



Involvement of lytic enzymes and secondary metabolites produced by *Trichoderma* spp. in the biological control of *Pythium myriotylum*

Severin Nguemezi Tchameni^{1,2} · Mihaela Cotârleț³ · Ioana Otilia Ghinea² · Marie Ampere Boat Bedine⁴ · Modeste Lambert Sameza¹ · Daniela Borda³ · Gabriela Bahrim³ · Rodica Mihaela Dinică²

Received: 22 January 2019 / Revised: 20 May 2019 / Accepted: 18 June 2019 / Published online: 2 July 2019
© Springer Nature Switzerland AG 2019

Abstract

This investigation was aimed to evaluate the antimicrobial activities and involvement of extracellular lytic enzymes produced by four strains of *Trichoderma* in the inhibition of *Pythium myriotylum*. Antagonistic effects were tested by dual culture. Activities of lytic enzymes were evaluated from the filtrate of each strain after cultivation in selected media. Organic extracts were obtained from liquid media subsequent to the cultivation of *Trichoderma* in potato dextrose broth (PDB). Non-volatile organic compounds such as polyphenols and flavonoids were evaluated spectrophotometrically while volatile organic compounds (VOCs) were analyzed by gas chromatography coupled with mass spectrometry (GC-MS). The antimicrobial activity of the organic extracts was determined using the poisoning method. Results have shown that all the strains were antagonists against *P. myriotylum*. *T. erinaceum* (IT-58), *T. gamsii* (IT-62), *T. afroharzianum* (P8), and *T. harzianum* (P11) that were found to produce cellulase, protease, and xylanase. Over 20 compounds were identified in each extract, including esters, lactones, and organic acids. The organic extracts also contained high amounts of polyphenolic compounds and flavonoids and significantly inhibited the mycelial growth of *P. myriotylum*. The minimal inhibition concentrations were 80 µg/µL, 40 µg/µL, 20 µg/µL, and 10 µg/µL, for extracts obtained from *T. erinaceum* (IT-58), *T. gamsii* (IT-62), *T. afroharzianum* (P8), and *T. harzianum* (P11), respectively. There was significant correlation between the production of total polyphenol and flavonoid content and the antagonistic effects of the tested strains.

Keywords *Trichoderma* sp. · Lytic enzymes · Secondary metabolites · Biological control · *Pythium myriotylum*

Introduction

Root rot of cocoyam (*Xanthosoma sagittifolium*) caused by *Pythium myriotylum* is a serious disease in sub-tropical countries including Cameroon (Gomez-Alpizar et al. 2011).

Infection occurs in the high humidity conditions during the rainy season. The first symptoms are yellowing and wilting of leaves, root rot, drying, and the infection often results in the death of the whole plant (Adiobo et al. 2007). In the absence of control measures, yield loss could reach up to 80–100%. The most practical methods employed by farmers are the use of resistant cultivars and chemical fungicide. Since the pathogen is soil borne, application of fungicide is difficult in humid conditions, expensive, and their repeated application could result in soil contamination, fungicide resistance, and could be harmful to non-target organisms (Tambong and Hofte 2001).

Hence, alternatives to reduce the use of chemical fungicides, aimed to enhance the productivity with less negative environment and ecological consequences, are needed. Biological control by using beneficial soil, belonging microorganisms, bacteria, and fungi species, are promising methods (Woo et al. 2014). Several species of *Trichoderma* have been successfully used for biological

✉ Rodica Mihaela Dinică
Rodica.Dinica@ugal.ro

¹ Laboratory of Biochemistry, Faculty of Science, University of Douala Cameroon, PO Box 24157, Douala, Cameroon

² Faculty of Science and Environment “Dunărea de Jos”, University of Galati, Romania, Domnească Street, 47, 800008 Galati, Romania

³ Faculty of Food Science and Engineering, “Dunarea de Jos”, University of Galati, Romania. Domnească Street, 47, 800008 Galati, Romania

⁴ Laboratory of Phytobiochemistry, Faculty of Science, University of Yaounde I, PO Box 812, Yaounde, Cameroon

control of soil borne pathogens (Vinale et al. 2008; Lorito et al. 2010; Tchameni et al. 2017). Mechanisms used by fungi as biological control agents against plant pathogens include competition for nutrients, induced systemic resistance, mycoparasitism, production of antibiotics, and extracellular lytic enzymes. During antagonism, *Trichoderma* produces volatile and non-volatile organic compounds such as polyphenols, flavonoids, terpenes, pyrones, sesquiterpenes, ketones, thioesters, cyclohexanes, and alcohols (Reino et al. 2008; Vinale et al. 2014). Many studies have shown that the production of these secondary metabolites was associated with inhibition of mycelial growth and spore germination of many plant pathogens (Zhang et al. 2014; Vos et al. 2015; Nieto-Jacobo et al. 2017). In addition, the antagonistic effect of *Trichoderma* is correlated with the production of extracellular lytic enzymes such as cellulases, proteases, chitinases, and xylanases (Harman 2006). However, successful antagonism often results from synergistic effects from different mechanisms responsible for an antimicrobial interaction.

This work was aimed to evaluate the antimicrobial activity of organic extracts of 4 strains of *Trichoderma* and the involvement of their proteases, cellulases and xylanases in the inhibition of *Pythium myriotylum*.

Materials and methods

Pathogen

Pythium myriotylum was isolated from the root of cocoyam showing the typical symptom of root rot disease. Infected roots were rinsed in distilled water and disinfected with sodium hypochlorite (1%) for 5 min. Roots were cut (3–5 mm) and inoculated on a potato dextrose agar (PDA) medium supplemented with penicillin (250 mg/mL) and ampicillin (250 mg/mL) and incubated at 28 °C. Plates were monitored daily, and the suspect pathogen mycelia were transferred in the fresh medium. *Pythium myriotylum* was identified using the protocol described by Agrios (2005). The pathogen was maintained in PDA medium and stored at 4 °C.

Trichoderma strains

Four strains of *Trichoderma* were used in this study. These strains came from core culture of the mycothèque of the Laboratory of Biochemistry, University of Douala. These isolates had been previously characterized using microscopic and molecular techniques. The obtained nucleotide sequences were submitted to the NCBI (National Center Biotechnology Information) GenBank data (www.ncbi.nlm.gov/BLAST) for annotation. Sequences are available under accession numbers:

JN157757, KX009499, KP115287, and KF040478 for *Trichoderma erinaceum* (IT-58), *Trichoderma gamsii* (IT-62), *Trichoderma afroharzianum* (P8), and *Trichoderma harzianum* (P11), respectively.

