Antifungal, anti-oomycete and phytotoxic effects of volatile organic compounds from the endophytic fungus Xylaria sp. strain PB3f3 isolated from Haematoxylon brasiletto

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Abstract

Aims: To determine the antifungal, anti-oomycete and phytotoxic activity; and chemical composition of the volatile organic compounds (VOCs) produced by endophytic fungus Xylaria sp. PB3f3 isolated from Haematoxylon brasiletto Karst.

Methods and Results: Bioactivity and chemical composition of the VOCs from Xylaria sp. PB3f3 were established by using simple and multiple antagonism bioassays, and gas chromatography/mass spectrometry, respectively. The results showed that Xylaria sp. PB3f3 inhibited the growth of the oomycetes Pythium aphanidermatum (78.3%), Phytophthora capsici (48.3%), and the fungi Alternaria solani (24.5%) and Fusarium oxysporum (24.2%), in multiple antagonism bioassays. Volatile organic compounds, produced at 20 and 30 days of fungal growth, inhibited root elongation on Amaranthus hypochondriacus (27.6%) and Solanum lycopersicum (53.2%). Forty VOCs were identified at 10, 20 and 30 days in Xylaria sp. PB3f3 cultures. The compounds with the highest fibre affinity were: 3-methyl-1-butanol and thujopsene, at 10 days of fungal growth; an unidentified amine and 2-methyl-1-butanol at 20 days; and 2-methyl-1-propanol at 30 days. In the gas phase assay method 2-methyl-1-propanol and 2-methyl-1-butanol showed significant inhibitory effects on root elongation and germination of Am. hypochondriacus and S. lycopersicum.

Conclusions: Xylaria sp. PB3f3 and its VOCs showed significant phytotoxic effects on root elongation and germination of Am. hypochondriacus and S. lycopersicum.

Significance and Impact of the Study: The genus Xylaria produces a great variety of secondary metabolites, but, up date, there are no reports of the identification of bioactive volatile compounds. Thus, Xylaria sp. PB3f3 and its VOCs are a possible candidate for the biological control of weeds.

Introduction

Endophytic fungi are micro-organisms that colonize the tissue of its host plant in an inter, or intra, cellular way without apparently causing symptoms of disease (Aly et al. 2011). These micro-organisms produce a large number of secondary bioactive metabolites, useful in medicine (Sette et al. 2006; Yu et al. 2010; Cui et al. 2011), industry and agriculture (Suryanarayanan et al. 2009; Wang and Dai 2010).
In agriculture, endophytic fungi and their volatile or non-volatile metabolites have potential as biocontrol agents against pathogenic micro-organisms of crops, insects and weeds (Mercier and Jiménez 2004; Macías-Rubalcava et al. 2010, 2014; Brum et al. 2012; Suwannarach et al. 2013). One example is the case of the genera Muscodor and Trichoderma. They are producers of volatile organic compounds (VOCs) with a large biological potential, such as antimicrobial compounds against different fungal species (Botrytis cinerea, Monilinia fructicola, Penicillium expansum, Rhizoctonia solani, Sclerotinia sclerotiorum) and oomycetes (Aphanomyces cochlioides, Pythium ultimum) (Stinson et al. 2003; Mercier and Smilanick 2005; Schotsmans et al. 2008; Hung et al. 2013; Chen et al. 2015). These volatile compounds are used as agents in mycofumigation or biofumigation (Daisy et al. 2002).

The genus Xylaria Hill (ex Schrank, 1789, Xylariaceae) covers several fungal species ubiquitously present in vascular plants (Fournier et al. 2010). The endophytic species of Xylaria produces a large variety of secondary metabolites with different chemical structures, such as cytochalasins, terpenoids, alkaloids, coumarins and benzoquinones; with several biological activities such as phytotoxic, antifungal, antimarial and antibacterial, among others (Tansuwan et al. 2007; Liu et al. 2008; Silva et al. 2010; Santos Filho et al. 2011; Isaka et al. 2012; Ratnaweera et al. 2014; Wu et al. 2014).

According to phylogenetic studies, the genera Muscodor and Xylaria are anamorphs of the Xylariaceae family and belong to the Xylarioid group (Yuan et al. 2011); because of this, it is probable that Xylaria also produces volatile compounds with possible application as biocontrol agents. However, there are no reports of volatile compounds with biological activity produced by Xylaria species.

The objective of this study is to determine the antifungal, anti-oomycete and phytotoxic activity, and the chemical composition of the VOCs produced by the endophytic fungus Xylaria sp. PB3B isolated from Haematoxylon brasiletto Karst, employing simple and multiple antagonism bioassays against fungal and oomycetes plant pathogens; and to evaluate its phytogrowth-inhibitory activity on seed germination, root elongation and seedling respiration on two model plants.

Materials and methods

Fungal isolation

The endophytic fungus PB3B was isolated from healthy H. brasiletto Karts leaves (Fabaceae), collected at the ‘Reserva de la Biósfera Sierra de Huautla’ (REBIOSH) at Quilamula (18°30’4” N – 98°51’52” W and 18°32’12.2” N – 99°02’05” W; 1080–1230 m.a.s.l.) Morelos, Mexico, in September 2010.

