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Detection of viridiofungin A and other antifungal metabolites excreted by *Trichoderma harzianum* active against different plant pathogens

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Abstract Antibiosis is assumed to be an essential mechanism exerted by potential biocontrol agents (BCAs) of *Trichoderma* spp. Therefore, in the present study, we report for the first time on the elucidation and production of viridiofungin A (VFA) from *T. harzianum* isolate T23 cultures and investigate the antifungal potential of VFA and some other secondary metabolites purified from *T. harzianum* cultures against *Fusarium moniliforme*. The bioautography assay revealed that *T. harzianum* isolates T16 and T23 excreted several secondary metabolites with antifungal activity. Following isolation and purification of the antifungal zones, three fractions (F223, F323 and F423) from extracts of isolate T23 and two fractions (F416 and F516) from extracts of isolate T16 exhibited pronounced fungitoxic activity in the bioautography and antibiotic disk assays against *Cladosporium* spp. and *F. moniliforme*, respectively. The structure of the antifungal metabolite in fraction F323 was identified as viridiofungin A (VFA), the first report of production of VFA by isolate T23 of *T. harzianum*. Following cultivation of isolate T23 in PDB medium for 9 days, 94.6 mg l^{-1} of VFA were determined. VFA and fraction F516 retarded the mycelial growth of *F. moniliforme* in the non-volatile phase assay by >90% for each 250 μg ml^{-1} 7 days post-inoculation (dpi). While VFA and fraction F416 showed both volatile and non-volatile effects, fraction F516 seemed to exhibit mainly non-volatile activity. Microscopic examination revealed that hyphae of *F. moniliforme* grown on VFA-amended medium were less branched and appeared thicker than untreated hyphae. Furthermore, in the presence of VFA, formation of chlamydospores by *F. moniliforme* was increased. Finally, the antifungal spectrum of VFA towards various important plant pathogens was evaluated. Germination of propagules of a variety of fungal pathogens *in vitro* was differentially inhibited by VFA. While in the presence of 100 μg ml^{-1} VFA conidial germination of *V. dahliae* was completely inhibited, a slightly higher concentration (150 μg ml^{-1}) of the inhibitor was required to suppress germination of *Phytophthora infestans* sporangia or sclerotia of *Sclerotinia sclerotiorum*. Contrary to several reports in the literature, VFA proved to be fungistatic rather than fungicidal. However, neither VFA nor the other *Trichoderma* metabolites, such as 6PAP, F416 and F516, exhibited any antibacterial activity against Gram-positive and Gram-negative bacteria.

Keywords Antibiosis · Bioautography · *F. moniliforme* · Fungistatic · *T. harzianum* · Viridiofungin A

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Introduction

In the last decades, plant pathologist and commercial companies have shown increasing interest and have made great research efforts to develop alternative strategies for controlling plant diseases (Cook 1991). Hence, a substantial number of biocontrol agents (BCAs) have been released commercially and are available especially to control fungal plant pathogens causing root diseases (Lumsden et al. 1995; Fravel et al. 1998). The BCAs are an essential complement to commercially available fungicides which are predominantly applied to control foliage diseases, and have proved unsuitable for several reasons for combating root diseases (Buchenauer 1998). *Trichoderma* species are the most thoroughly studied potential BCAs that have attracted considerable attention due to their mycoparasitic properties (Weindling 1932), competition for nutrients (Chet 1987), cell wall-degrading capabilities (Metcalf and Wilson 2001), induction of systemic resistance in host plants against pathogens (Yedidia et al. 2001) and antibiosis (Howell and Stipanovic 1983; Verma et al. 2007; Bailey et al. 2008). Numerous species of the genus *Trichoderma* have been successfully developed as effective and commercially-applied formulations of biopesticides against various plant pathogens worldwide (Harman et al. 2004). The antagonistic properties of *Trichoderma* spp. in terms of antibiotic production have been described by Dennis and Webster (1971) and this has stimulated the investigations in this field. Subsequently, an increasing number of the antimicrobial metabolites produced by *Trichoderma* spp. have been the subject of intensive studies and consequently several compounds with antimicrobial properties have been elucidated (Moffatt et al. 1969; Collins and Halim 1972; Fujiwara et al. 1982; Almassi et al. 1991). Compounds produced by *Trichoderma* spp. include for example gliotoxin, viridin, gliovirin, glisoprenin, hepteledic acid, 6-pentyl-α-pyrone, koninginins, anthraquinones, trichodermamides, peptaibols, polyketides, terpenoids, polypeptides, trichothecenes, trichodermaides, azaphilones, harzialactones and metabolites derived from alpha-amino acids (Howell 1998; Vey et al. 2001; Reino et al. 2008).

Harris et al. (1993) reported the isolation and structure elucidation of several members of a new group of antibiotics from *T. viride* which have been termed viridiofungins; they belong to a family of the amino alkyl citrate antibiotics possessing several biological properties. They may act as inhibitors of squalene synthase, the first enzyme in the ergosterol biosynthesis pathway. Inhibitors of this enzyme have been described as potential antifungal agents (Nakayama et al. 2000). On the other hand, Onishi et al. (1997) reported that the antifungal mode of action of this group of metabolites is unrelated to the inhibition of ergosterol synthesis. They found that viridiofungins specifically interfered in the inhibition of serine palmitoyltransferase, a key enzyme in *de novo* synthesis of sphingolipids (Mandala et al. 1997). Viridiofungins lack antibacterial activity (Onishi et al. 1997).

Another pharmaceutical characteristic of the viridiofungins is the ability to inhibit the farnesyltransferase, a Ras-protein which is commonly abnormally active in cancer and found to be involved in the transformation of normal cells into abnormal cells (Meinz et al. 1993). Thus, the inhibition of the farnesyltransferase by viridiofungins may block this transformation and might be potentially used in cancer treatment (Wittinghofer and Waldmann 2000).

Even though viridiofungins have been elucidated for more than a decade studies dealing with the antifungal potency of this group of antibiotics against plant pathogenic fungi are extremely limited. In addition, no report on the production and isolation of viridiofungins from other species of *Trichoderma* except *T. viride* has been described. In the present study, we report for the first time on the production, isolation and structure elucidation of viridiofungin A (VFA) from *Trichoderma harzianum* isolate T23 cultures. Furthermore, the antifungal potential of VFA and some other secondary metabolites purified from cultures of *T. harzianum* isolates T23 and T16 in the non-volatile and volatile phases against *F. moniliforme* is described. Finally, the antifungal spectrum of VFA towards various important plant pathogens was evaluated.

