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In vitro EFFECT OF *Trichoderma asperellum* METABOLITES ON *Fusarium oxysporum* AND *Fusarium equiseti*

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ABSTRACT

The control of phytopathogenic fungi in agricultural crops requires the use of synthetic chemical fungicides, which have damaged the environment for decades. Biocontrol with microorganisms is one option to reduce their use, with the fungi of the *Trichoderma* genus standing out for their ability to interact with soil pathogens through different control mechanisms through antibiosis or production of substances harmful to other microorganisms. The objective of this work was to evaluate the biological control mechanism using *Trichoderma asperellum* antibiotics on the growth of *Fusarium oxysporum* and *F. equiseti*. Antibiosis bioassays were performed using the cellophane test (diffusible metabolite assay), the reverse plate technique (volatility assay), and poisoned foods (*T. asperellum* mycelium extracts and extracellular metabolite assays). The diffusible metabolites of *T. asperellum* presented the greatest inhibition of growth. The highest percentage of inhibition was observed on *F. oxysporum* in plates where *T. asperellum* developed for 72 h (>25 %), while *F. equiseti* inhibition was more effective in plates with 48 h (>40 %). In both species, no significant inhibitory effect was observed in volatility tests (>10 %), while extracellular metabolites showed no inhibition. In contrast, metabolites extracted from *T. asperellum* mycelium with ethyl acetate inhibited *Fusarium* between 18 and 40 %; with hexane, between 9 and 20 %; and with methanol, no inhibition was observed. The direct analysis in real-time mass spectrometry (DART-MS) analysis showed the presence of pyrones, fatty acids, alcohols, and carbohydrates in extracts and liquid culture of *T. asperellum*, which suggests that the control mechanism through antibiotics on *F. oxysporum* and *F. equiseti* is fungistatic.

Keywords: biocontrol, fungistatic, inhibition, mechanism, metabolite, phytopathogen.

INTRODUCTION

Soil-borne pathogens are a problem in agriculture worldwide, causing yield and quality losses in many crops (Madbouly and Abdelbacki, 2017). Among the pathogens that contribute the most to yield losses, the *Fusarium* genus stands out (Rivera-Jiménez *et al*., 2018). *Fusarium oxysporum* causes wilting and subsequent death due to colonization of the roots and vascular bundles (Zheng *et al*., 2022), while *F. equiseti* is associated with leaf spot, crown, and root rot, which causes seed deterioration and seedling infection (Gilardi *et al*., 2017).

Capsicum is a crop generally associated with infection by phytopathogenic fungi of the *Fusarium* genus (Hami *et al*., 2021), affecting its yield and commercial value. Currently, pathogenic fungi are effectively controlled with synthetic fungicides, but these are harmful to the environment (Shafique *et al*., 2018) and toxic to farmers, communities, and consumers; in addition, the indiscriminate use of these fungicides results in residual toxicity due to accumulation (Pathak *et al*., 2022). Therefore, there is a motivation to find fungicides that are safe, effective, and do not cause environmental damage (Wu *et al*., 2023).

Biological control with antagonistic microorganisms has increased and is successful as a green, low-cost, and agroecological alternative to synthetic fungicides since they colonize the rhizosphere without leaving toxic residues, unlike chemical fungicides used in conventional agriculture to control soilborne pathogens (Palmieri *et al*., 2022). Several microorganisms are used as biocontrol agents, including *Trichoderma*, a genus of avirulent hyperparasitic fungal species symbionts of plants that act against phytopathogenic fungi through several antagonistic mechanisms, including antibiosis, induction of plant defense, mycoparasitism, and competition for nutrients in the rhizosphere (Marques *et al*., 2018).

The antibiosis process of *Trichoderma* spp. is based on the production of antibiotic chemical compounds associated with competition for nutrients in the rhizosphere (Contreras-Cornejo *et al*., 2016). These fungi use antibiosis as a generalized strategy used for defense (Tyskiewicz *et al*., 2022) and produce a spectrum of antifungal secondary metabolites that inhibit microbial growth without physical contact (Dawidziuk *et al*., 2016), such as polyketides, gliotoxin, anthraquinones, pyrones, and peptaibols. Other metabolites associated with *Trichoderma* are enzymes that degrade the cell wall, siderophores, iron chelators, and volatile and non-volatile metabolites (Tyskiewicz *et al*., 2022).