The leaves were washed with running and distilled water and the surface was sterilized with the following sequence of solutions: 75% ethanol for 60 s; sterile distilled water, 3-4% sodium hypochlorite (65% Clorox®) (The Clorox company, Tlahnepantla Estado de México, México) for 60 s; and sterile distilled water (Rodrigues 1994). The surface-sterilized leaves were dried using sterile filter paper (Whatman® qualitative, Grade 1), after that the leaves were cut into 2 × 2 mm pieces at the central vein level, and four pieces were placed (per plate) in two different growth conditions, distilled water agar (WA) and natural potato-dextrose–agar (PDA) plates. Both media were added with chloramphenicol (500 mg l⁻¹) (Sigma-Aldrich, St Louis, MO), in order to inhibit bacterial growth. Plates were incubated at 25°C, and in 12:12 h light–dark photoperiod with a T12 30 W fluorescent light (Phillips, Chihuahua, México). They were observed daily. Fungal colonies emerging from the leaf pieces were isolated and re-cultured on PDA until pure cultures were obtained.

The purified PB3B strain was preserved in PDA slants at the Instituto de Química, UNAM, as well as in the Laboratorio de Micología C006, Instituto de Biología, UNAM in WA (0-2%) at 4°C, and in 30% glycerol–potato dextrose broth (PDB) at −80°C. Dried PDA and oatmeal agar (OA) cultures were deposited at the Herbario Nacional de México (MEXU), UNAM, with the collection number MEXU 27-559.

Identification of the strain PB3B

Morphological characteristics and sequencing of the ITS1-5-8-ITS2 region were used for taxonomic identification. For examination of the macro and micro morphological characteristics the strain PB3B was grown onto PDA plates at 27°C, in 12:12 h light–dark photoperiod for 8 weeks. Cultures were observed daily to measure the growth rate, for colony description and observation of morphological structures in a brightfield microscope. Colony and pigment colours were determined with the Methuen Handbook of Colors (Kornerup and Wanscher 1978).

For sequence analysis, the total genomic DNA was extracted from mycelium collected from a 7-old-day PDA culture with a FT71415 Rapid Fungal Genomic DNA isolation kit (Bio Basic Inc, Markham, ON, Canada). The ITS-5-8 S region was amplified with the universal ITS primers ITS5 (5’-GGAAGTAAAGTGTCGTAACAAAG-3’) and ITS4 (5’-TCCTCCGCTTATTGATATGC-3’) (White et al. 1990). The PCR reaction was performed in a Thermal
Cycler Gerndt 2720 (Applied Biosystems, Foster City, CA) with a reaction volume of 50 μl containing 25 μl GoTaq® Master Mix (Promega, Madison, WI), 2.5 μl of each primer (10 pm μl⁻¹) and 5 ng genomic DNA. The PCR programme, modified from Naumova et al. (2004), consisted of an initial denaturing step at 94°C for 1 min, followed by 30 cycles of 1 min at 94°C, 2 min at 58°C, 1 min at 72°C, and a final extension step for 5 min at 72°C. The amplification product was separated by electrophoresis in 0.8% (w/v) agarose gel (Invitrogen, Carlsbad, CA), stained with gel red (Biotium, Hayward, CA) in 1× TAE (Tris-acetate-EDTA) buffer at 90 V for 40 min; and, visualized under Benchtop Dual-Intensity Transilluminator (UVP Inc., Upland, CA). The sequence database. The closest resulting sequences (12 references and the strain PB3f3 isolated in this study) were obtained from the National Center for Biotechnology Information (NCBI) nucleotide sequence database. The sequence was aligned using the BLASTN program (Altschul et al. 1997) and deposited. The GenBank accession number of the strain PB3f3 is depicted in the results section.

Evolutionary relationship of the endophytic fungus PB3f3

The sequence of the endophytic fungus PB3f3 was analysed using BLAST searching against the NCBI nucleotide sequence database. The closest resulting sequences (12 references and the strain PB3f3 isolated in this study) were aligned, with gaps treated as missing data. A distance genetic tree was generated using the Neighbor-Joining method (Saitou and Nei 1987). Evolutionary distances were computed using the p-distance method (Nei and Kumar 2000) and evolutionary analyses were conducted in MEGA V (Tamura et al. 2011).

Multiple direct antagonism bioassay

Four 5 mm diameter inocula of Xyalaria sp. PB3f3, obtained from a 8-day-old PDA culture at 28°C, were placed at the centre of the Petri dish with a 3.5 cm separation between them, and incubated at 28°C for 1, 3, 5 and 7 days with 12:12 h (light–dark) photoperiod. Then, two 5 mm inocula obtained from a 10-day-old PDA culture at 28°C of the economically important plant pathogenic oomycetes Pythium aphanidermatum and Phytophthora capsici, and the fungi Alternaria solani and Fusarium oxysporum were placed at 2.5 cm on each side of each inoculum of the Xyalaria sp. PB3f3, respectively. The plant pathogenic micro-organisms used in the bioassays were donated by the Instituto de Fitosanidad, Colegio de Postgraduados, Montecillo, Estado de México.

