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ORIGINAL ARTICLE

Antifungal activity of *Trichoderma* VOCs against *Pyrenophora teres*, the causal agent of barley net blotch

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Abstract

Many species of *Trichoderma* produce secondary metabolites such as volatile organic compounds (VOCs) that reduce plant diseases and promote their growth. In this work we evaluated the antagonistic effects of VOCs released by eight strains of two *Trichoderma* species against *Pyrenophora teres* Drechsler, the causal agent of barley net blotch. Antagonism was estimated based on the percentage of mycelial growth inhibition according to the confronted cultures method. VOCs extraction and identification were performed by gas chromatography and mass spectrometry, through different methodologies for VOCs emitted by antagonists and pathogens alone or when confronted. VOCs produced by all *Trichoderma* strains inhibited mycelial growth of the pathogen in a range of 3 to 32%, showing weak and unpigmented mycelia with vacuolization. In addition, *P. teres* stimulated the release of VOCs by both *Trichoderma* species. The major groups of VOCs detected were sesquiterpenes, followed by diterpenes, terpenoids and eight-carbon compounds. This is the first report about characterization of volatiles emitted by *Trichoderma* in the presence of *P. teres*.

Key words: biocontrol, biopesticides, fungi, gas chromatography, mass spectrometry, pathogens, *Pyrenophora teres*, *Trichoderma*, VOCs

Introduction

Trichoderma (teleomorph *Hypocrea*) is one of the most widely studied genera of Hypocrealean fungi due to its numerous applications in agriculture, industry, and environmental issues (Błaszczyk *et al*. 2004; Schuster and Schmoll 2010; Mukherjee *et al*. 2013). To date, more than 60% of the registered biopesticides are based on *Trichoderma* species (Verma *et al*. 2007). Many species of this genus are able to reduce plant diseases and promote their growth and productivity through induced systemic resistance, antibiosis, enhanced nutrient efficiency, and myco-parasitism (Howell 1998; Harman *et al*. 2004; Hoitink *et al*. 2006; Contreras-Cornejo *et al*. 2009; Mathys *et al*. 2012; Szabó *et al*. 2012; Vos *et al*. 2015; Khaledi and Taheri 2016; Jalali *et al*. 2017). Antibiosis includes production of secondary metabolites, volatile and non-volatile compounds that are frequently used in the fungal ecosystem as chemical signals in inter- and intra-species communication, but also as weapons to inhibit growth of competing organisms (Demain and Fang 2000). Volatile metabolites, known as volatile organic compounds (VOCs), are low-molecular weight lipophilic compounds that easily evaporate at room temperature and pressure, and for which a role in long distance communication between organisms has been suggested (Kottb *et al*. 2015). VOCs are chemically diverse and include mono- and

sesquiterpenes, alcohols, ketones, lactones, esters, thioalcohols, thioesters and cyclohexenes (Schenkel *et al*. 2015). Over 300 distinct VOCs have been identified from fungi (Chiron and Michelot 2005; Korpi *et al*. 2009; Lemfack *et al*. 2013), many of which are known to be produced by different species of *Trichoderma* (Reino *et al*. 2008; Stoppacher *et al*. 2010; Siddiquee *et al*. 2012; Shahiri Tabarestani *et al*. 2016). Volatiles from *Trichoderma* have been shown to act antibiotically against pathogenic fungi and thereby promote plant growth (Bruce *et al*. 2000; Reino *et al*. 2008; Vinale *et al*. 2008; Zhang *et al*. 2014; Hung *et al*. 2015; Kottb *et al*. 2015; Lee *et al*. 2016; Nieto-Jacobo *et al*. 2017). Several studies show the effect of these metabolites not only as inhibitors of mycelial growth, but also as inhibitors of synthesis of deoxy-nivalenol (DON) and aflatoxin production in some phytopathogenic fungi of agronomic interest such as: *Aspergillus flavus* Link (Trichocomaceae), *Fusarium graminearum* Schwabe, *F. moniliforme* J. Sheld. and *F. oxysporum* E.F. Sm. & Swingle (Nectriaceae) (Cooney *et al*. 2001; Agüero *et al*. 2008; El-Hasan *et al*. 2008; Sánchez *et al*. 2015). VOCs produced by *Trichoderma aureoviride* Rifai (Hypocreaceae), primarily aldehyde and ketone components, were associated with the greatest inhibition of *Neolentinus lepideus* (Fr.) Redhead & Ginns, *Coriolus versicolor* (L.) Quél. (Polyporaceae) and *Gloeophyllum trabeum* (Pers.) Murrill (Gloeophyllaceae), three species of wood decay fungi (Bruce *et al*. 2000). Recent studies also show the antagonistic effect of *T. asperellum* Samuels, Lieckf. & Nirenberg VOCs on reduction of spore germination of *Alternaria* species (Kottb *et al*. 2015). All these results support the suggestion that *Trichoderma* volatiles can be a potentially useful tool in a context of sustainable agriculture.

