

Phenolics mediate suppression of *Fusarium oxysporum* f. sp. *cubense* TR4 by legume root exudates



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ABSTRACT

Fusarium oxysporum Schlecht f. sp. *cubense* (E.F. Smith) Snyder & H.N. Hansen (Foc) is a notorious soil-borne fungal pathogen that causes Fusarium wilt disease of bananas (*Musa* spp. L.). Foc, especially Tropical Race 4 (TR4), is particularly devastating due its ability to infect diverse banana cultivars including Cavendish bananas. Fusarium wilt is difficult to manage, but intercropping has been reported to efficiently suppress Fusarium wilt through exudation of root metabolites with antagonistic effects on Foc. Yet, the metabolites that inhibit Foc and the underlying mode of action remain unclear. Hydroponic culture and targeted metabolite profiling were used to investigate the potential of root-exuded phenolic acids and flavonoids of two legume species, *Desmodium uncinatum* (Jacq.) DC. and *Mucuna pruriens* (L.) DC., to inhibit growth and the biosynthesis of the phytotoxins beauvericin and fusaric acid (FA) in Foc TR4. Of the twelve metabolites targeted, four phenolic acids (benzoic, t-cinnamic, p-coumaric, p-hydroxybenzoic) were common in root exudates of both legumes, whereas vanillin and the flavonoid quercetin were only detected in root exudates of *M. pruriens*. Bioassays using synthetic benzoic-, t-cinnamic-, or p-hydroxybenzoic acid, or a mixture thereof, showed a concentration-dependent suppressive effect on Foc TR4. Low concentrations of phenolic acids (0.01 and 0.1 mM) inhibited chlamydospore germination and the production of macro- and micro-conidia, as well as the biosynthesis of FA. Mycelial growth of Foc TR4 was only inhibited at high concentrations (1 mM) of benzoic acid, t-cinnamic acid, and a mixture of the three phenolics. This study demonstrates that phenolics in root exudates of *D. uncinatum* and *M. pruriens* suppress Foc TR4 by directly inhibiting the early stages of pathogen development. Intercropping banana with *D. uncinatum* and *M. pruriens* may thus be considered as a valuable option for suppression of Foc TR4.

1. Introduction

Fusarium oxysporum Schlecht f. sp. *cubense* (E.F. Smith) Snyder & H.N. Hansen (Foc) is a devastating root-infecting fungal pathogen that causes Fusarium wilt disease of banana (*Musa* spp. L.) (Viljoen et al., 2020). Three races of Foc exist, of which Foc Tropical Race 4 (TR4) is considered the most destructive. Foc TR4 is particularly damaging to Cavendish bananas, which comprise about 50% of all bananas produced worldwide, but also affects several banana varieties that are susceptible to Foc races 1 and 2. Foc TR4 thus poses a serious threat to the livelihoods of nearly 400 million people that depend on banana for food security and income (Pegg et al., 2019). Fusarium wilt can be managed successfully by replacing susceptible with resistant banana varieties (Dita et al., 2018), if the fruit of such varieties are acceptable to growers

and consumers. Unfortunately, most banana hybrids produced through conventional (classical) breeding are often undermined by inferior fruit characteristics such as taste and cycle time (Tenkouano et al., 2011; Viljoen et al., 2020). Although genetic engineering could overcome such limitations (Paul et al., 2011; Dale et al., 2017), a major concern of consumers and regulators is the possible adverse effects of transgenes on human health and/or the environment (Ishii and Araki, 2016).

Manipulation of the banana rhizosphere has shown promising results and may provide a prospect for management of Fusarium wilt. Greenhouse and *in vitro* studies have reported microorganisms that are antagonistic against Foc (Bubici et al., 2019). For instance, Yadav et al. (2021) reported that *Bacillus licheniformis* strain CSR-D4 strongly inhibited the growth of Foc TR4 *in vitro*. The inhibitory effect was attributed to the production of antifungal metabolites such as

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bacillomycin, fengycin, surfactin, and iturin (Yadav et al., 2021). Non-pathogenic strains of *F. oxysporum* were also reported to colonise banana roots, establish asymptotically as endophytes, and suppress Foc (Belgrave et al., 2011; Nel et al., 2006). Besides, the disease can also be suppressed by intercropping banana with cassava (*Manihot esculenta* L. Crantz), Chinese leek (*Allium tuberosum* Rottler) and pinto peanut (*Arachis pintoi* Krap. et Greg. nom. nud.), or by rotation with pineapple (*Ananas comosus* [L.] Merr.), or rice (*Oryza sativa* L.) (Buddenhagen, 2009; Huang et al., 2012; Pattison et al., 2014; Wang et al., 2015; Li et al., 2020). Disease suppression was linked to root-secreted compounds (primary and secondary metabolites), but the underlying mode of inhibition remains unclear.

Foc inoculum survives in infested soil primarily as hardy thick-walled spores called chlamydospores. Chlamydospores remain dormant and survive in Foc-infested soils for decades, but germinate when susceptible banana varieties are planted in such soils (Rishbeth, 1955; Ploetz, 2015). Germination represents the early developmental stage of Foc, and results in the production of actively growing hyphae that produce two other types of asexual spores, called macroconidia and microconidia. During its development, Foc produces virulence factors such as plant cell wall-degrading enzymes and the phytotoxins beauvericin and fusaric acid (FA) (Liu et al., 2020). Beauvericin and FA are essential for infection and colonisation of banana roots (Li et al., 2013; Portal González et al., 2021), and possess antimicrobial activity that may enhance the ecological fitness of *F. oxysporum* spp. through the inhibition of competing soil microorganisms (Notz et al., 2002; Spraker et al., 2018). Thus, the early stages of Foc development are essential for host infection and are the target of interventions for the suppression of Fusarium wilt through manipulation of the rhizosphere (Peng et al., 1999; Dita et al., 2018; Bubici et al., 2019).

