

ORIGINAL ARTICLE

Characterization of cultural traits and fungicidal activity of strains belonging to the fungal genus *Chaetomium*

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Keywords

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Abstract

Aims: Compare and characterize *Chaetomium* strains with special regard to their potentialities as biocontrol agents.

Methods and Results: Twelve strains of the fungal genus *Chaetomium* from diverse ecological niches were identified as belonging to six different species. Large differences were observed between the strains with regard to temperature requirements for mycelial growth and pigmentation of culture filtrates. Culture filtrates and ethyl acetate extracts were assayed for fungicidal effects against important phytopathogens both on agar media and in multiwell plates. The samples from *Chaetomium globosum* were particularly active against *Botrytis cinerea*, *Pyrenophora graminea* and *Bipolaris sorokiniana*, while those from *C. cochliodes* and *C. aureum* were inhibitory towards *Phytophthora infestans*, and *P. infestans* and *Fusarium culmorum* respectively. To narrow down the active principle, the most promising extracts were separated by preparative HPLC and the resulting fractions tested in bioassays. Chaetoglobosins were identified as active compounds produced by *C. globosum*.

Conclusions: The bioassays revealed *C. aureum* and *C. cochliodes* as promising candidates for use in biocontrol. Both showed remarkably good activity against the prominent plant pathogen *P. infestans*.

Significance and Impact of the Study: We provide the first systematic study comparing six different *Chaetomium* species with regard to their use as biocontrol agents.

Introduction

Members of the fungal genus *Chaetomium* are generally cosmopolitan and reside in soil on cellulose-rich materials or on dung (Ahmed *et al.* 2016). *Chaetomium* species are, however, also present in many other compartments of the environment. They are, for example, found associated with living plants, for example, as endophytes in *Ginkgo biloba* and *Pinus* sp. (Skolko and Groves 1953; Qin *et al.* 2009; Martínez-Álvarez *et al.* 2016), on water-damaged building-materials (Wang *et al.* 2016a), on

ancient works of arts (Coronado-Ruiz *et al.* 2018), and in extreme habitats such as deserts or salt lakes (Murgia *et al.* 2018; Abdel-Azeem 2020). Since the establishment of the genus more than 300 species have been described (Asgari and Zare 2011). In 2019, the Index Fungorum (www.indexfungorum.org) listed 273 accepted species names (Abdel-Azeem 2020). Both a high degree of competitiveness towards other micro-organisms (Chang and Kommendhal 1968; Hubbard *et al.* 1982) and the ability to form a large number of bioactive metabolites, including some with fungicidal activity, indicate that certain

species of the genus may be interesting candidates for the biological control of plant pathogens (Madbouly and Abdel-Wareth 2020).

Indeed, suppression of plant pathogens by spore suspensions, filtrates, biochemical fractions or formulated materials from different *Chaetomium* species has been recorded not only *in vitro*, but also on detached leaves, in the greenhouse as well as in the field. For example, an isolate of *C. cochliodes* controlled *Fusarium* blight of oat seedlings when added to seeds or to the soil, both in greenhouse and field experiments (Tveit and Wood 1955). Strains of *C. globosum* reduced the development of tan spot (causal agent *Pyrenophora tritici-repentis*; Istifadah and McGee 2006; Larran *et al.* 2016) and spot blotch (*Bipolaris sorokiniana*) on potted wheat, and of Northern Corn Leaf Blight (*Setosphaeria turcica*) on detached maize leaves (Zhang *et al.* 2013). A commercially available product based on pellet and powder formulations of both *C. cupreum* and *C. globosum* suppressed a number of pathogens on different crops (Soytong *et al.* 2001). Under greenhouse conditions, application of spores and methanol extracts of *Chaetomium globosum*, *Chaetomium lucknowense* and *Chaetomium cupreum* to pomelo seedlings inoculated with *Phytophthora nicotianae* reduced the extent of root rot and increased plant weight (Hung *et al.* 2015a), and a bioformulation of *C. elatum* controlled *Fusarium* wilt of tomato (Soytong 2015).

Parasitism and antibiosis were identified as causative for the suppression of *Cochliobolus sativum* (= *B. sorokiniana*) by *C. globosum* (Aggarwall *et al.* 2004). *Phytophthora nicotianae* was inhibited by three different species of *Chaetomium* (Hung *et al.* 2015b) although no evidence of direct parasitism was recorded for *C. globosum* suppressing *Botrytis aclada* (Köhl *et al.* 1997). *Chaetomium* species are known to produce more than 300 secondary metabolites with enormous structural variation (Hamed *et al.* 2020) and several of these have been implicated in the biocontrol of plant pests and pathogens (Madbouly and Abdel-Wareth 2020). Among this variety of metabolites several types of bioactive compounds have been identified, such as the chaetoglobins (Ge *et al.* 2008), xanthenone (Pontius *et al.* 2008), orsellides (Schlörke and Zeeck 2006), polyhydroxylated steroids (Zhang *et al.* 2009), armochaetoglobins (Chen *et al.* 2015) and azaphilones (Yamada *et al.* 2011). Some of these natural products possess not only antifungal activity, but also anti-inflammatory, cytotoxic and enzyme inhibiting properties (Li *et al.* 2016). In addition to bioactive secondary metabolites, extracellular enzymes produced by *Chaetomium* species are suspected to contribute to the antagonism towards plant pathogens (Sun *et al.* 2006; Raguchander *et al.* 2014). More information on

secondary metabolites of *Chaetomium* species and compounds suspected to be involved in the interaction with plant pathogens are presented in recent research papers and reviews (e.g. Yan *et al.* 2018, 2019; Abdel-Azeem 2020).

For the legal registration of biocontrol agents, information on the biological properties of their active ingredients must be supplied. This includes the potential toxicity and pathogenicity towards humans and animals. Both may be critical especially in the case of *Chaetomium*, since some of its metabolites are potentially harmful (Ohtsubo *et al.* 1978; Sekita *et al.* 1981), and several species of the *Chaetomiaceae* have been reported to cause serious opportunistic infections in immunocompromised patients. Species able to grow at body temperature may break the thermal exclusionary zone of the human body and emerge as potential pathogens in immunocompromised humans (Ahmed *et al.* 2016). Furthermore, biocontrol agents based on living organisms can only function under appropriate temperature conditions. For this reason, candidate biocontrol strains should be checked for their temperature requirements early in the development process.

In this study, 12 strains of *Chaetomium* isolated from different habitats and environments were evaluated. The strains were taxonomically identified and characterized regarding cultural characteristics and temperature requirements for mycelial growth. The potential as biocontrol agents was assessed based on the inhibition of phytopathogens by culture filtrates of the *Chaetomium* strains. Ethyl acetate extracts from culture filtrates were separated by semi-preparative HPLC and the antifungal activity of the resulting fractions was determined in bioassays. The UV spectra of fractions with antifungal activity were analysed for the presence of known natural products.

Materials and methods

Fungal isolates

Twelve strains of *Chaetomium* were tested (Table 1). Of these, six were supplied by the JKI Fungal Culture Collection (maintained by JKI Institute for Epidemiology and Pathogen Diagnostics, Braunschweig). They were originally isolated from different substrates and deposited in the collection under the species names listed (Table 1). The other six *Chaetomium* strains were isolated in the context of this analysis from plant material after surface-disinfection and placement on PDA. The disinfection procedure included immersion in 96% EtOH for 1 min, followed by 1% NaOCl for 5 min (potato slices, leaves) or 10 min (wheat kernels) and several washes in sterile

distilled water. All *Chaetomium* strains were maintained at room temperature on potato dextrose agar (PDA; Sigma Aldrich, Darmstadt, Germany).

The phytopathogens used were provided by the JKI Culture Collection (*F. oxysporum* f. *lycopersici* (Sacc.) W.C. Snyder & H.N. Hansen, BBA 62060; *F. solani* (Mart.) Sacc., BBA 65129) or taken from the collection of the Institute for Biological Control (*Fusarium graminearum* Schwabe; *F. culmorum* (Wm.G. Sm.) Sacc.; *Botrytis cinerea* Pers.; *B. sorokiniana* Shoemaker, *Phytophthora infestans* (Mont.) de Bary). In addition, an isolate of *Pyrrenophora graminea* S. Ito & Kurib. (syn. *Drechslera graminea* Rabenh. ex Schltdl.) freshly isolated from a barley leaf expressing the leaf stripe symptom was used. Throughout this manuscript, these pathogens are collectively termed 'phytopathogenic fungi'.