In vitro antagonism assay

Five-millimeter plug of each strain of *Trichoderma* taken from 2-day-old culture was inoculated at one side of Petri dish containing PDA medium. A 3–5-mm mycelial plug from 5-day-old PDA culture of *P. myriotylum* was inoculated on the perpendicular opposite side. Plates inoculated only with pathogen served as control. All the plates were incubated at 30 °C for 7 days. The growth of pathogen in both the test and control plates was recorded. The inhibition of mycelial growth of the pathogen was evaluated according the formula: $I (\%) = ((D_o - D_t)/D_o) \times 100$, where D_o is the diameter of growth of the pathogen and D_t is the diameter of growth of the pathogen in paired culture. Each treatment consisted of three replicates, and the experiment was repeated twice.

Assay of proteases

The culture medium (25 mL) used for protease production was a mineral medium containing the following (g/L): $MgSO_4$ 0.52, KCl 0.52, KH_2PO_4 1.52, $FeSO_4 \times 7H_2O$ 0.01, $ZnSO_4 \times 7H_2O$ 0.01, and casein 5. The culture medium was first inoculated with a 0.5-cm mycelial plug and incubated at 25 ± 2 °C for 7 days. The culture was then centrifuged (5000 rpm, 10 min, 4 °C), and the supernatant (crude enzyme) was collected for analysis. A proteolytic activity assay, using casein as the substrate, was performed according to the method described by Ramakrishna and Pandit (1998) with some modifications. Enzyme activity was determined by incubating 250 μ L of the culture supernatant with 500 μ L 1% (w/v) casein sodium salt in 50 mM buffer pH 7.0 for 2 h at 30 °C. The reaction was stopped by the addition of 375 μ L 20% (w/v) trichloroacetic acid. The tubes were placed in an ice bath for 30 min and then centrifuged at 5000 rpm for 15 min at 4 °C, and the absorbance of the supernatant was measured at 280 nm versus an appropriate blank. The specific protease activity was defined as the amount of enzyme that hydrolyzes casein to produce equivalent absorbance to 1 μ mol of tyrosine/min/mg of protein, with tyrosine as standard. The soluble protein content in supernatant was estimated by the method described by Bradford (1976).

Assay of cellulases

The cellulase activity was assayed based on the method described by Gautam et al. (2011). To initiate the cellulase assay, 25 mL of culture broth (3 g $NaNO_3$, 0.1 g KH_2PO_4 , 0.5 g $MgSO_4 \times 7H_2O$, 0.5 g KCl, 1% CMC) was first inoculated with a 0.5-cm mycelial plug and incubated at 25 ± 2 °C for 7 days. The culture was then centrifuged (5000 rpm, 10 min,

4 °C), and the supernatant (crude enzyme) was collected for analysis. The supernatant (0.5 mL) was mixed with 1.5 mL reaction mixture (0.5 mL of 1% CMC, 1 mL of 0.1 M sodium citrate buffer at pH 4.8) and incubated for 60 min at 50 ± 2 °C. The reducing sugar produced was determined using dinitrosalicylic acid (DNS) (Miller 1959) where 3 mL of DNS reagent was added to the reaction mixture, heated at 90 °C for 10 min, followed by the addition of 1 mL of 40% potassium sodium tartrate. After cooling, the absorbance was read at 540 nm. The amount of glucose released was calculated from the glucose standard curve (0–35 $\mu\text{mol/L}$). The specific cellulase activity was defined as the amount of enzyme needed to release 1 μmol of glucose per min per mg of protein under assay conditions described.

Assay of xylanases

Five-millimeter mycelial plug (7 days) of each strain of *Trichoderma* was incubated into 250-mL Erlenmeyer flasks containing 50 mL of isolation media (devoid of agar) during 7 days under agitation at 180 rpm. The culture was filtered (Whatman no. 1), the filtrate was then centrifuged (5000 rpm, 10 min, 4 °C), and the supernatant (crude enzyme) was collected for analysis. Xylanase activity was assayed in 2% corn-cob powder, 0.4% NH_4NO_3 , 0.05% K_2HPO_4 , 0.05% $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 0.05% KCl, and 0.001% $\text{FeSO}_4 \times 7\text{H}_2\text{O}$. The xylanase activity was measured using 3,5-dinitrosalicylic acid (DNS). At 50 °C and pH 5.0, activity of xylanase was defined as the amount of enzyme required to release 1 μmol xylose per minute per mg of protein. Xylose was used as standard for xylanase analysis.

Liquid culture and extraction of organic compounds

Two 5-mm-diameter plugs of each *Trichoderma* strain, obtained from actively growing margins on PDA cultures, were inoculated into 1-L conical flasks containing 250 mL of sterile potato dextrose broth (PDB). Cultures were incubated for 14 days at 30 °C and 120 rpm. The cultures were filtered under vacuum through filter paper (Whatman No. 4; Brentford, UK), and the filtrates stored at 4 °C for 24 h. The organic metabolites were extracted exhaustively with ethyl acetate (3 times), and the combined organic fraction was dried (Na_2SO_4) and evaporated under reduced pressure at 40 °C. The red-brown residue recovered was stored at 4 °C until further analysis.

Evaluation of total phenol and total flavonoid

Total Polyphenol Content

The total polyphenol content was determined by applying the Folin-Ciocalteu method (Cudalbeanu et al. 2018) to a 96-well plate analysis. Twenty-five microliters of Folin-Ciocalteu reagent

was added to 10 μL of extract. After 5 min of incubation, 25 μL of a 20% aqueous sodium carbonate solution and then ultrapure water was added until the final volume reached 200 μL . Blanks were also prepared for each sample by replacing the Folin-Ciocalteu reagent with ultrapure water. A freshly prepared gallic acid solution was used as a standard reference, and the results are given in equivalents of gallic acid per 1 g of sample. After 30 min, the absorbance values of the samples at 760 nm were recorded using a multiwell plate reader (Tecan Pro 200).

Total Flavonoid Content

The total flavonoid content was quantified through a 96-well plate analysis using aluminum chloride. One hundred microliters of a 2% aqueous aluminum chloride solution was added to 100 μL of sample. After 15 min of incubation, the absorbance values at 415 nm were determined using the Tecan Pro 200 multiwell plate reader. Quercetin was used as a reference standard, and the results are given in equivalents of quercetin per 1 g of sample.