Materials and methods

Fungal materials

Due to their antagonistic activity against *Fusarium moniliforme*, two *Trichoderma* isolates (T16 and T23) were selected by El-Hasan et al. (2007) and identified

as *T. harzianum* using the specific internal transcribed spacer (ITS1, ITS2) sequences (unpublished data). In the present study the antifungal activity of both *T. harzianum* isolates were tested against numerous important plant pathogens including: *F. moniliforme* isolate F8, *F. oxysporum* f. sp. *fabae*, *Phytophthora infestans*, *Sclerotinia sclerotiorum*, *Botrytis cinerea*, *Verticillium dahliae*, *Gliocladium roseum*, *Alternaria alternata*, *Cladosporium* spp. and against the economically significant bacterial plant pathogens, *Clavibacter michiganense* and *Erwinia amylovora*. *Phytophthora infestans* was maintained on V8-medium while the other fungal isolates were stored in soil culture at 4°C and subcultured bimonthly. The bacterial pathogens were preserved in 30% glycerol culture. All microorganisms were obtained from the culture collection of the Institute of Phytomedicine, University of Hohenheim.

Cultivation in liquid medium and production of secondary metabolites

Two agar plugs from the edges of actively growing *T. harzianum* isolates T23 or T16 were transferred to 100 ml conical flasks containing 30 ml potato dextrose broth (PDB; Sigma, Steinheim, Germany). The fungal isolates were incubated on a rotary shaker at 150 rpm and at 25°C in the dark. After 2, 5, 8 and 12 days of incubation, cultures in triplicate were filtered through Whatman filter paper (Schleicher & Schuell GmbH, Dassel, Germany) and the filtrates repeatedly extracted with equal volumes of 99.5% ethyl acetate (EtOAc; Merck, Darmstadt, Germany). Combined organic phases were dried over anhydrous sodium sulphate (Applichem, Darmstadt, Germany) and evaporated under reduced pressure. The residue was resuspended in 1 ml EtOAc.

Thin-layer chromatography

Volumes of each 20 µl of the crude EtOAc extracts of the *Trichoderma* isolates T23 or T16 were applied on analytical pre-coated thin-layer chromatography (TLC) plates (ALUGRAM® SIL G/UV$_{254}$ [0.20× 100×100 mm] Macherey-Nagel, Düren, Germany) and chromatographed with either a mixture of tertiary butyl methyl ether (TBME; Roth, Karlsruhe, Germany):isooctane (Merck):butanol (Merck; 6:2:2; *v*:*v*:*v*) or TBME:MeOH (3:7; *v*:*v*) at approximately 25°C. 6-Pentyl-α-pyrone (6PAP) purified from *T. harzianum* isolate T23 and analytical EtOAc were co-chromatographed in the reference and control treatments, respectively. The resulting bands were visualised under UV-light at 254 nm and 366 nm. Developed TLC plates were air-dried overnight to remove the remaining solvents and the compounds with antifungal activities detected by the bioautography assay. The retention factors (R_f) and the corresponding inhibition zones were recorded.

Bioautography assay

For direct detection of antifungal zones, the developed TLC plates were first sprayed with a thin layer of malt extract agar at approximately 50°C and thereafter with a conidial suspension of *Cladosporium* spp. (approx. 2.3×10^{-6} conidia ml^{-1}). The inoculated TLC plates were then transferred into sterile plastic boxes, sealed with Parafilm to maintain 100% relative humidity (RH) and incubated at 25°C in the dark for 3–4 days. A reference non-sprayed TLC plate was prepared and kept for further isolation of the bioactive substances.

Isolation and purification of the crude fractions by Varian® preparative HPLC

The silica gel bands on the reference uninoculated TLC plate corresponding to the bioactive zones were gently scraped off and resuspended in acetone. The active compounds were extracted repeatedly with EtOAc and the combined organic solvents were evaporated under a vacuum of 150–170 mbar in a rotary evaporator at 40°C. Subsequently, the residues were redissolved in a mixture of acetonitrile (ACN) and water (1:1; *v*:*v*), centrifuged and then fractionated using analytical HPLC to identify the bioactive compounds. All data obtained from the retention time in HPLC/DAD and DAD-UV of these compounds were employed and scaled up to a preparative HPLC-system.

The bioactive fractions (four fractions from isolate T23 and six fractions from isolate T16) were separately collected, pooled and extracted twice with EtOAc. The combined organic phases were filtered over anhydrous sodium sulphate and the solvents evaporated using a rotary evaporator at 150–170 mbar and 40°C. The small remaining solvents evaporated

completely under a constant flow of nitrogen at 40°C until the pure fraction crystallised in the glass tube. The crystallised fractions were dissolved in analytical acetone to give stock solutions with a final concentration of each of 10 mg ml^{-1}.

Determination of antifungal activity of the fractions

In order to estimate the antifungal potential of the fractions, two assays were performed.

Bioautography assay Volumes of each 15 µl from the stock solutions of the purified bioactive fractions (F116, F216, F316, F416, F516, F616, F123, F223, F323 and F423) isolated from the crude extracts of the isolates T23 or T16 as well as acetone (1.5%) as control were applied on TLC plates (RP-2 F_{254}, 10× 10 cm) and developed in mixtures with different polarity of EtOAc:chloroform:isooctane:butanol (4:4:5:4; *v*:*v*:*v*:*v*) or TBME:MeOH (*3*:*7*; *v*:*v*). Developed TLC plates were used for detection of the antifungal activities in the fractions using the bioautography test as previously outlined. To quantitatively evaluate the inhibition zones caused by the fractions, the plates were gently enclosed in nylon foils and photocopied. The clear zones on the sheets representing the inhibition zones of the fractions on the TLC plate were individually cut out from the sheet and weighed.

Antibiotic disk assay The antifungal effects of the fractions toward *F. moniliforme* were further assessed using the antibiotic disk assay. Volumes of each 15 µl from the stock solutions of the separated fractions and 6PAP were then applied to sterilised filter paper disks (Whatman No. 1; 9 mm ∅) to give compound concentrations each of 150 µg/disk. Acetone (1.5%)-saturated disks were included as the control. The impregnated disks were placed in the middle of malt extract agar (MEA) plates and incubated at 4°C for 24 h to allow a prolonged diffusion of the compounds into the agar medium. Subsequently, the plates were inoculated at ca. 2 cm from the edge of the plate with three agar plugs (5 mm ∅) of an actively growing *F. moniliforme* culture and incubated at 25°C in the dark for 10 days. Each treatment was set up in triplicate. Mycelial growth inhibition around the disks indicated the antifungal potential of the individual fractions.