Molecular species determination of *Chaetomium* sp. isolates

Fungal isolates of *Chaetomium* sp. were grown on 2% (w/v) PDA at 24°C in darkness for 14 days. Fungal mycelia were scraped from the Petri dishes and ground with 0.25 mm glass beads in 2 ml tubes in the Precellys24 (Peqlab, Erlangen, Germany), followed by DNA extraction using the DNeasy Plant mini kit (Qiagen, Hilden,

Germany) according to the manufacturer's instructions. The DNA concentration was determined photometrically using the Nanodrop (Peqlab). For differentiating *Chaetomium* sp., three barcodes were used, based on the results of Wang *et al.* (2016a). In detail, β -tubulin (primers T1 and T2; O'Donnell and Cigelnik 1997) and RNA polymerase II second largest subunit (primers RPB2AM-1bf and RPB2AM-7R; Miller and Huhndorf 2005) were used. Additionally, the internal transcribed spacer region was included with the primers ITS-1 and ITS-4 as described in White *et al.* (1990). PCR conditions were set as follows: 5 min at 95°C, 40 cycles of 30 s at 95°C, 30 s at 55°C and 45 s at 72°C, followed by a final extension of 5 min at 72°C. PCR fragment size and single product amplification was checked by gel electrophoresis. PCR products were cleaned up with the ExoSAP-IT PCR Product Cleanup Reagent (Thermo Fisher, Berlin, Germany) and sent for sequencing to LGC Genomics (Berlin, Germany). For tree construction and alignment purposes each PCR reaction was done in three independent replicates. After removal of sequence parts of minor quality, a consensus sequence was determined that was used for further analyses. For genotyping, one PCR reaction of good quality was sufficient that was sequenced in both directions. The highest BLAST hit was taken for species determination. In case more than one hit showed equal high probability, all most likely results were used, as shown in the results section (Table 2).

Table 1 Origin of the *Chaetomium* strains analysed in this study

Isolate number	Origin	Substrate/site of isolation
1	JKI culture collection; deposited as BBA 62109: <i>C. globosum</i>)	Horse radish
2	This study	Wheat leaves (greenhouse)
3	This study	Wheat leaves (greenhouse)
4	This study	Potato tuber
5	This study	Wheat kernels (greenhouse)
6	This study	Barley leaves (greenhouse)
7	This study	Wheat kernels (greenhouse)
8	JKI culture collection; deposited as BBA 63353: <i>C. cochliodes</i>	<i>Abies/Rhododendron</i> Forest (Ghorepani, Nepal, altitude 2800 m)
9	JKI culture collection; deposited as BBA 63132: <i>C. aureum</i>	<i>Guzmania</i>
10	JKI culture collection; deposited as BBA 62111: <i>C. nozdrenkoae</i>	Soil (<i>Lolium multiflorum</i>)
11	JKI culture collection; deposited as BBA63377: <i>C. indicum</i>	<i>Abies/Rhododendron</i> Forest (Ghorepani, Nepal, altitude 2800 m)
12	JKI culture collection; deposited as BBA70564: <i>C. elatum</i>	Oil painting

Microscopy

Ascospores of the *Chaetomium* strains were scraped from sporulating cultures, mounted in water and viewed under differential interference contrast optics of a Zeiss Axio-scope 2 light microscope. Ascospores were photographed with a Zeiss AxioCam 105 color digital camera. The width and length of 120 ascospores per strain were determined using the camera software.

Determination of effect of temperature on mycelial growth

Mycelial plugs (7 mm diameter) were cut from 7- to 14-day-old actively growing colonies of each of the *Chaetomium* strains on PDA using a cork borer and placed in the centre of fresh PDA plates. The inoculated plates were incubated in darkness in microbiological incubators set at 15, 20, 25, 30 or 35°C. Three plates were evaluated per strain-temperature combination. Every other day, colony diameters were recorded along two perpendicular lines until the mycelium reached the edge of the plate, or for the more slowly growing colonies for a duration of maximal 14 days. For each plate, mean colony diameters

Table 2 Results of the molecular identification of the analyses of *Chaetomium* sp. strains. Shown is the BLAST hit with the highest probability for the three tested coding regions. A consensus sequence of three individual PCR reactions was determined for analysis

Isolate number	Primer/identity			Identity/designation used in this study
	ITS-1-4	Tubulin	rpb-2	
1	<i>C. globosum</i>	<i>C. globosum</i>	<i>C. globosum</i> , <i>C. fusum</i>	<i>C. globosum</i> (I)
2	<i>C. globosum</i>	<i>C. globosum</i>	<i>C. globosum</i>	<i>C. globosum</i> (II)
3	<i>C. globosum</i>	<i>C. globosum</i>	<i>C. globosum</i> , <i>C. fusum</i>	<i>C. globosum</i> (III)
4	<i>C. globosum</i>	<i>C. globosum</i>	<i>C. globosum</i>	<i>C. globosum</i> (IV)
5	<i>C. globosum</i>	<i>C. globosum</i>	<i>C. globosum</i>	<i>C. globosum</i> (V)
6	12 hits, among them <i>C. cochliodes</i>	<i>C. cochliodes</i> , <i>C. pseudocochlioides</i>	<i>C. cochliodes</i>	<i>C. cochliodes</i> (I)
7	<i>C. cristatum</i> , <i>C. coarctatum</i> , <i>C. cochliodes</i> , <i>C. megalocarpum</i> , <i>C. globosum</i>	<i>C. cochliodes</i> , <i>C. pseudocochlioides</i>	<i>C. cochliodes</i>	<i>C. cochliodes</i> (II)
8	<i>C. globosum</i> , <i>C. megalocarpon</i> , <i>C. cochliodes</i> , <i>C. coarctatum</i> , <i>C. cristatum</i>	<i>C. cochliodes</i>	<i>C. cochliodes</i>	<i>C. cochliodes</i> (III)
9	<i>C. aureum</i>	<i>C. aureum</i>	<i>C. aureum</i>	<i>C. aureum</i>
10	<i>C. megalocarpum</i> , <i>C. madrasense</i> , <i>C. ascotrichoides</i> , <i>C. grande</i>	<i>C. nozdrenkoae</i>	<i>C. nozdrenkoae</i>	<i>C. nozdrenkoae</i>
11	<i>C. bostrychodes</i>	<i>C. ramosissimum</i>	<i>C. ramosissimum</i> , <i>C. erectum</i>	<i>C. ramosissimum</i>
12	<i>C. elatum</i> , <i>C. rectangulare</i> , <i>C. globosum</i>	<i>C. elatum</i>	<i>C. elatum</i> , <i>C. rectangulare</i>	<i>C. elatum</i>

(minus the diameter of the inoculum plug) were calculated and used to compute the means and standard deviation for the three plates. The experiments on effect of temperature on mycelial growth as well as all other experiments described below were performed at least twice.

Dual cultures

Four mycelial plugs (7 mm diameter) from actively growing colonies on PDA of *P. graminea* or *B. sorokiniana* respectively were placed equidistantly on three plates each with synthetic nutrient poor agar (SNA) (Nirenberg and O'Donnell 1998), about 2 cm from the edge of the plates. One mycelial plug of each of the *Chaetomium* strains was placed in the centre of the plates. The plates were incubated in the dark at room temperature and photographs were taken after 5 days (*P. graminea*) or 10 days (*B. sorokiniana*) after inoculation.

Production of culture filtrates and assessment of antifungal activities

Culture filtrates were produced on a rotary shaker in potato dextrose broth (PDB; Roth, Karlsruhe, Germany). Erlenmeyer flasks (300 ml) containing 50 ml autoclaved PDB were inoculated with three mycelial plugs from sporulating *Chaetomium* plates and placed for 14 days on

a rotary shaker at 25°C and 150 rev min⁻¹. Following separation of the culture filtrate from the mycelium with a Büchner funnel using paper filter and vacuum suction, the filtrates were sterilized by passing through Rotilabo[®] syringe filters (0.45 µm pore size) (Roth) and added to molten PDA shortly before solidification to obtain a final concentration of 10%. Three plates each were inoculated in the centre with mycelial plugs from actively growing cultures of the phytopathogenic fungi listed in the section 'Fungal isolates'. The controls were PDA plates not amended with culture filtrate. The inoculated plates were incubated at 20°C, and mycelial diameters were determined at regular intervals as described above. Inhibitory activity (%) of the culture filtrates was computed in relation to the mycelial diameters on the control plates using the formula: (diameter on control plate–diameter on amended plate)/diameter on control plate × 100%.