Plants secrete up to 50% of their photosynthesized carbon and 15% of their nitrogen into the rhizosphere in the form of rhizodeposits and root exudates (root metabolome) (Haichar et al., 2016). Root exudates contain a myriad of primary and secondary metabolites of low (<1000 Da) and high (>1000 Da) molecular weight (Oburger and Jones, 2018; Vives-Peris et al., 2019). Root-secreted metabolites of intercrops are important in the suppression of Fusarium wilt, partly by inhibiting the early stages of Foc development (Buddenhagen, 2009; Huang et al., 2012). Zhang et al. (2013) identified five organic volatiles and organosulfur compounds, including 2-methyl-2-pentenal, and four organosulfur compounds (dimethyl trisulfide, dimethyl disulfide, dipropyl disulfide, dipropyl trisulfide) from Chinese leek (*Allium tuberosum*) that inhibited Foc. The strongest inhibition was obtained with 2-methyl-2-pentenal and dimethyl trisulfide. Zuo et al. (2015) suggested that the inhibitory effect of *A. tuberosum* volatiles could be intensified by root-secreted phenolic compounds, but the effect of phenolic compounds on the early development of Foc is not completely understood.

Phenolic compounds are ubiquitous plant secondary metabolites and a class of natural chemicals containing one or more hydroxyl groups attached to an aromatic ring (Cheynier et al., 2013). They are divided into flavonoids (including flavones, flavanones, and flavanols) and phenolic acids (Cheynier et al., 2013). Phenolic compounds possess antioxidant properties and antimicrobial activity, and can suppress fungal pathogens *in vitro*, suggesting a role in the suppression of soil-borne pathogens such as *F. oxysporum* spp. (Michielse et al., 2012; Yuan et al., 2018). Phenolic compounds can suppress fungal pathogens by inhibiting spore germination, hyphal growth, and the synthesis of virulence factors such as toxins (Michielse et al., 2012; Ling et al., 2013; Schöneberg et al., 2018; Gautier et al., 2020; Oufensou et al., 2020). Phenolic compounds may be synthesized during normal growth and development, or may accumulate in plant tissues in response to various abiotic (e.g., heat, drought) and biotic (e.g. beneficial and antagonistic microorganisms) stress (Haichar et al., 2016). Legumes (Fabaceae) can secrete large quantities of phenolic compounds due to their root biomass, root density, and synergistic interaction with beneficial microorganisms (Duchene et al., 2017; Sasse et al., 2018). *Desmodium*

uncinatum (Jacq.) DC and *Mucuna pruriens* (L.) DC secrete flavonoids and phenolic acids that have been demonstrated to play an important role in the suppression of weeds and nematodes (Tsanuo et al., 2003; Arim et al., 2006; Hooper et al., 2015). Consequently, legumes could be useful intercrops when developing sustainable crop production systems, especially for banana (McIntyre et al., 2001; Wink, 2013; Ocimati et al., 2019; Muoni et al., 2019; Blomme et al., 2020).

Despite the importance of phenolic compounds in plant defense against fungal pathogens, there is limited knowledge about the effect of root-secreted phenolics on the early stages of Foc development. We hypothesized that the root metabolome of legumes contains phenolics with antimicrobial activity that may inhibit development of Foc TR4. On the basis of this hypothesis, the specific objectives of this research were to (i) assess the cytotoxicity of root exudates from *D. uncinatum* and *M. pruriens* against Foc TR4, (ii) fingerprint the profiles of phenolic acids and flavonoids in root exudates of *D. uncinatum* and *M. pruriens*, and (iii) assess the effect of identified metabolites on chlamydospore germination, mycelial growth, production of macro- and microconidia, and the synthesis of beauvericin and FA by Foc TR4.

2. Materials and methods

2.1. Fungal strain and plant material

An isolate of *Fusarium oxysporum* Schlecht f. sp. *cubense* (E.F. Smith) Snyder & H.N. Hansen Tropical Race 4 (Foc TR4) (VCG 01213/16) was obtained from the Department of Plant Pathology, Stellenbosch University, South Africa. The fungus was preserved at -80 °C on 30% (v/v) glycerol, and revived by culturing on potato dextrose agar (PDA) at 28 °C for 5 days. Seeds of *Desmodium uncinatum* (Jacq.) DC. (CIAT 728) were provided by the International Centre for Tropical Agriculture (CIAT) in Cali, Colombia. Seeds of *Mucuna pruriens* (L.) DC. were purchased from Gardengoods (Johannesburg, South Africa).

2.2. Reference standards and other chemicals

Twelve reference standards (10 phenolic acids and two flavonoids) were used for the fingerprinting of root-exuded phenolics of *D. uncinatum* and *M. pruriens* (Table 1). All solvents for metabolite

Table 1

Metabolite concentration of phenolic compounds and flavonoids in root exudates of *Desmodium uncinatum* and *Mucuna pruriens* cultured in a hydroponic system.

Metabolite	R _T (min)	UVλ _{max} (nm)	Metabolite concentration in root exudates (μg g ⁻¹ fresh root)	
			Desmodium uncinatum	Mucuna pruriens
Benzoic acid	6.13	229	0.324 ± 0.068 ^{ab}	0.057 ± 0.025 ^b
Caffeic acid	4.29	322	nd	nd
t-cinnamic acid	7.75	275	0.674 ± 0.285 ^a	0.228 ± 0.091 ^a
p-coumaric acid	5.34	309	0.056 ± 0.027 ^b	0.013 ± 0.006 ^b
t-ferulic acid	5.64	322	nd	nd
Gallic acid	1.27	270	nd	nd
p-hydroxybenzoic acid	3.94	254	0.081 ± 0.021 ^b	0.002 ± 0.002 ^b
Protocatechuic acid	2.56	258	nd	nd
Naringenin	8.4	288	nd	nd
Quercetin	7.64	370	nd	0.051 ± 0.008 ^b
Umbelliferon	5.8	324	nd	nd
Vanillin	5.48	280	nd	0.032 ± 0.007 ^b

R_T= Retention time; nd = not detected. Means in the same column followed by the same letter are not significantly different according to Tukey's range test.

analysis were of HPLC-MS grade. Other chemicals, unless otherwise stated, were purchased from Carl Roth (Karlsruhe, Germany). For metabolite fingerprinting, mix-standards with concentrations of 1, 5, and 10 mg L⁻¹ were prepared. For analysis of beauvericin the concentrations of standards were 0.1 and 1 mg L⁻¹, and for FA it was 1, 5 and 10 mg L⁻¹.