VOC analysis by GC-MS

For the identification of volatile compounds (VOCs) from *Trichoderma* spp., the concentrated extracts were dissolved in 100 μL of methanol, passed through a 0.45- μm disposable PTFE filter, and used for GC-MS analysis. The GC-MS analyses were performed on a Varian 4000 electron impact mass spectrometer using a Varian CP-8400 injector. A 30 m \times 0.25-mm factor four capillary column with a particle size of 0.25 μm (Varian) was used. The injection temperature was set at 250 °C. The helium gas flow rate through the column was 1 mL/min, ions were generated at an electron impact (EI) of 70 kV, the ion source temperature was set at 200 °C, and the mass range was m/z 50–1000. The column temperature was maintained isothermally at 70 °C for 2 min and then raised to 300 °C at a rate of 10 °C/min. Authentic analytical standards were employed for retention time and retention index comparison with the VOCs detected. VOCs were identified against a library of mass spectra (National Institute of Standard and Technology v2.1 mass spectral database).

Antimicrobial assay of organic extract

The organic crude extracts were tested in vitro against *P. myriotylum* to evaluate their antimicrobial properties. Pathogen plugs (5-mm diameter) from growing edges of colonies were placed in the center of Petri dishes containing PDA. Ten microliters of crude extracts, at concentrations varying from 10 to 80 $\mu\text{g}/\mu\text{L}$, was applied on the top of each plug. Controls were obtained by applying 10 μL of DMSO. The solvent was evaporated in a laminar flow cabinet, and the plates were incubated at 30 °C for 5 days. The pathogen

growth was measured daily as colony diameter. Each treatment consisted of three replicates and the experiment was repeated twice. The growth inhibition (GI) was determined on the third day using the following equation: $GI = ((Do - Dt)/Do) \times 100$ where Do is the diameter of mycelial growth of pathogen and Dt is the diameter of mycelial growth of pathogen during antagonism.

Statistical analysis

Statistical analyses of the data were performed using STATGRAPHICS Computer Software, version 5.0. The differences among treatments for all the studied characteristics mentioned above were analyzed for statistical significance using variance analysis (ANOVA). Treatments were compared by using Duncan's multiple range test at the $p < 0.05$ significance level.

Results

In vitro antagonism of *Trichoderma*

Four strains of *Trichoderma* were tested for their ability to inhibit mycelial growth of *P. myriotylum* in vitro by the paired culture technique. The results showed that all the strains used inhibit the mycelial growth of *P. myriotylum* (Table 1). The degree of inhibition was 48.68%, 50.67%, 57.0%, and 63.33%, respectively, for *T. erinaceum* (IT-58), *T. gamsii* (IT-62), *T. afroharzianum* (P8), and *T. harzianum* (P11). According to these results, statistical analysis revealed that there is significant difference ($p \leq 0.05$) between the inhibitions percentage.

Effects of organic crude extract of *Trichoderma* spp. on mycelial growth of *P. myriotylum*

All the organic extracts from the strains of *Trichoderma* used significantly inhibited the mycelial growth of *P. myriotylum* (Table 2). The total inhibition growth was

Table 1 Inhibitory effects of *Trichoderma* against *P. myriotylum* in dual culture

Strains of <i>Trichoderma</i>	Inhibition of mycelial growth (%)
IT-58	48.67a ± 1.2
IT-62	50.67a ± 3.9
P8	57.00ab ± 1.6
P11	63.33b ± 1.01

IT-58: *Trichoderma erinaceum*; IT-62: *Trichoderma gamsii*; P8: *Trichoderma afroharzianum*; P11: *Trichoderma harzianum*. Means with same letters are not significantly different at $p \leq 0.05$. Each treatment was made by three replicates, and the experiment was repeated twice

evaluated at four different concentrations for extracts from *T. erinaceum* (IT-58), *T. gamsii* (IT-62), *T. afroharzianum* (P8), and *T. harzianum* (P11), respectively. Negative and significant correlation was obtained between the antagonistic activity and the concentration of inhibition of mycelial growth of crude organic extract. The organic extract from *T. harzianum* (P11) which had the highest antagonistic activity also had the minimum inhibitory concentration among all the organic extracts.

Activity of extracellular lytic enzymes

Activity of cellulase

Trichoderma harzianum (P11) recorded the highest activities of cellulase followed by *T. afroharzianum* (P8), *T. gamsii* (IT-62), and *T. erinaceum* (IT-58) (Fig. 1). *Trichoderma harzianum* (P11), which recorded the highest inhibition, exhibited the highest cellulase activity; *T. erinaceum* (IT-58), which recorded least inhibition, produced the lowest cellulase activity.

Activity of xylanase

Results presented in Fig. 2 showed that *T. erinaceum* (IT-58) exhibited the highest xylanase activity followed by *T. harzianum* (P11), *T. afroharzianum* (P8), and *T. gamsii* (IT-62). *T. erinaceum* (IT-58) which had the lowest antagonistic effect recorded the highest xylanase activity.

Activity of protease

The results of protease activity are presented in the Fig. 3. *T. harzianum* (P11) recorded the highest activity followed by *T. afroharzianum* (P8), *T. gamsii* (IT-62), and *T. erinaceum* (IT-58). *T. harzianum* (P11) which exhibited the highest antagonistic activity had the highest activity of protease.

Total phenolic compounds and flavonoid content

The highest total phenolic compounds were produced by *T. harzianum* (P11) followed by *T. afroharzianum* (P8), *T. gamsii* (IT-62), and *T. erinaceum* (IT-58) (Fig. 4). The flavonoid production was also maximum with *T. harzianum* (P11) followed by *T. afroharzianum* (P8), *T. erinaceum* (IT-58), and *T. gamsii* (IT-62) (Fig. 5). A significant correlation was observed between inhibitory effect of secondary metabolites and the production of total phenols and flavonoids. *T. harzianum* (P11) which has the lowest minimal inhibitory concentration exhibited the highest production of phenols and flavonoid compounds.