The plates were sealed with Parafilm® (Probiotek, San Nicolás de los Garza, México) and incubated in the conditions described above. The average diameter measurement for each mycelium plant pathogen was recorded after 3 and 6 days of antagonistic interaction. The percentages of growth inhibition of plant pathogenic micro-organisms were determined, compared with their respective controls. Four replicate plates were used for each multiple antagonism bioassay. The controls consisted of each plant pathogen growing under the same conditions, without the presence of Xyalaria sp. PB3f3. The antagonistic interactions were observed until day 40 (Macías-Rubalcava et al. 2008; Sánchez-Fernández et al. 2016).

Simple direct antagonism bioassay

Simple antagonism bioassays were performed using 9 cm PDA plates. Xyalaria sp. PB3f3 was pre-inoculated for 1, 3, 5 and 7 days on one side of the plate and incubated under an artificial 12:12 h (light–dark) photoperiod at 28°C. Then, three inocula of 5 mm diameter from the plant pathogenic fungus and oomycete cultures were placed on the shore on the other side of the plate after each pre-inoculation date of Xyalaria sp. PB3f3, sealed with Parafilm® and incubated under the same conditions as mentioned above. The percentages of growth inhibition were measured after 3 and 6 days of antagonistic interaction by measuring the diameter of each mycelium of the plant pathogenic fungi and oomycete, and compared with their respective controls. Four replicates were used for each simple antagonism bioassay. The controls consisted of each fungus growing individually under the same conditions.

The recovery of the micro-organisms was evaluated after 20 days of antagonistic interaction by transferring a plug of the fungi and oomycete colonies to a new PDA plate, and incubating them under the same conditions. The growth of the plant pathogens was compared with controls (Macías-Rubalcava et al. 2010; Sánchez-Fernández et al. 2016).

Antifungal effect of VOCs in divided Petri dishes

Bioassays were performed in 9 cm two-compartment divided Petri plates, each containing 10 ml PDA. The plate-dividing wall prevented the diffusion of any soluble compounds produced by Xyalaria sp. PB3f3, but allowed the free exchange of VOCs (Macías-Rubalcava et al. 2010). The antifungal activity of the VOCs was tested against plant pathogenic micro-organisms five times: at 1, 3, 5, 10, 20 and 30 days. An inoculum of the endophyte was placed in one of the compartments of the plate, and
incubated at 28°C in a 12:12 h light–dark photoperiod, before the bioassay. The inoculum from the plant pathogenic fungi and oomycete cultures was then placed in the other dish compartment. The plate was sealed with Parafilm® and incubated as described above. The bioassays were carried out in quadruplicate. Control cultures were obtained by growing each plant pathogen alone, under the same conditions. The results of the bioassays were observed for 40 days. The percentage growth inhibitions were calculated after 3 and 6 days by measuring the colony diameter of each plant pathogen, and comparing it with their respective control (Macías-Rubalcava et al. 2010; Sánchez-Fernández et al. 2016).

Culture and extraction of organic compounds produced by endophytic fungus Xylaria sp. strain PB3f3

Fifty 10 cm Petri plates, containing 15 ml PDA medium, were inoculated with a 1 cm² agar plug from the growing periphery of an 8-day mycelium of Xylaria sp. PB3f3 growing in PDA, and placed in the centre of a Petri dish. The Petri dishes were incubated at room temperature for 30 days in a 12:12 h light–dark photoperiod under fluorescent light.

At the end of the incubation period, the mycelia and culture medium were mechanically fragmented and, subsequently macerated with 21 of CH₂Cl₂ (×5). The organic phases were filtered over anhydrous Na₂SO₄ and concentrated in vacuo to yield 2 g of a reddish solid.

Antifungal and anti-oomycete effect of organic extracts

The inhibitory effect of the organic extract (culture medium and mycelium) from the endophytic fungus Xylaria sp. PB3f3 was tested on the radial growth of the fungi and oomycetes used in the antagonism bioassays described above. The extract was dissolved in maximum 0.5% MeOH; and then evaluated at 250 µg ml⁻¹ in sterile PDA, and before the agar had solidified added to 6 cm Petri dishes. The positive controls were the commercial fungicide Prozycar® (carbendazim: methyl benzimidazol-2-yl-carbamate) (Promotora técnica industrial S.A. de C.V. Morelos, México), and the anti-oomycete Ridomil Gold 4E®, (Metalaxyl: 2-(2,6-dimethyl-phenyl)-(2-methoxy-acetyl)-amino]-propionic acid methyl ester) (Syngenta Crop protection, Inc., Greensboro, NC), both were added to agar at 250 µg ml⁻¹. Plates with only PDA, and PDA plates with 0.5% MeOH, were used as a negative control. An inoculum (5 mm² agar plug) of each test micro-organism was placed in a Petri dish, and incubated at 28°C in a 12:12 h light–dark photoperiod. The average of two perpendicular diameter measurements of each colony was recorded after 1, 6 and 10 days of incubation, depending on the growth rate of the tested micro-organisms. The experiment used a complete randomized design with four replications per treatment (Macías-Rubalcava et al. 2010; Meléndez-González et al. 2015).