2.3. Plant hydroponic culture, root exudate sampling, and extraction of metabolites

Seeds of *D. uncinatum* and *M. pruriens* were surface sterilized (Paradiso et al., 2017) and germinated at 28 °C for 5 days. After germination, 25 seedlings of similar size were selected and cultured in half-strength Hoagland's nutrient solution in a growth chamber (Percival Scientific, Boone, IA, USA) under a 16-h light cycle (irradiance at 100 µmol m⁻² s⁻¹, 25 °C, and 50% relative humidity) for 14 days. The nutrient solution was then changed to full-strength, and the plants cultured for an additional 28 days. The nutrient solution was replenished every 3 days and aerated every 15 min using a membrane pump (KNF Neuberger, Freiburg, Germany). The composition of macronutrients in the nutrient solution was 5 mM KNO₃, 2 mM KH₂PO₄, 1 mM NH₄NO₃, 7 mM Ca(NO₃)₂ × 4H₂O, and 2 mM MgSO₄ × 7H₂O. The composition of micronutrients was 90.4 mM H₃BO₃, 0.5 mM MnSO₄, 1.5 mM ZnSO₄, 0.8 mM Cu SO₄, 0.34 mM (NH₄)₆Mo₇O₂₄, and 63 mM Fe-EDTA.

To collect root exudates, plants were removed from the hydroponic system and their roots gently immersed in sterile distilled water (at 25 °C) for 1 min, followed by dipping in 0.2 mM CaCl₂ (pH 6.2) to remove the nutrient solution. A sponge wrapped around the stem of each plant was used to stabilise the plants on an acrylic lid mounted on a 1-L glass tank (Assistant, Sondheim, Germany). The glass tank was filled with 0.2 mM CaCl₂ (pH 6.2) into which the plant roots were entirely immersed for 6 h to collect root exudates. Collection of root exudates started 3 h after the onset of light (Pramanik et al., 2000). The CaCl₂ solution was filtered using filter paper (Grade 595 ½; 185 mm, Schleicher & Schuell, Dassel, Germany) and the root exudate solution was then transferred to 1-L polypropylene bottles (Kautex Textron, Bonn, Germany), and stored at -20 °C. Roots were removed and weighed to determine fresh root mass.

Root-exuded phenolic metabolites were extracted from the CaCl₂ solution using liquid-liquid phase extraction with ethyl acetate. To do this, samples were mixed with 300 mL of ethyl acetate in 2-L separatory funnels and shaken vigorously for 3 min. After 1 min of repose, the ethyl acetate-phase was filtered over anhydrous Na₂SO₄ to remove any residual water. This process was repeated with 200 mL of ethyl acetate. Extracts were pooled and concentrated at 40 °C using a rotary evaporator (Büchi, Flawil, Switzerland). Extracts were then transferred to pre-weighed 2-mL tubes and reduced to a dried pellet under a nitrogen gas stream on a thermoblock at 40 °C (Barkley, Leopoldshoehe, Germany). For metabolite fingerprinting, the pellet was suspended in 1 mL of acetonitrile:water (1:1). For assessing the cytotoxic potential of root exudates, the pellet was weighed and re-suspended in dimethyl sulfoxide (DMSO) to a final concentration of 1 mg mL⁻¹. The resulting solution was filtered into 50-mL Falcon tubes using a syringe filter (0.2 µm; Sartorius, Göttingen, Germany), and stored at 4 °C in the dark.

2.4. Cytotoxicity bioassay of crude root exudates

The cytotoxic potential of *D. uncinatum* and *M. pruriens* root exudates was assessed using Foc TR4 chlamydospores. The chlamydospores were produced according to the methodology described by Goyal et al. (1973) and purified using density gradient centrifugation. For density gradient centrifugation 20 mL of Percoll gradients (25, 50, and 80% w/v), prepared in 0.1% (v/v) Triton X-100, were gently layered in 50-mL Falcon tubes. The tubes were centrifuged at 3000 revolutions per minute (r.p.m.) for 10 min at 4 °C (Allegra X-15R centrifuge, Beckman Coulter, Fullerton, CA, USA). The chlamydospore suspension was then gently

overlaid onto the upper 80% gradient, and the tubes further centrifuged for 40 min. Gradients were collected separately and washed thrice with 30 mL of cell wash buffer [phosphate-buffered saline (pH 7.6); MP Biomedicals, Irvine, CA, USA] containing 0.01% (v/v) Triton X-100. Following collection, chlamydospores were pooled and resuspended in 10 mL of Tris buffer (50 mM Tris-HCl, pH 7.4).

Cytotoxicity assays were conducted using the Alamar Blue kit (Bio-Rad, Hercules, CA, USA) that measures activity of the mitochondrial respiratory chain as a readout of cell viability. The assay was performed following the manufacturer's protocol. Briefly, root exudates were serially diluted in Barz broth (Hutner et al., 1950; Barz, 1971) to final concentrations of 0, 0.01, 0.1 and 1 µg mL⁻¹, in 24-well cell culture plates (Greiner Bio-One, Frickenhausen, Germany). About 10⁴ chlamydospores were added to each well. Plates were incubated on an orbital shaker (Edmund Bühler, Hechingen, Germany) rotating at 120 r.p.m. in the dark at 28 °C for 24 h. Absorbance was measured using a microplate reader (Tecan, Maennedorf, Switzerland), and readings were normalized to the DMSO control where cell viability was set to 100%.

2.5. Effect of crude root exudates on radial mycelial growth of Foc TR4

A high concentration of root exudates (100 µg mL⁻¹) was used to assess the effect on mycelial growth of Foc TR4. Bioassays were conducted in 90-mm Petri plates, where PDA was mixed with root exudates (100 µg mL⁻¹). For the controls, PDA was mixed with DMSO [10% (v/v)]. Plates were inoculated with a 5-mm-diameter mycelial disc taken from the periphery of a 5-day-old Foc culture, and incubated at 28 °C. After 10 days, the colony diameter was measured using a digital Vernier calliper (NeikoTools, Chesterton, IN, USA) (Hendricks et al., 2017). In addition, the morphology of the colonies and hyphae was examined with bright-field microscopy using a Leica DM750 microscope equipped with a Leica ICC50 HD camera (Leica Microsystems, Heerbrugg, Switzerland).

2.6. Metabolite fingerprinting

Fingerprinting of root-exuded phenolics of *D. uncinatum* and *M. pruriens* was conducted using the Accela HPLC system coupled with the Accela Photodiode Array (DAD)-detector (Thermo Fisher Scientific, San Jose, CA, USA). Separation of metabolites was performed on a YMC-Triart C18 column (3.0 × 100 mm, 3 µm particle size) (YMC, Kyoto, Japan). Eluents were 10 mM formic acid at pH 3.7 (eluent A) and acetonitrile (eluent B). The applied eluent gradient is provided in the supporting information (Table S1). The flow rate was 0.5 mL min⁻¹, and the injection volume was 6 µL. The selected DAD wavelength range was 215–600 nm. Reference standards were run as a standard mixture with concentrations of 1, 5, and 10 mg L⁻¹. Peaks were identified by the specific absorbance maximum of each metabolite (Table 1). Identified metabolites were quantified by linear regression analysis of the corresponding peak areas using the Quanbrowser software (Thermo Fisher Scientific, San Jose, CA, USA). Metabolite concentration was expressed as µg g⁻¹ fresh root weight.