Table 2 Inhibitory effects of organic extract from *Trichoderma* against *P. myriotylum*

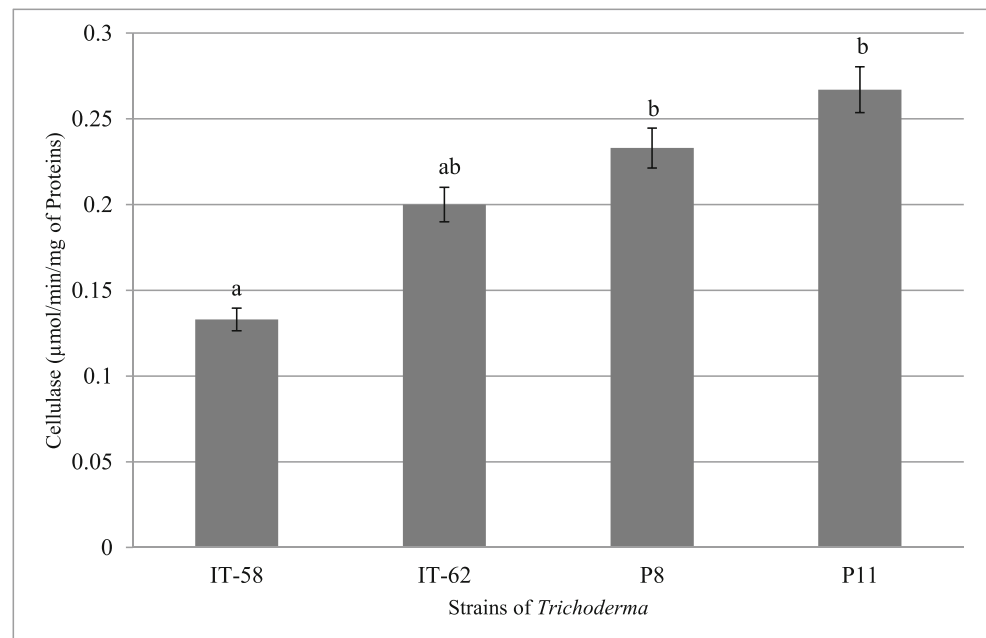
Crude extracts ($\mu\text{g}/\mu\text{L}$)	Inhibition of mycelial growth (%)			
	IT-58	IT-62	P8	P11
0.0	0.0a \pm 0.0	0.0a \pm 0.0	0.0a \pm 0.0	0.0a \pm 0.0
10.0	0.0a \pm 0.0	30.0b \pm 2.2	30.66b \pm 2.8	100.0b \pm 0.0
20.0	30.25b \pm 0.9	53.50c \pm 1.6	100.0c \pm 0.0	100.0b \pm 0.0
40.0	60.75c \pm 0.7	100.0d \pm 0.0	100.0c \pm 0.0	100.0b \pm 0.0
80.0	100.0d \pm 0.0	100.0d \pm 0.0	100.0c \pm 0.0	100.0b \pm 0.0

IT-58: *Trichoderma erinaceum*; IT-62: *Trichoderma gamsii*; P8: *Trichoderma afroharzianum*; P11: *Trichoderma harzianum*. In the same column, the means with same letters are not significantly different at $p \leq 0.05$. Each treatment was made by three replicates, and the experiment was repeated twice

Analysis of volatile organic compounds

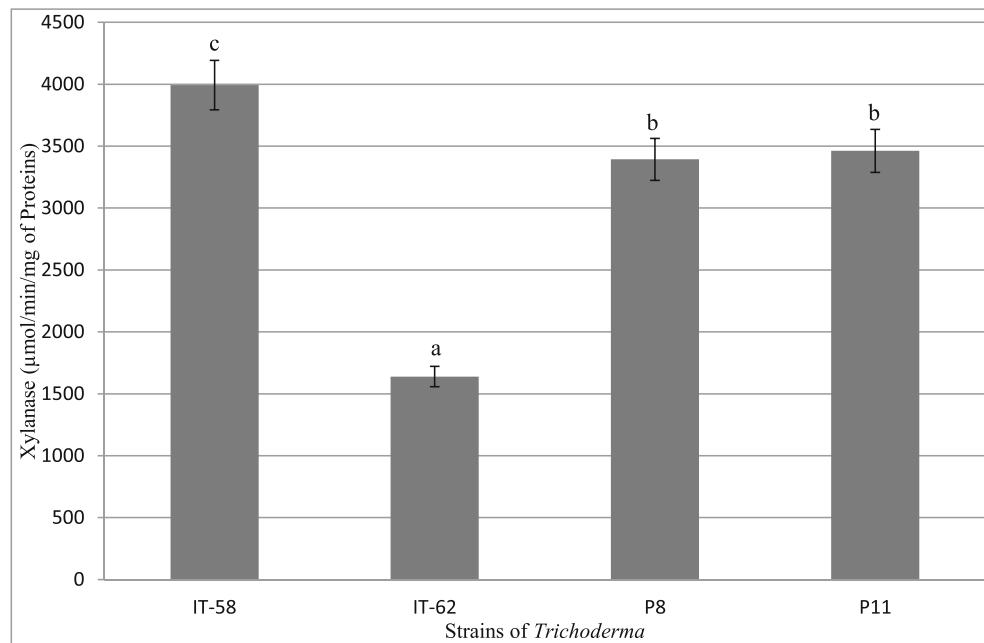
Volatile organic compounds were analyzed by GC-MS. Results showed that the quality and the quantity of volatile compounds produced are variable for each strain of *Trichoderma* (Table 2). GC-MS analysis of extract from *T. erinaceum* (IT-58) showed the presence of 22 different components representing 98.75%. The major components were 2-(3-acetoxy-4,4,10,13,14-pentamethyl-2,3,4,5,6,7,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl) propanoic acid (34.19%); 15,17,19-nonacosatriynoic acid (14.70%); and 6 β -hydroxyfluoxymesterone (11.60%). Results of analysis of *T. gamsii* (IT-62) revealed the presence of 20 different compounds representing 98.33%. The main components were 1,3-dioxolane-2-(1-hydroxyethyl)-methylate (24.64%) followed by

2,6-Bis(1,1-dimethylethyl)-4-(1-oxopropyl) phenol (12.06%) and 2-myristynoyl pantetheine (10.11%). Chemical analysis of volatile compounds from *T. afroharzianum* (P8) extract revealed the presence of 23 different compounds (97.8%), with ethyl iso-allocholate (33.76%) and 2-(3-acetoxy-4,4,10,13,14-pentamethyl-2,3,4,5,6,7,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl) propanoic acid (11.84%) as major components. Twenty-two compounds were obtained from GC-MS of *T. harzianum* (P11) organic extract (98.41%). Among them, 2-(3-acetoxy-4,4,10,13,14-pentamethyl-2,3,4,5,6,7,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl) propanoic acid (20.9%), 1,3-dioxolane-2-(1-hydroxyethyl)-methylate (14.50%), and harzianolide (12.13%) were the main compounds (Table 3).

