Endophytic *Trichoderma* strains isolated from forest species of the Cerrado-Caatinga ecotone are potential biocontrol agents against crop pathogenic fungi

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**Abstract**

The indiscriminate use of chemical pesticides increasingly harms the health of living beings and the environment. Thus, biological control carried out by microorganisms has gained prominence, since it consists of an environmentally friendly alternative to the use of pesticides for controlling plant diseases. Herein, we evaluated the potential role of endophytic *Trichoderma* strains isolated from forest species of the Cerrado-Caatinga ecotone as biological control agents of crop pathogenic fungi. Nineteen *Trichoderma* strains were used to assess the antagonistic activity by *in vitro* bioassays against the plant pathogens *Colletotrichum truncatum*, *Lasiodiplodia theobromae*, *Macrophomina phaseolina*, and *Sclerotium delphinii* isolated from soybean, cacao, fava bean, and black pepper crops, respectively. All *Trichoderma* strains demonstrated inhibitory activity on pathogen mycelial growth, with maximum percent inhibition of 70% against *C. truncatum*, 78% against *L. theobromae*, 78% against *M. phaseolina*, and 69% against *S. delphinii*. Crude methanol extracts (0.5 to 2.0 mg mL\(^{-1}\)) of *Trichoderma* strains were able to inhibit the growth of *C. truncatum*, except *Trichoderma* sp. T3 (UFPIT06) and *T. orientale* (UFPIT09 and UFPIT17) at 0.5 mg mL\(^{-1}\), indicating that the endophytes employ a biocontrol mechanism related to antibiosis, together with multiple mechanisms. Discriminant metabolites of *Trichoderma* extracts were unveiled by liquid chromatography-tandem mass spectrometry-based metabolomics combined with principal component analysis (PCA), which included antifungal metabolites and molecules with other bioactivities. These results highlight the biocontrol potential of *Trichoderma* strains isolated from the Cerrado-Caatinga ecotone against crop pathogenic fungi, providing support for ongoing research on disease control in agriculture.
Introduction

Diseases caused by fungi are among the most harmful to plants due to their rapid spread and the ability to adapt to various environmental conditions [1]. The most commonly used methods for the control of plant diseases caused by fungi include the use of chemical fungicides [1, 2], which have disadvantages such as the potential risk of soil and water contamination, damage to human health, and the development of resistance against fungicides by plant pathogens [2, 3].

Research on alternative methods of controlling fungal diseases has been widely developed, often requiring the integrated implementation of several methods, known as integrated disease management (IDM) [2, 4]. Among these methods are the use of disease-resistant cultivars, adequate water and soil management, fertilization, crop rotations, and biological control agents (BCA), aiming to maintain or increase agricultural production with a reduced application of chemical agents [5]. Many studies still need to be developed for the use of biological control of plant pathogens on a global scale, since biopesticides represent only approximately 2% of all pesticides sold in the world [6, 7].

A promising alternative method for plant pathogen control is based on the use of antagonistic microorganisms, such as endophytic fungi, capable of protecting their hosts from the action of pathogens [8, 9]. These microorganisms live inside plant tissues without causing damage in a complex mutualistic relationship, where endophytes receive nutrients and protection, while plants have advantages, such as greater resistance in environments with intense stress caused by biotic (insects, herbivores, nematodes, and phytopathogenic microorganisms) or abiotic factors (pH, temperature, drought and saline stresses, etc.) [10, 11].

Recent research has revealed that although endophytic microorganisms have received global interest, there are still several gaps in knowledge, such as the different biomes explored [12]. Considering that endophytes depend on host species and environmental conditions, the diversity of biomes and endemic plants found in Brazil represents a potential source of new beneficial microbial resources [13]. In this context, the Cerrado-Caatinga ecotone stands out [14], which corresponds to the transition area where ecological communities or ecosystems from the Cerrado and Caatinga biomes coincide [15].

The Cerrado and Caatinga biomes are recognized for their great importance. The Cerrado, also known as the Brazilian savanna, is one of the 25 biodiversity hotspots for conservation priorities in the world [16, 17], and the Caatinga is the only uniquely Brazilian biome, in which most of its biological heritage cannot be found anywhere else in the world [18]. The Cerrado-Caatinga ecotone occupies 1.3% of the Brazilian territory, extending over regions of the Piauí, Bahia, and Minas Gerais states [14], and it presents great species richness, whether from the biomes that formed them or endemic species [15]. Few studies have been carried out to explore the biocontrol potential of endophytic fungal biodiversity in this transition zone, which also requires attention due to increasing anthropogenic degradation with the expansion of agricultural production areas [19].

Trichoderma species have been tried as BCA and used as an alternative to synthetic pesticides to control a variety of plant diseases [20]. The biocontrol mechanisms of Trichoderma are based on the activation of multiple mechanisms, either indirectly, by competing for space and nutrients, promoting plant growth and plant defensive mechanisms, and antibiosis, or directly, by mycoparasitism [21, 22]. They are found in rhizospheric and non-rhizospheric soils, in addition to their endophytic relationships with many plants [22, 23]. Their biodiversity has been extensively investigated in various geographical locations, and their distribution varies with ecosystems [24, 25]. Therefore, it is fundamental to explore the biocontrol potential of
Trichoderma strains isolated from native areas, since they represent a tool for sustainable food production.

In this study, we investigated the potential role of endophytic Trichoderma strains isolated from forest tree species of the Cerrado-Caatinga ecotone [26, 27] as biological control agents of crop pathogenic fungi. First, we evaluated the interaction between endophytes and pathogens by *in vitro* antagonism bioassays. The biocontrol factor related to antibiosis was examined using *in vitro* bioassays with crude methanolic extracts of Trichoderma strains and liquid chromatography-tandem mass spectrometry-based metabolomic approaches. Such data will support ongoing research to find new beneficial microbial resources to control plant diseases.