Trichoderma spp. inhibit phytopathogenic fungi *in vitro* by producing diffusible, volatile, and non-volatile secondary metabolites. Although most *Trichoderma* species produce toxic volatile metabolites that affect the growth and development of phytopathogens (Stracquadanio *et al*., 2020), Sánchez-García *et al*. (2017) reported inhibition percentages of over 70 % of diffusible metabolites in two isolates of *T. asperellum* (Tri-3 and Tri-5) on three root phytopathogenic fungi in *Phaseolus vulgaris* L. (*F. oxysporum*, *F. verticillioides*, and *F. solani*). Likewise, Mishra *et al*. (2018) evaluated the ability of *T. asperellum* to produce volatile and non-volatile metabolites against *F. oxysporum* f. sp. *capsici* and

found that volatile metabolites are less effective in reducing mycelial growth (22 %) of the pathogen than non-volatile compounds, with inhibition percentages higher than 25 %.

There is evidence that metabolites produced by *T. asperellum* have an inhibitory effect on the development of phytopathogens in relation to their mode of action. *In vitro* studies could be a useful tool for identifying sources of antifungal compounds. Therefore, the objective of this work was to evaluate the biological control mechanism by antibiosis of *T. asperellum* metabolites on two pathogenic strains (*F. oxysporum* and *F. equiseti*).

MATERIALS AND METHODS

The biological control agent *Trichoderma asperellum* ITC17 Ta13-17 (GenBank number: MH015346) and the pathogen strains *Fusarium oxysporum* FCHJ-T6, *F. oxysporum* FCHA-T7, and *F. equiseti* FCHE-T8 (GenBank numbers MG020428, MG020429, and MG020433, respectively) were provided by Dr. Jairo Cristóbal Alejo and belong to the microbial collection of the Technological Institute of Conkal, Mexico. The *T. asperellum* strain was isolated from the root and stem crown of sweet pepper (*Capsicum annuum* L.), and *F. oxysporum* and *F. equiseti* were isolated from habanero pepper root (*C. chinense* Jacq.).

Diffusible metabolites assay

The assay was carried out in two stages using the cellophane test as described by Sánchez-García *et al*. (2017). Cellophane circles were placed on plates with potato dextrose agar medium (PDA, BD Bioxon, México), one per plate; then, a 6 mm diameter mycelial disc of *T. asperellum* (three-day culture) was centrally inoculated for growth on the cellophane and removed after 24, 48, and 72 h. To assess metabolite diffusion, the plates were incubated at 30±2 °C in the dark. A sterile filter paper disk (6 mm diameter) (Whatman™, Grade 1) was used as a control.

In the second stage, a 6 mm diameter mycelial disc with *F. oxysporum* FCHJ-T6, *F. oxysporum* FCHA-T7, and *F. equiseti* FCHE-T8 (seven-day culture in PDA medium) was placed on the central position where the antagonist was inoculated. The plates were incubated at 30±2 °C in the dark, and radial growth (mm) was recorded every 24 h until the control reached the edge of the plate. Three repetitions were established for each treatment and a control. The diameter was measured with a ruler, and the percentage inhibition of radial growth (PIRG) of the pathogen was calculated according to the following formula:

$$PIRG = \frac{(D1 - D2)}{D1} \times 100$$

where $D1$ is the diameter of the pathogen on the control plate and $D2$ is the diameter of the pathogen on the plate with *T. asperellum*.

