotia. These were lightly inserted into the PDA medium. In another Petri dish (90 × 15 mm) containing 20 mL of PDA, a PDA disk (5 mm Ø) colonized by *T. azevedoi* CEN1241 was deposited at different growth periods: 0 h, 24 h, 48 h, 72 h, and 96 h. The plates containing antagonist and sclerotia were superimposed, forming a shared atmosphere. The set was sealed with plastic paraffin and transparent plastic film, so that the plates containing the sclerotia were in the upper position. The control treatment was represented by plates also with sclerotia, but without the antagonist at the bottom. The system was maintained at a temperature between 23 and 25°C and a photoperiod of 12 h. At the end of five days, the number of germinated sclerotia was quantified. The experiment was carried out in a completely randomized design with four replications per treatment and repeated twice.

Effects of VOCs emitted by *T. azevedoi* CEN1241 on *S. sclerotiorum* infection using split-plate bean fragments. Fragments of bean plants grown in a greenhouse, 21-day-old and eight centimeters long, were disinfected with ethanol (70%) for 1 min, followed by sodium hypochlorite (50%) for 3 min and then washed three times in sterilized water for 30 s. In one of the compartments of the divided plate, moistened filter paper was added and a bean fragment was added to it. With the aid of a platinum needle, a hole was made in the center of the fragment and, then, a disc of mycelium (3 mm Ø) colonized by *S. sclerotiorum* was inoculated. In the center of the other compartment, containing 10 ml of PDA medium, a PDA disk (5 mm Ø) was placed, colonized by *T. azevedoi* CEN1241, in different growth periods: 0 h, 24 h, 48 h, 72 h, and 96 h. The control treatment was represented by plaques also with *S. sclerotiorum*, but without the antagonist in the other compartment. The set was sealed with plastic paraffin and transparent plastic film and kept at a temperature between 23ºC and 25ºC and a photoperiod of 12 h. At the end of five days, with the aid of a digital caliper (Digimess), measurements were taken of the length of the lesions caused by *S. sclerotiorum* in the bean fragments. The experiment was carried out in a completely randomized design with four replications per treatment and repeated twice.

Effects of VOCs emitted by *T. azevedoi* CEN1241 on the severity of white mold in a greenhouse. Due to the high rate of mycelial inhibition of *S. sclerotiorum* exposed to *T. azevedoi* CEN1241 VOCs at 48 and 72 h, these two periods could not be included in this assay. The experiment was conducted as follows: three common bean seeds were sown in plastic bags, with a capacity of 3 l, filled with a mixture of soil, fine sand and cattle manure, in a 3:1:1 ratio. At seven days after sowing, thinning was performed, leaving only one plant per bag. The bags received daily irrigation. At 21 days after sowing, with the aid of a platinum needle, a hole was made in the lower third of the plants and a mycelium disc (5 mm Ø) colonized by *S. sclerotiorum* exposed to the VOCs of *T. azevedoi* CEN1241 was inoculated in a double-plate, in different growth periods: 0 h, 24 h, and 96 h. The mycelium discs were fixed with the aid of adhesive tape. The positive control was represented by the inoculation of plants with mycelia of *S. sclerotiorum* without previous exposure to VOCs and the negative control represented by plants without inoculation of *S. sclerotiorum*. At the end of seven days, with the aid of a digital caliper (Digimess), measurements were taken of the length of the lesions caused by *S. sclerotiorum* in the bean plants. The experiment was carried out in a completely randomized design with seven replications per treatment and repeated twice.

Statistical analysis. Data concerning mycelial inhibition in a double-plate, inhibition of sclerotia in a double-plate, infection in bean fragments in a split-plate and white mold severity in a greenhouse were submitted to analysis of variance and the means were compared by Tukey's test ($P \leq 0.05$). All of these analyses were performed using the Sisvar 5.6 software (Ferreira, 2011). For the quantitative analysis of the VOCs emitted by *T. azevedoi* CEN1241, generalized linear models (GLM) of gamma family with identity linkage function were built, and then a multiple comparison of the means was performed using Tukey's test ($P \leq 0.05$), with Holm adjustment. These analyses were performed using the R software 3.6.3 (R Core Team, 2019). In the GLM analysis, the stats package was used and in the multiple comparison of averages, the multcomp.

Results

Chemical analysis of collected VOCs. Chemical analysis by GC-MS detected 37 VOCs produced by *T. azevedoi* CEN1241, covering seven major chemical classes: alcohols, aldehydes, aromatics, esters, hydrocarbons, ketones, and terpenes (Table 1, Fig. 1). Among the 37 VOCs detected, 26 were identified and 11 inferred.

