


# Bacteria-induced production of the antibacterial sesquiterpene lagopodin B in *Coprinopsis cinerea*

Martina Stöckli,<sup>1,†</sup> Brandon I. Morinaka,<sup>1,‡</sup>  
Gerald Lackner,<sup>1,§</sup> Anja Kombrink,<sup>1,¶</sup> Ramon Sieber,<sup>1,\*\*</sup>  
Céline Margot,<sup>1</sup> Claire E. Stanley,<sup>2,††</sup>  
Andrew J. deMello,<sup>2</sup> Jörn Piel<sup>1</sup> and  
Markus Künzler <sup>1\*</sup>

<sup>1</sup>Institute of Microbiology, Department of Biology, ETH Zurich, Vladimir-Prelog-Weg 4, Zürich, CH-8093, Switzerland.

<sup>2</sup>Institute for Chemical and Bioengineering, ETH Zurich, Vladimir-Prelog-Weg 1, Zürich, CH-8093, Switzerland.

## Summary

Fungi defend their ecological niche against antagonists by producing antibiosis molecules. Some of these molecules are only produced upon confrontation with the antagonist. The basidiomycete *Coprinopsis cinerea* induces the expression of the sesquiterpene synthase-encoding gene *cop6* and its two neighboring genes coding for cytochrome P450 monooxygenases in response to bacteria. We further investigated this regulation of *cop6* and examined if the gene product is involved in the production of antibacterials. Cell-free supernatants of axenic cultures of the Gram-positive bacterium *Bacillus subtilis* were sufficient to induce *cop6* transcription assessed using a fluorescent reporter strain. Use of this strain in a microfluidic device revealed that the *cop6* gene was induced in all hyphae directly exposed to the supernatant and that induction occurred within less than one hour. Targeted replacement of the *cop6* gene demonstrated the requirement of the encoded synthase for the biosynthesis of the sesquiterpene lagopodin B, a previously reported antibacterial

compound from related species. Accordingly, lagopodin B from *C. cinerea* inhibited the growth of several Gram-positive bacteria including *B. subtilis* but not Gram-negative bacteria. Our results demonstrate that the *C. cinerea* vegetative mycelium responds to soluble compounds of a bacterial culture supernatant by local production of an antibacterial secondary metabolite.

## Introduction

Saprobic fungi acquire their nutrients by secretion of hydrolytic enzymes into their substrate and absorption of the hydrolysis products. These fungi have evolved various defense strategies to protect these hydrolysis products from being absorbed by other microorganisms and to establish a secure ecological niche (Brakhage *et al.*, 2005). The main defense strategy used by fungi is chemical defense that involves the production of antibiosis molecules such as proteins, peptides or secondary metabolites (SMs). These molecules are either constitutively produced or their production is induced in response to the antagonist (Spiteller, 2015; Sandargo *et al.*, 2019). It has been suggested that inducible production allows the fungus to save resources in the absence of the respective antagonist. In the coprophilous model, basidiomycete *Coprinopsis cinerea*, both types of regulation for the production of antibiosis molecules have been described. The antibacterial peptide copsin was produced constitutively by axenically cultivated vegetative mycelium (Essig *et al.*, 2014), whereas the expression of a paralogous gene and some lysozyme-encoding genes in the same tissue were dependent on the co-cultivation with bacteria (Kombrink *et al.*, 2019).

The genomes of filamentous fungi are rich in gene clusters coding for biosynthetic pathways of SMs with various bioactivities, yet only few of them are expressed under laboratory conditions (Brakhage and Schroeckh, 2011; Chiang *et al.*, 2013). The production of some SMs is induced in co-cultures of fungi with bacteria (Cueto *et al.*, 2001; Oh *et al.*, 2007; Schroeckh *et al.*, 2009; Ola *et al.*, 2013; Spraker *et al.*, 2018) and it has been hypothesized that these SMs function, depending on

Accepted 9 May, 2019. \*For correspondence. E-mails: mkuenzle@ethz.ch, markus.kuenzler@micro.biol.ethz.ch; Tel. +41 44 632 49 25 Present addresses: <sup>†</sup>rqmicro AG, Brandstrasse 24, Schlieren, CH-8952, Switzerland; <sup>‡</sup>Department of Pharmacy, National University of Singapore, 18 Science Drive 4, Singapore, 117543, Singapore; <sup>§</sup>Leibniz Institute for Natural Product Research and Infection Biology, Hans Knöll Institute, Beutenbergstrasse 11a, Jena, D-07745, Germany; <sup>¶</sup>HLB bv, Kampsweg 27, Wijster, 9418PD, The Netherlands; <sup>††</sup>Lonza AG, Lonzastrasse 3, Visp, CH-3930, Switzerland; <sup>\*\*</sup>Agroscope, Reckenholzstrasse 191, Zürich, CH-8046, Switzerland.