Identification of the fraction F323 (viridiofungin A)

The identification of viridiofungin A (VFA) in the fraction F323 was performed using HPLC/DAD, LC-MS, and ^{1}H- and ^{13}C-NMR techniques, respectively. Based on the fast and high resolution hybrid LTQ LC/ESI-Orbitrap™ MS technology (Thermo Fisher Scientific, Inc. Waltham, MA, USA) in analysing F323, the molecular formula of $C_{31}H_{45}NO_{10}$ could be accurately elucidated (methods described below). The structure of viridiofungin A could be identified by comparing the data obtained from the above mentioned analytical techniques with those in the literature (Harris et al. 1993).

Liquid chromatography-electrospray ionisation tandem mass spectrometry

LC-ESI-MS was performed on a HP1100 (Agilent, Waldbronn, Germany) modular HPLC-system coupled to a Micromass VG Platform-II (Manchester, UK) quadrupole mass spectrometer. The HPLC-system was combined with a HP1100 autosampler, HP1100 gradient pump, HP1100 thermoregulator and HP1100 diode array detector module. Separation was carried out with a Luna® synergy polar column (Phenomenex®; 250×3 mm, 5 µm) at a temperature of 25°C and a flow rate of 0.6 ml min^{-1} using a mixture of ACN:water at (8:2; *v*:*v*). The tuning parameters for positive ion spray (ES^{+}) were 3.5 kV for capillary and 40 V for cone at a source temperature of 120°C. Full-scan mode was employed in the *m*/*z* range of 200–700.

^{1}H and ^{13}C NMR (nuclear magnetic resonance) and HH-COSY (correlation spectroscopy) spectra were recorded using a Varian® (NMR-INOVA-300 Mhz) instrument as a gated spin echo in deuteroacetone. Shifts were recorded in parts per million (ppm) downfield from tetramethylsilane using the central resonance of deuteroacetone as an internal standard.

Determination of the optimal medium and harvesting time for VFA production

In the course of our studies concerning the optimisation of VFA production, four liquid media [PDB, Czapek Dox broth (CDB), malt extract broth (MEB) and Richard's solution (RS)] were tested;

200 ml of each medium were inoculated with two agar plugs (5 mm ∅) of the isolate T23 in 500 ml flasks in triplicate. Controls included liquid medium inoculated with isolate T16 and medium uninoculated. At stated incubation periods, 900 µl samples of the crude culture filtrates were transferred into vials, 100 µl ACN:H_2O (3:7; *v*:*v*) added and the VFA concentrations analysed by HPLC/DAD.

Effects of the non-volatile phases of viridiofungin A, F416 and F516 on mycelial growth of *Fusarium moniliforme*

To assess the antifungal activity of the bioactive metabolites purified from both *T. harzianum* isolates against *F. moniliforme*, the technique described by Carpenter (1942) was used. The pure compounds dissolved in acetone were individually diluted with sterile distilled water (SDW) and incorporated into MEA at ca. 60°C to give a series of concentrations ranging from 50 to 250 µg ml^{-1}. Control medium contained a corresponding concentration of analytical acetone. After solidification of the agar medium, agar disks, (5 mm ∅) taken from the margin of a 14 day-old *F. moniliforme* culture grown on MEA, were transferred to the centre of the Petri plates (5 cm ∅), and the plates were then incubated at 25°C in the dark. The radial colony growth of the fungus was recorded daily and mycelial growth inhibition was expressed in percent of growth of the corresponding control treatments. In order to estimate the effect of the *Trichoderma* metabolites on conidial production of *F. moniliforme*, the procedure outlined in our earlier study was used (El-Hasan et al. 2007). Direct microscopic examination of the hyphae served to monitor the morphological characteristics and chlamydospores produced by *F. moniliforme*. The experiments were repeated twice.

Detection of the volatile phase effects of VFA, F516 and F416 on mycelial growth of *F. moniliforme*

The antifungal potency of the vapour phase of the metabolites isolated from both *Trichoderma* isolates was investigated as described by El-Hasan et al. (2007). Whatman filter papers (90 mm ∅), soaked with aqueous solutions of the different compounds at 250 µg ml^{-1}, were fixed inside the lids of Petri plates containing MEA already inoculated with agar disks (5 mm ∅) of an actively growing *F. moniliforme* culture. The plates were closed with two layers of Parafilm and kept upside down and mycelial growth retardation compared to the controls was measured over a period of 9 days. Filter papers soaked with 2% acetone (according to the acetone concentration in the test solution) or 6PAP at 250 µg ml^{-1} were used as negative and reference control treatments, respectively.

Assessment of the antimicrobial spectrum of viridiofungin A

The antifungal effects of VFA on germination of conidia/sporangia and elongation of germ tubes were tested against *F. oxysporum* f. sp. *fabae*, *B. cinerea*, *G. roseum*, *A. alternata*, *V. dahliae and P. infestans*. In this assay, 0.3 ml PDB/V8 medium in 0.6 ml Eppendorf tubes containing VFA concentrations ranging from 50 to 200 µg ml^{-1} and conidial/sporangial suspensions of the target fungi were prepared. Control medium contained 2% acetone. Cultures were incubated on a shaker at 100 rpm at 25°C in the dark for 24 h. Subsequently, aliquots were taken from the cultures. Germination rates of conidia/sporangia and germ tube elongations were determined. Conidia/sporangia were considered as germinated if germ tubes extended at least 50% of the length/diameter of the propagation units. To determine the fungicidal/fungistatic effect of VFA, the remaining conidia/sporangia were washed with fresh PDB by centrifugation and further incubated as mentioned above. The germination rates of conidia/sporangia and germ tube elongations were evaluated again after an additional 24 h incubation.