The statistical analysis of total VOCs emitted by *T. azevedoi* CEN1241 showed that the 96 h period was quantitatively superior to the other growth periods. The average total amount emitted in this time period was approximately

Table 1. Mean amount ± standard error in ng/h of VOCs emitted by *Trichoderma azevedoi* CEN1241 in different growth periods

Compound	RI	*Trichoderma* growth time (h)				
		0	24	48	72	96
Benzaldehyde	968	0.00 ± 0.00	6.74 ± 4.83	1.30 ± 0.29	0.95 ± 0.38	0.00 ± 0.00
1-Octen-3-one[a]	977	0.00 ± 0.00	0.00 ± 0.00	9.08 ± 1.45	23.07 ± 5.92	0.00 ± 0.00
1-Octen-3-ol	983	6.03 ± 1.33	12.08 ± 5.02	70.87 ± 10.58	240.63 ± 65.14	748.50 ± 163.63
3-Octanone	988	0.00 ± 0.00	0.00 ± 0.00	5.80 ± 1.19	28.33 ± 12.64	90.20 ± 15.85
3-Octanol	998	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	4.32 ± 0.13
Octanal	1,005	2.32 ± 0.63	2.86 ± 0.40	2.26 ± 0.96	2.94 ± 1.41	3.31 ± 1.04
2-Ethylhexan-1-ol	1,030	2.50 ± 0.80	6.62 ± 1.89	7.55 ± 1.83	6.06 ± 3.28	8.30 ± 3.79
Limonene	1,033	0.00 ± 0.00	0.00 ± 0.00	1.24 ± 0.70	1.18 ± 0.35	1.51 ± 0.77
Phenylacetaldehyde	1,049	13.18 ± 5.20	10.32 ± 6.44	4.02 ± 1.12	2.91 ± 1.49	0.00 ± 0.00
(*E*)-2-octen-1-ol	1,069	0.00 ± 0.00	0.00 ± 0.00	7.93 ± 1.48	18.45 ± 7.79	0.00 ± 0.00
Undecane	1,100	0.00 ± 0.00	3.28 ± 1.16	3.30 ± 0.86	3.58 ± 1.64	3.76 ± 1.95
Nonanal	1,105	10.00 ± 3.15	18.96 ± 3.15	11.24 ± 5.18	14.65 ± 8.04	14.42 ± 7.61
Phenylethanol	1,114	0.00 ± 0.00	6.79 ± 5.15	77.79 ± 4.08	89.53 ± 52.39	0.00 ± 0.00
Carvomenthone[a]	1,131	0.00 ± 0.00	8.01 ± 4.34	62.63 ± 8.05	36.99 ± 16.94	58.48 ± 16.88
Dodecane	1,200	0.00 ± 0.00	2.49 ± 0.69	2.03 ± 0.39	2.24 ± 0.88	2.16 ± 1.45
Decanal	1,206	10.32 ± 3.51	15.75 ± 2.14	11.34 ± 3.85	12.31 ± 5.32	14.12 ± 6.71
Tridecane	1,300	0.00 ± 0.00	6.04 ± 5.64	2.08 ± 0.32	2.68 ± 0.19	3.16 ± 1.12
Undecanal	1,308	1.39 ± 0.46	2.22 ± 0.63	1.58 ± 0.44	1.73 ± 0.43	1.95 ± 0.69
Tetradecane	1,400	0.00 ± 0.00	2.97 ± 0.79	1.75 ± 0.47	2.13 ± 0.19	1.71 ± 0.90
Sesquiterpene 1[a]	1,421	0.00 ± 0.00	6.10 ± 2.75	3.48 ± 0.55	1.39 ± 0.82	0.00 ± 0.00
β-Caryophyllene	1,421	0.00 ± 0.00	3.72 ± 1.64	2.04 ± 0.33	0.87 ± 0.29	2.67 ± 0.74
β-Cedrene	1,428	0.00 ± 0.00	10.09 ± 5.56	7.54 ± 1.07	2.72 ± 1.39	1.97 ± 0.50
α-Bergamotene[a]	1,434	0.00 ± 0.00	1.63 ± 0.37	4.56 ± 0.37	3.60 ± 2.05	0.00 ± 0.00
Sesquiterpene 2[a]	1,442	0.00 ± 0.00	0.00 ± 0.00	1.29 ± 0.21	0.00 ± 0.00	0.00 ± 0.00
Geranylacetone	1,448	3.47 ± 1.77	0.00 ± 0.00	4.12 ± 1.17	3.40 ± 0.34	4.27 ± 0.57
α-Himachalene	1,458	0.00 ± 0.00	0.00 ± 0.00	1.54 ± 0.24	1.50 ± 0.33	0.00 ± 0.00
Acoradiene[a]	1,478	0.00 ± 0.00	3.04 ± 1.08	1.37 ± 0.20	1.99 ± 0.45	0.00 ± 0.00
Sesquiterpene 3[a]	1,484	0.00 ± 0.00	3.94 ± 2.04	3.87 ± 0.57	1.88 ± 0.65	1.58 ± 0.45
Pentadecane	1,500	0.00 ± 0.00	1.68 ± 0.64	2.44 ± 0.83	3.43 ± 1.65	2.26 ± 1.00
β-Bisabolene	1,508	0.00 ± 0.00	1.86 ± 0.67	3.52 ± 1.05	2.48 ± 0.91	1.97 ± 0.89
Sesquiterpene 4[a]	1,512	0.00 ± 0.00	4.79 ± 1.76	4.66 ± 0.76	2.40 ± 1.02	5.82 ± 1.63
β-Sesquiphellandrene	1,524	0.00 ± 0.00	0.00 ± 0.00	2.69 ± 0.50	1.19 ± 0.76	0.00 ± 0.00
Hexadecane	1,599	0.00 ± 0.00	1.75 ± 0.52	1.29 ± 0.08	1.24 ± 0.05	1.26 ± 0.37
Methyl dihydrojasmonate[a]	1,649	0.00 ± 0.00	2.68 ± 0.85	1.64 ± 0.53	1.44 ± 0.16	1.23 ± 0.22
1-Isopropyl-1-Isopropyl-4,8-dimethylspiro[4.5]dec-8-en-7-ol[a]	1,669	0.00 ± 0.00	21.20 ± 4.34	13.20 ± 5.58	2.78 ± 2.15	0.00 ± 0.00
1-Isopropyl-4,8-dimethylspiro[4.5]dec-8-en-7-one[a]	1,690	0.00 ± 0.00	7.82 ± 1.84	8.19 ± 3.49	3.97 ± 1.67	14.06 ± 3.11
Heptadecane	1,700	0.00 ± 0.00	1.70 ± 0.20	0.72 ± 0.23	0.56 ± 0.10	0.56 ± 0.06
Total VOCs	-	49.25 ± 14.62 d	177.27 ± 45.29 c	352.10 ± 27.86 b	527.34 ± 153.20 b	993.68 ± 222.60 a