their concentration, either as signaling molecules or as growth inhibitors in the chemical defense of these fungi against bacterial competitors and antagonists (Brakhage *et al.*, 2005; Andersson and Hughes, 2014; Netzker *et al.*, 2015). Besides biotic stress, abiotic stress can also trigger expression of silent SM gene clusters (Scherlach and Hertweck, 2009; Gressler *et al.*, 2015). However, there are only few cases where the regulation of SM gene clusters involved in bacterial-fungal interactions (BFIs) was studied at a molecular level (Schroeckh *et al.*, 2009; Nützmänn *et al.*, 2011; Spraker *et al.*, 2018). The SM gene clusters of filamentous fungi include non-ribosomal peptide synthetases (NRPSs), polyketide synthases (PKSs) and terpene synthases (TSs) (Lackner *et al.*, 2012; Bills *et al.*, 2013). Genomes of Basidiomycota encode more TS genes than NRPS and PKS and homologs of sesquiterpene synthase encoding genes are widespread in this phylum (Schmidt-Dannert, 2015). These synthases catalyze the cyclization of farnesyl pyrophosphate to different compounds with diverse structures. The genome of the model basidiomycete *C. cinerea* encodes for six sesquiterpene synthases (Agger *et al.*, 2009). Only one of these genes, termed *cop6*, was thought to belong to a biosynthesis gene cluster since it is flanked by two P450 cytochrome monooxygenase encoding genes (Agger *et al.*, 2009). COP6 was functionally characterized as an  $\alpha$ -cuprenene synthase by heterologous expression in yeast. Expression of *cop6* together with its two neighboring P450 cytochrome monooxygenases, led to the production of oxygenated  $\alpha$ -cuprenene derivatives in this heterologous system (Agger *et al.*, 2009). Based on these results, it has been suggested that *cop6* is involved in the production of the antibacterial sesquiterpene lagopodin B, but so far experimental proof for this is missing and no expression data in *C. cinerea* are available (Agger *et al.*, 2009; Schmidt-Dannert, 2015). Lagopodin B was isolated from the supernatant of axenic cultures of different *Coprinus* species; *C. lagopus* Fries (Bollinger, 1965) and *C. cinereus* (Bu'Lock and Darbyshire, 1976) and was chemically characterized in 1965 (Bollinger, 1965). These fungal species were transferred to the genus *Coprinopsis* after it had become clear that the *Coprinus* type species (*C. comatus*) is more closely related to parasol mushrooms (Redhead *et al.*, 2001). Twenty years after their isolation and chemical characterization, the antibacterial activity of lagopodins against Gram-positive bacteria was demonstrated as part of a PhD thesis (Bastian, 1985). Later on, the compounds were also shown to inhibit platelet aggregation (Lauer and Anke, 1991).

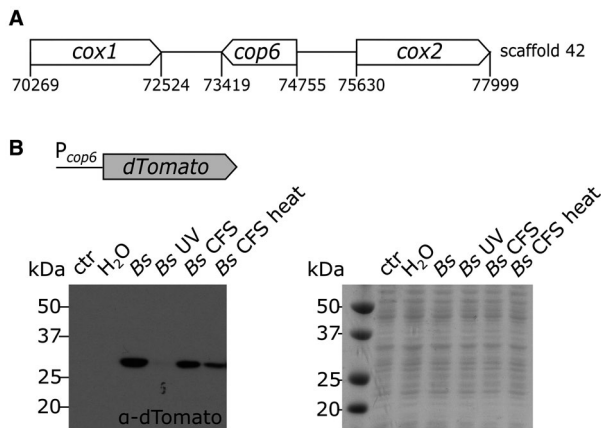
In a recent study, we found that the expression of the *cop6*-containing gene cluster was significantly induced in a laboratory strain of *C. cinerea* upon co-culture with the Gram-positive bacterium *Bacillus subtilis* NCIB 3610 and the Gram-negative bacterium *Escherichia coli* Nissle

1917 (Kombrink *et al.*, 2019). The terpene synthase-encoding *cop6* gene was among the most highly induced genes in the entire *C. cinerea* genome. Here, we further characterized the biotic and abiotic regulation of this gene cluster and demonstrate that cell-free supernatants of axenic bacterial cultures are sufficient for the induction of the gene cluster. Furthermore, we show that the *cop6* gene is required for the production of lagopodin B and that lagopodin B is active against a variety of Gram-positive but not against Gram-negative bacteria.