Material and methods
Strains and materials
The nineteen *Trichoderma* spp. isolates (S1 Table) were obtained from leaves of forest tree species (S2 Table), located in a Cerrado-Caatinga ecotone in Southwest Piauí, Brazil (8°51’7.48” S and 44°11’39.95” W) [26], and maintained in potato dextrose agar (PDA) culture medium (Himedia). This area comprised a fragment of one hectare within the legal reserve [26]. For the identification of the *Trichoderma* isolates, the gene regions for the translation elongation factor (tef1) and the second largest RNA polymerase subunit (rpb2) were amplified and sequenced, and the construction of phylogenetic trees was performed by comparing the sequences available in GenBank (National Center for Biotechnology Information, NCBI) [27].

The UFPI01, UFPI09, UFPI12, UFPI14, UFPI15, UFPI17, and UFPI18 isolates were previously identified as *T. orientale* (Samuels & Petrini) Jaklitsch & Samuels; the UFPI02 isolate was previously identified as *T. longibrachiatum* Rifai; and the UFPI03, UFPI07, UFPI10, UFPI16, and UFPI19 isolates were previously identified as *T. koningiopsis* Samuels, Carm. Suárez & H.C. Evans [27]. The UFPI04, UFPI05, UFPI06, UFPI08, UFPI11, and UFPI13 isolates were not identified by comparing the sequences available in GenBank and may likely constitute new species; therefore, they were named *Trichoderma* sp. T1, *Trichoderma* sp. T2, *Trichoderma* sp. T3, *Trichoderma* sp. T4, *Trichoderma* sp. T5, and *Trichoderma* sp. T6, respectively [27]. The strains were registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) by n˚ A7580C1 and A1B50F7, as recommended by the Brazilian Biodiversity Law (n˚ 13.123/15).

For plant pathogenic fungi tested for antagonism bioassays, *Colletotrichum truncatum* (Schwein.) Andrus & W.D. Moore strain was isolated from infected soybean pods, located in the same mesoregion where *Trichoderma* strains were found, through the cultivation of infected material in PDA medium incubated at 25˚C under a 12 h photoperiod [28]. The *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. strain was isolated from cacao fruit with symptoms of Lasiodiplodia canker. *Macrophomina phaseolina* (Tassi) Goid. COUFPI 10 and COUFPI 11 strains were isolated from the seeds and roots of fava bean, respectively, placed in PDA medium and incubated at 25˚C for seven days [29]. *Sclerotium delphinii* Welch COUFPI 209 and COUFPI 249 strains were isolated from black pepper with symptoms of concentric leaf spots by inoculating sclerotia in PDA medium [30]. All strains were maintained in PDA culture medium at 28˚C in the absence of light and preserved using Castellani’s method.

Liquid chromatography–mass spectrometry (LC–MS)-grade methanol and acetonitrile were purchased from J.T. Baker (Center Valley, PA, USA). Analytical grade formic acid and sodium formate encephalin were purchased from J.T. Baker (Center Valley, PA, USA), and leucine enkephalin from Waters (Manchester, UK).
**In vitro antagonism bioassays against plant pathogenic fungi**

Culture medium fragments (1 cm$^2$) with *Trichoderma* spp. mycelia and fungal plant pathogens (1 cm$^2$), previously cultivated in PDA at 28˚C, were transferred to PDA medium 5 cm apart from each other [31]. The plates were incubated at 28˚C, and mycelial growth was evaluated daily for seven days. The experimental design was completely randomized with 20 treatments and 3 replications, totaling 60 experimental units for the bioassays of each plant pathogen. The treatments consisted of 19 *Trichoderma* isolates plus a control sample containing only the plant pathogen.

The antagonistic potential was measured as the percent inhibition, according to the formula: % inhibition = (DC-DT/DC)′100, where DT is the growth radius of the plant pathogen colony toward the antagonist and DC is the growth radius of the control [32]. The mycelial growth rate index (MGRI) was obtained from the averages of the daily values of mycelial growth for each treatment, according to the formula MGRI = Σ(D-Da)/N, where D = current average colony diameter, Da = average colony diameter from the previous day, and N = number of days after inoculation [33]. Analysis of variance followed by the Scott–Knott test at the 5% significance level was conducted using R v.3.5.2 software (R Core Team, Vienna, Austria).

**Inhibitory activity bioassay of *Trichoderma* spp. organic extracts against *C. truncatum***

For the extraction of bioactive compounds, *Trichoderma* strains were inoculated on PDA at 28˚C in the absence of light for four days. Subsequently, the culture media (60 x 15 mm) containing the fungal colonies were cut into small pieces, and cold methanol (15 mL) was added. The samples were vortexed for 1 min, allowed to rest for 5 min, and vortexed again for 1 min. Subsequently, the extracts were centrifuged at 4,000 g for 15 min at 4˚C, and the supernatants were concentrated under a flow of nitrogen gas [34]. Then, the extracts were weighed, and dimethylsulfoxide (DMSO) was added to prepare a 100 mg mL$^{-1}$ stock solution.

Methanolic extracts of *Trichoderma* spp. were used to evaluate the inhibitory activity against *C. truncatum*. For this purpose, fragments of the phytopathogen (9 mm$^2$) were inoculated in the center of Petri dishes (60 x 15 mm) containing PDA with increasing concentrations of the extract (0.0, 0.5, 1.0, and 2.0 mg mL$^{-1}$). For the control (0.0 mg mL$^{-1}$), only DMSO was added. Plates were kept in B.O.D. incubator (Bio-Oxygen Demand) at 28˚C in the absence of light, and colony diameters were measured daily for 10 days with the aid of a digital caliper. The experimental design was completely randomized with extracts from 19 isolates at concentrations of 0.0, 0.5, 1.0, and 2.0 mg L$^{-1}$, with three replications for each concentration. Data were subjected to analysis of variance, followed by regression analysis using R software and SigmaPlot v11.0 (Systat Software Inc. Chicago, USA). Additionally, Pearson’s correlation analysis of the percent inhibition of *Trichoderma* strains against *C. truncatum* in co-culture and crude extract bioassays was performed.