Additionally, fungicidal activity of the culture filtrates was assessed in microplates at concentrations of 10 and 30%. Conidia suspensions from *B. cinerea*, *B. fabae*, *B. sorokiniana* or *F. culmorum* for inoculation of the microplates were obtained from sporulating cultures on PDA. About 5 ml of PDB were pipetted onto the cultures, followed by rubbing of the surface with a spatula. The resulting conidial suspensions were passed through gauze (MULLRO[®]; Hartmann) and the concentration of conidia was adjusted to 5 × 10⁴ ml⁻¹ with a haemocytometer. In the case of *P. infestans*, suspensions of sporangia were

obtained from cultures on rye agar and also adjusted to $5 \times 10^4 \text{ ml}^{-1}$. For preparation of rye agar 100 g of rye seeds were autoclaved, left to soak overnight and passed through cheesecloth. The mucilage was resuspended in water to 500 ml, and 2.5 g glucose and 6 g agar were added. The medium was autoclaved and poured into Petri dishes. Filtrates of 14-day-old *Chaetomium* cultures were diluted in a ratio 3 : 7 with PDB containing conidia of the respective pathogens to obtain a final concentration of 30% culture filtrate. Filtrate concentrations of 10% were prepared by adding to concentrated culture filtrates PDB (*P. infestans*: pea-juice medium) and PDB containing conidia (*P. infestans*: pea juice medium containing sporangia) in a ratio 1 : 2 : 7. Aliquots of 200 μl were pipetted into three wells each of 96-well microplates. The controls were wells with 200 μl PDB or pea-juice medium containing fungal conidia or sporangia of *P. infestans*; respectively without culture filtrate added. The microwell plates were incubated for 2–7 days at room temperature before fungal growth was rated visually or under a dissecting microscope according to the following differentiation: no inhibition (as in controls); amount of mycelium in wells increasingly reduced; conidia (*P. infestans*: sporangia) ungerminated or with only short mycelial strands.

Determination of fungicidal activity of ethyl-acetate extracts in multi-well plates

Cultures of the strains *C. globosum* 1, *C. cochliodes* 3, *C. aureum*, *C. nozdrenkoae*, *C. ramosissimum* and *C. elatum* were raised by adding 12 agar plugs from freshly grown plates to 200 ml PDB in 1-l flasks and passed through a Büchner funnel as described above. Using a separating funnel, the filtrates were extracted three times with ethyl-acetate (volumes: 150, 100, 100 ml). The ethyl-acetate fractions were combined and evaporated to dryness with a rotary evaporator. The dried crude extracts thus obtained (corresponding to 200 ml liquid fungal culture) were dissolved in 4 ml 99% ethanol (stock solution). Aliquots from stock solutions were diluted with ethanol in a ratio 1 : 4, and 4 μl each were pipetted into three wells each of 96-well microtiter plates (well volume 300 μl), and 296 μl PDB or pea juice medium containing fungal conidia or sporangia of *P. infestans* respectively were added to each well. After incubation for 2–7 days at room temperature, fungal growth was assessed as described above.

HPLC fractionation and profiling of crude extracts

The dried crude extracts of the fermentation of *C. globosum* 1, *C. cochliodes* 3, *C. aureum* and *C. nozdrenkoae*

were dissolved in acetonitrile and analysed by HPLC (Agilent 1100 Series) equipped with a LiChrospher RP 18 column (4 \times 125 mm; 5 μm ; Merck, Darmstadt, Germany). The column was used at 40°C with a flow rate of 1 ml min^{-1} . An elution gradient was used composed of H₂O and acetonitrile, starting from 99% H₂O to 100% acetonitrile over a period of 20 min and then the 100% acetonitrile-flow was continued for further 4 min. The compounds were detected via a diode array detector.

For separation of the crude extracts, the same method was used and a fraction collector (Agilent 1100 Series) was used to collect eluted samples. The use of 96-well plates for collecting the flow through resulted in 92 different fractions of 250 μl from each extract. The solvent of each fraction was then evaporated. In the bioassays, 6 μl methanol (*P. infestans*: 4 μl ethanol) were pipetted into each well and 194 μl PDB containing fungal conidia (*P. infestans*: 196 μl pea juice medium containing sporangia) were added. Incubation of the plates and assessment of inhibitions were as described above.

The molecular weight of the selected peaks was determined using a HPLC-MS (Agilent 1260 Series LC and 6130 Series Quadrupole MS System; Agilent, Santa Clara, CA). The mass spectra were recorded using atmospheric pressure chemical ionization with positive and negative polarization. A Superspher RP 18 (125 \times 2 mm; 4 μm ; Merck) column was used at 40°C. For every run, 1 μl of a sample at a concentration of 1 mg ml^{-1} was injected. The elution was performed with a gradient of H₂O and acetonitrile and a flow rate of 0.45 ml min^{-1} .

Results

Molecular species determination and biology of the *Chaetomium* isolates

Molecular species determination of *Chaetomium* sp. was done with three different genotyping markers. When BLAST results named several species with a similar high probability, all hits were listed (Table 2). Except for the *C. globosum* isolates, species identification with the ITS-marker was rather unspecific, leading to up to twelve equally high hits in BLAST. The primer pairs for the tubulin and rpb-2 (RNA polymerase II second largest subunit) loci were much more specific and mostly resulted in only one or two distinct most likely hits in BLAST. Combining the results obtained with the three primer pairs allowed us to assign the strains to the species *C. globosum*, *C. cochliodes*, *C. aureum*, *C. nozdrenkoae* and *C. ramosissimum*.

The ascospores of *C. globosum* and *C. cochliodes* were very similar in shape and size (*C. globosum*: minimal width 7.3 μm , maximal length 10.0 μm ; *C. cochliodes*:

minimal width 7.2 μm , maximal length 9.3 μm) (Fig. 1). Based on their morphology, the ascospores of the single isolates of *C. aureum*, *C. ramosissimum* and *C. elatum* were distinguishable from each other as well as from those of *C. globosum* and *C. cochliodes*. The ascospores of *C. globosum*, *C. cochliodes*, *C. elatum* and *C. ramosissimum* were limoniform and bilaterally flattened, while those of *C. aureum* were rather spindle shaped (Fig. S1). The isolate of *C. nozdrenkoae* failed to produce perithecia and ascospores on PDA, malt extract agar and a number of other agar media tested (not shown).

On PDA plates (Figs S2a–e and S3a), as well as in shake culture in PDB (Fig. 2) all isolates of *C. globosum* produced a typical brown pigment. *C. aureum* produced a red pigment that stained PDA plates red (Figs S2i and S3b) and made the culture filtrate appear dark red to almost black. The culture filtrates of *C. cochliodes*, *C. nozdrenkoae* and *C. ramosissimum* were more or less yellow, while the filtrate from *C. elatum* appeared translucent with no pigmentation (Fig. 2).

Temperature affected the growth of *Chaetomium* strains on PDA differently (Fig. 3 and Fig. S3). For *C. ramosissimum* the speed of mycelial growth increased with incubation temperature, accordingly the slowest and fastest growth was at 15 and 35°C respectively (Fig. 3a). The growth pattern of *C. nozdrenkoae* was similar to that of *C. elatum*, but the maximal growth rate was recorded at 25/30°C (Fig. 3b). *C. elatum* (Fig. 3c and Fig. S3d) and strains *C. cochliodes* 1–3 (not shown) were unable to grow at 35°C and reached their maximal speed of growth at 20/25°C (*C. elatum*, *C. cochliodes* 1) or at 25°C (*C. cochliodes* 2 and 3). All five isolates of *C. globosum* had their slowest mycelial growth at 35°C (Fig. S3a). Mycelial growth was fastest at 30°C (*C. globosum* 1; Fig. 3d) or at

20°C (*C. globosum* 2–4; Fig. 3e). *C. aureum* was unique in that it grew comparatively slow at all incubation temperatures (Fig. 3f).