2.7. Selection of metabolites for bioassays

Three metabolites (benzoic acid, *t*-cinnamic acid, and *p*-hydroxybenzoic acid), which were common in root exudates of *D. uncinatum* and *M. pruriens* (Table 1), were selected and assessed for their effect on Foc TR4. Stock solutions (100 mM) of each compound, and a mixture of the three compounds, were prepared in DMSO. The solutions were filter-sterilized as described earlier, and stored at 4 °C in the dark. For bioassays, concentrations of 0.01, 0.1, and 1.0 mM were selected based on a previous study (Siqueira et al., 1996) and measurements in this study.

2.8. Chlamydospore germination, radial mycelial growth, and conidiation bioassays

Chlamydospore germination assays were conducted in 24-well cell culture plates containing 900 µL of Barz broth and 10^4 chlamydospores per well, as described earlier. The broth was supplemented with phenolic acids, and DMSO was used as control. After incubation for 7 h, samples were retrieved in 2 mL Eppendorf tubes and chilled on ice. Germinated chlamydospores were counted using a haemocytometer and a Leica DM750 microscope equipped with a Leica ICC50 HD camera (Leica Microsystems). Micrographs were acquired using Leica Application Suite Software, converted to 8-bit grayscale or 24-bit RGB, and annotated using CorelDraw 12.0 (Corel, Ottawa, Canada). Fine details of germinating chlamydospores were captured using scanning electron microscopy (SEM).

For SEM analysis, chlamydospores were suspended in 1 mL of ice-cold fixative [2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2)] and kept at 4 °C for at least 1 h. After post-fixation in 1% (v/v) osmium tetroxide for 1 h, samples were bloc-stained overnight in 1% (v/v) aqueous uranyl acetate at 4 °C. Samples were then gradually dehydrated in a series of ethanol solutions of 50, 70, 90, and 100% (v/v), and finally dried using hexamethyldisilazane. Dried pellicles were mounted onto 12-mm aluminium SEM stubs using conductive carbon tape. To enhance conductivity, samples were sputter-coated with 8 nm of gold using a low vacuum coater (EM ACE200; Leica, Vienna, Austria). SEM was performed using the Zeiss Merlin scanning electron microscope (Carl Zeiss, Jena, Germany) with a Gemini-type field emission gun electron column (FEG-SEM) equipped with two Oxford Instruments X-MaxN 150 SDDs. Typical imaging conditions were magnification of $1-3 \times 10^4$, a working distance of 5–10 mm, 2–3 kV, a beam current 100–200 pA and using an In Lens secondary electron detector. Micrographs were captured in TIF format using a pixel averaging noise reduction algorithm and SmartSEM software (Carl Zeiss).

The experiment for assessing radial mycelial growth was conducted in 90-mm-diameter Petri dishes containing PDA mixed with phenolic acids, as described earlier. Colony diameter was measured using a digital Vernier calliper (NeikoTools). Production of macroconidia and microconidia was assessed as described by Ohara et al. (2004). Macroconidia and microconidia were enumerated using a haemocytometer and a bright field microscope (Axioskop; Zeiss, Oberkochen, Germany) (Fey et al., 2007).

2.9. Analysis of beauvericin and fusaric acid biosynthesis

Cultures were prepared in 250-mL Erlenmeyer flasks containing 50 mL of Czapek-Dox broth (Sigma-Aldrich, Sternheim, Germany). The broth was inoculated with Foc TR4 and incubated as described earlier. Culture filtrates were harvested by filtering using Grade 595 ½; 185 mm diameter filters (Schleicher & Schuell, Dassel, Germany). The pH of the filtrate was determined, and the samples stored at 4 °C until analysis. To extract toxins from the filtrate, the samples were homogenized for 1 min in acetonitrile-water-glacial acetic acid (79:20:1, v/v/v) using a homogenizer (Workcenter T10 basic, IKA®, Wilmington, NC, USA). The mixture was re-homogenized after 2 min of repose and centrifuged for 10 min at 12000 r.p.m before reducing to dryness under a nitrogen gas stream. Dried crude extracts were reconstituted in acetonitrile/water (50% v/v) prior to analysis.

Analysis of beauvericin was performed by HPLC/ESI-MS using the Accela HPLC-LTQ Velos mass spectrometer (Thermo Scientific, San Jose, CA, USA). The LC separation was performed with a YMC Triart C18 column (3.0 × 100 mm, 3 µm particle size) (YMC, Kyoto, Japan). The eluents were: water with 0.2% (v/v) formic acid in 10% (v/v) acetonitrile (eluent B) and 0.2% (v/v) formic acid (eluent C). The flow rate was 0.5 mL min⁻¹, and the injection volume was 1 µL. The applied eluent gradient is provided in the supporting information (Table S2). The mass spectrometer was operated in the full scan mode with the range of *m/z*

100–900. Beauvericin was identified as protonated molecular ion with *m/z* 784 ($M + H$)⁺. Quantification of beauvericin was performed by linear regression of the corresponding peak areas using the Quanbrowser software (Thermo Fisher Scientific, San Jose, CA, USA).

Analysis of FA was conducted using the Accela HPLC system coupled with an Accela DAD-detector (Thermo Fisher Scientific, San Jose, CA, USA). The eluents were: water with 0.2% (v/v) formic acid in 10% (v/v) acetonitrile (eluent B) and 0.2% (v/v) formic acid (eluent C). The flow rate was 0.5 mL min⁻¹, and the injection volume was 3 µL. The applied gradient is shown in the supporting information (Table S3). The selected DAD wavelength range was 220–350 nm. FA was identified by its specific absorbance maximum of 271 nm. Quantification of FA in the samples was performed by linear regression of the corresponding peak areas using the Quanbrowser software (Thermo Fisher Scientific, San Jose, CA, USA).