**Metabolic fingerprinting by liquid chromatography–high resolution mass spectrometry**

**Sample preparation.** For extract preparation of the 19 *Trichoderma* isolates, the culture media (60 x 15 mm) containing the fungal colonies, previously cultivated on PDA at 28˚C for four days, were cut into small pieces and extracted with methanol (15 mL), vortexed for 1 min, maintained at rest for 5 min, and vortexed again for 1 min. After filtration, the supernatants were concentrated to approximately 1 mL, lyophilized, and stored at -47˚C until use.
analyses by ultra-high-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC-ESI-Q-TOF-MS), lyophilized samples were reconstituted in a solution containing water/methanol/acetonitrile (1:2.2 v/v/v, 1 mL). The samples were vortexed for 1 min, sonicated for 30 min at room temperature, filtered using a 0.22 μm PTFE syringe filter (Millipore, USA), and transferred to vials for LC–MS analysis [35].

UHPLC-ESI-Q-TOF-MS analysis. An ACQUITY UPLC connected to a XEVO-G2XS QTOF mass spectrometer (Waters, Manchester, UK) equipped with an electrospray ion source was used. Liquid chromatography was performed using a Titan™ C18 UHPLC column (2.1 x 100 mm, 1.9 μm, Supelco). The column temperature was maintained at 45˚C. The separation was performed at a flow rate of 0.4 mL min⁻¹ under a gradient program in which the mobile phase consisted of (A) 0.1% formic acid (v/v) and (B) pure methanol. The gradient program was applied as follows (in % B): (t) = 0 min, 1%; t = 2.0 min, 1%; t = 8.0 min, 38%; t = 20 min, 99.5%; t = 25 min, 99.5%; t = 25.1 min, 1%; and t = 28 min, 1%, for a total analysis time of 28 minutes. The injection volume was 0.2 μL. Positive ion mode was recorded, and the instrument was operated in data-independent acquisition mode (MS²). The m/z range was 100–1700, with an acquisition rate of 0.5 sec per scan. The following instrumental parameters were used: capillary: 3.0 kV; cone: 40,000 V; desolvation temperature: 550˚C; cone gas flow: 10 L h⁻¹; desolvation gas flow: 900 L h⁻¹. The collision energy was 20 to 60 eV for fragmentation. Leucine encephalin (molecular weight = 555.62; 200 pg μL⁻¹ in 1:1 acetonitrile:water) was used as the lock mass for accurate mass measurements, and a 0.5 mM sodium formate solution was used for calibration. Samples were randomly analyzed.

Data processing and statistical data analysis. LC–MS raw data were processed using Progenesis QI 2.0 software (Nonlinear Dynamics, Newcastle, UK), which enabled the selection of possible adducts, peak alignment, deconvolution, and putative metabolite identification based on MS² experiments. Progenesis QI generates a table of the ions labeled according to their nominal masses and retention times as a function of their intensity for each sample. The MassBank database (https://massbank.eu) and Vaniya/Fiehn Natural Products Library (https://mona.fiehnlab.ucdavis.edu/) were used to perform the identification using the following search parameters: precursor mass error < 5 ppm and fragment tolerance ≤ 10 ppm.

The list of extracted ion chromatograms by retention time was uploaded to the MetaboAnalyst 5.0 web platform (http://www.metaboanalyst.ca) for principal component analysis (PCA). Ions detected in at least 10% of the samples were retained for analysis, and an interquartile range (IQR) filter was used. Data were sum-normalized, and Pareto scaling was used. A heatmap and unsupervised hierarchical clustering were performed using 50 features with the lowest adjusted p value < 0.05 depicting differential peaks.

Results and discussion
Endophytic Trichoderma strains from forest species inhibit several crop pathogenic fungi
The antagonistic potential of the endophytic Trichoderma spp. strains was investigated against different plant pathogens. The 19 Trichoderma spp. isolates demonstrated inhibitory activity against mycelial growth, ranging from 50 to 70% for C. truncatum (Fig 1A), 30 to 78% for L. theobromae (Fig 1B), 49 to 78% for M. phaseolina COUFPI 10 (Fig 1C), 58 to 74% for M. phaseolina COUFPI 11, 6 to 62% for S. delphinii COUFPI 209 (Fig 1D), and 2 to 69% for S. delphinii COUFPI 249.

Regarding the inhibition of C. truncatum (Fig 1A), the fungi T. orientale (UFPIT01, UFPIT09, UFPIT14, UFPIT15, and UFPIT17), T. longibrachiatum (UFPIT02), T. koningiopsis...
Trichoderma sp. T3 (UFPIT06), Trichoderma sp. T4 (UFPIT08), and Trichoderma sp. T6 (UFPIT13) stood out with the highest percent inhibition, from 63 to 70%, and Trichoderma sp. T5 (UFPIT11) and T. orientale (UFPIT12), with the lowest values ranging from 50 to 54%.

Fig 1. In vitro percent inhibition of Trichoderma spp. isolates against C. truncatum (A), L. theobromae (B), M. phaseolina COUFPI 10 and COUFPI 11 (C), and S. delphinii COUFPI 209 and COUFPI 249 (D). Averages followed by the same lowercase or capital letter are not significantly different by the Scott–Knott test at the 5% confidence level. The variation coefficients (CVs) were 4.67% for L. theobromae, 4.55% for M. phaseolina COUFPI 10, 5.96% for M. phaseolina COUFPI 11, 8.37% for S. delphinii COUFPI 209 and 6.02% for S. delphinii COUFPI 249.
and UFPIT18) showed the highest percentage inhibition against *L. theobromae* (Fig 1B) of 73–78%, while *T. orientale* (UFPIT09 and UFPIT12) had the lowest performance of 30–32%.