The isolates produce biological active natural products

To determine the ability of the different *Chaetomium* strains to produce antifungal compounds, filtrates from shake cultures were added to PDA in Petri dishes. The growth of different phytopathogenic fungi on these media was then compared to growth on unamended plates (Fig. 4). For the filtrates from *C. globosum*, a clear inhibitory activity (>50%) was observed towards *B. cinerea* and *P. graminea*. Inhibition of *B. sorokiniana* was below 20% and around 30% in the case of *F. culmorum* and *P. infestans* (Fig. 4a). This pattern was strikingly similar for all five isolates of *C. globosum* tested. As an example, inhibition by the filtrates from isolate *C. globosum* 1 is shown in Fig. 4a. The effect of the culture filtrates was also similar among the three isolates of *C. cochliodes* that all caused a moderate inhibition of the growth of *P. infestans* by around 35–40%, as exemplified by isolate *C. cochliodes* 3 (Fig. 4b). Growth of the other fungi was either not affected or the degree of inhibition was below 15%. The culture filtrates from *C. aureum* had a similar broad antifungal spectrum as those from *C. globosum*. However, the activity was strongest against *P. infestans* but only moderate against *B. cinerea* and *D. graminea*. In addition, the filtrates from *C. aureum* caused a considerable inhibition of growth of *F. culmorum* and *F. oxysporum* (Fig. 4c). Interestingly, *F. solani* was always less affected than *F. culmorum* and *F. oxysporum*. The filtrates from *C. nozdrenkoae* showed activity only against the mycelial growth of *P. infestans* (Fig. 4d), whereas those from *C.*

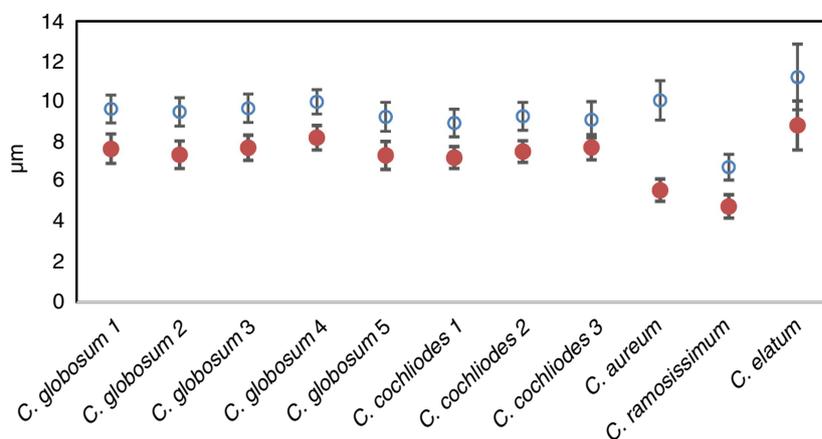


Figure 1 Size of the ascospores of the studied strains of *Chaetomium globosum*, *C. cochliodes*, *C. aureum*, *C. ramosissimum* and *C. elatum* (means and standard deviation of 120 ascospores per strain); open circles: ascospore length, filled circles: ascospore width. [Colour figure can be viewed at wileyonlinelibrary.com]



Figure 2 Filtrates from shake cultures of the *Chaetomium* strains after 14 days of culturing in PDB. (a–e) *C. globosum* 1–5; (f–h) *C. cochliodes* 1–3; (i) *C. aureum*; (j) *C. nozdrenkoae*; (k) *C. ramosissimum*; (l) *C. elatum*. [Colour figure can be viewed at wileyonlinelibrary.com]

ramosissimum failed to inhibit the growth of any of the fungi tested (Fig. 4e). In the tests with the filtrates from *C. elatum* mycelial growth of *P. infestans* was inhibited by around 30% (Fig. 4f). Inhibition of the other tested fungi was below 20% and a high variability was observed in the case of *F. culmorum*.

Inhibition of the mycelial growth of *P. graminea* by *C. globosum* was also seen in dual culture plates. In these tests, the inhibitory activity of all five strains of *C. globosum* was very similar (Fig. S4a–e). The moderate inhibition of *D. graminea* by *C. aureum* observed on filtrate-amended agar plates was not apparent in dual cultures (Fig. S4i). *Bipolaris sorokiniana* was strongly inhibited in dual culture with *C. globosum*. *Bipolaris sorokiniana* was equally strongly affected by all five isolates of *C. globosum* (exemplified by isolate *C. globosum* 1 in Fig. S4).

In further tests performed in 96-well plates, culture filtrates at concentrations of 30 or 10% were inoculated with conidia of *B. fabae*, *B. cinerea*, *F. culmorum* and *B. sorokiniana* (first set of experiments; Table 3) or sporangia of *P. infestans* (second set of experiments; Table 4). In these tests, filtrate of *C. globosum* 1 served as an example of the *C. globosum* strains and the filtrates of *C. cochliodes* 1 and 3 respectively were used as representatives of the *C. cochliodes* strains. In the first set of experiments, a moderate to strong effect on *B. fabae*, *B. cinerea*, *F. culmorum* and *D. graminea* was recorded for *C. globosum* 1. The filtrate from *C. aureum* caused a clear inhibition of *F. culmorum* and *D. graminea*, but had just a mild effect on *B. fabae* and *B. cinerea*. A mild effect on *F. culmorum* was also recorded for the filtrate from *C. cochliodes*. Generally, the inhibitory effects observed in these experiments were dose-dependent. No or only very weak effects were observed with all other filtrates (Table 3). Dose dependency of the effects of the culture

filtrates was also observed with *P. infestans*. All culture filtrates tested caused a moderate to strong inhibition of *P. infestans*. Autoclaving of the filtrates tended to reduce the inhibitory activity (Table 4).

Ethyl acetate extracts of *C. globosum* 1 caused a moderate to strong inhibition of *B. cinerea* and *P. infestans* and a low inhibition of *F. culmorum* (Table 5). The *C. cochliodes* extract showed a high activity only against *P. infestans*, while the extract from culture filtrate from *C. aureum* inhibited the growth of all the three test organisms. The extracts from *C. nozdrenkoae*, *C. ramosissimum* and *C. elatum* were inactive against *B. cinerea* and *F. culmorum* and had only a very low inhibitory effect on *P. infestans*.

Fractionation of biological active extracts

Ethyl acetate extracts were separated by HPLC and the fractions were deposited into 92 wells of micro plates. Four wells served as controls. In the bioassays with the HPLC fractions from *C. globosum*, inhibition of the growth of *B. cinerea*, *B. sorokiniana*, *F. culmorum* and *P. infestans* was recorded within wells D1–D2 and E4–E6 (Figs 5a,c and 8a). The strongest and highly reproducible activity was found by inhibition of *B. sorokiniana* and *B. cinerea* and in wells E4–6 (Fig. 5a,c). The HPLC signals corresponding to the wells D1–D2 and E4–E6 were found to be related to the group of the chaetoglobosins and these are responsible for the antifungal activity in the crude extracts of *C. globosum* (Fig. 5b). The assumption that the chaetoglobosins are the biologically active compounds within the wells D1–D2 and E4–E6 from the fractionated extract was supported by the 3D-chromatogram (Fig. 5d). Apart from the UV-spectra of the chaetoglobosin class of compounds, there were no other signals