Fig. 1 Activity of cellulases produced by *Trichoderma*

IT-58 *Trichoderma erinaceum*, IT-62 *Trichoderma gamsii*, P8 *Trichoderma afroharzianum*, P11 *Trichoderma harzianum*. According to DUNCAN test, the same letters are not significantly different at $p < 0.05$. Each treatment was made by three replicates and the experiment was repeated twice.

Fig. 2 Activity of xylanases produced by *Trichoderma*



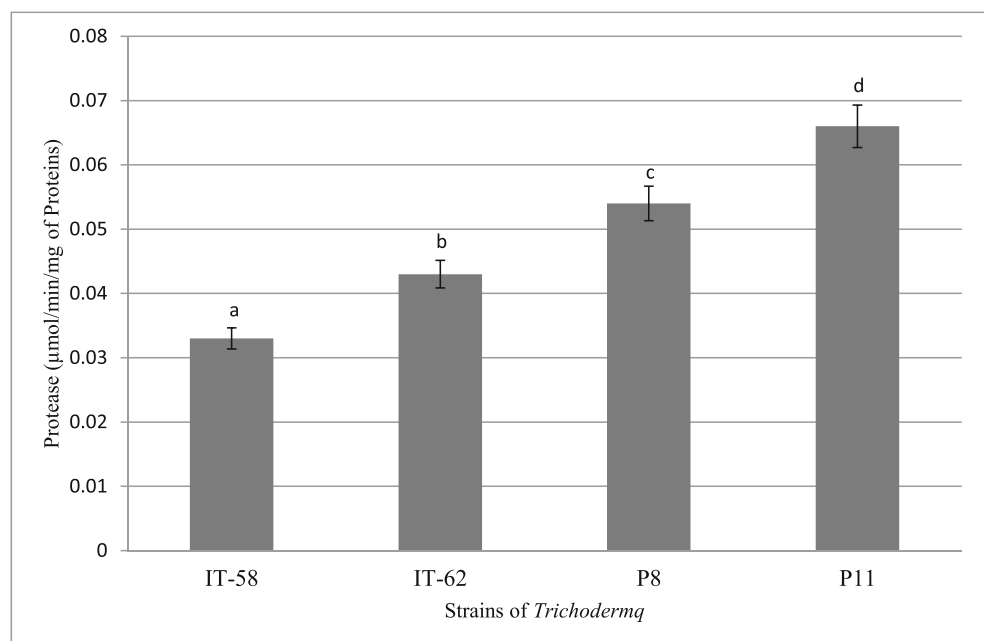
IT-58 *Trichoderma erinaceum*, IT-62 *Trichoderma gamsii*, P8 *Trichoderma afroharzianum*, P11 *Trichoderma harzianum*. According to DUNCAN test, the same letters are not significantly different at $p < 0.05$. Each treatment was made by three replicates and the experiment was repeated twice.

Discussion

Trichoderma spp. have been successfully used in agriculture for the control of soil borne disease. In the present study, the four strains of *Trichoderma* tested were found to be effective in

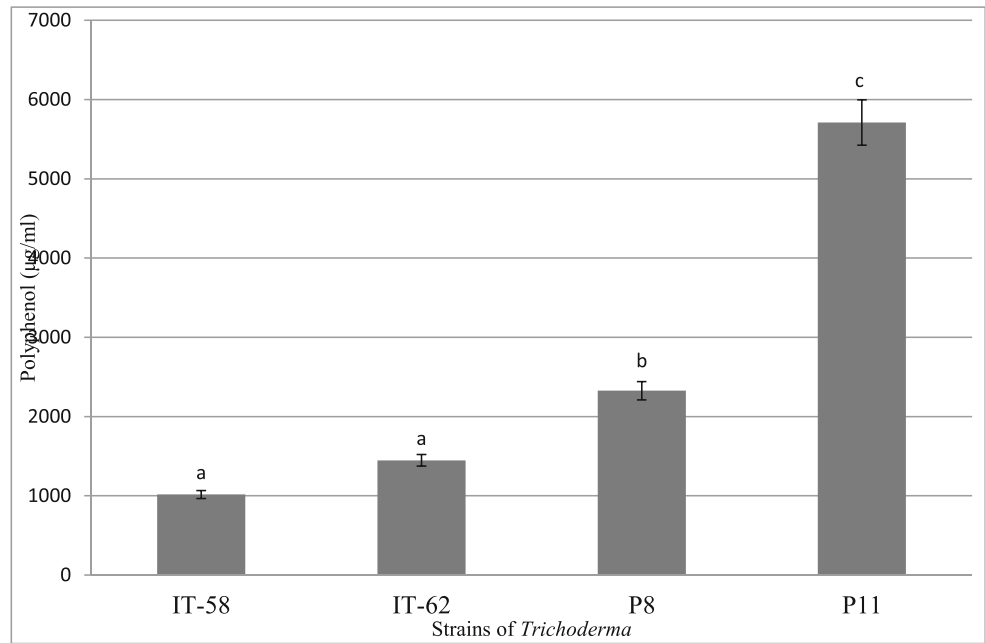
inhibiting the mycelial growth of *Pythium myriotylum* and the inhibiting percentage was variable for each of the species of *Trichoderma* used. *T. harzianum* (P11) performed the best in the antagonistic test. These results suggest that *Trichoderma* could produce different antimicrobial components. One of the

Fig. 3 Activity of proteases produced by *Trichoderma*



IT-58 *Trichoderma erinaceum*, IT-62 *Trichoderma gamsii*, P8 *Trichoderma afroharzianum*, P11 *Trichoderma harzianum*. According to DUNCAN test, the same letters are not significantly different at $p < 0.05$. Each treatment was made by three replicates and the experiment was repeated twice.