Against *M. phaseolina* COUFPI 10 (Fig 1C), *T. orientale* UFPIT01 and UFPIT12 yielded the highest (78%) and lowest (49%) percent inhibition, respectively, while the other isolates showed intermediate values, above 50% inhibition. *T. orientale* (UFPIT01, UFPIT09, UFPIT14, UFPIT15, UFPIT17, UFPIT18), *T. longibrachiatum* (UFPIT02), *Trichoderma* sp. T6 (UFPIT13), and *T. koningiopsis* (UFPIT16 and UFPIT19) stood out in inhibiting the growth of *M. phaseolina* COUFPI 11, from 67 to 74%, and the others, with lower values, ranging from 58 to 66% inhibition, did not differ statistically.

The inhibitory activity against *S. delphini* COUFPI 209 (Fig 1D) was highest in *Trichoderma* sp. T3 UFPIT06 (61%) and *T. koningiopsis* UFPIT16 (62%) and lowest in *T. orientale* UFPIT09 (6%) and *T. orientale* UFPIT17 (8%), the others ranged from 22 to 56%. In *S. delphini* COUFPI 249 (Fig 1D), the fungi *T. orientale* (UFPIT14 with 67% and 69%, respectively) and *Trichoderma* sp. T3 UFPIT06 (69%) presented the best results, with *T. orientale* (UFPIT09, UFPIT12, and UFPIT17 with 3, 2 and 3%, respectively) showing less effectiveness.

Most endophytes reduced the MGRI of the plant pathogen colonies, differing statistically from the control treatments (S1 Fig), except for *S. delphini* COUFPI 249 paired with *T. orientale* UFPIT09. All isolates stood out in reducing the MGRI for *C. truncatum* (S1A Fig), with the highest indices observed for *Trichoderma* sp. T1 (UFPIT04), *T. orientale* (UFPIT12), *Trichoderma* sp. T6 (UFPIT13), and *T. koningiopsis* (UFPIT19) strains. Against *L. theobromae*, only the isolate *T. orientale* (UFPIT12) showed the highest MGRI (S1B Fig).

*T. longibrachiatum* (UFPIT02), *T. koningiopsis* (UFPIT03 and UFPIT10), *Trichoderma* sp. T2 (UFPIT05), *Trichoderma* sp. T3 (UFPIT06), *T. orientale* (UFPIT09, UFPIT14, UFPIT15, and UFPIT17), *Trichoderma* sp. T5 (UFPIT11), and *Trichoderma* sp. T6 (UFPIT13) stood out in reducing the MGRI of *M. phaseolina*, while *T. koningiopsis* (UFPIT03 and UFPIT16) stood out against *M. phaseolina* COUFPI 11 (S1C Fig). The MGRI of *S. delphini* COUFPI 209 was reduced for all isolates, and the highest indices were obtained when paired with *T. orientale* (UFPIT09, UFPIT12, and UFPIT17), while for *S. delphini* COUFPI 249, *Trichoderma* sp. T1 (UFPIT04) and *Trichoderma* sp. T6 (UFPIT13) showed the highest MGRI (S1D Fig).

Several studies have shown the efficacy of *Trichoderma* strains against *C. truncatum*. The species *T. harzianum* and *T. asperellum* showed percent inhibition of 75 and 73%, respectively, against this pathogen [36]. *T. virens, T. longibrachiatum*, and *T. koningii* also inhibited the growth of *C. truncatum* with %inhibition of 54 to 81% [37]. Commercial formulations based on *T. viride, T. harzianum*, and *T. hamatum* promoted %inhibition ranging from 67 to 81% [38]. In our work, similar results were obtained against this pathogen for the species *T. orientale, T. longibrachiatum, T. koningiopsis* and unidentified *Trichoderma* isolates.

The species *T. harzianum*, *T. asperellum*, *T. atroviride*, and *T. virens* showed percent inhibition in the range of 29 to 54% against *L. theobromae* [39], while *T. koningii* and *T. virens* reached 75 to 80% [40]. *T. pseudokoningii, T. hamatum, T. koningii*, and *T. reesei* also significantly inhibited pathogen growth by 62 to 90% [41]. These values corroborate the %inhibition observed in our study; however, to our knowledge, there are no reports of studies about the biocontrol potential of *T. orientale, T. koningiopsis* and *T. longibrachiatum* against *L. theobromae*.

In previous studies, *T. longibrachiatum* showed a percent inhibition of 58% against *M. phaseolina* [42], while *T. koningiopsis* strains ranging from 15 to 70% [43]. Similar results were obtained in our study for these species; however, there are no reports about *T. orientale* against *M. phaseolina*. Swain et al. (2021) investigated the biocontrol potential of *T. erinaceum* and *T. hebeiensis* against *S. delphini* and found a percent inhibition of approximately 75%, which is the
only study of growth inhibition of this pathogen using *Trichoderma* strains [44]. This is the first report that demonstrates the biocontrol potential of the species *T. orientale, T. koningiopsis* and *T. longibrachiatum* against *S. delphii*.

The high percentage of inhibition may be related to the rapid growth of *Trichoderma* spp., which often completely overlap the colonies of *C. truncatum* (S2 Fig), *L. theobromae* (S3 Fig), and *M. phaseolina* COUFPI 10 (S4 Fig) and COUFPI 11 (S5 Fig). The inhibition may also be related to the efficacy of *Trichoderma* spp. in competing for space and nutrients and in parasitizing pathogens [45]. *S. delphii* COUFPI 209 (S6 Fig) and COUFPI 249 (S7 Fig) were very aggressive when competing with *Trichoderma* spp. by space and nutrients; in some cases, they even grew on endophyte colonies. Antibiosis is also an action mechanism present in endophytic fungi of the *Trichoderma* genus, which produce several secondary metabolites with antimicrobial activity used to inhibit the development of plant pathogens [46]. Thus, this action mechanism may also be occurring, justifying the high % inhibition achieved by *Trichoderma* spp.