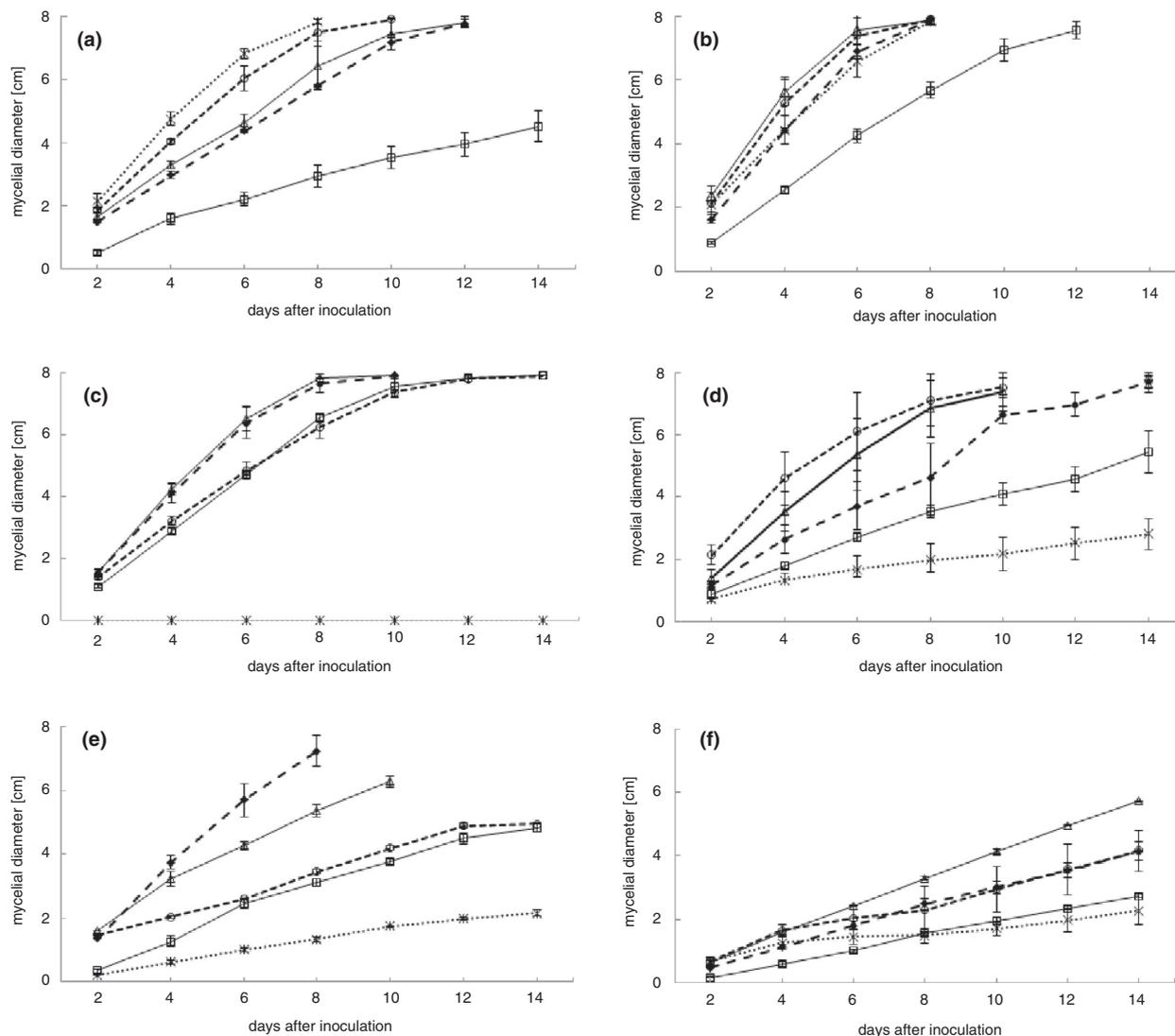


Figure 3 Effect of temperature on radial mycelial growth of *Chaetomium* strains on PDA plates. (a) *C. ramosissimum*; (b) *C. nozdrenkoae*; (c) *C. elatum*; (d) *C. globosum* 1; (e) *C. globosum* 4; (f) *C. aureum*. Line markings are identical for graphs a–f, with: open square, continuous line = 15°C; filled rhombus, interrupted line = 20°C; open triangle, continuous line = 25°C; open circle, interrupted line = 30°C; asterisk, dotted line = 35°C.

detectable in the range between 200 and 600 nm wavelength at the respective retention times and thus in the wells D1–D2 and E4–E6. The fractions from *C. cochliodes* 3 caused a strong inhibition of *P. infestans* in wells E1–10 and inhibited *F. culmorum*, *B. sorokiniana* and *B. cinerea* within wells E3–E9 (Fig. 8b). In the bioassays with fractionated extracts from *C. aureum*, the test organism *P. infestans* was strongly inhibited in the wells A9–A12 and B10–B12 (Figs 6a and 8c). The signals and UV-spectra, which were found to correspond to the compounds responsible for the biological activity, did not match to any of the natural products in our libraries (Fig. 6b,c).

Thus, the compounds could not yet be identified. The reddish-brown colour on the well bottom suggested that the dark red pigment of *C. aureum*, reported previously as oosporein (Taniguchi *et al.* 1984), was deposited in these wells. The UV-spectra indicated the presence of oosporein, but due to smearing and interference with signals from other compounds an unequivocal identification of oosporein was not possible. The involvement of oosporein in the observed inhibition of *P. infestans* appears nevertheless likely.

In case of the bioassay with the fractionated extracts from *C. aureum* against *F. culmorum* the inhibition was

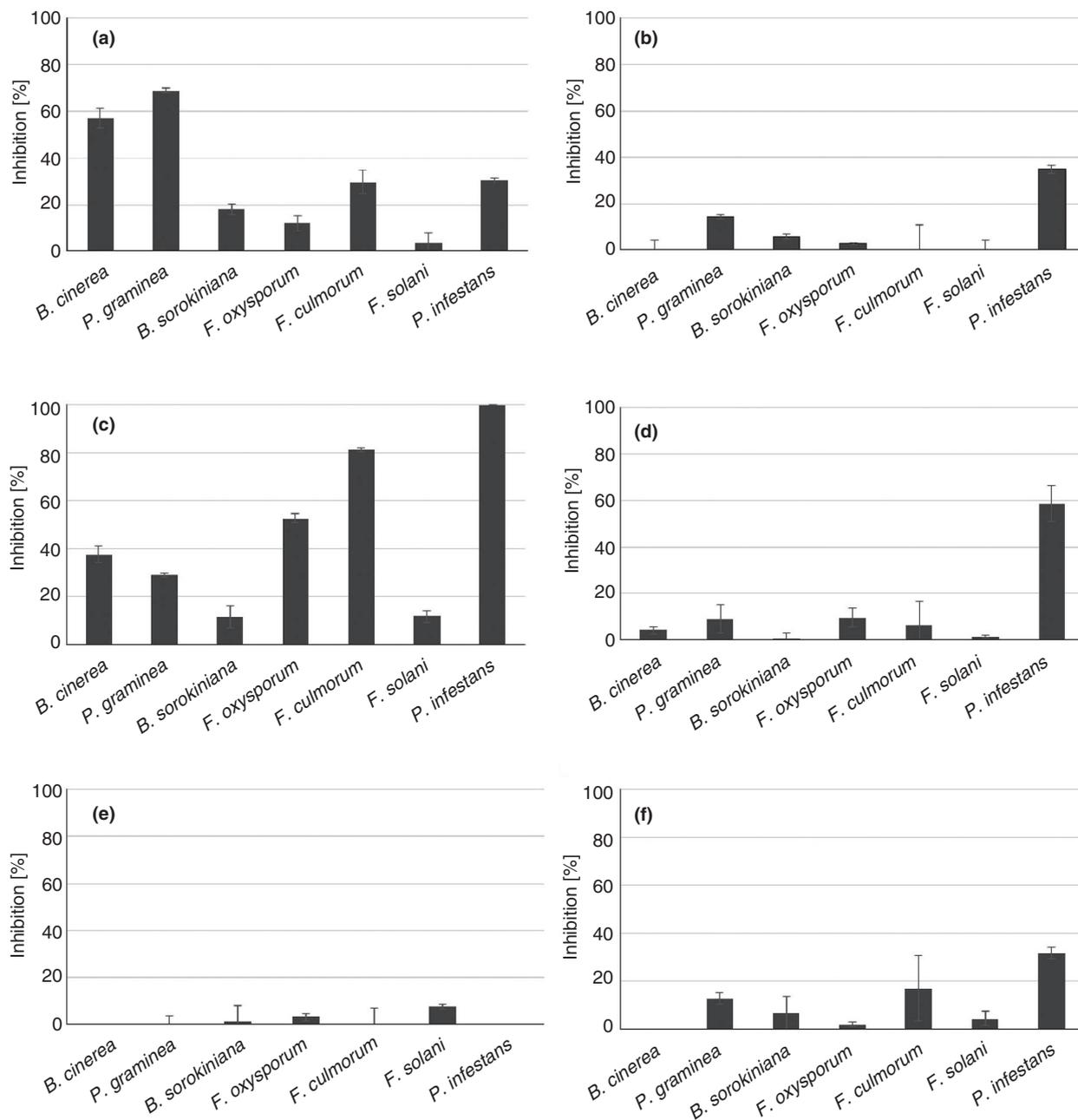


Figure 4 Mycelial growth of different phytopathogenic fungi on PDA plates amended with culture filtrate (10% v/v) of (a) *Chaetomium globosum* 1, (b) *C. cochliodes* 3, (c) *C. aureum*, (d) *C. nozdrenkoae*, (e) *C. ramosissimum* and (f) *C. elatum* in relation to growth on unamended plates. Means and standard deviation of three plates per filtrate/strain combination.

more or less weak. The sporulation of *F. culmorum* was reduced whereas mycelial growth was little affected, extended over several wells and was only visible under a dissecting microscope at the beginning of the incubation period, which made interpretation of the results difficult (Figs 7a and 8c). Identification of the compounds potentially responsible for the biological activity was not

possible since the signals and UV-spectra in the corresponding wells did not match with any natural product in our library (Fig. 7b,c).