## Results

### *The expression of cop6 encoding a sesquiterpene synthase is induced by a soluble compound in bacterial cell-free culture supernatant*

The sesquiterpene synthase-encoding gene *cop6* (COP6, JGI protein ID 394772) of the model mushroom, *Coprinopsis cinerea* (Kues, 2000), was found to be induced in co-culture with both the Gram-negative bacterium *Escherichia coli* Nissle 1917 and the Gram-positive bacterium *Bacillus subtilis* NCIB 3610 (Kombrink *et al.*, 2019). It was suggested that *cop6* belongs to an SM gene cluster consisting of *cop6* and two adjacent genes, *cox1* (COX1, JGI protein ID 201523) and *cox2* (COX2, JGI protein ID 420898) encoding two predicted cytochrome P450 monooxygenases (Agger *et al.*, 2009; Stajich *et al.*, 2010) (Fig. 1A). In agreement with this hypothesis, the expression of *cox1* and *cox2* was also induced in co-culture with bacteria (Kombrink *et al.*, 2019). In order to characterize the bacterial induction of the *cop6* gene, the promoter region of *cop6* was fused to the reporter gene *dTomato* and this construct was ectopically integrated into the genome of *C. cinerea* AmutBmut (wildtype, wt). Expression of *cop6* was assessed by cultivation of the *C. cinerea* *pcop6-dTomato* reporter strain under the same conditions as the wt strain (Kombrink *et al.*, 2019) and analyzing the fungal whole-cell protein (WCP) extract for the presence of the reporter protein dTomato by immunoblotting. As expected, the reporter gene was induced, as visualized by the presence of dTomato, in the presence of live *B. subtilis* NCIB 3610, but interestingly not in the presence of washed and UV-treated bacteria (Fig. 1B). Since saprobic bacteria and fungi feed both by osmotrophy and on some of the same nutrients (De Boer *et al.*, 2005), we set out to test if the mere growth of bacteria and thereby depletion of nutrients could induce the expression of the sesquiterpene synthase gene *cop6*. For this purpose, half of the medium was replaced by sterile double deionized water. This starvation condition did not induce the reporter gene (Fig. 1B). In some BFIs, physical contact of bacteria with fungal



**Fig. 1.** Induction of *C. cinerea* sesquiterpene synthase-encoding *cop6* gene by *B. subtilis* and its cell-free culture supernatant. A. Representation of the *C. cinerea* genomic region of the *cop6* gene cluster. *cox1* and *2* encoding cytochrome P450 monooxygenases flank the *cop6* gene coding for an  $\alpha$ -cuprenene synthase. The arrowheads indicate the direction of transcription. B. The promoter region of the *cop6* gene was fused to the reporter gene *dTomato*. The reporter strain was cultivated on glass beads and either co-cultivated with bacteria or their cell-free culture supernatant. WCPs were extracted from the mycelium of these cultures and 50  $\mu$ g assayed for the presence of dTomato protein with  $\alpha$ -dTomato antibodies on 12% SDS-PAGE transferred to a nitrocellulose membrane (left panel). An identical gel was run and stained with Commassie Brilliant Blue to control for the amount of loaded protein (right panel). ctr stands for control treatment where nothing was added, H<sub>2</sub>O for the exchange of half of the medium with sterile water, Bs for co-cultivation with *Bacillus subtilis* NCIB 3610, Bs UV for co-cultivation with UV-killed *B. subtilis* NCIB 3610 cells, Bs CFS for the addition of MeOH-extracted *B. subtilis* NCIB 3610 cell-free supernatant, Bs CFS heat for the MeOH-extracted *B. subtilis* NCIB 3610 cell-free supernatant that was heat treated (10 min, 100°C).