Several studies have demonstrated the ability of *Trichoderma* strains to inhibit the growth of plant pathogens through antibiosis mechanism [46, 47]. Among the secondary metabolites of *Trichoderma* with antimicrobial activity are syringaresinol [48], HT-2 toxin [49], trigonelline [50], *trans*-zeatin [51], koninginin A [52], koninginin D [53], koninginin E [52], 6-pentyl-α-pyrene [10], gliotoxin, gliovirin, crispanol, pyrone, 6-pentyl-2H-pyran-2-one, harzianic acid, koningic acid [53], alamethicin, and dermadin [53].

To investigate the antibiosis mechanism performed by *Trichoderma* spp. isolates, we also evaluated whether *Trichoderma* spp. methanolic extracts had inhibitory activity against one of the plant pathogens. For this purpose, we selected the fungus *C. truncatum*, the causal agent of anthracnose in soybeans, which is economically relevant. As a result, antifungal activity increased with increasing concentrations of the methanolic extracts, potentiating the % inhibition of the pathogen (S8 Fig). At concentrations of 0.5, 1.0, and 2.0 mg mL\(^{-1}\), there was an increase in the % inhibition of *C. truncatum* when compared to the dose of 0.0 mg mL\(^{-1}\) for all isolates. However, no significant difference from the concentration of 0.5 mg mL\(^{-1}\) was observed for four of the isolates (*T. longibrachiatum* (UFPIT02), *Trichoderma* sp. T3 (UFPIT06), *Trichoderma* sp. T4 (UFPIT08), and *T. orientale* (UFPIT17)).

The extracts of *T. koningiopsis* (UFPIT10) and *Trichoderma* sp. T5 (UFPIT11) showed the highest % inhibition, differing statistically from the other isolates at a concentration of 2 mg mL\(^{-1}\) (S8 Fig). Interestingly, *T. koningiopsis* (UFPIT10) and *Trichoderma* sp. T5 (UFPIT11) did not show the highest activities in the pairing co-culture bioassay (Fig 1A), although both presented % inhibition higher than 50%. The divergence of results may be explained by the variation between the performance of *Trichoderma* isolates of the same species in *in vitro* and *in vivo* bioassays, since the biological control mechanisms of fungi can occur simultaneously, affecting their action [21, 22].

Correlation analysis between co-culture and crude extract bioassays indicated that *Trichoderma* spp. (UFPIT05, UFPIT08, UFPIT11, and UFPIT13), *T. koningiopsis* (UFPIT07, UFPIT10, UFPIT16, and UFPIT19), *T. orientale* (UFPIT12 and UFPIT15), and *T. longibrachiatum* (UFPIT02) had a positive linear relationship, with emphasis on *Trichoderma* sp. T4 (UFPIT08) and *T. longibrachiatum* (UFPIT02), which presented r values of 0.97 and 0.96, respectively (S3 Table). On the other hand, *Trichoderma* sp. T1 (UFPIT04), *Trichoderma* sp. T3 (UFPIT06), *T. koningiopsis* (UFPIT03), and *T. orientale* (UFPIT01, UFPIT09, UFPIT14, UFPIT17, and UFPIT18) showed negative correlations, with emphasis on *Trichoderma* sp. T3 UFPIT06 (r = -1.00), indicating that other biocontrol mechanisms prevailed in relation to antibiosis.
Untargeted metabolomic analysis revealed antimicrobial metabolites of *Trichoderma* strains from forest species

The metabolic content of the methanolic extracts of all *Trichoderma* spp. isolates (S8 Fig) were explored by PCA to correlate with the efficiency of inhibition. The PCA showed that 54.9% of the total variation in the data were represented by the first two principal components (Fig 2A). Although a great overlap of the species was observed, samples of *Trichoderma* spp. isolates were clearly separated from the control samples. Clustering of species was performed according to the similarity of their metabolomic profiles and resulted in two large clusters. In the first, with negative scores for PC1, *T. longibrachiatum* UFPIT02 (T2) and *T. orientale* UFPIT01 (T1), UFPIT14 (T14), and UFPIT18 (T18) overlapped, and *T. orientale* UFPIT15 (T15) remained close to this group, overlapping with *T. longibrachiatum* UFPIT02 (T2) and *T. orientale* UFPIT14 (T14).

A second clustering, with majority positive scores for PC1, was formed by the species *T. orientale* (UFPIT12 and UFPIT17) and *T. koningiopsis* (UFPIT03, UFPIT07, UFPIT10, UFPIT16, and UFPIT19) and unidentified isolates *Trichoderma* sp. T1 (UFPIT04), *Trichoderma* sp. T2 (UFPIT05), *Trichoderma* sp. T3 (UFPIT06), *Trichoderma* sp. T4 (UFPIT08), *Trichoderma* sp. T5 (UFPIT11), and *Trichoderma* sp. T6 (UFPIT13). *T. orientale* UFPIT09 (T9), located near the zero value of PC1, remained intermediate between these two large clusters of species. The PC1 x PC3 score plot (Fig 2B) revealed some clusters similar to those observed in the PC1 x PC2 score plot; however, a new group stood out, with positive scores for PC1, formed by *T. koningiopsis* UFPIT03 (T3), UFPIT07 (T7), UFPIT10 (T10), and UFPIT16 (T16), partially overlapping with *T. koningiopsis* UFPIT19 (T19).

Altogether, PCA showed that *Trichoderma* spp. from the same species can produce different secondary metabolites, and isolates from different species can produce similar molecules. In the loading plot, the metabolites produced by *Trichoderma* spp. isolates are displayed, and the most distant points represent the metabolites that most influenced the clustering. Molecular signatures of *Trichoderma* spp. isolates were identified according to the elution order, MS/
MS fragmentation pattern, molecular formula, and database search. A total of 16 molecules were identified (Table 1 and S11 Fig).