Furthermore, in the bioassays with fractions of the extract from *C. nozdrenkoae* and *B. cinerea*, *F. culmorum* and *P. infestans*, fungicidal activity was not observed, which fits to the results obtained in the bioassays with

Table 3 Effect of filtrates from shake cultures (in PDB) of selected strains of *Chaetomium* sp. on growth of different phytopathogenic fungi. Test in microplates in PDB, 10/30% refers to the percentage (v/v) of culture filtrate in the medium. Inhibition was determined 2–5 days after inoculation

	<i>Botrytis fabae</i>		<i>Botrytis cinerea</i>		<i>Fusarium culmorum</i>		<i>Bipolaris sorokiniana</i>	
	30%	10%	30%	10%	30%	10%	30%	10%
<i>C. globosum</i> 1	++/+++	++	++/+++	++	+/+	+/+	+++	++
<i>C. cochliodes</i> 3	±	–	±	–	+	–	±	±
<i>C. aureum</i>	+	+	+	+	+++	++	+++	+
<i>C. nozdrenkoae</i>	–	–	–	–	–	–	–	–
<i>C. ramosissimum</i>	±	–	–	–	±	–	±	–
<i>C. elatum</i>	–	–	–	–	±	–	±	–

– no inhibition, as in controls; ±, +, ++ amount of mycelium in wells increasingly reduced; +++ conidia nongerminated or with only short mycelial strands.

Table 4 Effect of filtrates from shake cultures (in PDB) of selected strains of *Chaetomium* sp. on growth of *Phytophthora infestans*. Inhibition was determined in microplates with pea juice medium; 10/30% refers to the percentage (v/v) of culture filtrate in the medium, determined 5–7 days after inoculation

	<i>Phytophthora infestans</i>			
	Non-autoclaved filtrate		Autoclaved filtrate	
	30%	10%	30%	10%
<i>C. globosum</i> 1	++	+	+	–
<i>C. cochliodes</i> 1	++/+++	±	++	±
<i>C. aureum</i>	+++	++	++	±
<i>C. nozdrenkoae</i>	++	+	++	±
<i>C. ramosissimum</i>	++	±	++	–
<i>C. elatum</i>	++/+++	+	++/+++	±

– no inhibition, as in controls; ±, +, ++ amount of mycelium in wells increasingly reduced; +++ sporangia ungerminated or with only short mycelial strands.

the crude extracts from *C. nozdrenkoae* as shown in Table 5.

Discussion

Molecular identification of the 12 *Chaetomium* strains was achieved by PCR and sequencing of the amplicons using primers targeting the ITS, tubulin and rpb2 genes (Wang *et al.* 2016a). The specificity differed among the primers, where the ITS-primer is the least specific. Taken together, the use of three primer pairs based on three different loci was necessary and sufficient to resolve the identity of the different *Chaetomium* strains down to the species level. The results of the molecular identification were supported by microscopic observations on the size and shape of the ascospores. The morphological

Table 5 Effect of ethyl-acetate extracts from culture filtrates of selected strains of *Chaetomium* sp. on growth of *Botrytis cinerea*, *Fusarium culmorum* and *Phytophthora infestans*. Test in microplates in PDB (*B. cinerea*, *F. culmorum*) or pea juice medium (*P. infestans*). The amount of extract added per well corresponds to 0.04 ml culture filtrate. Inhibition was determined 2–7 days after inoculation

	<i>Botrytis cinerea</i>	<i>Fusarium culmorum</i>	<i>Phytophthora infestans</i>
<i>C. globosum</i> 1	++/+++	+	++/+++
<i>C. cochliodes</i> 3	+	±	+++
<i>C. aureum</i>	+/+	++/+++	++
<i>C. nozdrenkoae</i>	–	–	±
<i>C. ramosissimum</i>	–	–	±
<i>C. elatum</i>	–	–	±

– no inhibition, as in controls; ±, +, ++ amount of mycelium in wells increasingly reduced; +++ conidia/sporangia ungerminated or with only short mycelial strands.

characteristics match with descriptions in the taxonomic literature (Arx *et al.* 1986; Wang *et al.* 2016b). For five of the isolates obtained from the JKI Culture Collection, the previous identification based on morphology could be confirmed. However, the strain identified as *C. ramosissimum* in the present study was deposited in the collection in 1975 under the name *C. indicum*. This apparent discrepancy can be explained by taxonomic changes, since Wang *et al.* (2014) recognised *C. ramosissimum* as separate species within the *C. indicum* group. As optimum and maximum temperatures for growth, these authors state 24 and 30°C for *C. indicum* and 30 and 41°C for *C. ramosissimum* respectively. This is in agreement with the observation that growth of our *C. ramosissimum* strain was substantially faster at 30 and 35°C than at 25°C. Also, the limoniform shape of the ascospores and their size correspond well to the data given by Wang

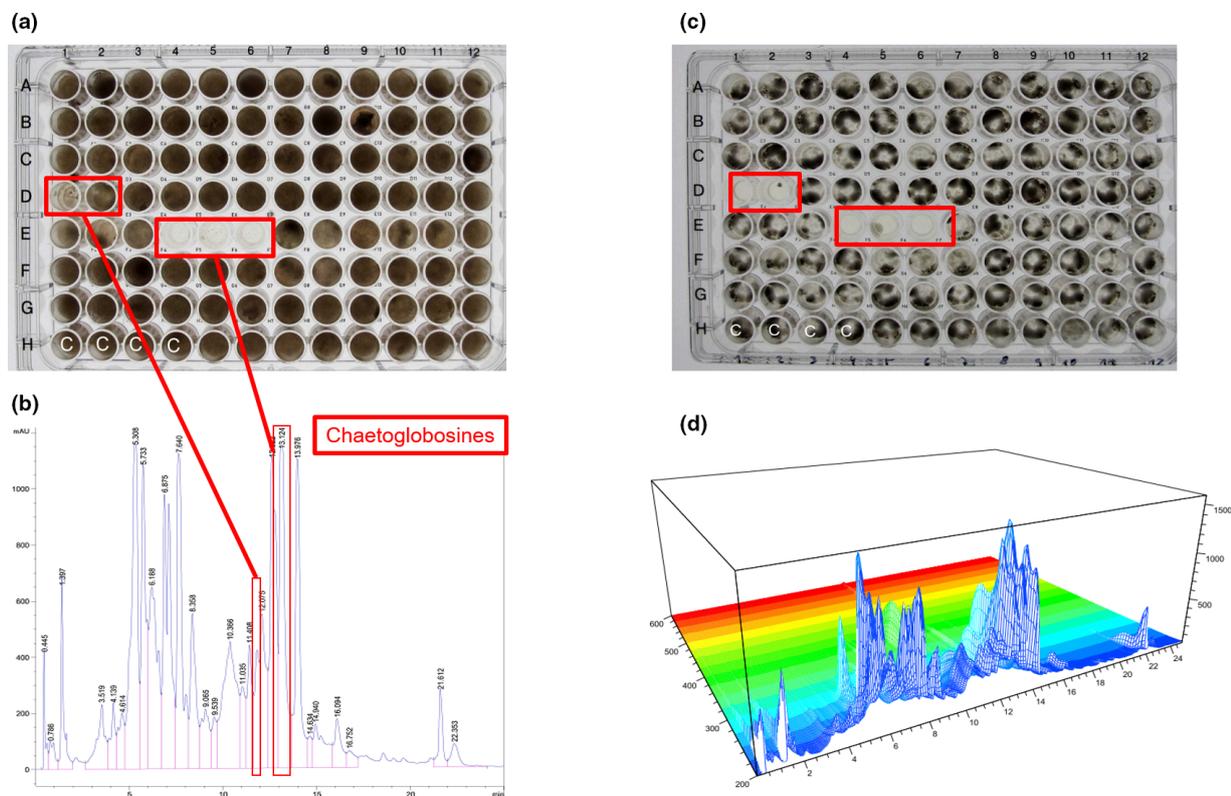


Figure 5 Biological activity assays of fractionated extracts of *Chaetomium globosum*. Antifungal activity of single fractions obtained after separation of the crude extract of *Chaetomium globosum* by semi-preparative HPLC. Wells H1–H4 are solvent controls. (a) Activity on *B. sorokiniana*, (b) HPLC chromatogram with fractions of the wells D1–D2 and E4–E6 highlighted in red. The extracts were produced after fermentation in PDB for 14 days on a rotary shaker at 25°C and 150 rev min⁻¹. (c) Activity on *B. cinerea*. (d) 3d-chromatogram of the fractionated crude extract from (b). [Colour figure can be viewed at wileyonlinelibrary.com]

et al. (2014) and indicated *C. ramosissimum* as the correct taxon.

The formation of extracellular pigments with various biological functions is known from many fungi (Caro *et al.* 2016) and has also been reported for strains and species of *Chaetomium* (Brewer *et al.* 1968; Cooke and Collins 1970; Rodríguez *et al.* 2002). In our study, the culture filtrates from *C. aureum* and from all five strains of *C. globosum* were stained deep red and brown respectively. Formation of a number of red acidic metabolites on PDA has also been reported for *C. aureum* strain MF-91 (Wang *et al.* 2013). According to Taniguchi *et al.* (1984), the red pigment produced by *C. aureum* is oosporein. The nature of the brown pigment from *C. globosum* is as yet unknown. Hu *et al.* (2012) reported interruption of pigment production of *C. globosum* by a specific inhibitor of the biosynthetic pathway for melanin.