In addition, germination of sclerotia of *S. sclerotiorum* in the presence of VFA was assessed. To ensure that the originating hyphae developed from the germinating sclerotia and not from hyphal fragments adhering to the sclerotia, sclerotia were surface-sterilised by soaking in an ethanol–sodium hypochlorite mixture (1:1; *v*:*v*) for 1 min. They were then rinsed in SDW and air-dried for 5 min. Sclerotia of 2–4 mm in length/diameter were placed individually in the middle of Petri plates (35 mm ∅) containing PDA medium amended with 50, 100, 150 and 200 µg ml^{-1} of VFA and incubated at 25°C in

the dark. After evaluation of sclerotial germination, the non-germinated sclerotia were washed with SDW for 5 min and incubated again on fresh PDA medium.

Investigation of the effect of VFA on bacteria

The activity of VFA and the other metabolites purified from both *T. harzianum* isolates was tested also against *C. michiganense* and *E. amylovora* using the disk diffusion assay outlined by Sholberg et al. (2001) with modifications. Briefly, 100 µl of the bacterial suspension from a 24 h-old culture grown in King B broth were spread on the surface of the King B agar plates. Sterile filter paper disks were treated with 50, 100, 150, and 200 µg ml^{-1} of each VFA, 6PAP, F416 and F516, respectively. The disks were then transferred into the middle of the agar plates; 2% acetone and streptomycin (10 µg/disk) solutions were applied to control disks.

Statistical analyses

All laboratory experiments were repeated at least twice assuming all factors were randomised. Statistical analyses were carried out by means of SPSS software (version 15.0). Significant effects were investigated by the post-hoc multiple comparison test. ANOVA, Duncan's new multiple range test (MRT) and LSD were used for comparing treatment means. The differences among treatments were determined at P=0.05.

Results

Detection of fungitoxic metabolites in culture filtrates of *T. harzianum* by bioautography assay

Two days post-inoculation (dpi) of *T. harzianum* isolate T23 in PDB, an antifungal compound was detected in the culture filtrate using the bioautography assay (Fig. 1). The compound with an R_f value of 0.65, accumulating with an extended incubation time corresponded to 6-pentyl-alpha-pyrone (6PAP). An additional inhibition zone with an R_f value of 0.21 was detected 5 dpi and this antifungal compound accumulated with an increased incubation time. In crude extracts of isolate T16 two inhibition zones with R_f values of 0.69 and 0.81 were detected 8 and 12 dpi, respectively. The antifungal compound with the R_f value of 0.81 produced a high activity at 12 dpi.

Determination of the antifungal activities of the fractions

Bioautography assay After isolation and purification of the antifungal zones, ten fractions from both isolates of *T. harzianum* were obtained following preparative HPLC. Among these fractions, three produced by isolate T23 and two by isolate T16 showed considerable antifungal activities according to the bioautography test (Fig. 2). Fraction F323 (R_f 0.79) from isolate T23 exhibited the most distinct inhibition zone against *Cladosporium* spp. followed

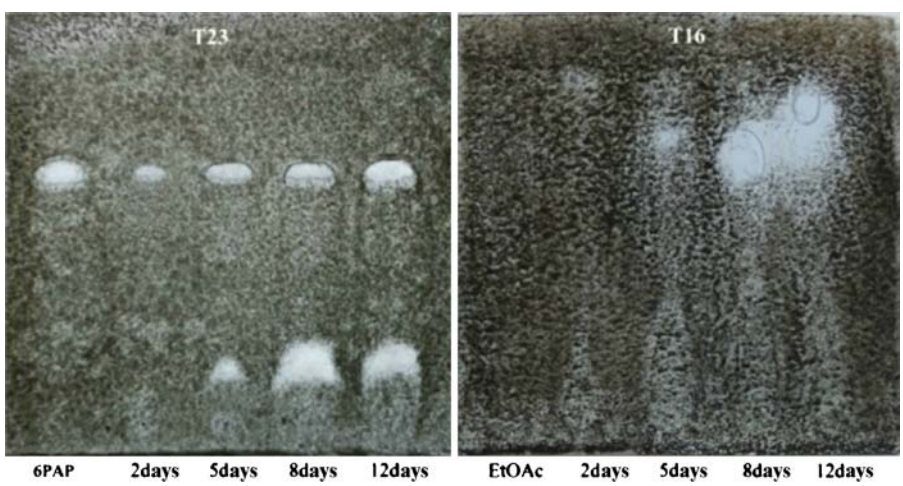

Fig. 1 Bioautography assay to detect antifungal active substances in crude culture filtrates of *T. harzianum* isolates T23 and T16 using *Cladosporium* spp. as a test organism. 6PAP and EtOAc were employed as controls. TLC plates containing the crude extracts of isolate T23 were developed with TBME:isooctane:butanol (6:2:2; *v*:*v*:*v*), those containing the extracts of isolate T16 were developed with TBME: MeOH (3:7; *v*:*v*)

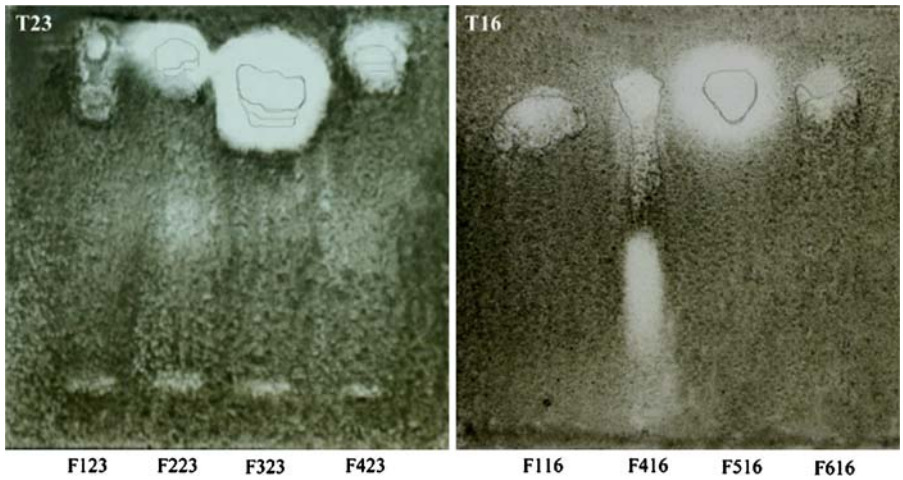

Fig. 2 Bioautography assay to detect the activity of different fractions purified from the culture filtrates of *T. harzianum* isolates T23 and T16 using *Cladosporium* spp. as a test organism. TLC plates containing the fractions isolated from the isolate T23 were developed with EtOAc:chloroform:iso-octane:butanol (4:4:5:4; *v*:*v*:*v*:*v*) and the mixture TBME:MeOH (3:7; *v*:*v*) was used for the fractions from isolate T16