The metabolite of m/z 264.10862 was found in all Trichoderma spp. isolates and identified as 2-O-methyladenosine (Table 1, metabolite 1), a member of the adenosine class that has

Table 1. Secondary metabolites identified in Trichoderma strains using UPLC-ESI-Q-TOF-MS.

| No. | m/z     | Retention time (min) | Adduct     | MS/MS Fragment masses | Molecular formula | Exact mass | Putative identification | Δ m/z (ppm) | Strains | Biological activity | References |
|-----|---------|----------------------|------------|-----------------------|-------------------|------------|-------------------------|-------------|---------|---------------------|------------|
| 1   | 264.1086 | 0.68                 | [M +H-H₂O]⁺ | 69.0328, 84.0443, 139.0881, 150.9268 | C_{11}H_{15}N_{5}O_{4} | 281.1119 | 2-O-Methyladenosine       | 1.78        | All     | Anti-inflammatory     | [54]       |
| 2   | 118.0863 | 0.86                 | [M+H]⁺     | 59.0705, 60.0838, 99.0061 | C_{6}H_{11}NO₂ | 117.0790 | Glycine-Betaine          | 0           | All     | Plant growth promoter | [55]       |
| 3   | 138.0553 | 0.91                 | [M+H]⁺     | 65.0380, 78.0338, 92.0496, 93.0572, 138.0562 | C_{7}H_{12}NO₂ | 137.0480 | Trigonelline             | -2.19       | UFPIT02-UFPIT05, UFPIT07-UFPIT11, UFPIT13-UFPIT19 | Plant growth promoter, antibacterial | [56, 57] |
| 4   | 220.1196 | 4.63                 | [M+H]⁺     | 97.0356               | C_{10}H_{13}NO   | 219.1123 | Trans-Zeatin             | -1.37       | UFPIT06, UFPIT07, UFPIT10, UFPIT12 | Plant growth promoter, antibacterial, antifungal | [51, 58–60] |
| 5   | 237.1126 | 6.80                 | [M +H-H₂O]⁺ | 215.0709, 235.1137, 249.0980 | C_{7}H_{16}O_{3} | 254.1159 | Phomalone                | -1.96       | All     | Antibacterial, antifungal, cytotoxic | [61]       |
| 6   | 247.0957 | 10.34                | [M+H]⁺     | 56.9367, 162.0252, 166.0648 | C_{14}H_{14}O_{4} | 246.0895 | Columbianetin            | -1.22       | UFPIT01, UFPIT02, UFPIT09, UFPIT14, UFPIT15, UFPIT18 | Antibacterial, antifungal | [62–65] |
| 7   | 265.1423 | 10.55                | [M+H]⁺     | 173.0774, 189.0489, 195.0886, 245.0917 | C_{15}H_{20}O_{4} | 264.1352 | Abscisic acid            | 3.78        | UFPIT04, UFPIT13 | Plant growth promoter, antioxidant, antibacterial, antifungal | [66–68] |
| 8   | 419.1713 | 10.83                | [M+H]⁺     | 186.0499, 204.1036, 441.1527 | C_{12}H_{26}O_{8} | 418.1640 | Syringaresinol           | -2.87       | UFPIT01, UFPIT02, UFPIT13, UFPIT14, UFPIT15, UFPIT18 | Antibacterial, antifungal, anti-inflammatory | [48, 69] |
| 9   | 281.1754 | 12.28                | [M+H]⁺     | 123.0814, 133.0655, 160.0524, 175.0431, 177.0254, 245.1556, 263.1660 | C_{16}H_{24}O_{4} | 280.1688 | Brefeldin-A              | -4.64       | UFPIT03, UFPIT07, UFPIT10, UFPIT16, and UFPIT19 | Antiviral, antifungal, antitumoral | [70, 71] |
| 10  | 305.1721 | 13.93                | [M+Na]⁺    | 147.0131, 153.0918, 161.0294, 225.0098, 255.1513, 259.1615, 276.1381 | C_{16}H_{26}O_{4} | 282.1837 | Koninginin E             | -2.13       | UFPIT03, UFPIT07, UFPIT10, UFPIT16, and UFPIT19 | Plant growth promoter, antifungal | [52]       |
| 11  | 307.1882 | 14.11                | [M+Na]⁺    | 133.0656, 267.1297, 289.1688 | C_{16}H_{28}O_{4} | 284.1993 | Koninginin A             | -1.76       | UFPIT03, UFPIT07, UFPIT10, UFPIT16, and UFPIT19 | Plant growth promoter, Antifungal | [52, 72] |

(Continued)
been isolated from the mycelium of *Cordyceps sinensis* [54]. The metabolite of m/z 118.0863 [M+H]+ was detected in all *Trichoderma* spp. isolates and identified as glycine-betaine (Table 1, metabolite 2), presenting in the MS/MS spectrum the fragment of m/z 59.0705 corresponding to (CH3)3N+ [55]. Betaines are naturally occurring metabolites fundamental to the mitigation of osmotic stress in plants and macro- and microorganisms [55]. The metabolite of m/z 138.0553 [M+H]+ was identified as trigonelline (Table 1, metabolite 3) and was detected in *T.* longibrachiatum (UFPIT02), *Trichoderma* sp. T2 (UFPIT05), *T.* koningiopsis (UFPIT07), *Trichoderma* sp. T5 (UFPIT11), *Trichoderma* sp. T6 (UFPIT13), and *T.* koningiopsis (UFPIT19) isolates. The MS/MS spectrum was characterized by the fragment of m/z 92.0496 [–HCOOH]+, referring to the carboxylic acid group [56, 57]. This alkaloid is widely used in medicine to protect the liver and heart and to treat hypercholesterolemia [81] and has already been identified in *T. asperellum* fermentation cultures [50].