Pyrenophora teres is the causal agent of net blotch disease of barley, one of the most important diseases that affect this crop, causing yield losses in all cereal-growing regions of the world. Two forms of the pathogen have been described based on the symptoms they produce on the host plant, namely the spot and the net form. The spot form is characterized by dark-brown oval spots and is caused by *P. teres* f. *maculata* while the net form, caused by *P. teres* f. *teres*, is characterized by dark-brown net-like spots (Tekauz and Mills 1974; Mathre 1982). Net blotch is one of the main diseases of barley crops in South America. In Argentina, the average yield loss is approximately 20%; the disease affects both the weight and number of grains per square meter, and may also decrease the amount of malt extract, which affects malting quality for beer production (Carmona *et al*. 2008). To date, net blotch disease is controlled primarily by using resistant cultivars or synthetic fungicides. However, chemical control could induce pathogen resistance, making it ineffective (Baturo-Ciesniewska *et al*. 2012). Global ecological awareness requires the use of natural products and microorganisms for the control of phytopathogens, which has led to the implementation of different strategies in the use of microorganisms with antagonistic properties. For this reason, the goal of this study was to assess the antagonistic effects of *T. harzianum* Rifai and *T. longibrachiatum* Rifai VOCs against the phytopathogenic fungus *P. teres* Drechsler [anamorph *Drechslera teres* (Sacc.) Shoemaker] (Pleosporaceae) in order to find environmentally-friendly alternatives for the control of this pathogen.

In addition to testing the biocontrol potential of *T. harzianum* and *T. longibrachiatum* VOCs against *P. teres*, we characterized the volatile profiles emitted by strains using headspace – gas chromatography-mass spectrometry (HS-GC-MS) analysis. This is the first report about characterization of volatiles emitted by *Trichoderma* in the presence of *P. teres*.

Materials and Methods

Fungal isolates

Trichoderma spp. strains used in this study were obtained from barley rhizosphere from different localities (Tres Arroyos, Bordenave, Bolívar and Barrow) within Buenos Aires province, Argentina. Among 40 isolates, eight of them (T0, T2, T3, T4, T7, T8, T9 and T10) were selected on the basis of their great speed of growth, a parameter related directly with an effective biological control agent (Harman *et al*. 2004; Heydari and Pessarakli 2010).

The pathogen *P. teres* was isolated from diseased barley leaves from crops located in the Experimental Station Ing. Agr. Julio Hirschhorn located in Los Hornos, Buenos Aires province, Argentina (34°52′S – 57°58′W). From a total of ten strains from foliar lesions one of them was selected (DtLPS2) according to the same parameter mentioned above.

All isolates had been previously characterized by means of morphological characteristics and molecular techniques (Moya 2017). The obtained nucleotide sequences were submitted to the National Center for Biotechnology Information (NCBI) GenBank database (www.ncbi.nlm.nih.gov) for gene annotation. Sequences are available under accession numbers: KX572904.1, KX572905.1, KX572906.1, KX572907.1, KX572908.1, KX572909.1, KX572910.1, KX572911.1 for T0–T10 and KF656729 for DtLPS2, respectively. All isolates were cultured on potato dextrose agar (PDA) and maintained at 4°C at Centro de Investigaciones de Fitopatología (CIDEFI), Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, until use.