Most *Chaetomium* species are mesophilic, with an optimal temperature range of 25–28°C (Prokhorov and

Linnik 2011). Among the incubation temperatures tested, 35°C was the optimum for *C. ramosissimum*. The ability of a *Chaetomium* species to grow near to the temperature of 37°C is used as an indicator for their potential to infect humans (Wang *et al.* 2014). From the toxicological point of view, the use as biocontrol agents of such strains must be evaluated critically. In contrast to *C. ramosissimum*, *C. elatum* and *C. cochliodes* ceased their growth at 35°C. For *C. elatum* this observation is in agreement with previous studies (Asgari and Zare 2011; Prokhorov and Linnik 2011). The isolates of *C. globosum* were able to grow at 35°C in our study, but growth was strongly restricted compared to the other temperatures employed. Based on the observation of a low radial growth, morphological changes in ascocarp development and absence of mature ascocarps, Prokhorov and Linnik (2011) concluded that temperatures of 33°C or higher were unfavourable for growth of *C. globosum*. With this in mind, the species *C. globosum* has been assigned to risk group I, the lowest of four groups categorizing the risk of

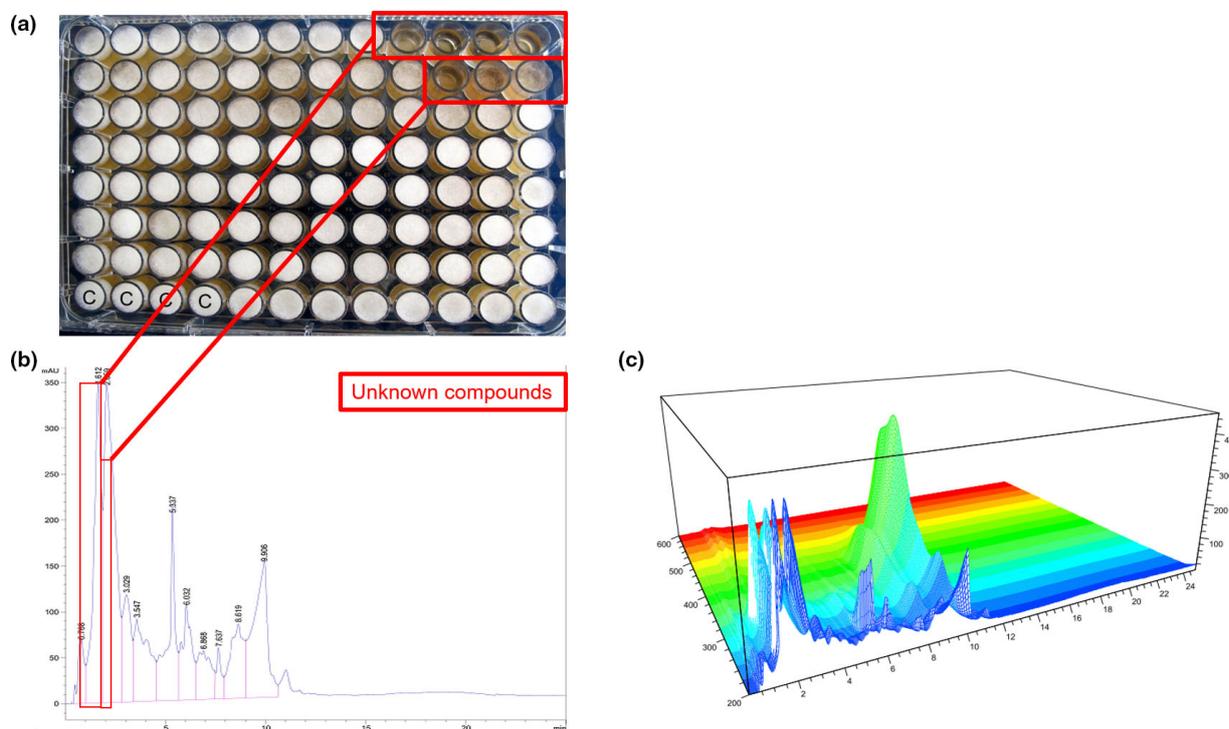


Figure 6 Biological activity assays of fractionated extracts of *Chaetomium aureum*. Antifungal activity of single fractions obtained after separation of the crude extract of *Chaetomium aureum* by semi-preparative HPLC. Wells H1–H4 are solvent controls. (a) Activity on *P. infestans*, (b) HPLC chromatogram with fractions of the wells A9–A12 and B10–B12 highlighted in red. The extracts were produced after fermentation in PDB for 14 days on a rotary shaker at 25°C and 150 rev min⁻¹. (c) 3d-chromatogram of the fractionated crude extract from (b). [Colour figure can be viewed at wileyonlinelibrary.com]

biological agents to cause infections in humans (BAUA). *C. aureum* grew comparatively slow at all temperatures in our study, with maximal growth at 25°C. This is in contrast to *C. aureum* MF-91 that was reported to grow rapidly on PDA with the maximum at 35°C (Wang *et al.* 2013).

On agar medium amended with culture filtrate, an inhibition of *P. infestans* was recorded for all *Chaetomium* strains except for *C. elatum*, whereas in microplates the inhibition was observed also for the filtrate from *C. elatum*. Components with activity against *P. infestans*, *B. cinerea* and *F. culmorum* could be extracted with ethyl acetate from the culture filtrates of *C. globosum*, *C. cochliodes* and *C. aureum*, whereas no or only low antifungal activity was present in the extracts from *C. nozdrenkoae*, *C. ramosissimum* and *C. elatum*. Based on these observations, the latter three strains were not or only partially included in the experiments with fractions of extracts separated by HPLC.

In our study, a broad spectrum of phytopathogenic fungi was inhibited on agar media amended with culture filtrates of *C. globosum* and *C. aureum*. For *C.*

globosum, our results fit well with the literature stating antifungal activity against several phytopathogens including oomycetes (Aggarwall 2015). Among the most named compounds reported to be involved in the biological activity are the chaetoglobosins, a family of macrocyclic polyketide alkaloids belonging to the class of cytochalasans. Unfortunately, some of them are highly cytotoxic and irreversibly bind to actin (Kretz *et al.* 2019), which may pose a hurdle for the use of *Chaetomium* species in biocontrol. In the bioassays of our study with the fractionated extracts from *C. globosum*, the HPLC and MS analysis of the active compounds identified the chaetoglobosins. Chaetoglobosins were found to be the main antifungal compounds for the activity of *C. globosum* and other *Chaetomium* species against *Bipolaris sorokiniana* (Biswas *et al.* 2012), *Saetosphaeria turcica* (Zhang *et al.* 2013), *Fusarium oxysporum* (Soytong 2015), *Sclerotinia sclerotiorum* (Zhao *et al.* 2017) and *F. sporotrichoides* (Jiang *et al.* 2017). Chaetoglobosins A and C, isolated from culture filtrates of a strain of *C. globosum*, inhibited the maize pathogen *Setosphaeria turcica in vitro*, and Chaetoglobosin A