| No. | m/z     | Retention time (min) | Adduct | MS/MS Fragment masses | Molecular formula | Exact mass | Putative identification | Δ m/z (ppm) | Strains | Biological activity | References |
|-----|---------|----------------------|--------|-----------------------|-------------------|-----------|------------------------|-------------|---------|---------------------|------------|
| 12  | 453.1914| 14.23                | [M+H-H2O]+ | 147.0929, 154.0670, 174.1137, 212.2395, 225.0931, 263.1654, 281.1769, 328.2860, 373.2012, 374.2914, 413.1963, 431.2048 | C26H30O6         | 470.1947  | Physodic acid           | -1.28       | All     | Antibacterial        | [73]       |
| 13  | 447.2001| 14.43                | [M+Na]+  | 215.0365, 233.0460, 263.0544, 285.1583, 303.1583, 429.1891, 447.1993 | C22H17O6         | 424.2110  | HT-2 Toxin              | -3.07       | UFPIT07 and UFPIT10  | Mycotoxin  | [74]       |
| 14  | 318.3013| 16.01                | [M+H]+   | 97.9455, 150.0253, 264.2691, 282.2798, 286.2755, 294.2803 | C14H15NO3        | 317.2940  | Phytosphingosine        | -3.15       | UFPIT01, UFPIT02, UFPIT09 and UFPIT17 | Anti-inflammatory, antibacterial | [75–77] |
| 15  | 163.0393| 16.54                | [M+H]+   | 120.9752, 121.0289, 135.0442, 163.0393 | C4H6O3           | 162.0321  | 4-Hydroxycoumarin       | -2.47       | All     | Antifungal, antibacterial, antioxidant, antitumoral | [78, 79] |
| 16  | 338.3413| 20.72                | [M+H]+   | 97.1011, 100.0764, 109.1018, 111.0814, 114.0919, 121.023, 123.0819, 125.0974, 128.1070, 135.1178, 139.0942 | C8H4O3           | 337.3341  | Erucamide               | 1.19        | All     | Antibacterial        | [80]       |
The metabolite of m/z 220.1196 was identified as trans-zeatin (Table 1, metabolite 4), and it was detected in *Trichoderma* sp. T3 (UFPIT06), *T. koningiopsis* (UFPIT07 and UFPIT10), and *T. orientale* (UFPIT12) isolates. This cytokinin, previously reported in *Trichoderma* strains, can be used for plant growth stimulation and affects host plant phytohormones to enhance plant resistance against pathogens [50, 58]. The metabolite of m/z 237.1126 [M+H]⁺, identified as phomalone (Table 1, metabolite 5) and detected in all *Trichoderma* spp., is a common metabolite in many fungal species, with anti-inflammatory, antibacterial, antifungal, and antialgal activities [61]. The metabolite of m/z 247.0957 detected in *T. orientale* (UFPIT01), *T. longibrachiatum* (UFPIT02), and *T. orientale* (UFPIT09, UFPIT14, UFPIT15, and UFPIT18) isolates was identified as columbainetin (Table 1, metabolite 6), a phytoalexin with diverse biological activities [61], and that has been extracted from cultures of an endophytic strain of *Annulohypoxylon ilanense* [48].

The metabolite of m/z 265.1423 was identified as abscisic acid (Table 1, metabolite 7), a phytohormone directly involved in plant-microorganism interactions, improving the defense system and plant development [66], and it was detected only in *Trichoderma* sp. T1 (UFPIT04) and *Trichoderma* sp. T6 (UFPIT13) isolates. The metabolite of m/z 419.1713 detected in *T. orientale* (UFPIT01), *T. longibrachiatum* (UFPIT02), *Trichoderma* sp. T6 (UFPIT13), and *T. orientale* (UFPIT14, UFPIT15, and UFPIT18) isolates were identified as syringaresinol (Table 1, metabolite 8), a lignan that has been found to be a secondary metabolite of an endophytic strain of *A. ilanense* [48]. The metabolite of m/z 281.1754, present in *T. koningiopsis* (UFPIT03, UFPIT07, UFPIT10, UFPIT16, and UFPIT19), was identified as brefeldin-A (BFA) (Table 1, metabolite 9) and showed MS/MS spectrum with fragments of m/z 263.1660 [M + H - H₂O]⁺ and 245.1556 [M + H - 2H₂O]⁺ formed by the BFA dehydration pathway [70]. This metabolite is an antibiotic already isolated in several fungal genera, such as *Alternaria, Ascochyta, Penicillium, Curvularia, Cercospora*, and *Phylllosticta*. BFA has been reported to have important bioactivities, such as antibiotics, antivirals, cytostatics, antimitotics and antitumors [70].

The metabolites of m/z 305.1721 and m/z 307.1882 were identified as koninginin E and koninginin A, respectively (Table 1, metabolites 10 and 11). Koninginins are secondary metabolites belonging to the group of polyketides that are bioactive against several plant pathogens. Koninginin E has already exhibited activity against *Gaeumannomyces graminis* var. tritici, while koninginin A already exhibited activity against *G. graminis* var. tritici, *F. oxysporum*, *F. solani* and *Alternaria panax* [52]. Koninginins A and E were detected in *T. koningiopsis* (UFPIT03, UFPIT07, UFPIT10, UFPIT16, and UFPIT19 isolates). The metabolite of m/z 453.1914 was identified as phytosphingosine (Table 1, metabolite 12) and was detected in all *Trichoderma* spp. Physodic acid is a metabolite belonging to the depside group, and its antibacterial activity against *S. aureus* has been previously reported [73]. The metabolite of m/z 447.2001, detected in *T. koningiopsis* (UFPIT07 and UFPIT10), was identified as HT-2 toxin (Table 1, metabolite 13). The MS/MS spectrum was characterized by fragments of m/z 215.0365 [HT2—isoval acid—acetic acid—H₂O - CH₂O + H]⁺, 233.0460 [C₁₄H₁₆O₃ + H]⁺ and 263.0544 [HT2—isoval acid—acetic acid + H]⁺ [74]. HT-2 toxin is a secondary metabolite found mainly in fungi of the *Fusarium* genus and is classified as a trichotheccene type A mycotoxin [74].