Volatility assay

The effect of volatile metabolites of *T. asperellum* was evaluated by the inverted plate technique as reported by Toghueo *et al*. (2016). In different Petri dishes, 6-mm mycelial discs (one per plate) of *T. asperellum* or *Fusarium* sp. were inoculated, taken from cultures of two and seven days in PDA medium, respectively; three replicates were established for each *Trichoderma-Fusarium* interaction and a control. Subsequently, the bases of both plates were placed opposite, sealed with adhesive tape and plastic film, and incubated at 30±2 °C in the dark. As a control, the base of the Petri dish inoculated with the pathogen was placed opposite another with a sterile filter paper disc. The pathogen was placed on top to avoid interference by *T. asperellum* spores. The diameters of the *Fusarium* colonies were measured every 24 h for five days (all measurements were taken in the same box) (Chen *et al*., 2016). The inhibition percentage was calculated using the following equation:

$$I = \frac{C - T}{C} \times 100$$

where *C* is the mycelial growth of the pathogen in the control plate, *T* is the mycelial growth in the tests with *Trichoderma*, and *I* represents the percentage of mycelial growth that has been inhibited.

Trichoderma asperellum mycelium extracts assay

Mycelium extracts were obtained as reported by Ibrahim *et al*. (2017), with some modifications. *Trichoderma asperellum* was cultivated in potato dextrose broth (PDB) at 30±2 °C for 30 days in the dark. Under sterile conditions, the culture medium with five days of growth was filtered with sterile gauze to recover the mycelium, which was then dried in an incubator at room temperature for eight days. Dried mycelial mats were ground, extracted with 200 mL hexane with shaking for 24 h at room temperature, and filtered through Whatman no. 1 paper. The solid residue was extracted again with hexane as described above. The filtrates were combined and evaporated to obtain the hexane extract. The solid residue was extracted with ethyl acetate and methanol, respectively, as described for the hexane extract.

For the test, 55 mm Petri dishes were used with PDA medium added with 1000 μg mL^{-1} of the extracts. The plates were inoculated by placing a *Fusarium* sp. mycelial disk from a seven-day culture in PDA medium in the center. Petri dishes without organic extract and with sterile distilled water (negative control) and dimethyl sulfoxide (DMSO ACS reagent, Sigma-Aldrich, France) (solvent test) were used as controls. Three replicates were used for each treatment. The plates were incubated at 30±2 °C and the colony diameter was measured with a ruler every 24 h until the control treatment reached the edge of the plate. The percentage of growth inhibition was calculated as described in the previous assay.

Extracellular metabolites assay

Flasks of 250 mL volume containing 100 mL of sterile PDB were inoculated with mycelial plugs taken from the edge of a seven-day culture of *T. asperellum* in PDA medium. Each flask was inoculated with three discs (6 mm) and incubated without shaking at 30±2 °C in the dark for seven days (Anees *et al*., 2018). For the elimination of spores, the liquid medium was filtered through a cloth, sterile gauze, and Whatman paper, and centrifuged twice at 4500 rpm for 25 min (Hermle Labortechnik™, Germany). A syringe filter (LINKTOR™ syringe filter, 25 mm diameter, 0.22 µm pore size) to obtain sterile culture liquid (Marques *et al*., 2018).

For the assay with extracellular metabolites, the culture filtrate was added to the sterile PDA medium in concentrations of 0, 5, 10, 15, 20, and 25 % (v/v) (Mishra *et al*., 2016; Petrisor *et al*., 2017; Anees *et al*., 2018; Mishra *et al*., 2018) using PDA plates. These were inoculated in the center with a 6 mm diameter mycelial disc of the pathogen and incubated at 30±2 °C until the fungus reached the edge of the plate in the control treatment. The trial treatments were completely randomized with three replicates. The radial growth of the phytopathogens was measured every 24 h with a ruler. The percentage inhibition of mycelial growth in relation to the growth of the controls was calculated as previously described.

Analysis of *T. asperellum* mycelium extract and sterile culture liquid

Two samples obtained from the culture of *T. asperellum* were analyzed and identified. The extract of dry mycelium was obtained with hexane and ethyl acetate. Samples were analyzed in the form of crude extract and diluted to a concentration of 1 mg mL^{-1} from the crude-dry extract in their corresponding extraction solvent (HPLC grade). The 1 mg mL^{-1} aliquots were kept refrigerated until analysis.