Effect of *Trichoderma* VOCs on *Pyrenophora teres*

To investigate the effects of *Trichoderma* VOCs on *P. teres*, antagonistic assays were made according to the method of confronted cultures outlined by Dennis and Webster (1971). Two Petri dish bottoms were assembled together (each containing 20 ml of PDA) and were inoculated with a 5 mm diameter disc of pathogen and antagonist. They were incubated for 3 days at 25 ± 1°C in darkness. After this period, both inoculated bottom plates were placed facing each other, sealed with Parafilm® and incubated at 25 ± 1°C for 7 days in darkness. The Petri plate containing PDA without antagonist served as control. Observations on the radial growth of *P. teres* in the presence of *Trichoderma* strains T0–T10 were recorded after 6 days of incubation. Antagonism was estimated based on the percentage of mycelial growth inhibition (MGI), which was calculated as suggested by Michereff *et al.* (1994). Each treatment consisted of three replicates and one control, and the complete assay was repeated three times. All data were statistically analyzed by the Kruskal-Wallis test ($p \leq 0.05$) in the software InfoStat version 2011. In addition, macroscopic and microscopic observations of the pathogen mycelium were made using a Nikon YS2 microscope fitted with a Nikon D40 digital camera.

Extraction and identification of VOCs

For extraction and identification of VOCs only three *Trichoderma* isolates, namely T0, T3 and T8 were selected. The isolates of *T. harzianum* (T3 and T8) were selected based on differences in the percentage of MGI, while *T. longibrachiatum* (T0) was selected because it belongs to a different taxonomic group.

Two different methods were used to determine VOCs emitted by antagonist and pathogen alone or when confronted. For the first case, 5-mm-diameter mycelial disks of *P. teres* and *Trichoderma* T0, T3 and T8 were inoculated into 3-cm-diameter glass vials contain -ing 5 ml of PDA and sealed with Teflon plugs. These vials were incubated for 6 days at 25 ± 1°C in darkness. The second method was performed using confronted cultures as described above. All treatments consisted of three replicates. VOCs were collected at 0 and 6 days using headspace solid-phase micro-extraction (HS-SPME) during 30 min using a 65 µm film thickness polydimethylsiloxane/divinylbenzene fiber (PDMS/DVB) (Supelco, Bellefonte, PA, USA). Fibers were previously conditioned according to manufacturer's instructions, and reconditioned before each analysis. Analyses were performed using a Hewlett Packard 6890 gas chromatograph employing a non-polar DB-5 capillary column (30 m length, 0.32 mm inner diameter, 0.25 µm film thickness) (J&W, Folsom, CA, USA). The injector was operated in the splitless mode at 250°C, oven temperature was programmed (40°C for 2 min, 10°C/min to 200°C, 15°C/min to 250°C, with a holding time of 5 min at the final temperature). The flame ionization detector (FID) temperature was set at 280°C. Gas chromatography-mass spectrometry (GC-MS) analysis was performed on a HP 5975C VL Agilent mass selective detector (MSD) coupled to the GC equipment. The MSD was set in the electron impact mode at 70 eV and operated in SCAN mode with a mass range of 35–650 amu (Atomic Mass Unit), with the transfer line at 320°C, the ionization chamber at 230°C, and the quadrupole set at 150°C.

The VOCs were tentatively identified by interpretation of their mass spectral fragmentation; spectra were also compared to data from MS libraries (NIST 05 Mass Spectral Library, National Institute of Standards and Technology; Adams 2007) as well as with spectra and Kovats retention index (KI) (Kovats 1965) values previously reported by Stoppacher *et al.* (2010). KI values were calculated after analysis of C8-C20 and C21-C40 n-alkane series (Fluka, Switzerland) under the same chromatographic conditions. The measurements of quantification of VOCs were determined by calculating the area under the chromatographic curve.