by fractions F516 and F416 produced by isolate T16. Fraction F223 from isolate T23 also caused a significant inhibition zone. The inhibition percentages were quantitatively expressed in milligram following photocopying of the TLC plates and determining paper weights of the clear zones. Accordingly, about 56% of the total antifungal activity caused by the purified fractions of the extract from isolate T23 was due to fraction F323. The areas of inhibition by the fractions F223, F423 and F123 were 25.6%, 18.4% and 7.1%, respectively. Among the fractions purified from the extract of isolate T16, fraction F516 exhibited the highest antifungal activity (43.4%). Fraction F416 revealed two inhibition zones with R_f values of 0.28 and 0.83, respectively; this was refractionated again into two fractions (F4116 and F4216). The antifungal activity against *F. moniliforme* could only be attributed to the inhibition zone with the R_f value of 0.83 (data not shown); therefore, in our further investigations only fraction F4216 of F416 was included.

Paper disk assay The antifungal potential of the different fractions purified from isolates T16 and T23 was evaluated against *F. moniliforme* using the paper disk assay and the results represented as inhibition areas (IA; Table 1). Fractions F516, F323 and F416 significantly retarded mycelial growth of *F. moniliforme* and produced inhibition zones of 125.9, 119.4 and 108.4 mm^2, respectively. While fraction F223 caused a moderate inhibition (IA: 63.5 mm^2), the other fractions did not markedly inhibit the mycelial growth of the target fungus.

Structure elucidation of viridiofungin A (VFA)

Viridiofungin A, a colourless oil with a retention time of 22.7 min in HPLC/DAD, was identified using LC–MS (ES+/ES−), ^1H NMR and ^{13}C NMR and high resolution hybrid LC/Orbitrap MS. The following data were obtained: The UV-spectrum of VFA gives two maxima at (λ_{max} 226 nm, 278 nm) and a minimum at λ_{min} 253 nm. LC/Orbitrap MS; *m*/*z*: 592.31116 was the result of using high resolution MS. The best match could be achieved with *m*/*z*: 592.31162, which gave an *m*/*z* error of −0.46 ppm

Table 1 Inhibition of *F. moniliforme* mycelial growth *in vitro* caused by different fractions purified from culture filtrates of *T. harzianum* isolates T23 and T16 detected in the antibiotic disk assay

T23 fractions		T16 fractions	
Treatment	Inhibition area (mm^2)	Treatment	Inhibition area (mm^2)
Acetone	0.0	F116	25.2^{d}
6PAP	87.8^{b}	F216	12.6^{d}
F123	12.6^{d}	F316	8.3^{d}
F223	63.5^{c}	F416	108.4^{ab}
F323	119.4^{a}	F516	125.9^{a}
F423	8.7^{d}	F616	34.8^{d}

The inhibition areas in square millimeter of mycelial growth as means of three replicates per treatment are shown. Different letters indicate different levels of significance at P=0.05 probability level

corresponding to the measured *m/z* mass signal of 592.31116 revealing $^{[12]}C_{31}H_{46}O_{10}N$ as $[M^+ + 1]$ ion.

Analysis of the ^{13}C NMR spectrum (300 MHz, $(CD_3)2CO$ referenced to solvent) of VFA showed signals of carbonyl groups at 211.9, 175.9, 173.7, 173.2, and 170.9 ppm. The significant downfield shift of the first of these five signals indicates the presence of the ketone group. Two aromatic carbon atoms were detected at 155.4 and 128.8 ppm. Further aromatic carbon signals were present at 130.4 and 115.5 ppm. Two unsaturated carbon atoms each bearing one hydrogen atom gave signals located at 135.8 and 124.0 ppm. Tertiary non-aromatic carbon atoms corresponded to signals at 74.5, 54.6 and 54.2 ppm. Three CH2-carbon atoms were detected adjacent to a carbonyl group at 43.3, 42.7 and 37.5 ppm. Additional non-aromatic carbon signals were found at 33.5, 32.9, 29.9, 29.6, 28.9, 28.6, 28.4, 24.9, 24.8, 23.7 and 14.0 ppm. The ^1H NMR spectrum: (300 MHz, $(CD_3)2CO$. referenced to solvent) δ 0.90 (t. 7.0, 3H), 1.29 (brm, 14 H), 1.55 (m, 4H). 1.98 (m, 2H). 2.44 (t. 7.2). 2.62 (d, 1H), 2.91 (dd, 1H,), 2.94 (d, 1H), 3.13 (dd, 1H), 3.38 (d, 1H), 4.70 (dd, 1H), 5.68 (m, 2H, 6.78 (d, 2H), 7.03 (d, 2H). These data were in complete agreement with those reported by Harris et al. (1993).

Determination of the optimal medium and harvesting time for VFA production

Viridiofungin A was produced by *T. harzianum* isolate T23 in three out of four media tested. VFA production was detected both in PDB and Richard's solution (RS) after 2 dpi (Fig. 3). In RS, VFA production increased slowly up until 5 dpi (16 mg l^{-1}); the content remained constant up until 9 dpi and then the compound concentration decreased. However, in PDB medium the VFA content increased up until 9 dpi and reached 94.6 mg l^{-1}; the concentration of VFA then only slowly increased. In CDB medium, VFA was produced at a low rate during the experimental period (33.7 mg l^{-1} at 16 dpi). In MEB, isolate T23 did not produce VFA during the incubation time.