Phytoinhibitory effect of VOCs in divided Petri dishes

The phytotoxic activity of VOCs from Xylaria sp. PB3f3 was evaluated against the seed germination, root elongation and seedling respiration of two dicotyledonous species, Amaranthus hypochondriacus Linneo (Amaranthaceae), and Solanum lycopersicum Linneo var. lycopersicum (Solanaceae). These plants have commonly been used in phytotoxic bioassays. These test plants were selected because of their rapid, homogenous and high frequency germination (Macías-Rubalcava et al. 2014). Am. hypochondriacus seeds were purchased at a local market at Tulyehualco, Mexico, D.F., and S. lycopersicum seeds at Semillas Bertens, Celaya, Guanajuato, Mexico.

In vitro bioassays were carried out in Petri dishes with two compartments, one containing 10 ml of PDA for Xylaria sp. PB3f3 growth; and the other containing 10 ml of WA for seed germination. The PDA was inoculated with a 5 mm diameter agar plug from a Xylaria sp. PB3f3 culture. This was incubated at 28°C in darkness for 1, 3, 5, 10, 20, 30 and 40 days. At each incubation time, 30 seeds of Am. hypochondriacus or S. lycopersicum were sown onto the WA compartment, following a complete randomized design, with four replications per treatment. The plates were wrapped with plastic film layers to avoid leakage of VOCs from the plates, and incubated at 28°C in darkness. Am. hypochondriacus and S. lycopersicum seed germination, root elongation and seedling respiration were evaluated after 24 or 48 h exposure to VOCs, respectively. Seed germination, root elongation and seedling respiration were compared with controls without Xylaria sp. PB3f3 inoculum (Macías-Rubalcava et al. 2010, 2014).

Seed respiration was measured polarographically as oxygen uptake during the germination process with a Clark-type electrode connected to an YSI 5300A biological oxygen monitor (YSI Incorporated, Yellow, Springs, OH). The seedlings were transferred into a glass chamber containing 4 ml of air-saturated deionized water at 28°C. Oxygen uptake, in the presence of Xylaria sp. PB3f3 VOCs, was measured for 3 min (Macías-Rubalcava et al. 2014).

Furthermore, the phytogrowth-inhibitory activity of (±)-2-methyl-1-butanol and 2-methyl-1-propanol was evaluated using a gas phase method. These compounds were purchased from Sigma-Aldrich (>98% purity). Bioassays were carried out in two-compartment Petri dishes; one containing 10 ml WA (1%) for seeds
germination; and the other containing a handmade aluminium cup located in the centre. Thirty seeds of *Am. hypochondriacus* or *S. lycopersicum* were sown onto the WA compartment following a complete randomized design, with four replications per treatment. To determine the 50% inhibitory concentration (IC50) values, the pure compound concentrations were 3, 5, 10, 20, 40, 80 and 160 μg ml⁻¹. The organic compounds were added directly to the aluminium cup per 50 cm³ of air space, above the culture medium in a standard Petri dish. The plates were sealed with Paraﬁlm®, and incubated at 28°C in darkness. Seed germination, root elongation and seedling respiration were measured 24 h after treatment for *Am. hypochondriacus*, and 72 h for *S. lycopersicum*. The results were obtained as described above.

### Analysis of volatile organic compounds

The volatiles were tentatively identified at 10, 20 and 30 days of fungal culture, in triplicate. A 5 mm diameter inoculum from a *Xylaria* sp. culture was placed in solid phase micro extraction (SPME) vials of 40 ml capacity, with a polypropylene hole-cap and PTFE/coated silicone septa containing 15 ml of PDA. The cultures were incubated at 28°C and a 12:12 h (light–dark) photoperiod. Three PDA non-inoculated vials were also evaluated.

The VOCs were obtained by headspace (HS) SPME with a DVB/Carboxen/PDMS fibre (2 cm, 50/30 μm; Supelco, Bellefonte, PA). The fibre was conditioned before use during 40 min at 300°C, and exposed to the vials HS for 60 min. After VOCs adsorption, the fibre was inserted into the Agilent 6890 N series GC inlet (Agilent Technology, Palo Alto, CA), at 300°C, in splitless mode. The compounds were desorbed for 2 min, and separated on a 20 m × 0.18 mm × 0.18 μm, DB-5MS capillary column, utilizing the following GC oven temperature programme: 3 min at 40°C up to 300°C for 15 min, at 20°C min⁻¹. The flow rate of the helium carrier gas was 1 ml min⁻¹. The GC was coupled to a LECO time of flight mass spectrometer (MS-TOF; Leco Corporation, St Joseph, MI), operating at low resolution, with electronic ionization energy of 70 Ev, and scanning at a rate of 20 spec s⁻¹ with a mass range of 33–500 uma. The transfer line and ionization chamber temperatures were 250°C and 200°C, respectively. A LECO CHROMATOFTM software system (St Joseph, MI, USA) was used for data acquisition and processing.

*Xylaria* sp. PB3β VOCs tentative identification was performed based on comparison of MS fragmentation patterns with those available in the National Institute of Standards and Technology (NIST) database and those reported by Adams (2007). Kovats retention indices were calculated in relation to the retention times of a series of alkanes (C₈–C₂₀), and also compared with NIST database and literature data. Only compounds with quality match scores of >80% are listed. Compounds identified in the non-inoculated PDA vials were removed from the data analysis. The relative amount of each compound was expressed as a percentage resulting from the average of the peak area of the three replicates, compared with the total peak area. Whenever possible, the compound identiﬁcation was conﬁrmed by comparison of mass spectra, and Kovats indices from the available commercial standards with ≥98% purity (Sigma-Aldrich).