Results

Effect of *Trichoderma* VOCs on *Pyrenophora teres*

VOCs emitted by all isolates of *Trichoderma* in the presence of *P. teres* inhibited mycelial growth of the pathogen in a range of 3 to 32% (Table 1). The statistical analysis of the data revealed significant differences among the levels of antagonism produced by the eight isolates of *Trichoderma* tested (K: 21.43, $p \leq 0.05$). The isolate *T. harzianum* T3 caused the highest percentage of MGI, although this isolate did not show statistically significant differences with T0, T2, T4 and T9.

Table 1. Mycelial growth inhibition (MGI) of *Pyrenophora teres* produced by volatile compounds emitted by *Trichoderma longibrachiatum and T. harzianum* after six days of incubation

Isolate	MGI [%]
T. longibrachiatum T0	21.08 ±2 .3 bc
T. harzianum T2	19.25 ± 3.1 bc
T. harzianum T3	31.92 ± 1.3 c
T. harzianum T4	27.08 ±1 .4 c
T. harzianum T7	2.83 ± 1.2 a
T. harzianum T8	9.17 ± 1.1 ab
T. harzianum T9	20.33 ± 1.7 bc
T. harzianum T10	8.67 ± 0.7 ab

Data are given as mean ± standard error; values followed by the same letters do not differ significantly (Kruskal-Wallis test, $p \leq 0.05$)