Non-volatile effect of VFA, F416 and F516 on *F. moniliforme*

The compounds VFA, F416 and F516 increasingly retarded mycelial growth of *F. moniliforme* with rising concentrations of the chemicals in malt extract agar (Fig. 4). The compounds VFA and F516 proved to be more effective in suppressing *F. moniliforme* than F416. At 7 dpi, in the presence of 250 μg ml^{-1} of VFA, F516 or F416, mycelial growth of *F. moniliforme* was retarded by 90.4, 92.8 or 76.3%,

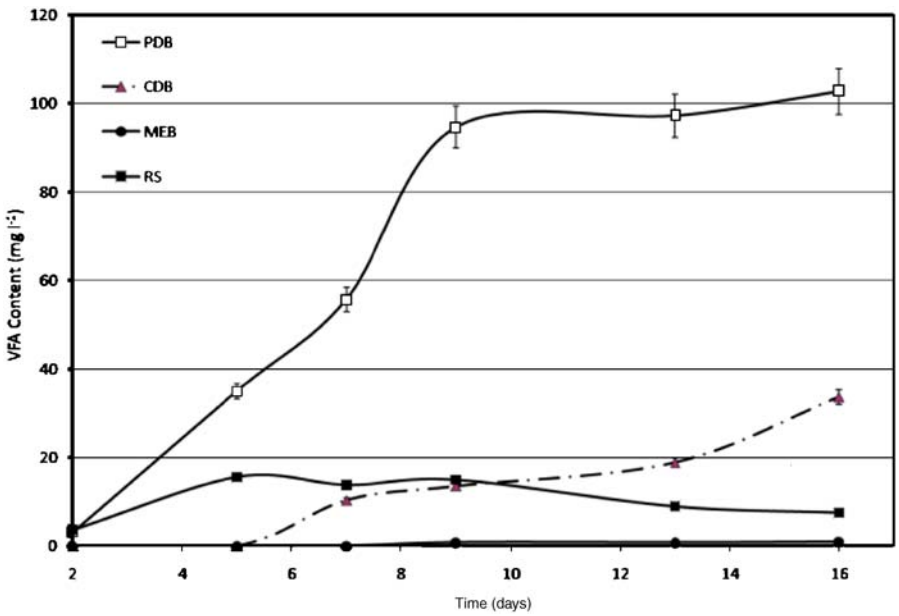

Fig. 3 Time-course for VFA production in four media: potato dextrose broth (*PDP*), Czapek Dox broth (*CDB*), malt extract broth (*MEB*) and Richard's solution (*RS*). VFA contents as means of three replicates are shown

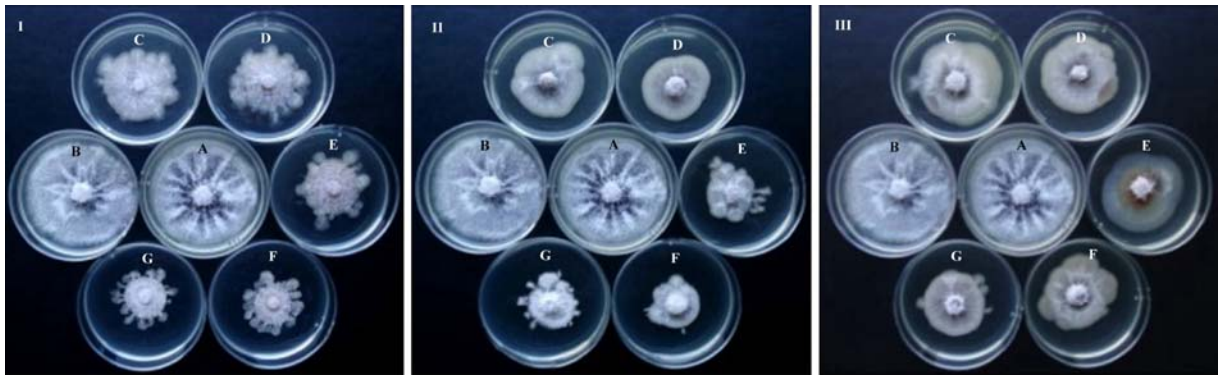

Fig. 4 Mycelial growth inhibition of *F. moniliforme* on malt extract agar containing 0 μg ml^{-1} (*A*), 2% acetone (*B*), 50 μg ml^{-1} (*C*), 100 μg ml^{-1} (*D*), 150 μg ml^{-1} (*E*), 200 μg ml^{-1} (*F*) and 250 μg ml^{-1} (*G*) of viridiofungin A (I), F516 (II) and F416 (III)

respectively. Light microscopic examination revealed that hyphae of *F. moniliforme* treated with the compounds were less branched and showed a wider diameter compared to control hyphae. Generally, conidial production sharply decreased with increasing concentrations of the metabolites. This effect was especially pronounced in the case of F416. In the presence of VFA, production of chlamydospores in *F. moniliforme* cultures was distinctly enhanced (data not shown).

Volatile phase effects of VFA, F516 and F416 on mycelial growth of *F. moniliforme*

The compounds VFA and F416 displayed distinct antifungal activity through their vapour phase (Fig. 5) and retarded at each 250 μg ml^{-1} mycelial growth of *F. moniliforme* by 66.0 and 60.8%, respectively, compared to the acetone control 9 dpi. The vapour phase of the metabolite F516 was less pronounced; colony diameter was retarded by 27.6% compared to the control.

Assessment of the antimicrobial spectrum of VFA

VFA presented a broad scope of antifungal action regarding both conidial germination and germ-tube elongation. In general, the degree of inhibition of both parameters correlated with VFA concentrations (Table 2). However, the various fungal species tested differed in their sensitivity to VFA. The most susceptible fungal species to VFA proved to be *V. dahliae*. Development of hyphae from sclerotia of *S. sclerotiorum* was also sensitively retarded in the presence of VFA. Furthermore, VFA exhibited a high activity on zoosporangia of *P. infestans* and germ-tube growth and the compound was somewhat less effective in the inhibition of conidial germination and germ-tube elongation of *F. oxysporum* f. sp. *fabae* and *G. roseum*. *Alternaria alternata* and *B. cinerea* showed the lowest sensitivity to VFA. The effect of VFA on the germination of propagules was obviously of a fungistatic nature and after the metabolite was removed by washing, the germination of propagules was restored.

The effect of VFA on bacteria

VFA and the other metabolites, including 6PAP, F416 and F516 isolated from *T. harzianum* isolates T23 and T16 exhibited no antibacterial activity against *C. michiganense* and *E. amylovora* when tested in the agar diffusion assay.