The volatiles were collected for a period of 4 h.
Values followed by the same letter in the columns do not differ, according to the Tukey's test ($P \leq 0.05$), with Holm adjustment.
VOC, volatile organic compound; RI, Retention Index calculated with a DB-5MS column.
[a]Tentatively identified compound.

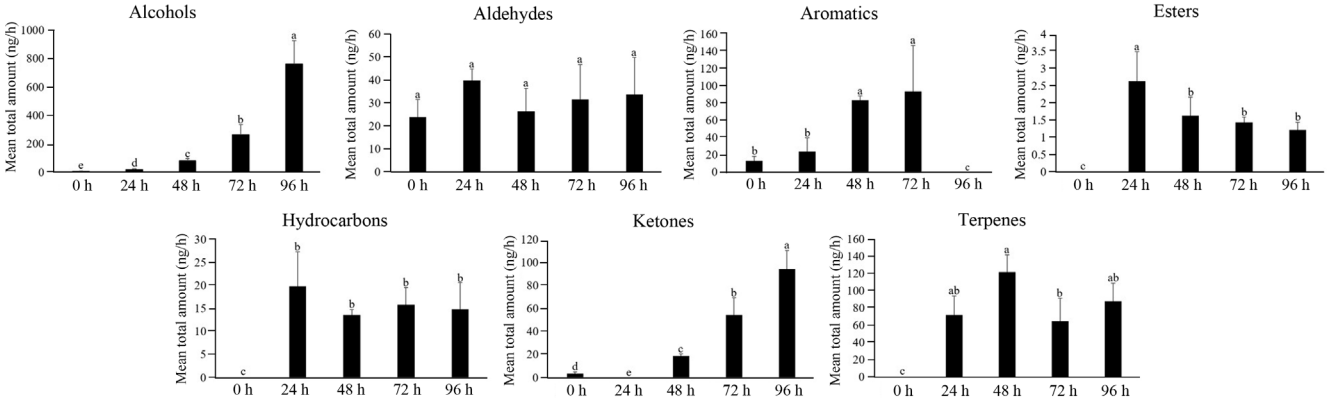

Fig. 1. Total amount (mean ± standard error) of volatile organic compound chemical classes emitted by *Trichoderma azevedoi* CEN1241 in ng/h. The bars followed by the same letter do not differ from each other, by Tukey's test ($P \leq 0.05$), with Holm adjustment.

993.68 ± 222.60 ng/h. The higher emission of VOCs presented for the period of 96 h is due to the large amount released from the compound 1-octen-3-ol and not to the diversity of VOCs released (Table 1). The time periods of 48 and 72 h did not differentiate between them, and formed the second statistical group, in terms of the total amount of VOCs. At time 0 h, the lowest emission of total VOCs was detected, with mean values of 49.25 ± 14.62 ng/h.

Phenylacetaldehyde was the most emitted compound in the 0 h period, with a mean of 13.18 ± 5.20 ng/h. In the period of 24 h, it was the compound nonanal, with an average of 18.96 ± 3.15 ng/h; within 48 h, the compound phenylethanol, with an average of 77.79 ± 4.08 ng/h. In the periods of 72 and 96 h, there was a significant increase in the compound 1-octen-3-ol, with averages of 240.63 ± 65.14 and 748.50 ± 163.63 ng/h, respectively, making this compound the majority for the last two analyzed time periods. It is important to emphasize that these high emission rates of 1-octen-3-ol were much higher than all other compounds in relation to all time periods analyzed.

The qualitative analysis of data showed that the 48 h period produced the highest diversity of VOCs in relation to the others, emitting 36 different VOCs, followed by the 72 h period, with emission of 35 VOCs. The 24 h period showed the emission of 28 VOCs, and the 96 h period, 25 VOCs. Time 0 h showed the lowest variability of VOCs released, with only eight compounds detected. The compounds 1-octen-3-one, (*E*)-2-octen-1-ol, α-himachalene and β-sesquiphellandrene were detected only at 48 and 72 h. The compound sesquiterpene 2 was verified only in the period of 24 h, while the compound 3-octanol, only in the period of 96 h.