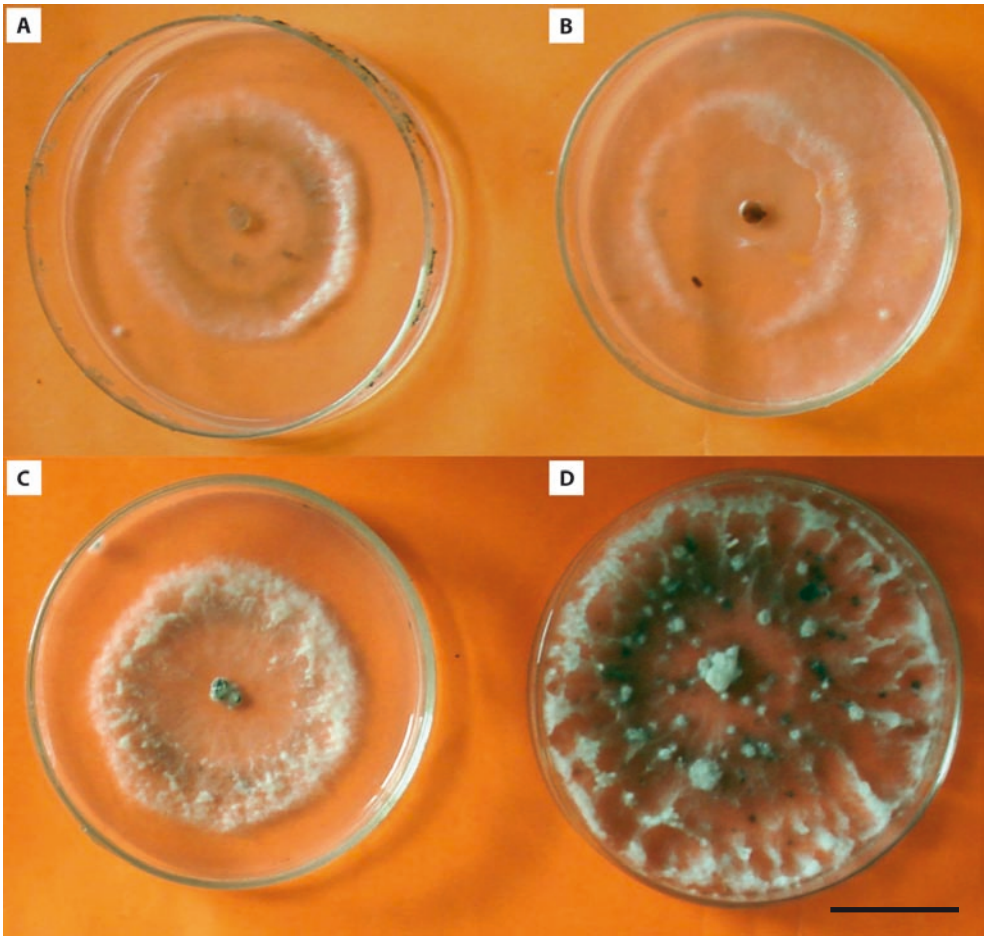

Fig. 1. Aspect of *Pyrenophora teres* colonies in the presence of VOCs emitted by *Trichoderma* T0 (A), T9 (B) and T2 (C). Note the unpigmented mycelium when compared with the control (D). Scale bar = 3 cm

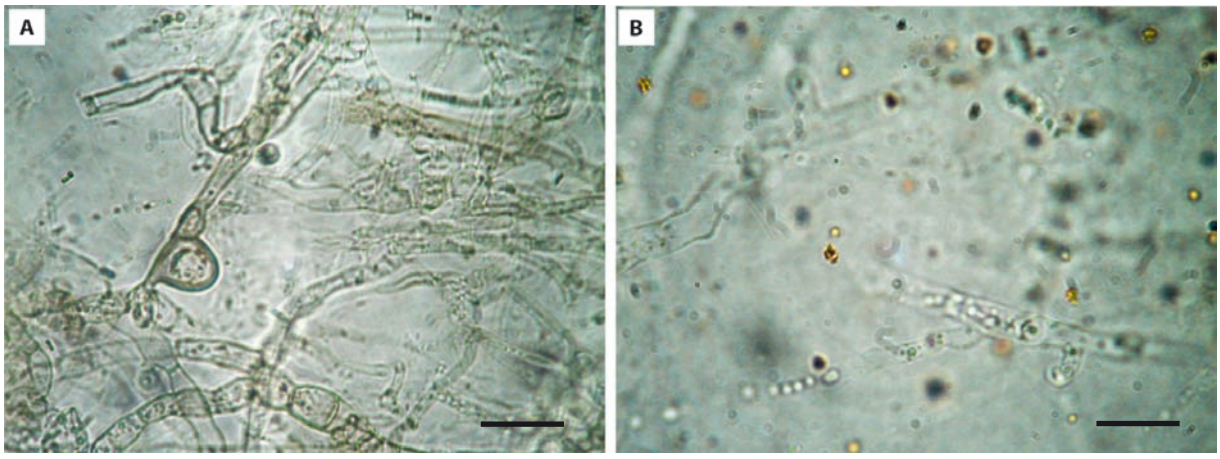

Fig. 2. Aspect of *Pyrenophora teres* mycelium: A – control; B – in the presence of *Trichoderma* VOCs. Image shows mycelium lacking its characteristic pigmentation and weakened, with numerous vacuoles inside the hyphae. Scale bar = 15 µm

Macroscopic observations revealed that all the colonies of the pathogen exposed to the *Trichoderma* VOCs showed unpigmented mycelia (Fig. 1) while microscopic observations of *P. teres* showed vacuolization and weak mycelium, and numerous vacuoles inside the hyphae in comparison with the control (Fig. 2).

Extraction and identification of VOCs

GC-MS analysis showed similar profiles for the volatile compounds emitted by monospecific cultures of *T. harzianum* isolates T3 and T8 and *T. longibrachiatum* T0 (Fig. 3), while GC-MS analysis of the VOCs