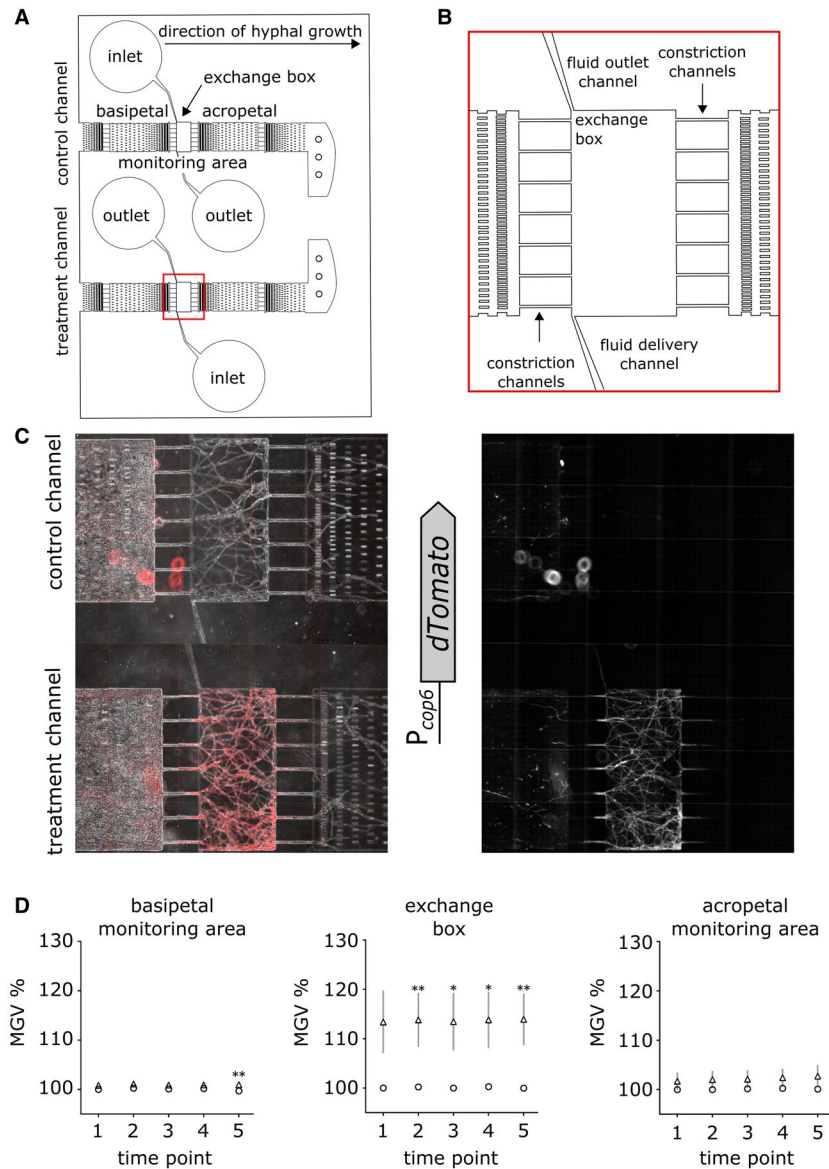
hyphae is required for the induction of fungal SM gene clusters (Schroeckh *et al.*, 2009). Since *B. subtilis* NCIB 3610 was found to physically interact with *C. cinerea* by binding in an end-on manner on the fungal hyphae (Stanley *et al.*, 2014), we tested if the presence of the bacteria is required for the induction of the *cop6* gene or if soluble compounds produced by the bacteria are sufficient to elicit the response. For this purpose, the reporter strain was co-cultivated with a methanol (MeOH) extract of bacterial cell-free culture supernatant. This extract was sufficient to induce the expression of the reporter gene (Fig. 1B). The water and MeOH-soluble compounds appeared to be heat stable as the cell-free supernatant subjected to heat (100°C, 10 min) retained their inducing activity. To test if induction of gene expression in the presence of the MeOH-extract of the bacterial cell-free culture supernatant is specific to *cop6*, analogous reporter strains, in which the expression of *dTomato* is driven by promoters of other bacterially induced genes, were analyzed. The induction appeared not to be specific for the

terpene synthase gene *cop6* since the reporter genes for the lysozyme-encoding gene *lys1* (LYS1, JGI protein ID 432813) and a gene encoding a secreted protein *psp* (PSP, JGI protein ID 417969) (Kombrink *et al.*, 2019) were also induced by the MeOH-extract of the bacterial cell-free culture supernatant (Fig. S1). In summary, we found that water and MeOH-soluble, heat stable compound(s) present in the supernatant of axenic *B. subtilis* cultures are able to induce an antibacterial (based on the antibacterial activity of LYS1 [Kombrink *et al.*, 2019]) response in the fungus *C. cinerea*.