The metabolite of m/z 318.3013 identified as phytosphingosine (Table 1, metabolite 14) presented MS/MS spectrum with fragments of m/z 264.2691 [M+H-DHO-2H₂O]⁺ and 282.2798 [M+H-DHO-H₂O]⁺, formed by the phytosphingosine dehydration pathway [75]. Phytosphingosine is a long-chain sphingolipid present in microorganisms, plants, and some mammalian tissues with antimicrobial and anti-inflammatory activity [76] and was produced by *T. orientale* (UFPIT01, UFPIT09, and UFPIT17) and *T. longibrachiatum* (UFPIT02) isolates. The
metabolite of m/z 163.0393 detected in all *Trichoderma* spp. was identified as 4-hydroxycoumarin (Table 1, metabolite 15), which is a fungal metabolite obtained from the precursor coumarin [79] that has important biological activities [78]. The metabolite of m/z 338.3413 detected in all *Trichoderma* spp. was identified as erucamide (Table 1, metabolite 16) and has been reported in *T. longibrachiatum* [80].

Altogether, the plethora of and the variety of secondary metabolites identified in the present study highlight how *Trichoderma* strains are capable of producing metabolites with different biological activities, which makes them very promising not only for the biocontrol of plant diseases but also for their application in medical, pharmaceutical and industrial biotechnology. Forest species from the Cerrado-Caatinga ecotone are rich in genetic resources and have diverse fauna and flora, with enormous biotechnological potential, including their diversity of endophytic fungi [26].

**Conclusions**

*Trichoderma* strains from the Cerrado-Caatinga ecotone revealed significant biocontrol potential against crop pathogenic fungi through antibiosis and multiple mechanisms, with possibilities of being used in formulations of biological products for the treatment of plant diseases. Metabolomic analysis proved to be effective in differentiating *Trichoderma* strains, in addition to identifying a variety of secondary metabolites with antimicrobial activity and other different bioactivities, demonstrating the importance of studying the biological resources of this area, which are still underexplored. Additionally, new bioactive metabolites can still be discovered, since this mutualistic association of endophytic fungi with their hosts is controlled by the genes of both organisms and modulated by the environment in which they live.

**Supporting information**

**S1 Fig.** Mycelial growth rate index (MGRI) of *C. truncatum* (A), *L. theobromae* (B), *M. phaseolina* COUFPI 10 and COUFPI 11 (C), and *S. delphini* COUFPI 209 and COUFPI 249 (D) paired with *Trichoderma* strains. Means followed by the same letter do not differ from each other by the Scott–Knott test at the 5% probability level. The coefficients of variation (CVs) were 20.51% for *L. theobromae*, 15.99% for *M. phaseolina* COUFPI 10, 19.13% for *M. phaseolina* COUFPI 11, 10.68% for *S. delphini* COUFPI 209, and 9.54% for *S. delphini* COUFPI 249. Different lowercase letters indicate a significant difference between *Trichoderma* spp. Different capital letters indicate a significant difference between *Trichoderma* spp. (TIF)

**S2 Fig.** *In vitro* antagonism bioassays of *Trichoderma* strains against *C. truncatum*. (TIF)

**S3 Fig.** *In vitro* antagonism bioassays of *Trichoderma* strains against *L. theobromae*. (TIF)

**S4 Fig.** *In vitro* antagonism bioassays of *Trichoderma* strains against *M. phaseolina* COUFPI 10. (TIF)

**S5 Fig.** *In vitro* antagonism bioassays of *Trichoderma* strains against *M. phaseolina* COUFPI 11. (TIF)
S6 Fig. *In vitro* antagonism bioassays of *Trichoderma* spp. against *S. delphinii* COUFPIT 209.
(TIF)

S7 Fig. *In vitro* antagonism bioassays of *Trichoderma* spp. against *S. delphinii* COUFPIT 249.
(TIF)

S8 Fig. Regression graph based on % inhibition of organic extracts of *Trichoderma* spp. against *C. truncatum*. The coefficients of variation (CVs) were 4.65% for the concentration of 0.5 mg mL\(^{-1}\), 8.45% for 1.0 mg mL\(^{-1}\) and 9.36% for 2.0 mg mL\(^{-1}\).
(TIF)

S9 Fig. PC1 x PC2 (A) and PC1 x PC3 (B) loading plots of metabolic fingerprints of *Trichoderma* spp. cultures generated using Meta baptist. Con = Control, UFPIT01 = T1, UFPIT02 = T2, UFPIT03 = T3, UFPIT04 = T4, UFPIT05 = T5, UFPIT06 = T6, UFPIT07 = T7, UFPIT08 = T8, UFPIT09 = T9, UFPIT10 = T10, UFPIT11 = T11, UFPIT12 = T12, UFPIT13 = T13, UFPIT14 = T14, UFPIT15 = T15, UFPIT16 = T16, UFPIT17 = T17, UFPIT18 = T18, and UFPIT19 = T19.
(TIF)

S10 Fig. UPLC-ESI-Q-TOF-MS chromatograms of *Trichoderma* spp. isolates.
(TIF)

S11 Fig. Structure of secondary metabolites identified in *Trichoderma* spp. using UPLC-ESI-Q-TOF-MS.
(TIF)

S1 Table. *Trichoderma* spp. isolates used in this study [29].
(DOCX)

S2 Table. *Trichoderma* spp. isolated from leaves of forest species in an area of the Cerrado-Caatinga ecotone [26].
(DOCX)

S3 Table. Pearson’s linear correlations between co-culture and crude extract bioassays based on percent inhibition of *Trichoderma* strains against *C. truncatum*.
(DOCX)

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