2.10. Statistical analysis

Statistical analysis and visualizations were performed using the R programming environment v4.0.2, (R Core Team, 2020). Data were visually inspected for normality and homoscedasticity using diagnostic residual plots and Q-Q plots to assess potential violation of linear modelling assumptions (Kozak and Piepho, 2018). In addition, the assumptions of normality and homoscedasticity were verified using Shapiro-Wilk test and Levene's test, respectively. When the assumptions for parametric tests were met, analysis of variance (ANOVA) and the post hoc Tukey's range test were used to test significant differences in the means. In case data were nonparametric, Mann-Whitney U and Kruskal-Wallis tests were applied. A subsequent post hoc Dunn's test was conducted for all pairs of comparisons between groups using the Benjamini-Hochberg adjustment for multiple testing (adjusted *p* value) (Benjamini and Hochberg, 1995). Where applicable, comparisons were made between treatments and the control. Unless otherwise stated, all values represent mean ± standard error of the mean (s.e.m) values.

3. Results

3.1. Root exudates of *D. uncinatum* and *M. pruriens* exhibit cytotoxicity against Foc TR4

Root exudates of *D. uncinatum* and *M. pruriens* stimulated cellular metabolic activity of Foc TR4 chlamydospores at a concentration of 5 µg mL⁻¹, but cellular metabolic activity was reduced as the concentration of the exudates increased (*p* < 0.0001) (Fig. 1A). The radial mycelial growth of Foc TR4 cultures was also reduced (*p* < 0.0001) in the presence of root exudates at a concentration of 100 µg mL⁻¹ (Fig. 1B). Aerial hyphae were notably reduced in the presence of root exudates (Fig. 1C), with compact masses of aberrant hyphae marked by hyperbranching and a pattern of repetitive bulges along the hyphal walls (Fig. 1C). Similar hyphal morphologies were not observed in the control cultures.

3.2. Identification of *D. uncinatum* and *M. pruriens* root-exuded phenolic metabolites

A total of twelve compounds (Table 1), including the phenolic acids benzoic, *t*-cinnamic, *p*-coumaric, and *p*-hydroxybenzoic, were targeted in root exudates of *D. uncinatum*. Of these, *t*-cinnamic acid and benzoic acid were predominant at 0.674 ± 0.285 and 0.324 ± 0.068 µg g⁻¹ fresh root weight, respectively. In contrast, the phenolic acids, benzoic, *t*-cinnamic, *p*-coumaric, *p*-hydroxybenzoic and vanillin, and the flavonoid quercetin, were detected in root exudates of *M. pruriens*. The predominant phenolic acids in *M. pruriens* were *t*-cinnamic acid and benzoic acid at 0.228 ± 0.091 and 0.057 ± 0.025 µg g⁻¹ fresh root weight, respectively. Unlike benzoic, *t*-cinnamic, *p*-hydroxybenzoic acid, *p*-coumaric was not detected in all root exudate samples of *D. uncinatum*.

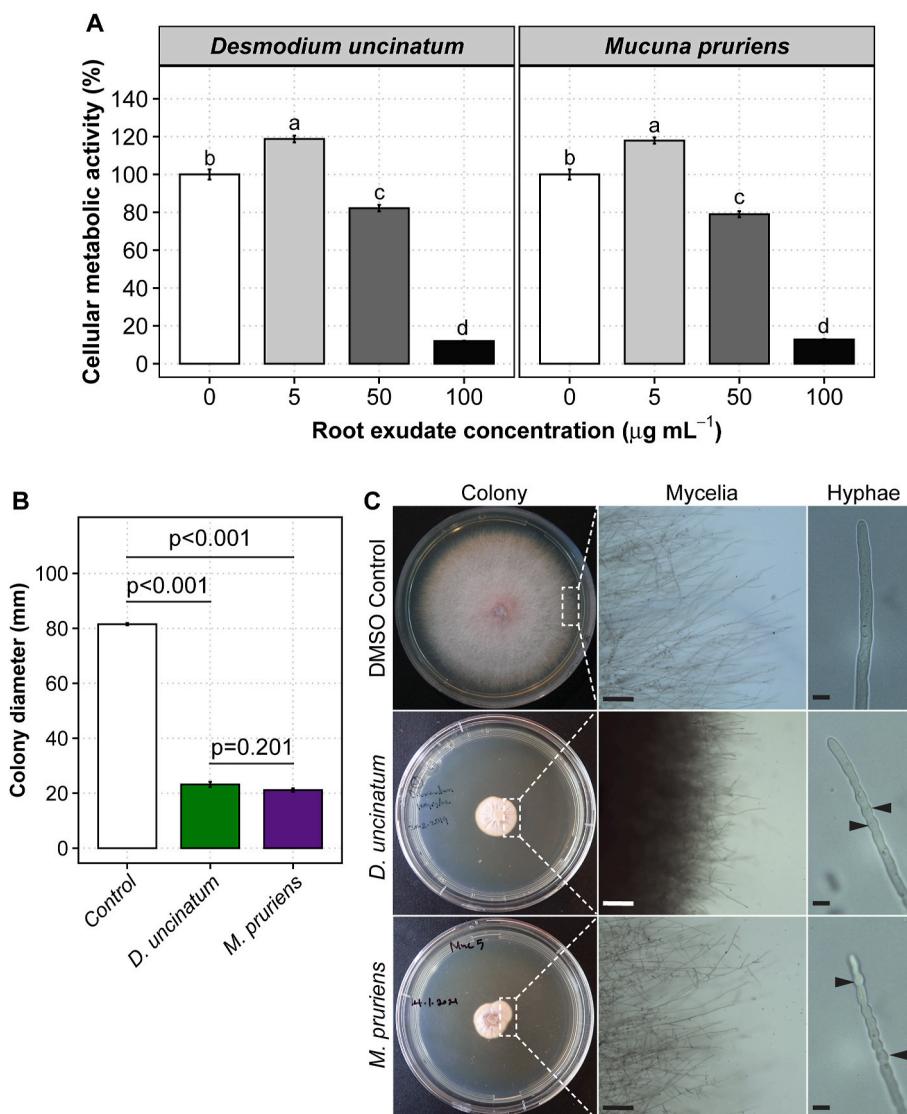


Fig. 1. The cytotoxic effect of *Desmodium uncinatum* and *Mucuna pruriens* root exudates on *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (Foc TR4). Cytotoxic effect of root exudates on chlamydospores assessed by measuring cellular metabolic activity (A). Mycelia growth inhibition (B) revealing compact masses of aberrant hyphae marked by hyperbranching (C) marked by a pattern of repetitive bulges along the hyphal walls (black arrowheads).