Direct analysis in real-time mass spectrometry (DART-MS) was carried out on a JMS-T100LP AccuTOF LC-PLUS spectrometer (JEOL, Tokyo, Japan) with a DART SVP100 ion source (Ionsense, Saugus, MA, USA). The DART ion source was operated with helium for analysis and nitrogen for standard mode; gas temperature was 300 °C, inlet pressure was 0.55 MPa, and voltage was ±600 V for positive and negative ion modes. The acquisition of the mass spectra was recorded with the Mass Center System Version 1.5.0k software in a mass range of m/z 50–1000 Da. Each sample was detected at least three to five times over 1–4 min. The analysis of the *T. asperellum* extracts at 1 mg mL^{-1} was carried out by placing 10 µL of volume in a capillary tube; subsequently, the capillary tube was placed between the helium stream from the DART ionization source and the vacuum interface to obtain DART mass spectra. Crude extracts were directly analyzed on DART-MS; the extract was taken with a capillary tube, and the sample adhered to the walls of the glass tube was analyzed (Procacci *et al*., 2021).

Statistical analysis

The data was analyzed using a completely randomized design of experiments with an analysis of variance (ANOVA) and Tukey's test at 95 % confidence using the Centurion XVII software (Statgraphics Technologies, VA, USA).

RESULTS AND DISCUSSION

In vitro antagonism of *T. asperellum* against *Fusarium* spp. by antibiosis

In the assay with diffusible metabolites, it was observed that the effect on the mycelial growth of the *Fusarium* strains was different in each treatment (Ta-24, Ta-48, and Ta-72). Inhibition was greater compared to the control at 192 h for *F. oxysporum* and at 216 h for *F. equiseti*, with Ta72 and Ta48 being the best treatments, respectively (Table 1). In an antibiosis assay, Sánchez-García *et al*. (2017) reported that *T. asperellum* inhibited radial growth (>75 %) of five *Fusarium* strains (two of *F. solani*, two of *F. oxysporum*, and one of *F. verticilloides*). In this work, growth inhibition was >25 % (192 h) in *F. oxysporum* and >40 % (216 h) in *F. equiseti*; this growth inhibition in the assay varied over time between strains, at days 8 and 9, respectively.

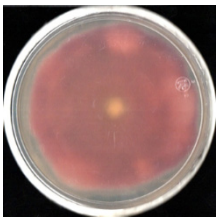
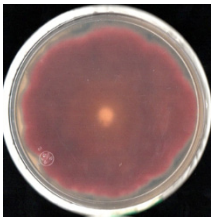
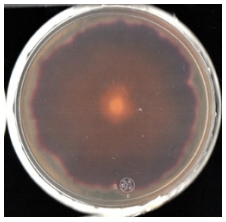
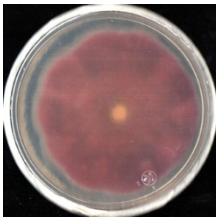
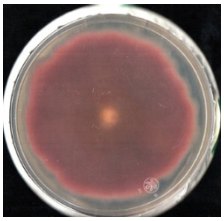
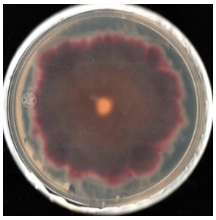
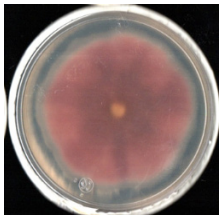
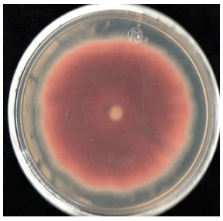
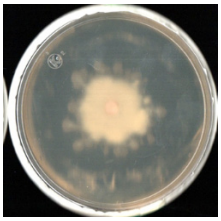
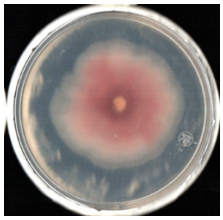
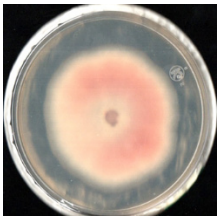
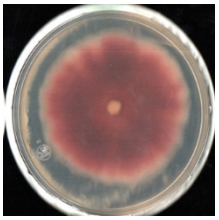
Trichoderma spp. have antagonistic activity against phytopathogens through multiple mechanisms that include mycoparasitism, competition for space and nutrients, induction of systemic resistance in plants, and antibiosis (Li *et al*., 2018). Regarding antibiosis, *Trichoderma* spp. produce secondary metabolites in the culture medium with potential antimicrobial activity. The identification and *in vitro* evaluation of these molecules against pathogens is important to identify strains with biocontrol potential. Different species of the *Trichoderma* genus can produce similar molecules (Morais *et al*., 2022) and different isolates of the same species can produce different secondary metabolites at various concentrations, which leads to the individuality of the species (Mesa-Vanegas *et al*., 2019).