### Statistical analysis

The experimental results relating to the inhibitory effect of *Xylaria* sp. strain PB3β on the mycelial growth of plant pathogenic micro-organisms, in the multiple and simple direct antagonism bioassays, were analysed with the paired t-test. The phytogrowth-inhibitory activity of VOCs and pure compounds, and the antifungal and anti-oomycete effects of the organic extract were analysed with one way analysis of variance (ANOVA, α = 0.05), followed by a Tukey statistical test (Mead et al. 2002), utilizing GRAPHPAD PRISM ver. 5.01 statistical computer software (GraphPad software, La Jolla, CA). Data are represented as mean ± standard deviation (SD). A P value of ≤0.05 (*) was employed to indicate statistical significance. The IC₅₀ value for pure compounds was calculated by probit analysis, based on the average percentage inhibition, obtained at each concentration with the previously mentioned statistical software.

### Results

#### Identification of PB3β strain

For identification of the PB3β strain the macro and micro-morphological characteristics were determined, as well as the sequence analysis of the ITS1-5.8S rDNA gene. Data were compared with those available in the literature (Chacko and Rogers 1981; Rogers 1985; Callan and Rogers 1993).

#### Culture description

The colony on PDA was incubated at 28°C in 12:12 h light–dark photoperiod, had a radial slow growth, covering a 9 cm Petri dish in 3 weeks. The growth rate was 1.75 cm² day⁻¹; plane, low and dense, with concentric rings and a regular margin; mycelium white (M4A1), velvety; exudate clear; reverse uncoloured becoming cream (M4A4) or reddish brown (M7E7) toward the centre, after 2 months incubation (Fig. 1). After 9 weeks of growth no stromata, conidiogenous cells, conidia or ascospores were observed, although treatments for spore...
producing inducements, including ultraviolet radiation, freezing and temperature difference were carried out.

The identity of the PB3f3 strain was determined by BLAST analysis of its ITS1-5′S-ITS2 sequence (GenBank accession number KP400260). The most similar sequences were those of *Xylaria* sp. TPO41010 (accession no. AB524023, with 97% similarity and 100% coverage) obtained from mass ascospores discharged from a fruiting body collected from a branch in Phayao, Thailand (Osono et al. 2011) and *Xylaria* sp. G30 (accession no. JQ623492, with 94% similarity and 99% coverage) an endophytic fungus isolated from *Garcinia hombroniana* (Bouthong et al. 2012, unpublished); followed by several other sequences of *Xylaria* species or of unidentified *Xylaria* strains (Table 1).

In the genetic distance tree (Fig. 2) inferred from the closest ITS1-5′S-ITS2 NCBI sequences, the endophytic fungus PB3f3 clustered with *Xylaria* sp. TPO41010 with a bootstrap support of 100%, and a genetic distance of 0.037. The results showed that the strain PB3f3 is a species of the genus *Xylaria*.

### Antifungal and anti-oomycete effect of *Xylaria* sp. PB3f3

As a preliminary test for the antifungal and anti-oomycete activity of the endophyte *Xylaria* sp. PB3f3, we performed multiple direct antagonism bioassays. Only the fungus previously incubated for 7 days showed a significant inhibitory effect on the radial growth of the plant pathogens after 3 and 6 days of antagonistic interaction, in which the endophytic fungus had 10 and 13 days of growth. From day 3 of antagonistic interaction, the endophytic fungus *Xylaria* sp. PB3f3 presented a significant inhibitory effect (*P* < 0.05) at a distance from the radial growth of the plant pathogens *F. oxysporum* (24.2%), *A. solani* (24.5%), *Ph. capsici* (48.3%) and *Py. aphanidermatum* (78.3%). The inhibitory effects were constant during the 40 days of observation.

On the other hand, simple direct antagonism bioassays were performed for determining whether *Xylaria* sp. PB3f3 could inhibit the plant pathogen’s growth in dual interactions. From day 3 of antagonistic interaction, in which the endophytic fungus had 10 days of growth, it produced a significant inhibitory effect at a distance of 90% (*P* < 0.05) over the growth of the four plant pathogens, this inhibition was constant during the 40 days of observation.

In order to confirm whether the plant pathogens could recover after the inhibition caused by the endophytic fungus *Xylaria* sp. in these simple direct antagonisms, each plant pathogen was re-inoculated in PDA plates after 20 days of antagonistic interaction. *Xylaria* sp. PB3f3, had a fungistatic effect on the *F. oxysporum* and *Ph. capsici* growth due to their recovery. In contrast, *Xylaria* sp. PB3f3 had a fungicide and oomicide effect on *A. solani*.
and *Py. aphanidermatum*, respectively, since they were not recovered. The growth inhibition observed in the bioassays can be mediated by volatile secondary metabolites and extracellular metabolites diffused into agar produced by *Xylaria* sp. PB3f3. However, there was not a significant inhibitory effect on the growth of any of the plant pathogens tested in the divided plate bioassay, which shows that the inhibition was caused by the metabolites diffused into the agar.