3.3. Effect of phenolic acids on chlamydospore germination, mycelial growth, and production of conidia

Chlamydospore germination was inhibited by benzoic acid ($p < 0.05$), *t*-cinnamic acid ($p < 0.05$) and a mixture of benzoic acid, *t*-cinnamic acid, and *p*-hydroxybenzoic ($p < 0.05$), but not *p*-hydroxybenzoic acid alone ($p = 0.0625$) (Fig. 2A). Inhibition of chlamydospore germination was dependent on metabolite concentration, with only benzoic acid reducing germination significantly at the lowest concentration (0.01 mM) compared to the control ($p < 0.05$). Scanning electron microscopy (SEM) revealed inhibition-associated abnormalities in the morphology of chlamydospores and germ tubes. Treated chlamydospores were remarkably shrivelled, deformed, and exhibited profoundly distorted or ruptured germ tubes (Fig. 2B), whereas no such effects were observed in the controls (Fig. 2C).

Radial mycelial growth was significantly stimulated by *t*-cinnamic acid at 0.01 mM ($p = 0.047$) and 0.1 mM ($p = 0.011$), whereas 1 mM had no effect on mycelial growth of Foc TR4 (Fig. 2A). *p*-Hydroxybenzoic acid had no effect on mycelial growth. In contrast, benzoic acid and a mixture of the three metabolites had no effect on mycelial growth at low concentrations, but exhibited a notable inhibition for benzoic acid ($p <$

0.0001) and a mixture of the three compounds ($p < 0.0001$) at 1 mM.

The number of macroconidia produced by Foc TR4 treated with benzoic acid, *t*-cinnamic acid, and *p*-hydroxybenzoic acid, or a mixture thereof, was lower than the control ($p < 0.0001$) (Fig. 2E). An average of $49.938 \pm 1.258 \times 10^3$ macroconidia mL^{-1} were found in the controls compared to less than $20 \times 10^3 \text{ mL}^{-1}$ in the metabolite-mixed treatments. A reduction of microconidia was found in treatments with benzoic acid at concentrations higher than 0.01 mM, and with *t*-cinnamic acid at 1 mM ($p < 0.0001$) (Fig. 2E). *p*-Hydroxybenzoic acid had no effect on the production of Foc TR4 microconidia. The phenolic acid mixture was at least two orders of magnitude more effective than individual compounds in suppressing the production of macro- and microconidia. The lowest concentration of the phenolic acid mixture (0.01 mM) reduced the production of macroconidia to an average of $13.0 \pm 0.632 \times 10^3 \text{ mL}^{-1}$ and of microconidia to $626.6 \pm 24.459 \times 10^3$ spores mL^{-1} , compared to $53.0 \pm 1.048 \times 10^3$ macroconidia and $999.4 \pm 18.859 \times 10^3$ microconidia mL^{-1} for the DMSO control.

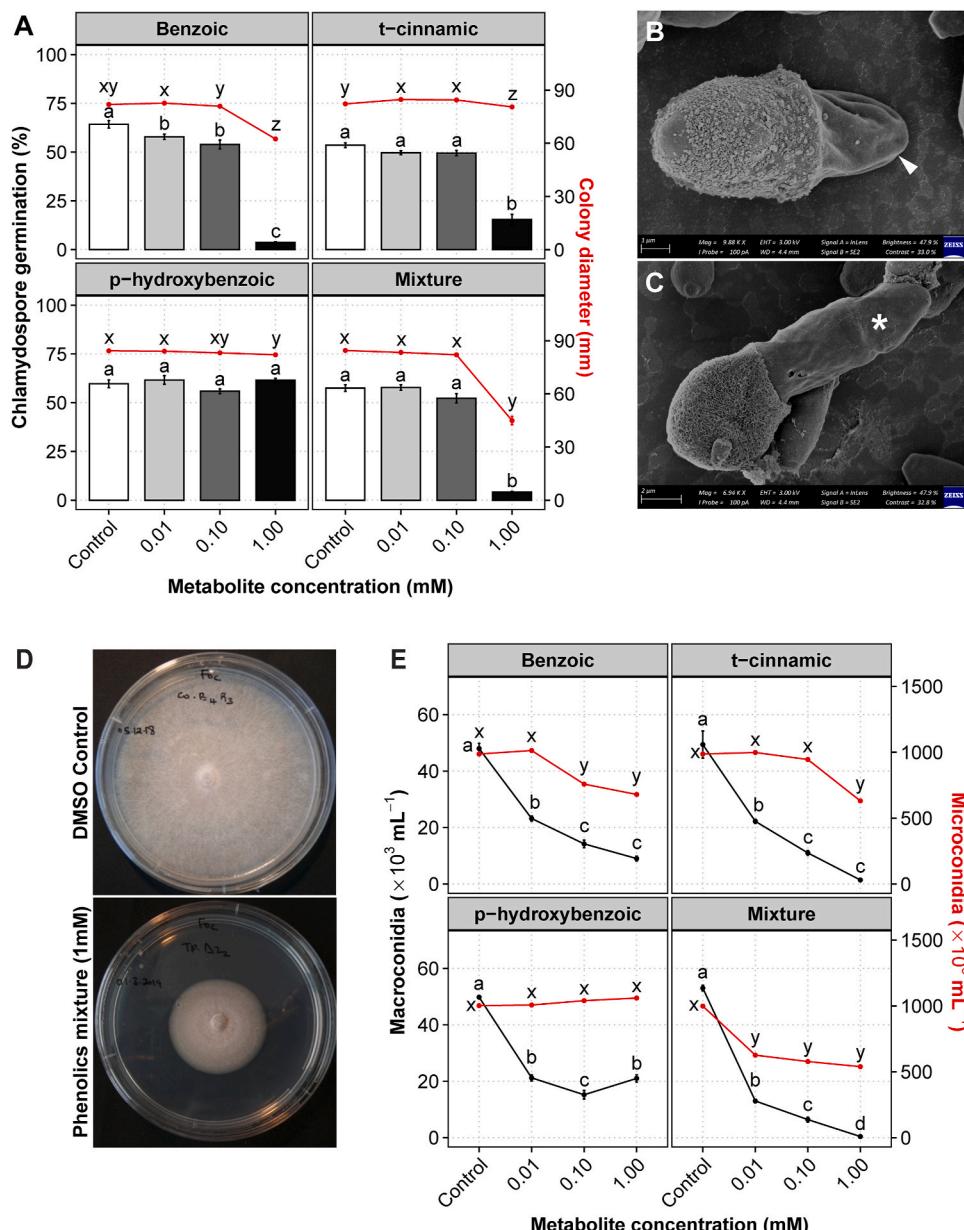


Fig. 2. Effect of phenolic acid metabolites detected in root exudates of *Desmodium uncinatum* and *Mucuna pruriens* on chlamydospore germination and radial mycelial growth of *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (Foc TR4) (A–D). (B) Electron micrographs of a germinating Foc TR4 chlamydospore with a protruding germ tube (white asterisk) and (C) a chlamydospore germinating in the presence of a mixture of phenolic acids at 1 mM revealing a distorted germ tube (white arrowhead). (D) A colony of Foc TR4 growing on potato dextrose agar (PDA), and (E) PDA supplemented with a mixture of phenolic acids at 1 mM (D). The phenolics mixture includes benzoic acid, t-cinnamic acid, and p-hydroxybenzoic acid.