The analysis of the average total amount of VOCs released by chemical class showed that the emission varies between the analyzed periods. The aldehyde classes were the only ones that did not show significant differences between the periods of VOC collection of *T. azevedoi* CEN1241 (Fig. 1). For the alcohol class, the period of 96 h was superior to the others, with an average of 761.13 ± 167.02 ng/h. In the aromatic compounds class, periods of 48 and 72 h were statistically different from the others, with averages of 83.13 ± 5.27 and 93.40 ± 53.64 ng/h, respectively. The 96 h period did not present any compounds of the aromatic class.

As for the ester class, the highest amount was observed in the 24 h period, whose emission average was of 2.68 ± 0.85 ng/h. The periods 48, 72, and 96 h did not differ from each other. In the period of 0 hours, no compound related to this chemical class was detected. The hydrocarbon class showed no significant difference between the periods of 24, 48, 72, and 96 h, with averages ranging from 13.64 ± 1.21 to 19.93 ± 7.42 ng/h. In the period of 0 h no hydrocarbons were detected.

The highest amount of ketones was detected at 96 h, with a mean of 94.48 ± 16.34 ng/h, statistically differing from the other periods. In the 24 h period no compound belonging to the ketone class was detected. In the terpene class, the 48 h period had the highest emission average, with 121.88 ± 20.16 ng/h, but not statistically different from the 24 and 96 h periods. Similar to esters and hydrocarbons, terpenes were also not emitted at 0 h.

Double-plate mycelial inhibition. The control treatment colonized the entire surface of the PDA medium after 96 h of cultivation. In treatments with exposure to *T. azevedoi* CEN1241 VOCs, a reduction in the mycelial growth of *S. sclerotiorum* was observed in relation to the control, without exposure to VOCs. The best results were obtained with

Table 2. Action of *Trichoderma azevedoi* CEN1241 volatile organic compounds in different growth periods on *Sclerotinia sclerotiorum*

Treatments[a]	DP (%)	GE (%)	FR (mm)	SV (mm)
CEN1241 - 0 h	40.84 ± 4.30 c	22.91 ± 7.97 b	18.21 ± 4.90 b	0.00 ± 0.00 c
CEN1241 - 24 h	61.86 ± 2.75 b	00.00 ± 0.00 c	21.08 ± 0.54 b	0.00 ± 0.00 c
CEN1241 - 48 h	100 ± 0.00 a	00.00 ± 0.00 c	2.85 ± 0.53 c	-
CEN1241 - 72 h	100 ± 0.00 a	00.00 ± 0.00 c	19.90 ± 8.05 b	-
CEN1241 - 96 h	62.26 ± 2.40 b	00.00 ± 0.00 c	15.11 ± 1.88 b	18.91 ± 3.38 b
Control	-	93.75 ± 7.90 a	62.21 ± 12.12 a	25.54 ± 4.18 a
Coefficient of variation (%)	4.90	25.15	27.57	23.69

Mycelial inhibition in double-plate (DP), percentage mycelogenic germination of sclerotia in double-plate (GE), lesions in split-plate bean fragments (FR) and white mold severity in bean plants in greenhouse (SV).
[a]Values followed by the same letter in the columns do not differ by Tukey's test ($P \leq 0.05$).

T. azevedoi CEN1241 cultivated for 48 and 72 h before exposure to the pathogen (Table 2). Under these conditions, the VOCs emitted inhibited the mycelial growth of the pathogen by 100%. The time-point 0 h had an inhibition of 40.84 ± 4.30% in relation to the control, and at 48 and 96 h the inhibition was 61.86% and 62.26%, respectively (Table 1). In addition to the reduction in mycelial growth of *S. sclerotiorum* when exposed to VOCs, a lower abundance of aerial mycelia was also detected in colonies of the pathogen.

Inhibition of mycelogenic germination in double-plate. After five days of incubation, some repetitions of the control treatment showed 100% mycelogenic germination, when the number of germinated sclerotia was evaluated. These had white, cottony mycelia and a vigorous appearance. There was a reduction in the mycelogenic germination of *S. sclerotiorum* (Table 2) in all conditions tested. However, the treatment with *T. azevedoi* CEN1241 at time-point 0 h allowed the germination of approximately 22.91% of the sclerotia, but differing significantly from