**Antifungal and anti-oomycete effect of organic extracts**

In order to evaluate whether the biological activity was attributable to volatile or non-volatile organic compounds from the endophytic fungus *Xylaria* sp. strain PB3f3, we also evaluated organic extracts from the culture medium and mycelium of *Xylaria* sp. PB3f3 on the same phytopathogens. The organic extract showed significant inhibitory activity (*P* < 0.05) on the radial growth of the oomycete *Py. aphanidermatum* (17.3% ± 3.7) and on the fungi *A. solani* (20.4% ± 2.4) and *F. oxysporum* (17.6% ± 1.9); however, the percentages of inhibition were low.

**Phytogrowth-inhibitory activity of VOCs**

The VOCs significantly inhibited (*P* < 0.05) the root growth of *Am. hypochondriacus* and *S. lycopersicum*. This inhibition varied depending on the *Xylaria* sp. PB3f3 growth. The VOCs production after 1, 3 and 5 days of fungal growth did not show significant inhibitory effects. For *Am. hypochondriacus* seeds the most phytotoxic effect was shown when the endophytic fungus had 30 days of growth (27.6% of inhibition); while in the *S. lycopersicum* seeds, the higher inhibition (53.2%) on the root growth
was after 20 days. In both cases, a decrease of the phytotoxic effect on *Am. hypochondriacus* (15–1% of inhibition) and on *S. lycopersicum* (20–5%) after day 40 of growth of *Xylaria* sp. PB353 (Fig. 3) was recorded. The VOCs did not significantly inhibit the germination and respiration processes of the two plants at any time of the endophyte growth.

Chemical composition

In this study, we tentatively determined a total of 40 VOCs in the samples at 10, 20 and 30 cultivation days (Table 1), and they belonged to different chemical families. The endophyte *Xylaria* sp. PB353 produced mainly alkanes (11). Likewise, other compounds were identified

### Table 2 GC/MS analysis of the volatile compounds produced by *Xylaria* sp. PB353 after 10, 20 and 30 days of culture

<table>
<thead>
<tr>
<th>Peak</th>
<th>Name</th>
<th>RI*</th>
<th>Total area (%)</th>
<th>Molecular formula</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>Dimethyl ether</td>
<td>587.32</td>
<td>1.71</td>
<td>46 C$_2$H$_6$O</td>
</tr>
<tr>
<td>2</td>
<td>Unknown</td>
<td>592.09</td>
<td>4.03</td>
<td>46 C$_2$H$_6$O</td>
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<tr>
<td>3</td>
<td>Unidentified amine</td>
<td>603.74</td>
<td>34.30</td>
<td>81 C$_2$H$_6$O</td>
</tr>
<tr>
<td>4</td>
<td>Unknown</td>
<td>620.84</td>
<td>2.52</td>
<td>86 C$_2$H$_6$O</td>
</tr>
<tr>
<td>5</td>
<td>2-Methyl-1-propanol</td>
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<td>–</td>
<td>25.43</td>
</tr>
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<td>–</td>
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</tr>
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<td>3-(Ethenoxy)-1-propene</td>
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<td>1-Pentanol</td>
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<tr>
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<td>38</td>
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<td>0.19</td>
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<td>Unknowns†</td>
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<td>2.96</td>
<td>3.11</td>
<td>2.89</td>
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</table>

Compounds found in the control PDA plate are not included in this table. Only compounds with quality match scores >80 are listed.

* Kovats indices calculated from retention time data on a DB-5MS capillary column.

† The values of the total area of VOCs were calculated considering all identified compounds before discriminating the peaks representing less than 1% of the total area.
in a lower proportion: esters (5), sesquiterpenoids (6), alcohols (6), amines (2), ketones (2), carboxylic acids (2) and one ether. From the total VOCs, 25, 20 and 22 compounds were produced at days 10, 20 and 30 of fungal growth, respectively (Table 2). Compounds with high fibre affinity were: 3-methyl-1-butanol and thujopsene after 10 days of growth; 2-methyl-1-butanol and an amine of unidentified structure after 20 days; and 2-methyl-1-propanol after 30 days of growth.

The Venn diagram shows the compounds identified at two or three dates of cultivation (Fig. 4). Two compounds were identified in common at 10 and 20 days of Xylaria sp. PB3f3 growth. Four compounds were produced at 10 and 30 days of fungal growth; and five compounds were produced commonly at 20 and 30 days of cultivation (Table 2). Figure 4 shows eight VOCs that were detected at the three dates of cultivation (10, 20 and 30 days): 2-pentyl-furan, 4-nonanone, acetic acid 2-ethylhexyl ester, trans-2-bergamotene, thujopsene, 1,8-methyl-4-(1-methyl,ethenyl)-spiro[4,5]dec-7-ene (acoradiene), 3,7,7-trimethyl-11-methylene-spiro[5,5]undec-2-ene and 1,2,3,5,6,7,8,8a-octahydro-1,4-dimethyl-7-(1-methylethenyl)-azulene. Such compounds were produced in a constitutive way with the fungus growth.