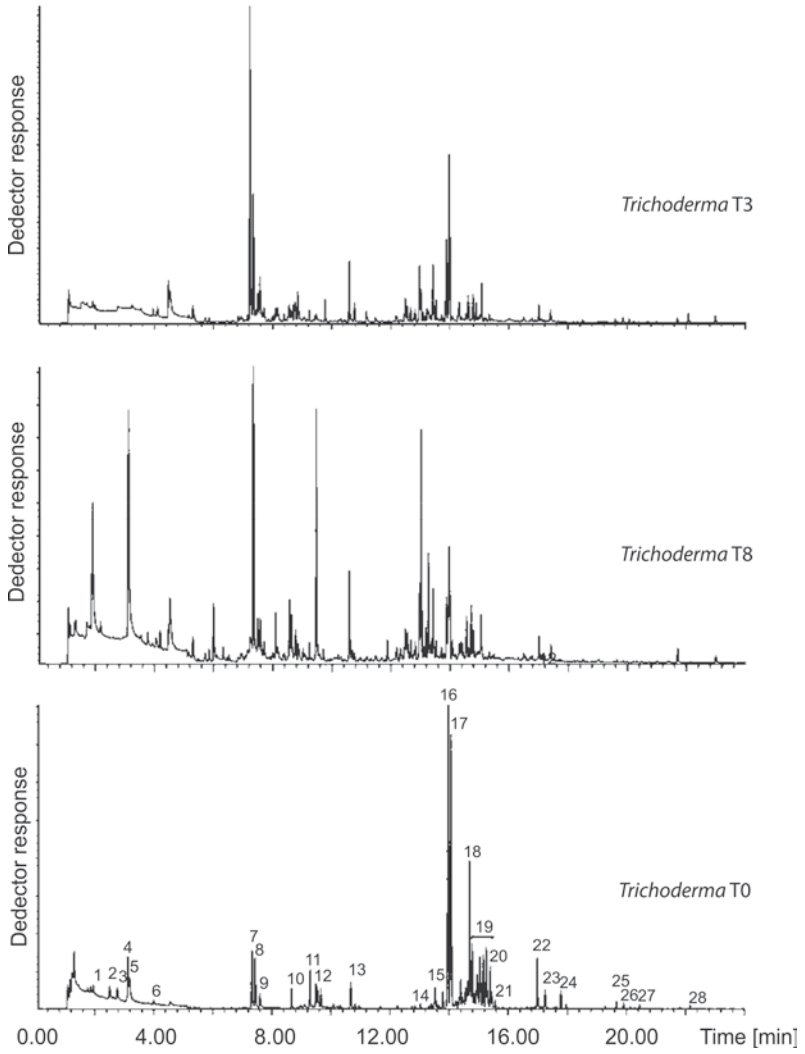

Fig. 3. Capillary gas chromatography profiles of VOCs emitted by the isolates *Trichoderma harzianum* T3 and T8, and *T. longibrachiatum* T0

produced by *P. teres* showed low values and scarcely recognizable profiles, which did not provide useful information. Regarding quantitative results, T0 produced 2, 3.5 and 5 times the amount of VOCs than T3, T8 and *P. teres* in monospecific cultures, respectively, and 5 and 7.5 times the amount of volatile compounds than T3 and T8 in confronted cultures, respectively. In all the confronted cultures tested, *P. teres* enhanced the production of VOCs by the antagonists. *Trichoderma* T0 produced 2.4 times more VOCs in confrontation with the pathogen than when it was cultured on its own. Similar results were observed for T3 (1.8 times) and T8 (1 time).

According to the NIST 05 mass spectra library of the GC-MS analysis, 28 volatile compounds were identified in the headspace of cultures (Table 2). VOCs emitted by the three *Trichoderma* isolates included 3-octanone, 1-octen-3-ol and some sesquiterpenes. Both T0 and T8 also produced other volatile compounds such as 2-methyl-1-propanol, 2-methyl-1-butanol and 3-methyl-1-butanol. The composition of VOCs produced by T0, T3 and T8 in monospecific cultures was similar to that recorded in the presence of the pathogen. However, some compounds such as alcohols released by *Trichoderma* in monospecific cultures, appeared as esters of acetic acid in the confronted cultures.

The qualitative analysis of the spectra showed that the major groups of compounds found were sesquiterpenes, followed by diterpenes, terpenoids and eight-carbon compounds.