The growth inhibition of *F. equiseti* depended on the development time of the biological control agent on the plate prior to culturing the pathogen, since the Ta48 treatment was more efficient with this strain than Ta72. The inhibition effect is due to the excretion of metabolites by *T. asperellum*, causing antibiosis (production of harmful substances for another organism) and inhibiting the growth of the pathogen. Metabolites excreted by antagonist agents may be volatile or non-volatile. In this case, *Trichoderma* spp. can produce both (Olmedo and Casas-Flores, 2014). The results demonstrated a fungistatic effect (that which prevents their growth) in the *Fusarium* strains caused by antibiosis (Table 1) through the synthesis and diffusion of *Trichoderma* metabolites in the plates with PDA medium.

The inhibitory effect of volatile metabolites produced by *T. asperellum* using the inverted plate technique has previously been reported (Morales-Rodríguez *et al*., 2018). However, in the volatility test, the results did not show statistical differences in the interactions between *T. asperellum* and *F. oxysporum* FCHA-T7 (<7 %) and *T. asperellum* and *F. equiseti* FCHE-T8 (<8 %) compared to the control.

For the poisoned food test, fungal extracts were obtained from *T. asperellum* mycelium using hexane (Hx-Ta), ethyl acetate (AcEt-Ta), and methanol (Me-Ta); these were tested at 1000 μg mL^{-1} (ppm) to evaluate their effect on the growth of *F. oxysporum* and *F. equiseti*, observing inhibition values less than 50 %. The AcEt-Ta extract presented the greatest growth inhibition on *F. oxysporum* (30.7 % for FCHJ-T6 and 18.1 % for FCHA-T7) and *F. equiseti* (38.1 %) at 120 and 168 h, respectively, followed by the Hx-

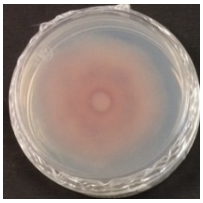
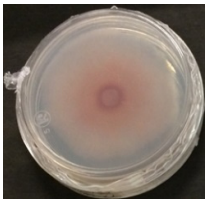
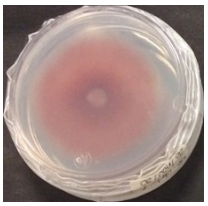
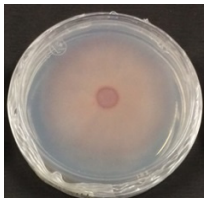
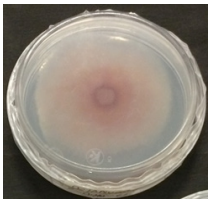
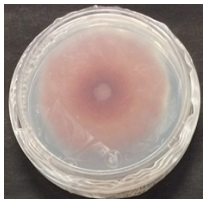
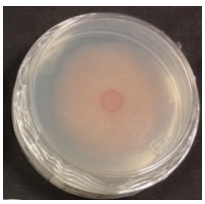
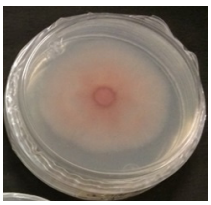
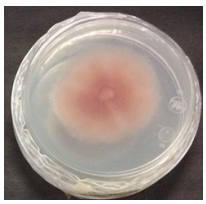
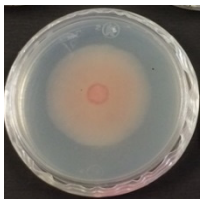
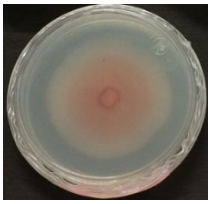
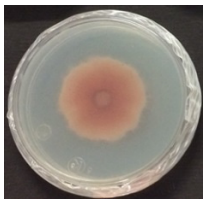
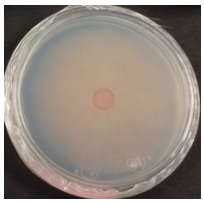
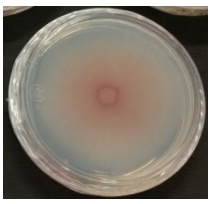
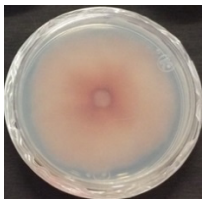
Table 1. Radial growth inhibition of *Fusarium oxysporum* FCHJ-T6, *F. oxysporum* FCHA-T7, and *F. equiseti* FCHE-T8 by *Trichoderma asperellum* in the diffusible metabolites[†] assay.