Phytogrowth-inhibitory activity of (±)-2-methyl-1-butanol and 2-methyl-1-propanol

Since 2-methyl-1-butanol (33.2%) and 2-methyl-1-propanol (25.4%) were two of the most abundant constituents present in the VOCs produced after 20 and 30 days, respectively, of Xylaria sp. PB3f3 growth, we evaluated their ability to inhibit the seed germination, root elongation and seedling respiration of Am. hypochondriacus and S. lycopersicum, using a gas phase bioassay.

Both compounds displayed a significant phytotoxic effect on the seed germination, and root elongation in a concentration-dependent manner. The (±)-2-methyl-1-butanol displayed high phytotoxic activity on the root growth of Am. hypochondriacus and showed an IC50 value of 4.59 μg ml⁻¹. Besides, the IC50 value on seed germination was found to be 26.45 μg ml⁻¹ with respect to S. lycopersicum seeds, the IC50 values on seed germination and root elongation were found to be 39.74 and 23.15 μg ml⁻¹, respectively. On the other hand, 2-methyl-1-propanol also significantly inhibited the tested seeds, it showed an IC50 value of 48.20 μg ml⁻¹ on germination and 26.45 μg ml⁻¹ on root growth of Am. hypochondriacus, while for S. lycopersicum the IC50 on germination and root growth were found to be 130.0 and IC50 54.25 μg ml⁻¹, respectively. Finally, both compounds significantly (P < 0.05) inhibited the seedling respiration process of the two plants tested at the maximum concentration evaluated, which was 160.0 μg ml⁻¹, in a range between 20% and 27% (Table 3).

Discussion

In the present study, we isolated a novel endophytic fungus identified as Xylaria sp. PB3f3 (GenBank ID: KP400260) from healthy leaves of H. brasiletto. We also showed, for the first time, that an endophyte of the genus Xylaria produces VOCs.

Regarding the antifungal activity, the multiple antagonistic bioassay showed significant inhibitory effects in a
range between 24 and 78% on the growth of *F. oxysporum*, *A. solani*, *Ph. capsici* and *Py. aphanidermatum*. However, in antagonism bioassays, in divided Petri dishes, the volatile organic compounds did not show significant inhibitory activity on the growth of pathogenic micro-organisms. In addition, the organic extracts showed significant inhibitory effects in a range between 17 and 20% on the growth of *F. oxysporum*, *A. solani* and *Py. aphanidermatum*. Therefore, the antifungal and anti-oomycete effect at a distance from the radial growth of the plant pathogens, observed in the multiple and simple direct antagonism bioassays, is generated by secondary metabolites produced by *Xylaria* sp. strain PB3f3; volatiles and non-volatile organic compounds acting individually or synergistically to increase the activity.

Concerning the composition of the identified VOCs of *Xylaria* sp. PB3f3, it changed qualitatively and quantitatively depending on the day of the fungus growth. The relative abundance of the 2-methyl-1-butanol, 2-nonenone, *trans*-2-bergamotene, thujopsene, 1,8-dimethyl-4-(1-methylthienyl)-spiro[4,5]dec-7-ene (acoradiene) and 3,7,7-trimethyl-11-methylene-spiro[5,5]undec-2-ene changed dramatically along the culture time; and as a consequence of the endophyte age (Fig. 5 and Table 2).

The most phytotoxic effect on the root development of *Am. hypochondriacus* and *S. lycopersicum* was obtained after 20 and 30 days of growth of *Xylaria* sp. PB3f3, respectively. The different phytotoxic effects on the root growth, on different ages of growth of *Xylaria* sp. PB3f3, was due to the sensitivity of each plant tested to the volatile compounds produced at the two dates. In addition, the VOC composition changed from 20 to 30 days of fungal growth. One of the most abundant compounds at 20 days was 2-methyl-1-butanol (33.28%); and 2-methyl-1-propanol (25.43%) at 30 days. These compounds showed significant inhibitory effects on the germination and root elongation of *Am. hypochondriacus* and *S. lycopersicum*, employing the gas phase bioassay, where *Am. hypochondriacus* was the most affected by both compounds (Table 3). This can explain why *Am. hypochondriacus* was the most inhibited at 30 days of fungal growth, since 2-methyl-1-propanol was the most abundant compound (25.43%), followed by 2-methyl-1-butanol (11.81%). On the other hand, 2-methyl-1-butanol also showed more phytotoxic activity on *S. lycopersicum* than 2-methyl-1-propanol and only the former was present at 20 days of fungal growth. In addition, 2-methyl-1-butanol in previous evaluations *in vitro* presented a significant phytotoxic activity on *Arabidopsis thaliana* causing 40% inhibition on seedling formation, at 250 μg ml⁻¹; and, 2-methyl-1-propanol also inhibited by more than 50% the seedling formation (Hung et al. 2014).