Fig. 4 Polyphenol content in organic extract produced by *Trichoderma*

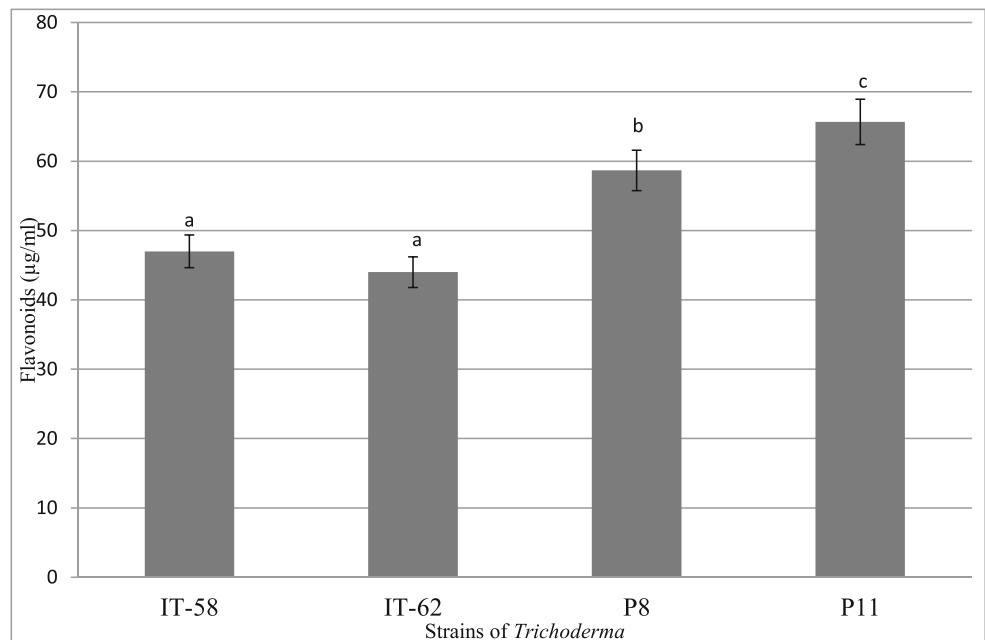


IT-58 *Trichoderma erinaceum*, IT-62 *Trichoderma gamsii*, P8 *Trichoderma afroharzianum*, P11 *Trichoderma harzianum*. According to DUNCAN test, the same letters are not significantly different at $p < 0.05$. Each treatment was made by three replicates and the experiment was repeated twice.

major mechanisms used by *Trichoderma* as biocontrol agent is mycoparasitism. Several cell wall degrading enzymes such as chitinase, cellulase, xylanase, and glucanase are involved in the process. Many studies have been showed that biocontrol agents including *Trichoderma* species secrete such enzymes able to

degrade cellulose, chitin, and glucan, which are the major constituents of cell wall of fungal and oomycete (Meena et al. 2001; Gruber et al. 2011; Saravanakuma et al. 2018). Viterbo et al. (2002) showed that, during the antagonism, *Trichoderma* produced chitinase, glucanase, and protease. Marco et al. (2003)

Fig. 5 Flavonoid content in organic extract produced by *Trichoderma*. IT-58 *Trichoderma erinaceum*, IT-62 *Trichoderma gamsii*, P8 *Trichoderma afroharzianum*, P11 *Trichoderma harzianum*. According to DUNCAN test, the same letters are not significantly different at $p < 0.05$. Each treatment was made by three replicates, and the experiment was repeated twice



IT-58 *Trichoderma erinaceum*, IT-62 *Trichoderma gamsii*, P8 *Trichoderma afroharzianum*, P11 *Trichoderma harzianum*. According to DUNCAN test, the same letters are not significantly different at $p < 0.05$. Each treatment was made by three replicates and the experiment was repeated twice.

Table 3 GC-MS analysis of volatile organic extract from *Trichoderma*

No.	RT (min)	Compounds	Relative percentage of volatile organic compounds			
			IT-58	IT-62	P8	P11
1	8.52	(4Z)-4-(Chloromethylene)-2-phenyl-1,3-oxazol-5(4H)-one	–	–	0.97	–
2	10.53	2-Octadec-1-enoxyethyl tetradecanoate	1.12	–	–	–
3	10.73	Harzianic acid	–	–	1.59	2.44
4	10.75	Bis(hydroxy methyl) carbamate of 7-chloro-1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-1,4-benzodiazepin-3-yl	–	4.12	–	–
5	10.92	2-[(5-Chloro-8-hydroxy-3-methyl-1-oxoisochroman-7-carbonyl)amino]-3-phenyl propionate	–	–	–	1.22
6	12.14	Acid-2-Chloro-5-sulfoaniline	–	–	0.62	–
7	14.83	Harzialactone	–	–	2.80	3.53
8	14.86	6-Pentyl- α -pyrane-1-one	1.59	3.82	–	–
9	15.65	3-(Benzyloxy) benzonitrile	–	0.52	–	–
10	18.04	Phthalic acid	0.32	1.12	0.93	1.02
11	26.15	2,6-Dimethyl-4-(methoxymethyl) phenol	2.71	–	–	–
12	26.90	5-Methyl-3-undecene	–	5.73	–	0.34
13	29.54	2-Chloroethyl isobutyrate of terephthalate	0.70	–	–	1.46
14	29.70	2-Bromo-1,3,5-triisopropylbenzene	1.40	2.92	–	3.19
15	29.71	2-Bromo-4,6-dimethylbenzamide	–	–	2.63	–
16	29.86	17-Acetyl-16-hydroxy-10,17-dimethylgona-4,13-dien-3-one	0.34	0.75	0.73	0.81
17	31.09	Gliotoxin	5.60	0.90	7.72	4.85
18	32.55	11,16, 22-triacetoxy androst-4-ene-3,20-dione	1.52	1.46	0.42	0.41
19	33.34	5-Androstenedione	0.80	–	–	–
20	34.25	Acide 2-(3-acetoxy-4,4,10,13,14- pentamethyl-2,3,4,5,6,7,10,11,12,13,14,15,16,17- tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl)propanoic	34.19	1.27	11.84	20.9
21	34.40	3-Hydroxyspirost-8-en-11-one	0.51	3.78	0.83	2.24
22	34.85	Dihydrocucurbitacin	–	0.30	–	–
23	35.44	Cholesta-7,22-diene-3b,5a,6b-triol	–	1.31	1.90	2.20
24	35.85	Heptatriacontanol	1.69	8.09	0.69	4.10
25	36.30	Thiocyanate of 11-hydroxy-3,17-dioxoandrost-4-en-9-yl	2.48	–	3.01	–
26	36.66	Aromadendrene	0.53	–	–	–
27	36.72	Pyrocalciferol	–	–	0.73	–
28	37.02	6 β -Hydroxyfluoxymesterone	11.60	3.93	1.84	2.24
29	37.19	Pregnenolone	–	–	0.24	–
30	37.39	Unknown	–	–	3.08	–
31	37.68	γ -Dodecalactone	–	–	1.32	–
32	37.83	Ethyl iso-allocholate	1.33	5.21	33.76	1.42
33	38.00	2,6-Bis (1,1-dimethylethyl)-4-(1-oxopropyl) phenol	6.82	12.06	9.04	8.38
34	38.77	Dermadine	3.64	6.29	–	4.14
35	38.79	17-Ethylenedioxy-5,19-cycloandrost-6-en-3-one	–	–	4.67	–
36	40.44	Inconnu	0.76	–	–	1.26
37	41.14	15,17,19-Nonacosatriynoic acid	14.7	–	6.48	–
38	41.15	2-Myristynoyl pantetheine	–	10.11	–	5.63
39	42.67	Harzianolide	–	–	–	12.13
40	44.12	1,3-Dioxolane-2-(1-hydroxyethyl)-methylate	4.40	24.64	–	14.50
		Total	98.75	98.33	97.8	98.41