#### Induction of *cop6* is restricted to hyphae that are directly exposed to the bacterial culture supernatant

The aforementioned analysis of fungal gene expression was conducted at colony level. Thus, it was not clear whether these genes were only induced in the hyphae that were exposed to the bacterial culture supernatant (local response) or whether the induction spreads also to non-exposed parts of the mycelium (systemic response). Propagation of defense gene induction from the area of interaction, both in acropetal and basipetal direction of specialized hyphae, was reported for *C. cinerea* in response to challenge with fungivorous nematodes (Schmieder *et al.*, 2019). In order to assess the spatial distribution of *cop6* expression in *C. cinerea* upon exposure to the bacterial culture supernatant, we performed the challenge of the *C. cinerea* *pcop6-dTomato* reporter strain in a custom-made microfluidic device and examined the fluorescence of dTomato in the single hyphae by fluorescence microscopy. We previously presented two microfluidic platforms that enable the study of BFI on a single cell (hyphal) level and the exchange of a fluid surrounding fungal hyphae in a controlled and rapid manner (Stanley *et al.*, 2014). The microfluidic device used in this study combines features of the two above-mentioned devices (Fig. 2A). The design allowed to spatially restrict the exposure of the fungus to the bacterial culture supernatant to a specific part of the mycelium, specifically the hyphae within the exchange box, and, at the same time, to monitor the fluorescence of hyphae outside of that area. The main features of this device were two independent growth channels, i.e. one control channel and one treatment channel. Both channels are composed of an exchange box and basipetal and acropetal monitoring areas with regard to growth direction of the fungal hyphae. The exchange box was connected to an inlet and outlet hole and separated from the basipetal and acropetal areas by seven constriction channels (Fig. 2B).

Importantly, growth of fungal hyphae into the microfluidic device blocked the constriction channels and therefore the flow of the exchanging solution into the



**Fig. 2.** Spatial restriction of bacterial *cop6* induction using a *C. cinerea pcop6-dTomato* reporter strain.

A. Representation of the microfluidic device design highlighting its main features.

B. The enlarged region illustrates the exchange box with the seven constriction channels on each side and the fluid delivery and outlet channels.

C. 5× concentrated MeOH-extract of cell-free *B. subtilis* NCIB 3610 culture supernatant was introduced after *C. cinerea pcop6-dTomato* hyphae reached the acropetal monitoring area of the device. Induction of *cop6* was indirectly assessed by detection of the fluorescent protein dTomato by epifluorescence microscopy. The first time point, recorded 20 min after addition of the supernatant, of one of the three biological replicates is shown. The bright field and fluorescence channels are merged in the left panel and the right panel represents the fluorescence channel only. Scale bar, 200 μm.

D. The mean gray value (MGV) of each area of the microfluidic device was determined as a measure of fluorescence intensity and, thus, *cop6* gene expression, using the segmentation method described in the supplementary Material and Methods. The mean and standard deviation of three independent replicates are represented (\*\* $p < 0.05$ , \* $p < 0.06$ , assessed by Welch's t-test).

basipetal and acropetal areas of the channel. A time-lapse experiment where the growth medium in the exchange box was replaced with a fluorescein containing solution demonstrated this functionality of the microfluidic device (Fig. S2). For the actual experiment, the hyphae of the reporter strain *C. cinerea pcop6-dTomato*