On the other hand, 3-methyl-1-butanol and 2-methyl-1-butanol, compounds with a high fibre affinity at 10 and 20 days of *Xylaria* sp. PB3f3 growth were also identified as volatile compounds produced by *Muscodor albus* which had antifungal activity against crop pathogens (Mercier and Jiménez 2004). This corroborates that micro-organisms belonging to the same phylogenetic group are able to produce similar bioactive compounds. Besides, volatiles from *Muscodor* can be used as biocontrol agents due to the bioactivity of its volatile compounds (Mercier and Manker 2005; Riga et al. 2008; Macías-Rubalcava et al. 2010). Among the identified compounds, the sesquiterpene thujopsene, the main compound identified at 10 days of fungal growth, has been previously identified in fungi and plants. It is worth mentioning that in the essential oil obtained by means of hydrodistillation, from the endophytic fungus *Xylaria* sp. NICL5, and isolated from *Capressus lusitanica*, one of its main components was *cis*-thujopsene (Santos Filho et al. 2011). Thujopsene has been also identified as the main component of the VOCs produced by the *Penicillium decumbens*, isolated from a building impaired by water. The *P. decumbens* VOCs have antifungal activity against *Eurotium herbarium* and *Gonytrichum macrocladum*. In addition, thujopsene also acted as a self-regulation mechanism against the growth of *P. decumbens* (Polizzi et al. 2011). Pure thujopsene isolated from *Thujaopsis dolabrata* presents antibacterial activity against gram-positive and gram-negative bacteria (Oh et al. 2011). Also, it is part of the essential oil of *Eugenia dysenterica*, which shows antibacterial activity against several strains of *Cryptococcus neoformans* (Costa et al. 2000). It is a volatile component

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**Figure 5** Relative area of common volatile metabolites produced after 10, 20 and 30 days culture of *Xylaria* sp. PB3f3. (A) 2-pentylfuran, (B) 4-nonanone, (C) acetic acid 2-ethylhexyl ester (at day 30 showed a relative abundance of 0.05), (D) *trans*-2-bergamotene (at day 30 showed a relative abundance of 0.16), (E) thujopsene (at day 30 showed a relative abundance of 0.16), (F) *trans*-2-bergamotene (at day 30 showed a relative abundance of 0.16), (G) 1,8-dimethyl-4-(1-methylthienyl)-spiro[4,5]dec-7-ene (acoradiene), (G) 3,7,7-trimethyl-11-methylene-spiro[5,5]undec-2-ene, (H) 1,2,3,5,6,7,8,8a-octahydro-1,4-dimethyl-7-(1-methylthienyl)-azulene.
of the heartwood extract of the cedar Callitropsis nootkatensis with activity against Phytophthora ramorum (Manter et al. 2007). Furthermore, the sesquiterpene trans-α-bergamotene is present in the Ocimum basilicum extract, which has antifungal activity against different species of Fusarium (Kocić-Tanackov et al. 2011); and, it is also a constituent of the essential oil of Cymbopogon citratus, which has antibacterial activity against Salmonella enterica (formerly Salmonella choleraesuis), Pseudomonas aeruginosa and Staphylococcus aureus (Falcão et al. 2012). The 1,8-dimethyl-4-(1-methylethenyl)-spiro[4,5]dec-7-ene (acordiene) is present in the VOCs generated by F. oxysporum, with nematicide effects on Meloidogyne incognita (Freire et al. 2012); and, as part of the VOCs of Salvia fruticose, which have antifungal activity against R. solani and Sc. sclerotiorum (Pitaroskili et al. 2003). Additionally, the 2-nonanone produced by Bacillus pumilus and Bacillus thuringiensis also has antifungal potential against Colletotrichum gloeosporioides (Zheng et al. 2013) and B. cinerea (Almenar et al. 2009). Likewise, it is used to increase the shelf life of the strawberry, due to its antifungal effects (Almenar et al. 2009). 3-Methyl-1-butanol, thujoepene, acordiene, trans-α-bergamotene and 2-nonanone can contribute to the antifungal activity observed in the multiple antagonism bioassays between Xylaria sp. PB3f3 and the four plant pathogens tested, which have economic importance in agriculture. These compounds could be the responsible for the antifungal and antioomycete activities shown in the antagonism bioassays, since they were the main compounds identified after 10 and 20 days of growth of Xylaria sp. PB3f3; and, they could be the compounds diffusing into agar, acting individually or synergistically to increase the activity.

Finally, it is important to say that in spite of the identification of different compounds with antifungal activity, previously reported in the literature, it is widely known that, in general, the antimicrobial effects caused by the microbial VOCs are the result of the synergistic effect (Strobel et al. 2001; Strobel 2010).

These results suggest that the endophytic fungus Xylaria sp. PB3f3 could be useful in the biological control of microorganisms, in particular, as a fungistatic and anti-oomycete agent on F. oxysporum and Ph. capsici, respectively; and, as a fungicide and oomicide agent on A. solani and Py. aphanidermatum, respectively. Furthermore, the VOCs, produced by Xylaria sp. PB3f3, could be useful in the biocontrol of weeds. Further studies in crop protection might be needed, especially to establish their potential efficiency. In addition, we will start research on the anti-oomycete, antifungal and phytotoxic potential of the non-volatile fungal secondary metabolites produced by the endophyte Xylaria sp. PB3f3; and, the VOC production in multiple antagonistic interactions and its biological importance.

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Conflict of Interest

No conflict of interest declared.

References

Bioactivity of volatile compounds from Xylaria sp.

B.L. Sánchez-Ortiz et al.


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