Treatment	*F. oxysporum* FCHJ-T6 (192 h)	*F. oxysporum* FCHA-T7 (192 h)	*F. equiseti* FCHE-T8 (216 h)
TST	 0.0±0.0 b	 0.0±0.0 b	 0.0±0.0 d
Ta-24	 0.7±2.0 b	 1.2±0.5 b	 5.9±0.5 c
Ta-48	 12.9±1.7 ab	 12.8±1.5 ab	 41.1±1.8 a
Ta-72	 25.4±7.3 a	 26.2±5.8 a	 15.2±0.2 b

[†]The values are presented as percentages of inhibition of radial growth and correspond to the average of three repetitions ± standard error. Values with different letters in the same column are statistically different ($p \leq 0.05$). TST: control; Ta-24, Ta-48, and Ta-72: treatments in which *T. asperellum* grew on cellophane for 24, 48, and 72 h, respectively.

Ta extract (9.3–18.6 %), while Me-Ta did not show a significant inhibitory effect in any strain compared to the control. The effect of Hx-Ta and AcEt-Ta on *F. oxysporum* was fungistatic; in contrast, Me-Ta promoted the mycelial growth of the phytopathogen while the inhibitory effect of the solvent could be dismissed (Table 2).

Table 2. Inhibition of radial growth of *Fusarium oxysporum* FCHJ-T6, *F. oxysporum* FCHA-T7, and *F. equiseti* FCHE-T8 by *Trichoderma asperellum* in the fungal mycelium extracts test†.

Treatment	*F. oxysporum* FCHJ-T6 (120 h)	*F. oxysporum* FCHA-T7 (120 h)	*F. equiseti* FCHE-T8 (168 h)
H_2O (-)			
	0.0±0.0 c	0.0±0.0 b	0.0±0.0 cd
DMSO			
	-2.7±0.0 d	3.0±1.3 b	0.9±0.4 c
Hx-Ta			
	9.3±0.0 b	15.5±0.4 a	18.6±0.9 b
AcEt-Ta			
	30.7±0.0 a	18.1±0.4 a	38.1±1.2 a
Me-Ta			
	-7.1±0.4 e	-5.2±0.9 c	-3.1±0.4 d

†The values are presented as percentages of inhibition of radial growth, correspond to the average of three repetitions ± standard error. Values with different letters in the same column are statistically different ($p \leq 0.05$). H_2O (-): sterile distilled water (negative control); DMSO: dimethyl sulfoxide (solvent test): Hx-Ta: hexane extract; AcEt-Ta: ethyl acetate extract and Me-Ta: methanolic extract.

Ibrahim *et al*. (2017), when evaluating the antifungal activity of crude extracts of ethyl acetate (AcET) of two isolates of *T. longibrachiatum* (MF1 and MF5) and two fractions of these extracts partitioned with 90 % hexane and methanol and applied at 1000 μg mL^{-1}, observed that the crude extract of ethyl acetate and the methanolic fraction of both isolates had less than 50 % inhibition on the growth of *F. oxysporum*. In contrast, the hexane fraction of *T. longibrachiatum* MF1 was slightly active against *F. oxysporum*, while the hexane fraction of *T. longibrachiatum* MF5 was inactive against the pathogen. Therefore, this study and other reports suggest that the inhibitory effect of *Trichoderma* metabolites contained in the organic extracts may vary from one microorganism to another.