Discussion

Several species of the genus *Trichoderma* belong to the most profoundly studied and extensively used BCAs (Butt et al. 2001). While the production of secondary metabolites is primarily considered to be the major mechanism for the bioactivity of a certain strain of a BCA, this may not be the case for others (Harman 2000). However, antibiosis is assumed to be an essential mechanism employed by various *Trichoderma* species (Ghisalberti and Sivasithamparam 1991; Howell 1998, 2003; Verma et al. 2007; Reino et al. 2008). Thus, our efforts in this study were focused on detection, isolation and identification of

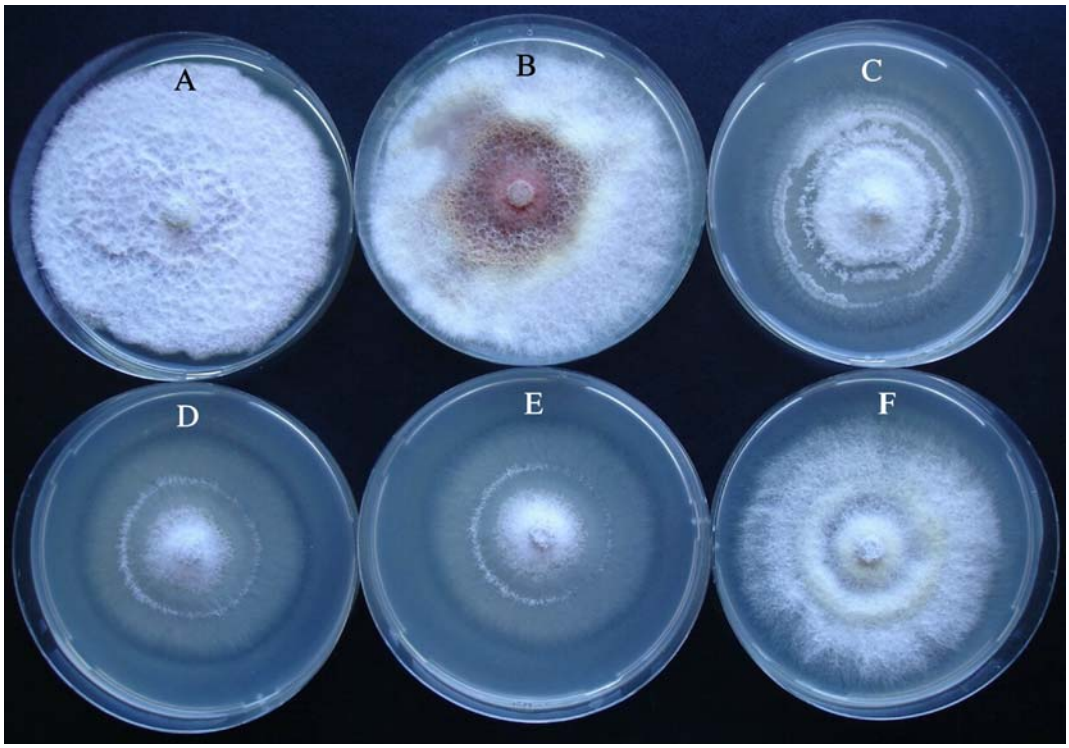

Fig. 5 Mycelial growth inhibition of *F. moniliforme* by 0 μg ml^{-1} (*A*), 2% acetone (*B*) and 250 μg ml^{-1} of 6PAP (*C*), VFA (*D*), F416 (*E*) and F516 (*F*), respectively, in the vapour phase activity test

significant secondary metabolites excreted by *T. harzianum* isolates T16 and T23. Our results obtained from direct bioautography, a powerful technique to localise antimicrobial active compounds on a chromatogram (Botz et al. 2001), indicate that isolate T23 excreted, in addition to the previously elucidated 6-pentyl-α-pyrone (6PAP; El-Hasan et al. 2007), further biologically-active metabolites that could be determined in organic crude extracts from 8 day-old culture filtrates. While 6PAP was produced by isolate T23 earlier than the new metabolites, the latter appeared to be more active in suppression of fungal growth at 8 dpi. The antifungal compounds produced by isolate T16 were detected in the bioautography assay using culture filtrates of 8 and 12 dpi. However, separation of the antifungal compounds produced by isolate T16 on TLC plates was more difficult than those produced by isolate T23. Detection of the metabolites produced by isolate T16 was achieved using a solvent mixture of TBME and methanol at a ratio of 3:7.

Following bioautography, fractionation of the inhibition zones by means of preparative HPLC resulted in several bioactive fractions which differed in their antifungal activities. While the fractions F323, F516 and F416 strongly retarded the mycelial growth of *Cladosporium* spp. on the TLC plate, the other fractions appeared to be either moderate (F223 and F423) or slight inhibitors. Of the metabolites produced by T23 and T16, F323 and F516 accounted for >55% and 43%, respectively, of the total antifungal activity. Similar results were obtained using the paper disk assay.

The antifungal fraction F323 from TLC plates was isolated and the elucidation of its structure proved to be viridiofungin A (VFA). The production of VFA by isolate T23 of *T. harzianum*, shown for the first time, was highly dependent on the liquid medium used. In PDB medium high amounts of VFA were secreted by isolate T23 up until 9 dpi (94.6 mg l^{-1}), while in CDB and RS media this isolate secreted only low amounts of VFA at comparable incubation times. However, in MEB medium no VFA was determined. Similarly, PDB was found to be optimal for 6PAP production by *T. harzianum* isolate T23 after a comparable incubation time (El-Hasan et al. 2007).

Table 2 Effect of different VFA concentrations on germination of conidia/sporangia/sclerotia and germ-tube growth of several fungal plant pathogens

Fungal plant pathogen	Propagule tested	VFA Concentration (mg/l)	Germination inhibition (%)	Germ tube inhibition (%)
Phytophthora infestans	Sporangia	0	−	−
		50	+	++
		100	++	+++
		150	++++	++++
		200	++++	++++
Fusarium oxysporum f. sp. *fabae*	Conidia	0	−	−
		50	+	+
		100	++	++
		150	+++	+++
		200	+++	++++
Verticillium dahliae	Conidia	0	−	−
		50	+++	+
		100	++++	+++
		150	++++	+++
		200	++++	++++
Botrytis cinerea	Conidia	0	−	−
		50	−	−
		100	+	+
		150	++	++
		200	+++	+++
Gliocladium roseum	Conidia		−	−
		50	+	+
		100	+	++
		150	++	++
		200	+++	+++
Alternaria alternate	Conidia	0	−	−
		50	−	−
		100	+	++
		150	++	++
		200	+++	+++
Sclerotinia sclerotiorum	Sclerotia	0	−	
		50	++	
		100	+++	
		150	++++	
		200	++++	

Inhibition ranges (in percent) of 0%, 1–25%, 26–50%, 51–75% and 76–100% are symbolised with (−), (+), (++), (+++) and (++++); respectively

Viridiofungin A could also be detected in mycelium of *T. harzianum* isolate T23 (data not shown); this compound can, therefore, be considered as an inter- and intracellular metabolite. VFA from T23 and both fractions F516, F416 from T16 were evaluated with respect to their antifungal potency against the maize pathogen *Fusarium moniliforme*. In the non-volatile assay, these metabolites effectively retarded mycelial

growth in the agar medium. The extent of the inhibition was generally concentration-dependent. Mycelial growth of *F. moniliforme* was inhibited by >90% in the presence of 250 μg ml^{-1} of either VFA or F516. An equivalent concentration of 6PAP retarded the mycelial growth of the same pathogen to a similar extent (El-Hasan et al. 2007).