3.4. Effect of phenolic acids on biosynthesis of fusaric acid and beauvericin

Biosynthesis of beauvericin was stimulated by benzoic acid, while t-cinnamic acid, p-hydroxybenzoic acid, and a mixture of the three metabolites, were stimulatory at low concentrations and inhibitory at 1 mM (Fig. 3A). Conversely, the biosynthesis of FA was progressively reduced by increasing concentrations of the phenolic acids individually and as a mixture (Fig. 3B). Biosynthesis of FA in Foc TR4 was completely abolished when treated with 1 mM of the metabolite mixture ($p < 0.0001$).

Interestingly, the pH (6.7 ± 0.2) of the broth medium was elevated during fungal growth. Hence, it was hypothesized that the repression of FA biosynthesis by phenolics was pH-dependent, and not due to effects brought on by impaired fungal growth after treatment. To prove this hypothesis, Foc TR4 was cultured in the same broth buffered at low (5.0) or high (7.0) pH using MES (2-(*N*-morpholino) ethanesulfonic acid). When FA concentration and fungal biomass were determined, an 8-fold increase in FA concentration was found at pH 7 compared to pH 5 ($p = 0.0008$). Fungal biomass was, however, not affected by pH ($p > 0.05$).

(Fig. 3C, D). The repression of FA biosynthesis by phenolic acids thus appears not to be linked to a reduction in fungal biomass. Rather, dissipation of cytosolic pH was assumed as a mechanism by which phenolic acids repress FA biosynthesis in Foc TR4.

4. Discussion

Plants release a myriad of primary and secondary metabolites into the rhizosphere through the process of root exudation (Haichar et al., 2016; Oburger and Jones, 2018). While root exudation was suggested to play an important role in the suppression of Fusarium wilt disease in banana intercropping systems (Buddenhagen, 2009; Pattison et al., 2014), the suppressive effect of root exudates and the metabolites involved remain poorly understood. Here, we show that root exudates of two legumes, *D. uncinatum* and *M. pruriens*, exhibit cytotoxicity against Foc TR4. The cytotoxicity can be attributed to metabolites present in root exudates of the two legumes. In support of this, targeted metabolite analysis revealed differences in phenolics (phenolic acids and flavonoids) present in root exudates of *D. uncinatum* and *M. pruriens*.

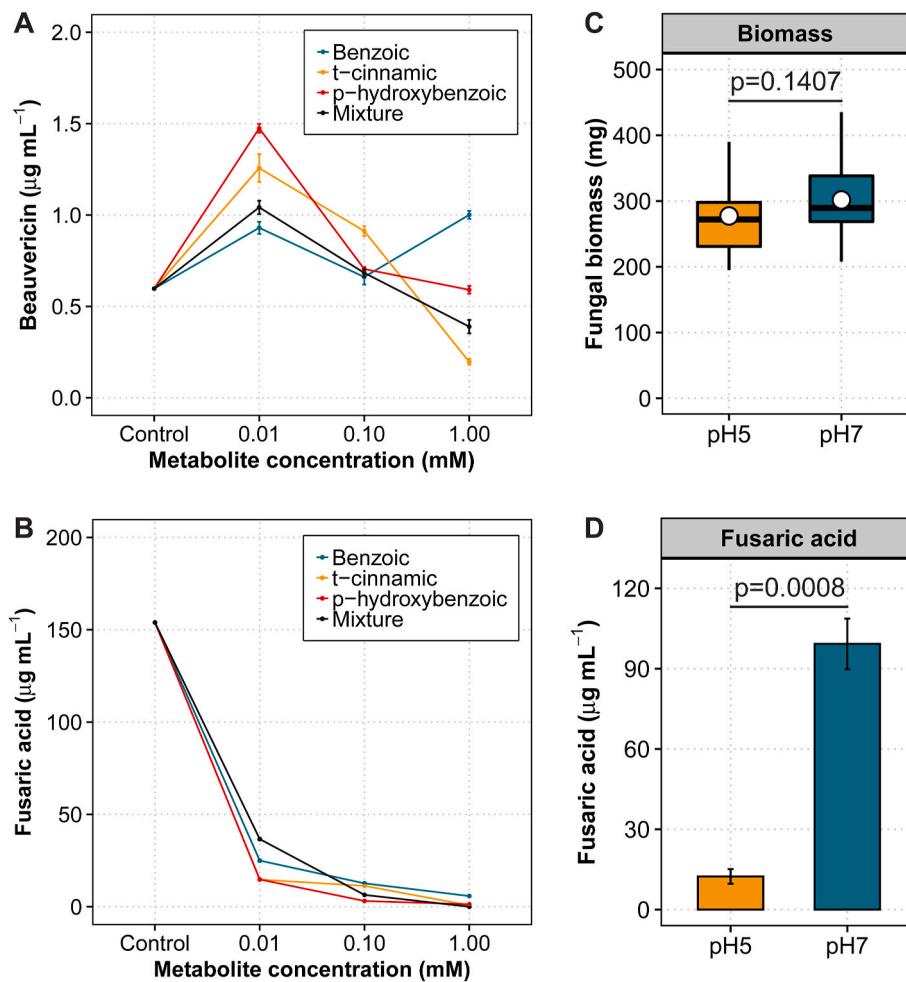


Fig. 3. Effect of phenolic acid metabolites on synthesis of beauvericin (A) and fusaric acid (FA) (B) mycotoxins in *Fusarium oxysporum* f. sp. *cubense* Tropical Tace 4 (Foc TR4). Influence of extracellular pH (pH 5 and pH 7) on fungal biomass (C) and FA biosynthesis biosynthesis (D) in Foc TR4. Boxplots show the upper and lower quartile, median (bold horizontal bar), mean (white circle), and whiskers (vertical lines).