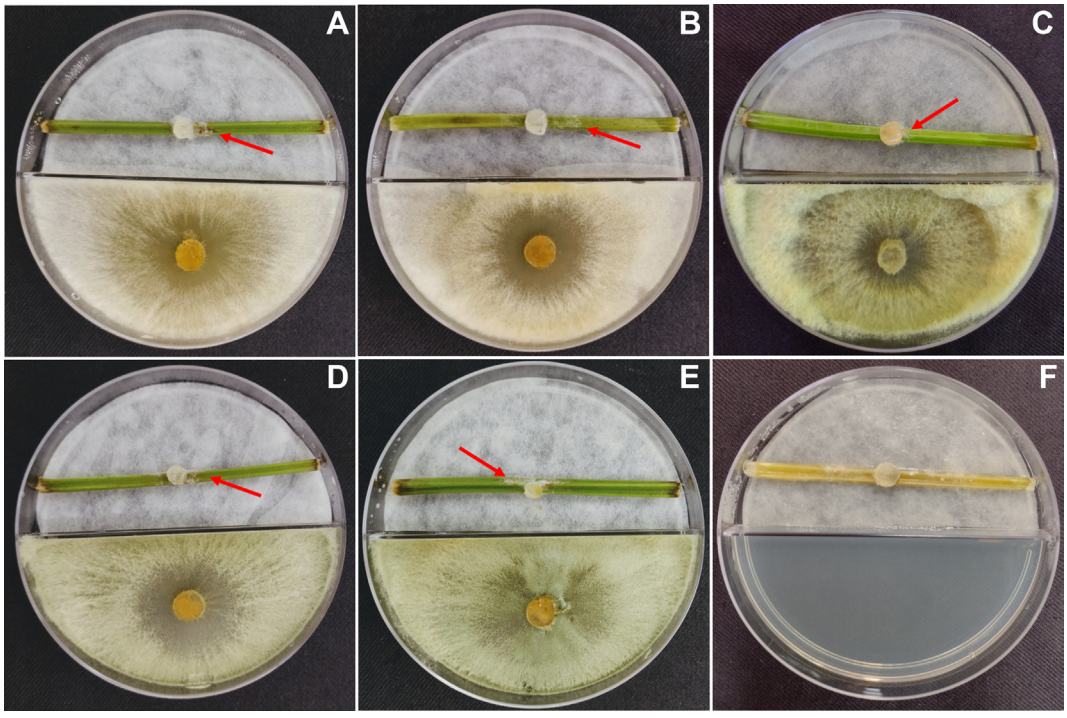

Fig. 2. Action of *Trichoderma azevedoi* CEN1241 volatile organic compounds at different growth periods on *Sclerotinia sclerotiorum* infection in bean fragments. (A) CEN1241 grown for 0 h. (B) CEN1241 grown for 24 h. (C) CEN1241 grown for 48 h. (D) CEN1241 grown for 72 h. (E) CEN1241 grown for 96 h. (F) Control. Arrows indicate the extent of lesions.

the control treatment, which presented 93.75% of mycelium germination. The other treatments with *T. azevedoi* CEN1241, previously cultivated for time-points of 24, 48, 72, and 96 h, inhibited the mycelogenic germination of *S. sclerotiorum* by 100%.

Effects of VOCs emitted by *T. azevedoi* CEN1241 on *S. sclerotiorum* infection using split-plate bean fragments. All treatments exposed to *T. azevedoi* CEN1241 VOCs were able to reduce the length of lesions caused by *S. sclerotiorum* in bean fragments (Table 2). The best result was obtained when *T. azevedoi* CEN1241 was previously cultured for 48 h. In this condition, the lesion lengths in the bean fragments were around 2.85 mm, which represents lesions approximately 21 times smaller than the lesions in the control treatment, which presented, on average, 62.21 mm in length. The other treatments with *T. azevedoi* did not differ statistically, forming an intermediate group, with lesion length ranging from 15.11 to 21.08 mm. The control treatment presented, in addition to larger lesions, a greater amount of mycelia in the fragments and symptoms of generalized wilt and onset of rot (Fig. 2).

Effects of VOCs emitted by *T. azevedoi* CEN1241 on the severity of white mold in a greenhouse. As mentioned before, due to the high rate of mycelial inhibition of *S. sclerotiorum* under exposure to VOCs of this *T. azevedoi* strain at 48 and 72 h, it was not possible to include these treatments in the assay.

White mold symptoms started 48 h after inoculation, both in the positive control and in the treatment with mycelia exposed to VOCs of *T. azevedoi* previously cultured for 96 h. Interestingly, treatments with mycelia exposed to VOCs of *T. azevedoi* with 0 and 24 h of previous cultivation did not show symptoms of white mold (Table 2). In the treatment with mycelia exposed to VOCs of *T. azevedoi* previously cultured for 96 h, the lesions caused by *S. sclerotiorum* measured approximately 18.91 mm in length, while in the positive control, 25.54 mm in length, differing significantly from the 96 h treatment. In addition, lesions in