Discussion

In nature, the volatile organic compounds released by *Trichoderma* mediate the interactions between the fungus and the surrounding environment, including other microorganisms and plant roots (Wheatley 2002). These compounds can promote plant growth and generate systemic resistance (Harman *et al*. 2004; Vinale *et al*. 2008).

Table 2. Volatile metabolites emitted by *Trichoderma* identified by headspace – gas chromatography-mass spectrometry (HS-GC-MS)

Peak No.	KI*	Compound	T0	T3	T8
1	613	2-Methyl-1-propanol	+	–	+++
2	659	2-Pentenone	+	–	–
3	679	3-Hydroxi-2-butanone	+	–	–
4	705	3-Methyl-1-butanol	++	–	+++
5	709	2-Methyl-1-butanol	+	–	–
6	777	2,3-Butanediol	+	+	+
7	975	1-Octen-3-ol	++	+++	+
8	985	3-Octenone	++	+++	+++
9	992	3-Octanol	+	+	+
10	1026	Limonene	+	+	+
11	1100	Undecane	++	+	+
12	1112	2-Phenylethanol	++	+	+++
13	1186	Naphthalene	+	++	++
14	1364	Unidentified	+	++	++
15	1396	Alpha-Cedrene	++	++	+++
16	1425	Unidentified sesquiterpene	+++	+++	+
17	1431	Betha-Cedrene	+++	+++	++
18	1488	Acora-2,4-diene	+++	+	+
19	1502	Alfa-Cuprenene	++	+	+
19	1504	Alpha-Bisabolene	+	+	++
19	1508	Betha-Bisabolene	++	+	+
19	1525	Betha-Alaskene	++	+	+
19	1532	Betha-Sesquiphellandrene	++	+	+
20	1546	Alpha-Longipinene	++	++	++
21	1569	Nerolidol	+	+	+
22	1683	Tricho-acorenol	++	+	+
23	1707	Acorenone	+	+	+
24	1757	Unidentified	+	–	–
25	1984	Cembrene	+	+	+
26	2014	Verticiol	+	+	+
27	2045	Unidentified	+	+	+
28	2383	Pimara-7,15-dien-3-one	+	–	–

* Kovats Index. Relative abundance of VOC was calculated from the integrated area data from GC-MS analysis of each isolated: absent (–), <1.0 % (+), 1.0–5.0% (++), >5% (+++)

In this study, the results from the method of confronted cultures showed that VOCs released by different *Trichoderma* isolates inhibited *P. teres* growth and induced the production of unpigmented mycelia. These results agree with those reported by numerous researchers who confronted diverse *Trichoderma* species with phytopathogenic fungi such as *F. graminearum, F. moniliforme*, *F. oxysporum, Rhizoctonia solani* J.G. Kühn (Ceratobasidiaceae), *Macrophomina phaseoli* (Maubl.) S.F. Ashby (Botryosphaeriaceae), *Phytophthora cactorum* (Lebert & Cohn) J. Schröt. (Peronosporaceae) and *A. flavus* (Calistru *et al*. 1997; Hajieghrari *et al*. 2008; Sánchez *et al*. 2015). Under a light microscope, *P. teres* hyphae with thin walls and no melanin were observed as effects of *Trichoderma* VOCs. Melanins have been reported to confer resistance to fungi against environmental stress factors such as radiant energy, water loss and extreme temperatures (Bell and Wheeler 1986; Butler and Day 1998). In addition, they are related to the virulence and/or pathogenesis of different fungi (Jacobson 2000). It is probable that one of their main functions is to protect cell walls from the action of hydrolytic enzymes released by the host and antagonistic microorganisms (Bell and Wheeler 1986; Jacobson 2000). In this sense, *Trichoderma* VOCs would weaken the cell walls of *P. teres*, which could

then be further affected by hydrolytic enzymes. Also, VOCs would affect pathogen survival by making it more susceptible to adverse environmental conditions. In addition, the growth VOCs production of isolates T0, T3 and T8 increased when they were confronted with *P. teres*. The presence of other microorganisms is known to enhance growth and induce activation of genes related to parasitism and competition in several *Trichoderma* species (Atanasova *et al*. 2013). It is probable that some of the above-mentioned mechanisms could explain the different behavior of T0, T3 and T8 isolates in monospecific and confronted cultures.