Phenolics represent one of the major classes of plant secondary metabolites with antioxidant properties, antimicrobial activity, and have been reported to suppress soil-borne pathogens including *F. oxysporum* spp. (Michielse et al., 2012; Yuan et al., 2018). The difference in the composition of phenolics in root exudates of the two legumes may be attributed to plant genotype. Plant genotype is a well recognized factor that strongly influences the metabolite composition and quantity of root exudates along with the microbiome present in the rhizosphere (Micallef et al., 2009; Zhalnina et al., 2018; Iannucci et al., 2021).

Benzoic acid exhibited the highest inhibitory effect on chlamydospore germination, production of conidia, and mycelial growth in Foc TR4. The difference in antimicrobial effect of phenolic acids has been previously reported for other *Fusarium* spp. (Schöneberg et al., 2018; Gautier et al., 2020), and may be attributed to the position of hydroxyl moiety on the aromatic ring or to the antioxidant capacities of the phenolic acids (Synowiec et al., 2021). Antioxidant compounds may act via nonspecific mechanisms such as disruption of plasma membrane function which can lead to a modification of membrane permeability (Pagnussatt et al., 2014; Morales et al., 2017).

The compact masses of aberrant hyphae with distinct structural aberrations could be due to disruption of the Spitzenkörper, a pleomorphic complex that regulates hyphal growth and morphogenesis (Riquelme et al., 2018). The Spitzenkörper is formed by aggregation of cytoskeletal components together with transport vesicles containing precursors for cell surface expansion and growth at hyphal tips (Riquelme et al., 2018). Phenolic acids such as *p*-coumaric acid and caffeic acid, where the

former was detected in root exudates of *D. uncinatum* and *M. pruriens*, have been reported to disrupt the Spitzenkörper and ultimately inhibit hyphal growth (Neves et al., 2005).

The reduced number of conidia suggests that phenolic acids inhibit conidia production in Foc TR4 which is consistent with previous reports on the suppression of conidia production in *Fusarium oxysporum* spp. by phenolics (Wu et al., 2008; Michielse et al., 2012). Generally, the prelude to conidia production in *F. oxysporum* spp. is the formation aerial hyphae. Macroconidia are mostly produced in sporodochia that occur on aerial hyphae (Ohara et al., 2004). The reduced number of macroconidia could be attributed to the reduction in aerial hyphae which was also observed in Foc TR4 cultures treated with phenolic acids. A reduction in the formation of aerial hyphae was reported to reduce the production of macroconidia in *Fusarium oxysporum* (Ohara and Tsuge, 2004). The biological significance of this finding would be that a decrease in macroconidia production could limit chlamydospore formation, because chlamydospores often develop from the cytological modification in segments of macroconidia (Ohara and Tsuge, 2004; Leslie and Sumnerell, 2006).

The dramatic decrease in FA in the filtrate of Foc TR4 cultures supplemented with phenolic acids suggests repression of FA biosynthesis. This finding apparently contrasts a report in *F. oxysporum* f. sp. *niveum*, in which *t*-cinnamic acid promoted mycotoxin biosynthesis (Wu et al., 2008) and could be attributed to the difference in fungal strains used. Suppression of FA biosynthesis in Foc TR4 may negatively affect the establishment of Foc TR4 in the rhizosphere, as FA plays an important

role in the inhibition of competing soil microorganisms during colonisation of the rhizosphere (Notz et al., 2002; Bacon et al., 2005; Spraker et al., 2018). Moreover, our findings suggest that biosynthesis of FA in Foc TR4 may be tightly linked to pH, where an alkaline pH is ideal for biosynthesis of FA, as previously reported in other *Fusarium* spp. (Masachis et al., 2016; Sánchez-Rangel et al., 2018; López-Díaz et al., 2018). Thus, the suppression of FA biosynthesis by phenolic acids could, in part, be attributed to the acidic, apolar, and planar characteristics allowing phenolic acids to readily traverse the plasma membrane and become protonated, thereby acidifying the cytosol (Pagnussatt et al., 2014; Morales et al., 2017). In support of this, buffering the growth medium at pH 5 dramatically reduced the amount of FA produced by Foc TR4 cultures.

The increased concentration of beauvericin in the supernatant of Foc TR4 cultures supplemented with low concentration of phenolic acids suggests the stimulation of beauvericin biosynthesis. A mechanistic explanation for this stimulation remains unclear, but could reflect a hormesis effect which is the protective response induced in cells and organisms exposed to low doses of toxic compounds (Calabrese et al., 2010; Belz and Sinkkonen, 2019). Stimulation of mycotoxin biosynthesis or fungal growth by low doses of phenolic acids has been reported previously (Wu et al., 2008) and in part attributed to catabolism of phenolic acids. However, the possibility that Foc TR4 is able to catabolise phenolic acids, releasing metabolic intermediates that stimulate fungal metabolism and growth, could not be corroborated in our experimental settings. Our findings highlight the role of legume root-exudate metabolites in pathogen suppression. With this, intercropping banana with *D. uncinatum* and *M. pruriens* may be considered for prospective development of sustainable strategies for management of Foc TR4.

5. Conclusion

This study examined the role of root-secreted metabolites in the suppression of Foc TR4, emphasizing the suppression of pathogen development and metabolism. Three phenolic acids; benzoic acid, *t*-cinnamic acid, and *p*-hydroxybenzoic acid; were common in root exudates of *D. uncinatum* and *M. pruriens*. Depending on the compound and its concentration, phenolic acids inhibited chlamydospore germination, hyphal growth, production of macroconidia and microconidia and the synthesis of beauvericin and FA toxins in Foc TR4. A key message of this study was the ability of Foc TR4 to alkalinize the growth medium (external environment), suggesting a metabolic adaptation for the production of virulence factors such as FA.

Author contributions

FR and AV conceived the project, sourced the funding, provided the resources, and supervised the research. EW designed performed the experiments, curated and analysed the data, and drafted the original manuscript. EW and JS performed metabolite analysis. FR, JS, and AV, critically revised and gave further inputs to the final manuscript.

Data availability statement

All data generated or analysed during this study are included in this article.

Declaration of competing interest

The authors declare no potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.rhisp.2021.100459>.

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