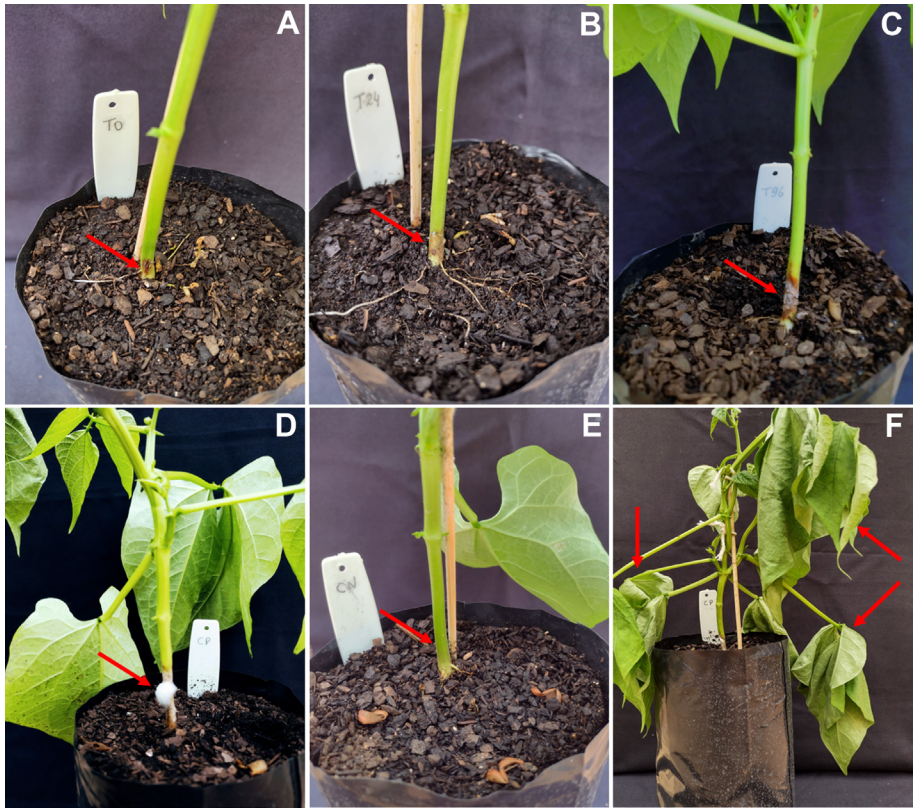

Fig. 3. Action of *Trichoderma azevedoi* CEN1241 volatile organic compounds (VOCs) on white mold severity in common bean plants. (A) Inoculation with mycelia exposed to the VOCs of CEN1241 cultured for 0 h. (B) Inoculation with mycelia exposed to the VOCs of CEN1241 cultured for 24 h. (C) Inoculation with mycelia exposed to the VOCs of CEN1241 cultured for 96 h. (D) Positive control. (E) Negative control. (F) Positive control, plant with symptoms of general wilting and early death. Arrows indicate the severity of the disease on different treatments.

the positive control showed greater virulence of the pathogen, translated into greater intensity of symptoms than plants inoculated with mycelia exposed to VOCs emitted by *T. azevedoi* cultivated for 96 h (Fig. 3). In all plants of the positive control, the presence of white, cottony mycelia, with a vigorous appearance typical of the pathogen, was observed. At the time of evaluation, some plants of this treatment had generalized wilting (Fig. 3F).

Discussion

This study was motivated by circumstantial observations that the age of *Trichoderma* colonies may produce different behaviors with regard to the quantitative and qualitative emission of VOCs, and that this may show better effectiveness of VOCs considering their effect on mycelial growth and severity of *S. sclerotiorum* in bean plants. Recently, we demonstrated *in vitro* that the mycelial inhibition of *S. sclerotiorum* by VOCs is more efficient with colonies of *Trichoderma* spp. in the sporulation phase, compared to colonies in the mycelial growth phase (da Silva et al., 2021a). Based on these results, new research was conducted to verify the effect of the age of the *Trichoderma* colony on its ability to suppress *S. sclerotiorum* by VOCs in a greenhouse.

The richness of genes that encode proteins responsible for the production of secondary metabolites has been verified in the genomes of several species of *Trichoderma*. Among these genes, those encoding VOCs form an important subset (Kubicek et al., 2011; Mukherjee et al., 2012). In this context, studying the profile of VOCs in different growth periods seems to be a promising strategy to better understand the action of these compounds during the fungus's role as a biological control agent. For example, would VOCs already participate in the mechanisms of action of *Trichoderma* since the beginning of its application in the field, or does the *Trichoderma* colony need to develop/sporulate so that these VOCs are emitted in large quantities and, consequently, contribute to biological control of the disease? This is one of the doubts as to how these VOCs can actually constitute one of the mechanisms for controlling plant diseases.

The results of the present study demonstrate that the VOC emission profiles of *T. azevedoi* CEN1241 vary with the time of sampling. It can, therefore, be postulated that the production and diversity of VOCs are altered according to the incubation period and are possibly related to the developmental stage of *T. azevedoi* CEN1241, and that this stage influences the action of VOCs on *S. sclerotiorum*. Similar to the present study, Lazazzara et al. (2021) analyzed the emission of VOCs from *Trichoderma* spp. in the periods of 48 and 72 h and identified greater diversity of VOCs in the time of 48 h. Lee et al. (2015) demonstrated that the age of the *T. atroviride* colony is related to the ability to promote the growth of *Arabidopsis thaliana* and that as the fungi mature, some compounds are lost, while new compounds are produced.