The GC-MS specters obtained for the three *Trichoderma* isolates tested were not identical regarding the abundance of each element. Nevertheless, their composition was similar, sharing at least three main compound groups: sesquiterpenes, monoterpenes and eight-carbon compounds. These profiles, particularly T0, were very similar to those found by Stoppacher *et al*. (2010) for *T. virens*, in which most of the VOCs released corresponded to sesquiterpenes, followed by eight-carbon compounds and finally monoterpenes. One of the first volatile compounds with antifungal activity isolated from *Trichoderma* species was 6-pentyl--α-pyrone (6PP), a lactone with a coconut-like aroma (Collins and Halim 1972). This compound is non-toxic and was synthesized as a flavoring agent with industrial purposes before being discovered as a natural product. Dennis and Webster (1971) demonstrated that 6PP inhibited the growth of phytopathogenic fungi such as *R. solani*, *Botrytis cinerea* Pers. (Sclerotiniaceae) and *Fusarium* species. Under the conditions assayed in this work, release of 6PP was not recorded in any of the isolates. Given that the production of *Trichoderma* VOCs generally depends on environmental factors, such as the sources of carbon and the microorganisms with which it interacts (Zeilinger and Schuhmacher 2013), future assays using different culture media should be carried out in order to investigate whether these isolates are able to produce 6PP under different conditions.

Several studies have shown the biological potential of sesquiterpenes regarding the self-regulation and suppression of growth in microorganisms that compete for space and/or nutrients (Hubbell *et al*. 1983; Minerdi *et al*. 2011; Polizzi *et al*. 2011). Many researchers have suggested that the mode of action of sesquiterpenes is based on the toxicity caused by loss of osmotic control in cell membranes, because they are mostly lipophilic compounds (Inoue *et al*. 2004). Other researchers have pointed out that these compounds facilitate the passage of toxins through the membranes, thus behaving as solvents (Kramer and Abraham 2012). Some sesquiterpenes found in this work, such as tricho-acorenol and acorenone, were also obtained by Citron *et al*. (2011) from *T. harzianum, T. longibrachiatum* and *T. viride* Pers. The β-bisabolene sesquiterpene present in this study was first found by Stoppacher *et al*. (2010) in the *Trichoderma* genus.

Volatile eight-carbon compounds are important elements that impart flavor and taste to mushrooms. They represent from 44.3 to 97.6% of the total volatile fraction produced by these microorganisms, in which 1-octen-3-ol appears as the most abundant (Combet *et al*. 2006). In this study, 1-octen-3-ol, 3-octenone and 3-octanol were recorded among the eight-carbon compounds obtained from the three isolates. These results agree with those found by Nemčovič *et al*. (2008) and Herrero-Garcia *et al*. (2011), who demonstrated that these compounds induce fungi conidiation and growth self-inhibition, depending on cell density. In addition, 1-octen-3-ol was reported as a plant gene defense activator on *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae) against *B. cinerea* (Kishimoto *et al*. 2007).

One of the alcohols released by T0 and T8 was 2-methyl-1-butanol. This compound was also found in *T. atroviride* P. Karst. (Nemčovič *et al*. 2008) and has been recorded as an inhibitor of mycelial growth and protein synthesis in wood decaying fungi (Humphris *et al*. 2002).

This study provides evidence that the VOCs emitted by *T. harzianum* and *T. longibrachiatum* have an antagonistic effect against the pathogen *P. teres* and could be used as a suitable alternative to synthetic fungicides for the control of net blotch disease of barley. The methodology used for the removal of VOCs in the confrontation of both microorganisms, reported for the first time in our work, allowed confirmation that the presence of *P. teres* stimulated greater production of VOCs by the antagonist. This VOCs increase could be due to changes in gene expression. The evaluation of VOCs impact in *in vivo* assays and genetic studies will be the subject of further research.

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