In the test with extracellular metabolites of the *T. asperellum* growth medium, none of the treatments with 0, 5, 10, 15, 20, and 25 % (v/v) of the filtrate in PDA medium inhibited the mycelial growth of the three *Fusarium* strains evaluated.

DART-MS analysis of mycelium extracts and sterile liquid culture of *T. asperellum*

The DART-MS analysis of the organic extracts allowed the identification of possible constituent compounds of diverse chemical nature. The hexane extract (Hx-Ta) produced signals associated with pyrans (1), pyrones (2 and 3), carboxylic acids (4), alcohols (5), fatty acids (6), and terpenes (7 and 8) (Table 3). The peaks associated with pyrones and fatty acids had the highest intensity and corresponded to the adducts $[M+O+H]^+$ and $[M+OH]^-$ and to the anion $[M-H]^-$.

The ethyl acetate (AcEt-Ta) extract contained 12 chemical groups, including furans (1), pyrans (2), pyrones (3, 4, and 5), carboxylic acids (6), alcohols (7), fatty acids (8 and 9), terpenes (10), polycyclic heteroarenes (11), and α-amino acid derivatives (12). The signals associated with fatty acids, pyrones, and alcohols presented the highest intensity and corresponded to the anion $[M-H]^-$ and the adducts $[M+O+H]^+$ and $[M+OH]^-$, respectively (Table 4).

In this study, the secondary metabolites extracted from *T. asperellum* mycelium with hexane and ethyl acetate caused inhibition of *Fusarium* spp. The signals with the highest intensity detected by DART-MS in both extracts corresponded to pyrone and fatty acid metabolites (Tables 3 and 4), while, in the ethyl acetate extract, a high-intensity peak attributed to a long-chain alcohol was detected (Table 4), which could explain the greater inhibitory activity of the AcEt extract on *Fusarium* spp. compared to the hexane extract. Sakpetch *et al*. (2018) had reported the presence of (2) 6-pentyl-2H-pyran-2-one (Table 3) in the hexane extract of the filtered liquid medium of *T. asperellum* and (1) 5-hydroxymethyl, 2-furancarboxaldehyde, (2) 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl, and (9) 9,12-octadecenoic acid (Table 4) in the ethyl acetate extract. These compounds were associated with antimicrobial and antifungal activity. The abundance and variety of secondary metabolites identified in the mycelium fungal extracts indicate that the *T. asperellum* strain is capable of producing metabolites with biological activity. Among the three extracts evaluated, the presence of pyrones and fatty acids was represented with greater intensity in the hexane and ethyl acetate

Table 3. Ions and adducts detected in the hexane extract of *Trichoderma asperellum* mycelium analyzed in mode (+) and (-) by direct analysis in real time mass spectrometry (DART-MS).

Suggested metabolite	Crude and dry extract [Ion]/(m/z)		Diluted extract (1 mg mL^{-1}) [Ion]/(m/z)	
	Mode (+)	Mode (-)	Mode (+)	Mode (-)
(1) 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	-----	$[M+Cl]^-$/ (179.068)	-----	-----
(2) 6-pentyl-2H-pyran-2-one	†$[M+O+H]^+$/ (183.127)	-----	†$[M+O+H]^+$/ (183.133)	-----
(3) 6-pent-1-enylpyran-2-one	-----	†$[M+OH]^-$/ (181.086)	$[M+H]^+$/ (165.117)	$[M+OH]^-$/ (181.083) $[2M-H]^-$/ (327.262)
(4) Benzeneacetic acid	-----	$[2M-H]^-$/ (271.121)	-----	-----
(5) 1-hydroxyheptadecane	-----	$[M-H]^-$/ (255.249)	-----	$[M-H]^-$/ (255.248)
(6) 9,12-octadecenoic acid	-----	-----	-----	†$[M-H]^-$/ (279.250)
(7) (3aR,4R,7R)-1,4-dimethyl-7-prop-1-en-2-yl-1,2,3,3a,4,5,6,7-octahydroazulene	$[M+O+H]^+$/ (221.240)	-----	-----	-----
(8) 2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene	-----	$[M-H]^-$/ (409.234)	-----	-----