The results of chemical analysis of VOCs emitted by *T. azevedoi* CEN1241 in different growth periods corroborate other studies that indicated the diversity of VOCs produced by species of this fungus genus, among which are: 3-octanone, nonanal, β-bisabolene, β-sesquiphellandrene (Lee et al., 2016); 1-octen-3-ol, 3-octanone (Nieto-Jacobo et al., 2017); 1-octen-3-one (Li et al., 2018); 1-octen-3-ol, β-bisabolene (González-Pérez et al., 2018); 1-octen-3-ol, 3-octanone, β-sesquiphellandrene (Estrada-Rivera et al., 2019); β-bisabolene, β-sesquiphellandrene and acoradiene (Guo et al., 2019); decanal, undecanal, β-cedrene, α-bergamotene, and heptadecane (Kamaruzzaman et al., 2021); 3-octanol, β-bisabolene (Eslahi et al., 2021), tetradecane (You et al., 2022).

Similar to the present study, Rajani et al. (2021) identified the same seven chemical classes with *T. longibrachiatum*. Wonglom et al. (2020) evaluated the emission profile of VOCs from *T. asperellum*, and identified six different chemical classes, but in common with this study, only the classes of alcohols and aldehydes. Guo et al. (2019), when investigating the VOC profiles of *T. harzianum*, *T. hamatum* and *T. velutinum*, demonstrated that the profiles of the three species were highly dependent on the species and predominance of sesquiterpenes. Previous studies have reported that the VOC profiles of different strains can vary significantly (Lee et al., 2016; Nieto-Jacobo et al., 2017; Siddiquee et al., 2012).

The specific action of VOCs synthesized and tested in isolation has already been reported by Intana et al. (2021). These authors demonstrated that synthetic VOCs 1-nonanol, 2-ethylhexanol, 2-methyl-1-butanol, 6-pentyl-2H-pyran-6-pentyl-2-pyrone and phenylethyl alcohol, already identified in *Trichoderma* spp., inhibited the mycelial growth of *Fusarium incarnatum*. Emphasis on 2-ethylhexanol with percentage of inhibition of 74.28%. In a similar way, Elsherbiny et al. (2020), individually demonstrated that VOCs 3-methyl-1-butanol, 6-pentyl-2-pyrone and 2-methyl-1-propanol, emitted by *T. atroviride*, are capable of inhibiting up to 100% *Phytophthora infestans* mycelial growth.

It seems reasonable to state that, in this work, the action of VOCs on the pathogen is not only related to the aging of colonies of *T. azevedoi* CEN1241. For example, a gradual

increase in the emission of 1-octen-3-ol and 3-octanone was observed after 48 h of cultivation. On the other hand, the phenylacetaldehyde compound showed a gradual decrease from time 0 h, not being detected in the period 96 h. In addition, the profile of some compounds, such as undecanal and octanal, kept the emission relatively constant in all the periods in which collections were made. For a large number of compounds, emission was detected only in 48 h cultures, as exemplified by 1-octen-3-one, 1-octen-3-ol, 3-octanone, (*E*)-2-octen-1-ol, phenylethanol, carvomenthone and α-bergamotene. Interestingly, during this period, we observed more pronounced effects of the action of *T. azevedoi* CEN1241 VOCs on *S. sclerotiorum*, mainly in the split-plate experiment. This demonstrates that the qualitative profile of VOCs can also be related to the action on the pathogen.

Inhibition of mycelial growth and mycelogenic germination of *S. sclerotiorum* by VOCs of *Trichoderma* spp. was reported in several studies previously conducted by different research groups: Castillo et al. (2011), Cruz-Magalhães et al. (2019), Ojaghian et al. (2019), Karimi and Altinok (2019), Rajani et al. (2021), and da Silva et al. (2021b). However, until the execution of this study, no reports had been found on the action of *Trichoderma* VOCs in five different periods of cultivation of the biocontrol agent. The choice of growth periods of 0, 24, 48, 72, and 96 h was inferred from previous experiments, in which the double-plate confrontation between *T. azevedoi* CEN1241 and *S. sclerotiorum* CEN1147 lasted approximately 72 to 96 h, compared to the control treatment, colonizing the entire diameter of the Petri dish (da Silva et al., 2020, 2021a).

The results of this study indicate that the mycelial inhibition of *S. sclerotiorum* by VOCs is more effective as the age of the biocontrol agent colony tested here increases. However, the effectiveness shows a downward trend after 96 h, compared mainly with the times of 48 and 72 h. The action of *Trichoderma* through the emission of VOCs can be strain-specific or affected by the substrate used, pH, light, water content, age of culture, antagonistic interaction with other microorganisms or combinations of these factors (da Silva et al., 2021a; González-Pérez et al., 2018; Guo et al., 2019; Lee et al., 2015). It was discussed earlier that toxic secondary metabolites from fungi are secreted by growing colonies near the onset of sporulation (Hicks et al., 1997; Trail et al., 1995). In fact, the data obtained here support this hypothesis, as the best results, regardless of the experiment, were found in the 24, 48, or 72 h periods. The morphological observation of the transition from mycelial growth to the sporulation phase of *T. azevedoi* CEN1241 is observed approximately after 72 h of growth, when it is possible to observe the color change of the antagonist mycelium, from white to greenish and, intense green, after 96 h of growth. The green color in *Trichoderma* colonies is a hallmark feature of the sporulation phase of many species of this genus (Bisset, 1984), including *T. azevedoi* (Inglis et al., 2020).