Generally, mycelial inhibition by the metabolites tested decreased with increasing incubation time. The outgrowth of sectors was especially observed in medium containing VFA and F516 (Fig. 4). This phenomenon may probably be attributed to degradation of the metabolites in the sectors or to genetic mutation shifting the sensitive isolate to metabolite-resistant isolates. However, both possibilities for the formation of sectors have not been tested in detail.

In addition, mycelial growth appeared very dense in the presence of VFA and F516 compared to F416. Furthermore, the increased chlamydospore formation by *F. moniliforme* in the presence of VFA is generally presumed to be the result of unfavourable growth conditions, which is consistent with reports in the literature (Venkat Ram 1952; Manzo and Claflin 1984; Mandal and Chaudhuri 1990). Pedersen et al. (1999) noted also that culture filtrates of *Burkholderia cepacia*, *Pseudomonas chlororaphis*, *P. fluorescens* and *P. involutus* increased the formation of chlamydospores in *F. moniliforme* and *F. oxysporum* cultures.

The antifungal metabolites tested differed in their volatile antifungal activity. While VFA and F416 exhibited pronounced volatile activity on the mycelial growth of *F. moniliforme*, the metabolite F516 was markedly less effective. The activity of VFA in the volatile phase has not been previously discussed in the literature. The suppressive influence of the vapour phase of both VFA and F416 was displayed on the aerial hyphae of *F. moniliforme*. Under the influence of the vapourised metabolites the hyphae appeared slight and scarce. However, the vapour phase activity of the metabolite F516 on the aerial hyphae was hardly detectable. Furthermore, VFA and F416 seemed to display a higher vapour phase activity than the metabolite 6PAP. The different volatile effects might reflect the chemical nature of these compounds. It might be assumed that volatile and non-polar metabolites with significant vapour pressure would rapidly saturate rhizosphere niches (Reino et al. 2008). Thus, they may enhance the activity of the BCA to suppress the target pathogens even before they come into direct contact with each other.

Viridiofungins were discovered more than a decade ago as metabolites produced by *T. viride*. Viridiofungin A has been described in the literature as a broad-spectrum antibiotic against *Candida*, *Cryptococcus* and *Aspergillus* species causing diseases usually in humans and animals (Harris et al. 1993). Onishi et al. (1997) also described the activity of this group of metabolites against *Saccharomyces* spp. and *Ustilago zeae*. To date, the potential of viridiofungins against important soil-borne fungi such as *Fusarium* spp., *Phytophthora* spp., *Sclerotinia* spp. and *Verticillium* spp. has not been reported. Thus, in the present study, various important plant pathogens were investigated with respect to their sensitivity to viridiofungin A *in vitro*.

Germination of asexually-formed propagules of a range of fungal pathogens representing the genera *Fusarium*, *Sclerotinia*, *Phytophthora*, *Verticillium*, *Botrytis*, *Gliocladium* and *Alternaria* proved to be differentially sensitive to VFA. In the presence of 150 μg ml^{-1} of VFA, the germination of sporangia and sclerotia was completely inhibited. Conidial germination of *V. dahliae* was most sensitive to VFA; germination was entirely suppressed at 100 μg ml^{-1} of the inhibitor, while *F. oxysporum* f. sp. *fabae* and *G. roseum* may be considered as moderately sensitive to VFA and *B. cinerea* and *A. alternata* were assumed to be less sensitive to VFA.

However, neither Gram-positive nor Gram-negative bacteria including *C. michiganense* or *E. amylovora* were suppressed even at higher concentrations of either viridiofungin A, F516, F416 or 6PAP. These findings agree with the observations of Onishi et al. (1997). The absence of VFA activity towards bacteria may be explained by the findings of Harris et al. (1993) assuming that VFA inhibits squalene synthase and consequently ergosterol synthesis. The inability of bacteria to synthesise ergosterol (Kadakal et al. 2005) may explain the lack of activity of VFA against bacteria. Ergosterol represents an essential constituent for function and stability of fungal membranes. VFA also inhibited *P. infestans*; since *Phytophthora* species usually lack the ability to synthesise ergosterol, it is likely that VFA may also interfere in other fungal metabolic pathways.

Onishi et al. (1997) found that VFA concentrations up to 32 μg ml^{-1} showed fungicidal effects against *C.*

albicans. Contrary to the reports of Harris et al. (1993) and Onishi et al. (1997) our results revealed that viridiofungin A exhibited fungistatic rather than fungicidal activity. Incubation of different fungal propagules for 24 h with high concentrations of VFA suppressed germination. After removing the inhibitor by washing, propagules started to germinate in a high percentage.

In conclusion, this study revealed that isolates T23 and T16 of *T. harzianum* produced numerous secondary metabolites exhibiting pronounced antifungal activities *in vitro*. The selection of the bioactive fractions for further chemical separation was based mainly on the bioautography assay. Among the antifungal compounds separated, the structure of metabolite F323 produced by isolate T23 was elucidated as viridiofungin A. Our results revealed that VFA proved to be highly active against species of *Fusarium*, *Verticillium*, *Phytophthora* and *Sclerotinia* which are considered to be important soil-borne fungal pathogens causing diseases in a variety of agricultural and horticultural crops. In a previous paper, the antifungal metabolite 6PAP produced by isolate T23 was identified (El-Hasan et al. 2007). The production of antifungal compounds may contribute to the potential of isolate T23 in controlling important soil-borne plant diseases. Insufficient information is currently available on which antifungal compounds produced by *Trichoderma* spp. are involved qualitatively and quantitatively in biocontrol during colonisation of the rhizosphere. Further studies for minimising crucial gaps in our understanding of biocontrol processes in the soil and rhizosphere are therefore urgently required.

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