†Most abundant ions in the DART-MS spectrum. The sample was detected at least three times. The metabolites were observed in the majority of the DART-MS spectra. m/z: mass to charge ratio; the letter M indicates the molecular weight of the metabolite.

extracts (Tables 3 and 4), as was the case of 6-pentyl-2H-pyran-2-one, which has been reported to have antimicrobial activity (Morais *et al*., 2022). According to the results, there is variability in the antimicrobial activity of this compound, since the fungal strains showed different degrees of sensitivity, which is dependent on the concentration of pyrone (Ismaiel and Ali, 2017). Given this evidence, and based on the inhibition results observed in the present study, it is suggested that the different secondary metabolites present in each extract and their concentrations exhibit various levels of antibiotic activity.

Table 4. Ions and adducts detected in the ethyl acetate extract of *Trichoderma asperellum* mycelium analyzed in mode (+) and (-) by direct analysis in real time mass spectrometry (DART-MS).

Suggested metabolite	Crude and dry extract [Ion]/(m/z)		Diluted extract (1 mg mL^{-1}) [Ion]/(m/z)	
	Mode (+)	Mode (-)	Mode (+)	Mode (-)
(1) 5-hydroxymethyl, 2-furancarboxaldehyde-	-----	-----	$[M+H]^+$/ (127.069)	-----
(2) 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	-----	$[M+OH]^-$/ (161.055)	$[M+H]^+$/ (145.087)	$[M+OH]^-$/ (161.055)
(3) 6-pent-1-enylpyran-2-one	-----	-----	$[M+H]^+$/ (165.121, 165.124)	-----
(4) 6-amyl-alpha-pyrone	-----	-----	†$[M+O+H]^+$/ (183.137, 183.140)	$[M-H+O_2]^-$/ (197.043)
(5) 2H-pyran-2-one, tetrahydro-4-hydroxy -4-methyl-	-----	$[M-H+O_2]^-$/ (161.055)	$[M+O+H]^+$/ (147.104)	$[M-H+O_2]^-$/ (161.055) $[2M-H]^-$/ (259.071)
(6) 2-furancarboxylic acid	-----	-----	$[M+O+H]^+$/ (129.087)	$[M+OH]^-$/ (129.026) $[2M-H]^-$/ (223.094)
(7) 1-hydroxyheptadecane	-----	-----	-----	†$[M+OH]^-$/ (273.092)
(8) n-octadecanoic acid	-----	$[M-H]^-$/ (283.279)	-----	-----
(9) 9,12-octadecenoic acid	-----	†$[M-H]^-$/ (279.251)	-----	-----
(10) (3R,3aS,7aR)-1,4-dimethyl-7-prop-1-en-2-yl-1,2,3,3a,4,5,6,7-octahydroazulene	-----	$[M+O_2]^-$/ (312.249)	$[M+H]^+$/ (205.130)	-----
(11) 1H-indole	-----	-----	-----	-----
(12) Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-	-----	M^-/ (117.027)	-----	$[2M-H]^-$/ (307.205)

†Most abundant ions in the DART-MS spectrum. The sample was detected at least three times. The metabolites were observed in the majority of the DART-MS spectra. m/z: mass to charge ratio; the letter M indicates the molecular weight of the metabolite.

CONCLUSIONS

Diffusible metabolites extracted with hexane and ethyl acetate from *Trichoderma asperellum* mycelium showed a fungistatic effect on the growth of *Fusarium oxysporum* and *F. equiseti*. On the contrary, the volatile, extracellular, and methanol-extracted metabolites of *Trichoderma* mycelium did not show a significant inhibitory effect on growth. This suggests that these compounds play a role as a signal or stimulus during pathogen-biological control agent confrontation and that the antibiosis mechanism. Inhibition values lower than 50 % could be synergistically associated with other antagonistic mechanisms of *T. asperellum*, such as competition for space and nutrients.

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