IT-58: *Trichoderma erinaceum*; IT-62: *Trichoderma gamsii*; P8: *Trichoderma afroharzianum*; P11: *Trichoderma harzianum*

RT retention time

demonstrated that *T. harzianum* produced a novel protease which inhibited the growth of *C. perniciosa*, the pathogen of witches' broom disease. In the same way, several studies have been shown that the antagonistic activities of *Trichoderma* against soil borne pathogens are correlated with the production of extracellular lytic enzymes (Zhang et al. 2014; Kredics et al. 2005; Szekeres et al. 2004; Gajera et al. 2012). De la Cruz-Quiroz et al. (2018) showed that cellulase and chitinase produced by *T. harzianum* and *T. asperellum* were involved in the suppression of mycelial growth of *Phytophthora capsici* and *Colletotrichum gloeosporioides*. In the present study, the production of these enzymes could be implicated in the in vitro inhibition of *P. myriotylum* mycelial growth.

During the antagonistic activity, *Trichoderma* species are known to produce non-volatile and volatile organic metabolites (Lorito et al. 2010; Vinale et al. 2014). These compounds could play a key role during the microbe attack. Many studies have demonstrated that non-volatile secondary metabolites, mainly phenolic compounds and flavonoids, can inhibit the mycelial growth and spore germination of soil borne pathogens (Mazzei et al. 2016; Pakora et al. 2017). The present study showed that the non-volatile organic compounds produced by the four strains of *Trichoderma* significantly reduced the mycelial growth of *P. myriotylum* in vitro. This suggests that during the antagonism, these compounds may be involved in the suppression of mycelial growth of *P. myriotylum*. Similar results were obtained by Pakora et al. (2017) who reported that crude organic extracts from *T. viride* and *T. harzianum* inhibited the mycelial growth and spore germination of *P. palmivora*, *P. megakarya*, and *P. capsici*, the oomycetes associated to cocoa black pod. Moreover, Leylaie and Zafari (2018) obtained a significant correlation between the anthraquinones (polyphenolic compounds) produced by *Trichoderma* and the antimicrobial activities against a panel of fungus pathogens. Our results also showed a significant correlation between the antimicrobial effects and the production of total polyphenols and flavonoids. Polyphenol compounds such as flavonoids could act by attaching to the cell wall of pathogens and disorganizing their structure. They could also be able to inhibit the activity of enzymes released by the pathogen (Vinale et al. 2014). The antimicrobial activities of secondary metabolites could be due to synergistic effects of non-volatile and volatile organic compounds. Many species of *Trichoderma* produce volatile organic compounds that have inhibited soil borne pathogens (Vinale et al. 2008, 2014). Volatile organic compounds confer resistance to *Trichoderma* against environmental stress factors and could protect its cell walls against the action of hydrolytic enzymes released by pathogens. In addition, in dual culture, the production of volatile compounds increased when *Trichoderma* was confronted with pathogens (El-Hasan et al. 2018). In the present study, all the strains used produced varying volatile organic compounds. Some of major compounds produced

such as harzianolide, 6 β -hydroxyfluoxymesterone, and 2,6-Bis (1,1-dimethylethyl)-4-(1-oxopropyl) phenol may have a role in the antimicrobial activity against phytopathogens [9]. In addition, some minor volatile compounds present in our extract, such as gliotoxin, 6-penthylypyrone, and harzianic acid, were previously isolated from many species of *Trichoderma* and have exhibited antimicrobial activity against soil borne pathogens (Vinale et al. 2014). The antimicrobial activities of our strains of *Trichoderma* could be due to the major and also the minor VOC produced. One of the mechanisms of action of these VOC could be due to their toxicity caused by loss of osmoregulation in cell membranes of *P. myriotylum*.

In conclusion, the results of this work revealed that *T. erinaceum* (IT-58), *T. gamsii* (IT-62), *T. afroharzianum* (P8), and *T. harzianum* (P11) significantly inhibited the in vitro development of *P. myriotylum*. This could be due to the combined effect of lytic enzymes, volatile and non-volatile organic components produced by *Trichoderma* in paired cultures. The activity of each enzyme and the nature of secondary metabolites implicated depend on the different *Trichoderma* species. However, the isolation and the test of secondary metabolites are further required in order to fully understand their roles.

Funding information Partial financial support for this work was received from AUF (Agence Universitaire de la Francophonie) through Eugeni-Ionescu Postdoctoral scholarship 2017–2018.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

References

- Adiobo A, Oumar O, Perneel M, Zok S, Hofte M (2007) Variation of Pythium-induced cocoyam root rot severity in response to soil type. *Soil Biol Biochem* 39:2915–2925
- Agrios GN (2005) *Plant pathology*, 5th edn. Elsevier Academic Press, Burlington, MA, pp 410–413
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254
- Cudalbeanu M, Ghinea OI, Furdul B, Dah-Nouvlessounon D, Raclea R, Costache T, Cuculea IE, Urfan F, Dinica MR (2018) Exploring new antioxidant and mineral compounds from *Nymphaea alba* wild-grown in Danube Delta biosphere. *Molecules* 23(6):1247
- De la Cruz-Quiroz R, Roussos S, Rodríguez-Herrera R, Hernandez-Castillo D, Aguilar NC (2018) Growth inhibition of *Colletotrichum gloeosporioides* and *Phytophthora capsici* by native Mexican *Trichoderma* strains. *Karb Int J Mod Sci* 4:237–243
- El-Hasan A, Schöne J, Höglinger B, Walker F, Voegel TR (2018) Assessment of the antifungal activity of selected biocontrol agents and their secondary metabolites against *Fusarium graminearum*. *Eur J Plant Pathol* 150:91–103
- Gajera HP, Bambharolia RP, Patel SV, Khatri TJ, Goalkiya BA (2012) Antagonism of *Trichoderma* spp. against *Macrophomina*