The methods to evaluate the unique effect of *Trichoderma* VOCs on biological control are, without a doubt, the greatest difficulty for studies on this topic, and many studies are restricted to the double-plate methodology, with the absence of *in vivo* tests. This work represents an advance in the knowledge about the real capacity of *Trichoderma* VOCs to protect bean plants from the pathogenic action of *S. sclerotiorum*. Initially, it was demonstrated, through the split-plate methodology, that VOCs reduce lesions in bean fragments caused by *S. sclerotiorum*, with emphasis on exposure to VOCs emitted at 48 h of cultivation.

We subsequently advanced even further with our inferences about the ability of VOCs to biocontrol white mold. We chose to identify whether the action of *T. azevedoi* CEN1241 VOCs in different growth periods *in vitro* could cause damage to the *S. sclerotiorum* mycelia that would be capable of reducing the severity of the disease in a greenhouse. Interestingly, the severity of white mold on bean plants was drastically affected when the pathogen mycelia were exposed to VOCs at time 0 and 24 h. It is worth noting that a previous study carried out in our laboratory demonstrated that *T. azevedoi* CEN1241 VOCs did not protect lettuce plants from infection caused by *S. sclerotiorum* in the condition in which the pathogen and antagonist were incubated at the same time in a double-plate and, after 96 h, the mycelium was inoculated in lettuce plants (da Silva et al., 2021b). These findings suggest that *S. sclerotiorum* infection may be related to specific characteristics of the plants used in the experiments. For example, lettuce plants have a greater amount of water in their structure and consequently more tender tissues, which would facilitate infection, even with the mycelia of *S. sclerotiorum* weakened by VOCs. Bean plants are more rustic, with a more robust morphological structure, which possibly hampered the pathogenic action of the *S. sclerotiorum* mycelia, already weakened by the action of VOCs *in vitro*. The results obtained support this hypothesis, as the symptoms in the control plants, without exposure to VOCs, were visually more severe, with some plants showing generalized wilting and onset of death seven days after inoculation.

In addition, we observed a curious fact for the time 96 hours. As in the mycelial inhibition experiment, which showed a decrease in the effectiveness of biocontrol in the 96 h period, the greenhouse experiment also showed

that the *S. sclerotiorum* mycelia exposed to *T. azevedoi* CEN1241 VOCs in the 96 h period were able to cause considerable rates of symptoms in inoculated plants, but with less intensity compared to the control treatment. The unique ability of *Trichoderma* spp. in reducing plant disease symptoms has already been verified *in vitro* in the interaction *Phytophthora infestans* × potato (Elsherbiny et al., 2020), *Fusarium incarnatum* × melon (Intana et al., 2021), *Plasmopora viticola* × grapevine (Lazazzara et al., 2021) and *Botrytis cinerea* × tomato (You et al., 2022).

The molecular response of *S. sclerotiorum* to VOCs emitted by *Trichoderma* had already been inferred by Ojaghian et al. (2019). These authors showed that the mechanism of inhibitory action against *S. sclerotiorum* exerted by VOCs from *Trichoderma* spp. alters the regulation of antioxidant enzymes, such as glutathione-S-transferase, concluding that the detoxification capacity of antimicrobial substances may be a key virulence and defense factor of *S. sclerotiorum*. A recent study demonstrated that *T. azevedoi* CEN1241 VOCs can alter genes related to different biological functions of *S. sclerotiorum*, such as melanin production, cell wall, defense, cell respiration, transmembrane transport, pathogenicity, vegetative incompatibility, and stress response (da Silva et al., 2022). It has also been reported that VOCs from *Trichoderma* spp. reduce the width and decrease the density of hyphae of *S. sclerotiorum* (Motlagh and Abolghasemi, 2022; da Silva et al., 2020), and those of *T. asperellum* cause anomalies in the hyphae of *Fusarium incarnatum* (Intana et al., 2021). From these results, it is postulated that VOCs can weaken the pathogen and hinder the infective process in plants and, consequently, reduce the severity of the disease.

Fungi of the genus *Trichoderma* are recognized worldwide as agents of biocontrol of plant diseases and their action is attributed to several mechanisms, which is why it has been nicknamed the "Swiss army knife" among agricultural biocontrol products (Elsherbiny et al., 2020). However, understanding and proving the exclusive action of VOCs is a new and open frontier in antimicrobial research, mainly because the available techniques have limitations that make it difficult to obtain results, from a practical point of view. By using an innovative methodology, the present study clearly demonstrated that the antibiosis mechanism exerted by *Trichoderma* can complement other mechanisms of action, such as parasitism and competition, thus contributing to a better efficiency in the control of white mold in the culture of beans.

The VOC profile of *T. azevedoi* CEN1241 changes with colony age and alters the ability of the biocontrol agent to suppress *S. sclerotiorum*. The greatest amount of VOCs was detected in colonies of *T. azevedoi* CEN1241 cultivated for 96 hours and the greatest diversity with colonies of 48 hours. The VOCs of *T. azevedoi* CEN1241 in the period of 0 hours are less effective for the inhibition of mycelia of the sclerotia of *S. sclerotiorum*. *T. azevedoi* CEN1241 VOCs are able to reduce *in vitro* lesions and white mold severity in common bean plants.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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