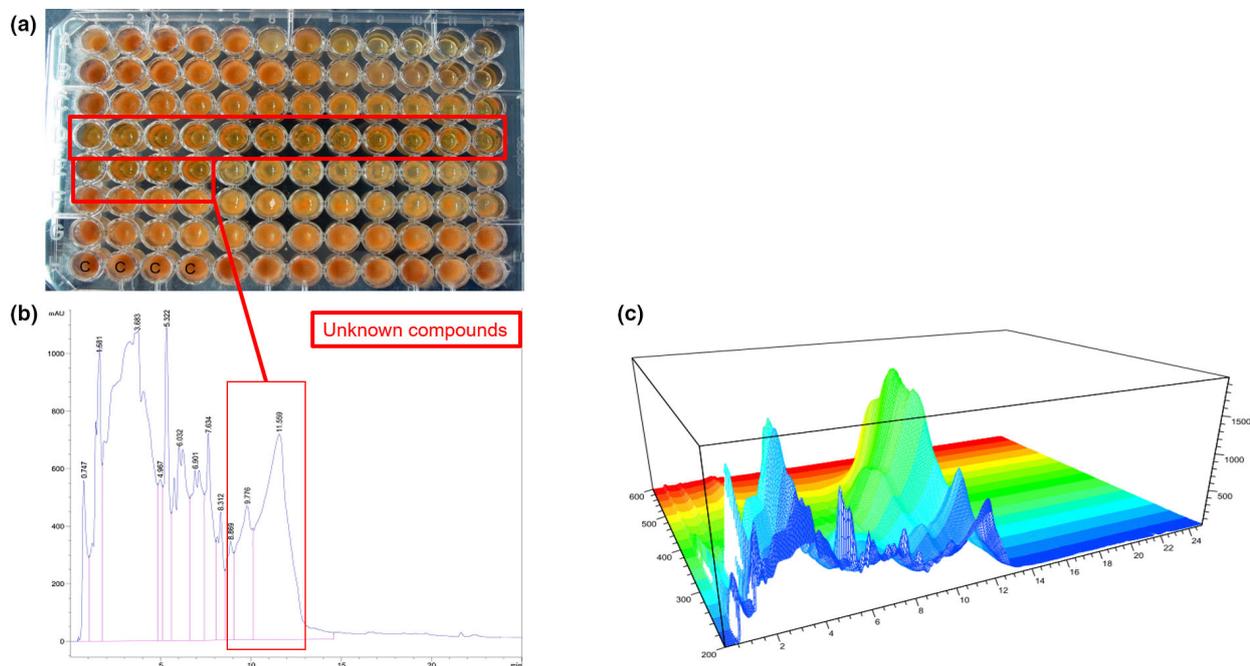


Figure 7 Biological activity assays of fractionated extracts of *Chaetomium aureum*. Antifungal activity of single fractions obtained after separation of the crude extract of *C. aureum* by semi-preparative HPLC. Wells H1–H4 are solvent controls. (a) Activity on *F. culmorum*, (b) HPLC chromatogram with fractions of the wells D1 D12 and E1–E4 highlighted in red. The extracts were produced after fermentation in PDB for 14 days on a rotary shaker at 25°C and 150 rev min⁻¹. (c) 3d-chromatogram of the fractionated crude extract from (b). [Colour figure can be viewed at wileyonlinelibrary.com]

displayed biocontrol activity also on detached leaves (Zhang *et al.* 2013).

Another hazardous compound shown to be present in extracts of *Chaetomium* is sterigmatocystin (Rank *et al.* 2011), which is biosynthetically related to the aflatoxins. However, by HPLC and MS analysis with pure sterigmatocystin as standard we could not detect this compound in our extracts. We were also not able to detect flavipin, a metabolite from *C. globosum* reported to inhibit the growth of several plant pathogenic fungi, especially *F. graminearum* (Xiao *et al.* 2013).

Compared to *C. globosum*, much less work has been done on the potential of *C. aureum* and *C. cochliodes* as biocontrol agents. Both showed a strong activity against *P. infestans*, *C. aureum* inhibited also *F. culmorum*. For *C. aureum*, *in vitro* fungicidal activity and biocontrol activity have been reported against *Pythium aphanidermatum* (Pornsuriya *et al.* 2010), *Fusarium circinatum* (Martínez-Álvarez *et al.* 2016), the rice blast pathogen *Magnaporthe grisea* and the sheath blight pathogen *Rhizoctonia solani* (Wang *et al.* 2013). *C. aureum* produces a number of bioactive secondary metabolites, including oosporein (Taniguchi *et al.* 1984) that was shown to have

antifungal activity against *R. solani*, *Botrytis cinerea* and *Pythium ultimum* (Mao *et al.* 2010). In previous studies crude extracts from *C. cochliodes* showed activity *in vitro* against *Phytophthora* sp. (Rujira *et al.* 2017) and *Drechslera oryzae* (syn. *Bipolaris oryzae*) (Soytong 2014). Chaetoglobosin A was isolated from culture filtrates of *C. cochliodes* and suggested to be responsible for the inhibition of *P. ultimum* and other pathogens (Kang *et al.* 1999).

In our bioassays with the fractionated extracts from *C. aureum* and *C. cochliodes* it was possible to determine active components, but the compounds are as yet unknown (Figs 6 and 7).

Overall, our results regarding the identification of antifungal metabolites produced by the *Chaetomium* strains are still preliminary, given the large number of biologically active compounds reported for the genus (Zhang *et al.* 2012). Structure elucidation was not accomplished since the amount of the highly active components was insufficient. In further studies the aim will be to find a way (optimization of fermentation conditions and/or genetic manipulation) to increase the production and to isolate enough pure compounds for

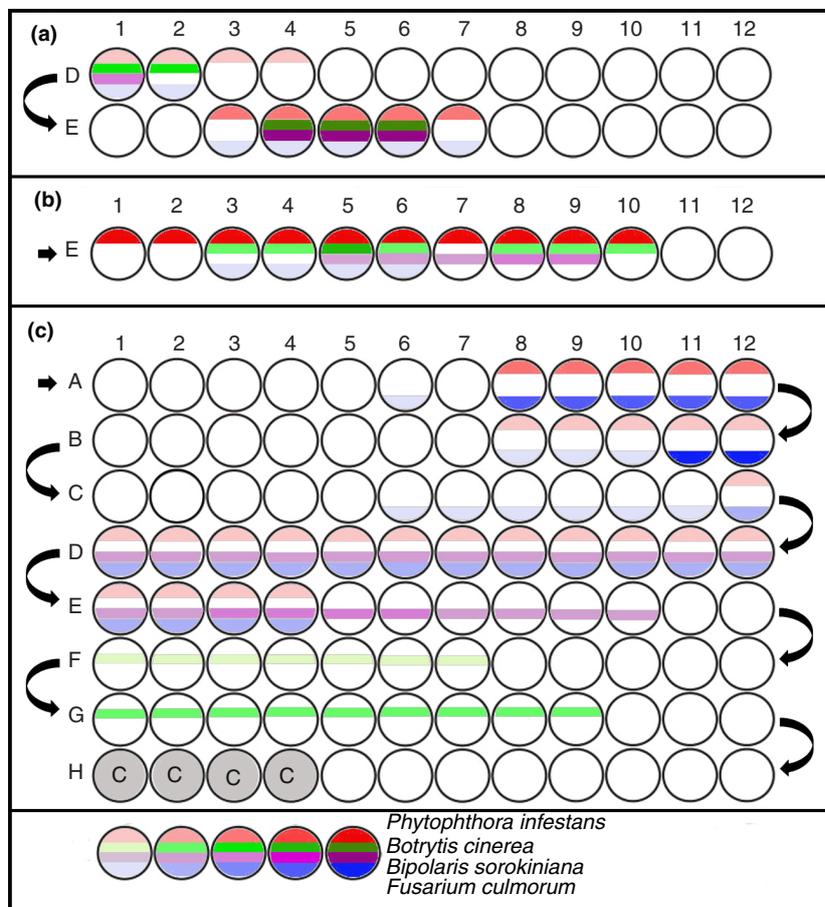


Figure 8 Schematic drawing of growth inhibition by fractionated extracts of *Chaetomium globosum* (a), *C. cochliodes* (b), and *C. aureum* (c) on *P. infestans* (upper row), *B. cinerea* (second row from top), *B. sorokiniana* (second row from bottom), and *F. culmorum* (bottom row). Colour intensity represents inhibition intensity in the individual wells. Arrows indicate the direction of loading. [Colour figure can be viewed at wileyonlinelibrary.com]

structure elucidation. This will include testing of media other than the standard laboratory medium PDB used here. Our present study can nevertheless serve as starting point for further elaboration of the compounds produced by our strains. With regard to the safety of potential biocontrol products based on *Chaetomium* the antifungal versus the cytotoxic properties of the compounds could be of specific interest.

Authors' contributions

A.L. performed molecular analyses of *Chaetomium* sp., S.J. carried out HPLC fractionation and profiling. P.Z. and M.M. determined antagonistic potential and temperature behaviour of the fungal isolates. W.M. maintained and provided the fungal strains and contributed to the molecular analyses. E.K. carried out microscopic analyses.

A.L., S.J. and E.K. wrote the article. This article was carefully reviewed by the other co-authors.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Ascospores of (a) *Chaetomium globosum*, (b) *C. cochliodes*, (c) *C. aureum*, (d) *C. ramosissimum* and (e) *C. elatum*. Bars = 10 μ m.

Figure S2. Colonies of *Chaetomium* sp. grown on PDA at 20°C.

Figure S3. Colonies of (a) *C. globosum* 4, (b) *C. aureum*, (c) *C. nozdrenkoae* and (d) *C. elatum* grown on PDA at 35°C.

Figure S4. Dual cultures on SNA between the *Chaetomium* strains (mycelial plugs in the center of the plates) and *Pyrenophora graminea* (a–m) (5 dpi) or *Bipolaris sorokiniana* (n–p) (10 dpi).