- phaseolina*: evaluation of coiling and cell wall degrading enzymatic activities. *J Plant Pathol Microbiol* 3:149
- Gautam SP, Bundela PS, Pandey AK, Jamaluddin K, Awasthi MK, Sarsaiya S (2011) Optimization for the production of cellulose enzyme from municipal solid waste residue by two novel cellulolytic fungi. *Biotech Res Int* 2011:1–8. <https://doi.org/10.4061/2011/810425>
- Gomez-Alpizar SE, Picado I, Tambong JT, Saborio F (2011) A PCR-RFLP assay for identification and detection of *Pythium myriotylum*, causal agent of the cocoyam root rot disease. *Lett Appl Microbiol* 52:185–192
- Gruber S, Kubicek CP, Seidl-Seiboth V (2011) Differential regulation of orthologous chitinase genes in mycoparasitic *Trichoderma* species. *Appl Environ Microbiol* 77(20):7217–7226
- Harman GE (2006) Overview of mechanisms and uses of *Trichoderma* spp. *Phytopathol* 96:190–194
- Kredics LZ, Antal A, Szekeres L, Hatvani L, Manczinger C, Vágvölgyi S, Nagy E (2005) Extracellular proteases of *Trichoderma* species. *Acta Microbiol Immunol Hung* 52:169–184
- Leylaie S, Zafari D (2018) Antiproliferative and antimicrobial activities of secondary metabolites and phylogenetic study of endophytic *Trichoderma* species from vinca plants. *Front Microbiol* 9:1484. <https://doi.org/10.3389/fmicb.2018.01484>
- Lorito M, Woo SL, Harman GE, Monte E (2010) Translational research on *Trichoderma*: from ‘omics to the field. *Annu Rev Phytopathol* 48(1):395–417
- Marco LJ, Valadares-Inglis MC, Felix RC (2003) Production of hydrolytic enzymes by *Trichoderma* isolates with antagonistic activity against *Crinipellis perniciosa* the causal agent of witches’ broom of cocoa. *Braz J Microbiol* 34:33–38
- Mazzei P, Vinale F, Woo LS, Pascale A, Lorito M, Piccolo A (2016) Metabolomics by proton high-resolution magic-angle-spinning nuclear magnetic resonance of tomato plants treated with two secondary metabolites isolated from *Trichoderma*. *J Agric Food Chem* 64:3538–3545
- Meena B, Marimuthu T, Vidyasekaran P, Velazhahan R (2001) Biological control of root rot of groundnut with antagonistic *Pseudomonas fluorescens* strains. *J Plant Dis Protect* 108:368–381
- Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem* 31:426–428
- Nieto-Jacobo MF, Steyaert JM, Salazar-Badillo FB, Nguyen DV, Rostás M, Braithwaite M, De Souza JT, Jimenez-Bremont JF, Ohkura M, Stewart A, Mendoza-Mendoza A (2017) Environmental growth conditions of *Trichoderma* spp. affects indole acetic acid derivatives, volatile organic compounds, and plant growth promotion. *Front Plant Sci* 8:102
- Pakora GA, Mpika J, Kone J, Daouda DM, Kebe I, Nay B, Buisson D (2017) Inhibition of *Phytophthora* species, agents of cocoa black pod disease, by secondary metabolites of *Trichoderma* species. *Environ Sci Pollut Res* 25:29901–29909. <https://doi.org/10.1007/s11356-017-0283-9>
- Ramakrishna T, Pandit MW (1998) Self-association of α -chymotrypsin: effect of amino acids. *J Biosci* 13(3):215–222
- Reino JL, Guerrero RF, Hernández-Galán R, Collado IG (2008) Secondary metabolites from species of the biocontrol agent *Trichoderma*. *Phytochem Rev* 7:89–123
- Saravanakuma K, Dou K, Lu Z, Wang X, Li Y, Chen J (2018) Enhanced biocontrol activity of cellulase from *Trichoderma harzianum* against *Fusarium graminearum* through activation of defense-related genes in maize. *Physiol Mol Plant Pathol* 103:130–136
- Szekeres A, Kredics L, Antal Z, Kevei F, Manczinger L (2004) Isolation and characterization of protease overproducing mutants of *Trichoderma harzianum*. *FEMS Microbiol Lett* 233:215–222
- Tambong JT, Hofte M (2001) Phenazines are involved in biocontrol of *Pythium myriotylum* on cocoyam by *Pseudomonas aeruginosa* PNA1. *Eur J Plant Pathol* 107:511–521
- Tchameni NS, Sameza ML, O’donovan A, Fokom R, Ngonkeu MEL, Nana Wakam L, Etoa FX, Nwaga D (2017) Antagonism of *Trichoderma asperellum* against *Phytophthora megakarya* and its potential to promote cacao growth and induce biochemical defence. *Mycology* 8(2):84–92
- Vinale F, Sivasithamparam K, Ghisalberti EL, Marra R, Woo SL, Lorito M (2008) *Trichoderma*–plant–pathogen interactions. *Soil Biol Biochem* 40:1–10
- Vinale F, Sivasithamparam K, Ghisalberti LE, Woo LS, Nigro M, Marra R, Lombardi N, Pascale A, Ruocco M, Lanzuise S, Manganiello G, Lorito M (2014) *Trichoderma* secondary metabolites active on plants and fungal pathogens. *Open Mycol J* 8(Suppl-1, M5):127–139
- Viterbo A, Ramot O, Chernin L, Chet I (2002) Significance of lytic enzymes from *Trichoderma* spp. in the biocontrol of fungal plant pathogens. *Antonie Van Leeuwenhoek* 81:549–556
- Vos CMF, DeCremer K, Cammue BPA, DeConinck B (2015) The toolbox of *Trichoderma* spp in biocontrol of *Botrytis cinerea* disease. *Mol Plant Pathol* 16(4):400–412
- Woo LS, Ruocco M, Vinale F, Nigro M, Marra R, Lombardi N, Pascale A, Lanzuise S, Manganiello G, Lorito M (2014) *Trichoderma*-based products and their widespread use in agriculture. *Open Mycol J* 8(Suppl-1, M4):71–126
- Zhang F, Yang X, Ran W, Shen Q (2014) *Fusarium oxysporum* induces the production of proteins and volatile organic compounds by *Trichoderma harzianum* T-E5. *FEMS Microbiol Lett* 359:116–123

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.