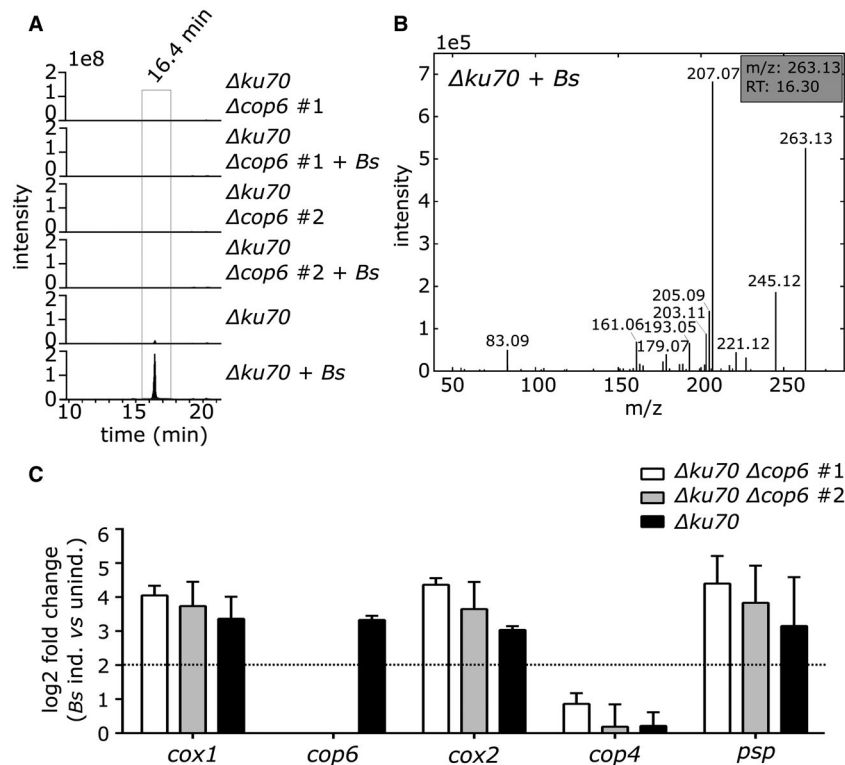
were grown until the constriction channels on both sides of the exchange box were blocked. The culture medium within the exchange box of the treatment channel was replaced with 5× concentrated MeOH-extract of the cell-free supernatant of *B. subtilis* NCIB 3610 dissolved in *C. cinerea* minimal medium (CCMM) pH 6.4. For the

control channel, we replaced the culture medium with 5× concentrated MeOH-extract of CCMM pH 6.4 dissolved in CCMM pH 6.4. Subsequently, red fluorescence in the device was monitored in a time-lapse experiment with a 30-min time interval. Fig. 2C represents the first time point taken 20 min after addition of the cell-free culture supernatant of such a time-lapse experiment. The presence of dTomato was restricted to the hyphae that were directly exposed to the *B. subtilis* NCIB 3610 cell-free culture supernatant in the exchange box. For quantification of the dTomato-mediated fluorescence, the area covered by hyphae within the exchange box and the basipetal and acropetal monitoring areas was determined by segmentation of the bright field microscopy images of these areas (Fig. S3). Subsequently, as a measure of fluorescence, the mean gray value (MGV) of the hyphae was compared between the treatment and the control channels over time. The MGV of the hyphae within the exchange box was significantly higher

in the treatment channel compared to the control channel (Fig. 2D). In contrast, the MGV of the hyphae within the acropetal and basipetal monitoring areas was not significantly different from the MGV in the same areas of the control channel for the first four time points (80 min). These results suggest a restriction of the induction of the *cop6* gene to hyphae that are directly exposed to the bacterial culture supernatant. No intrahyphal propagation of this induction to non-exposed hyphal compartments was observed, at least not within the examined time period.

#### *cop6* is involved in the production of compound 1

Next, we wanted to determine the biosynthesis product of COP6 in its native host. Based on the heterologous expression of the *cop6* gene in the yeast *S. cerevisiae*, COP6 was functionally categorized as an  $\alpha$ -cuprenene synthase and suggested to be involved in the biosynthesis



**Fig. 3.** *cop6*-dependent production of a specific metabolite (compound 1) in *C. cinerea* upon co-cultivation with *B. subtilis* NCIB 3610. The different *C. cinerea* strains were cultivated on bead plates in the absence (–Bs) and presence of *B. subtilis* NCIB 3610 (+Bs).

A. The culture broth was extracted with EtOAc and the crude extract analyzed by LC-MS/MS. Extracted ion chromatography for *m/z* 263.1265–263.1291 for compound 1 with the formula  $C_{15}H_{18}O_4$  and its theoretical *m/z* 263.1278 (for  $[M + H]^+$ ) is represented. The mass of compound 1 was not observed where the gene that encodes the sesquiterpene synthase COP6 was deleted ( $\Delta cop6$ ).

B. MS2 spectrum of compound 1 is depicted.

C. Induced expression of the *cop6* gene cluster in presence of *B. subtilis* NCIB 3610 was determined by qRT-PCR. The RNA was extracted from the same cultures and mean and standard deviation of three biological replicates are represented. A gene that codes for a secreted protein (*psp*) served as positive control, since it was also induced in presence of bacteria and *cop4* that encodes another sesquiterpene synthase as negative control since it was not induced in presence of bacteria (Kombrink et al., 2019). A dotted line is depicted at a log<sub>2</sub>-fold change of 2, which was considered as the threshold for significant expression induction. *C. cinerea*  $\Delta ku70$   $\Delta cop6$  #1 and #2 represent two independent transformants.

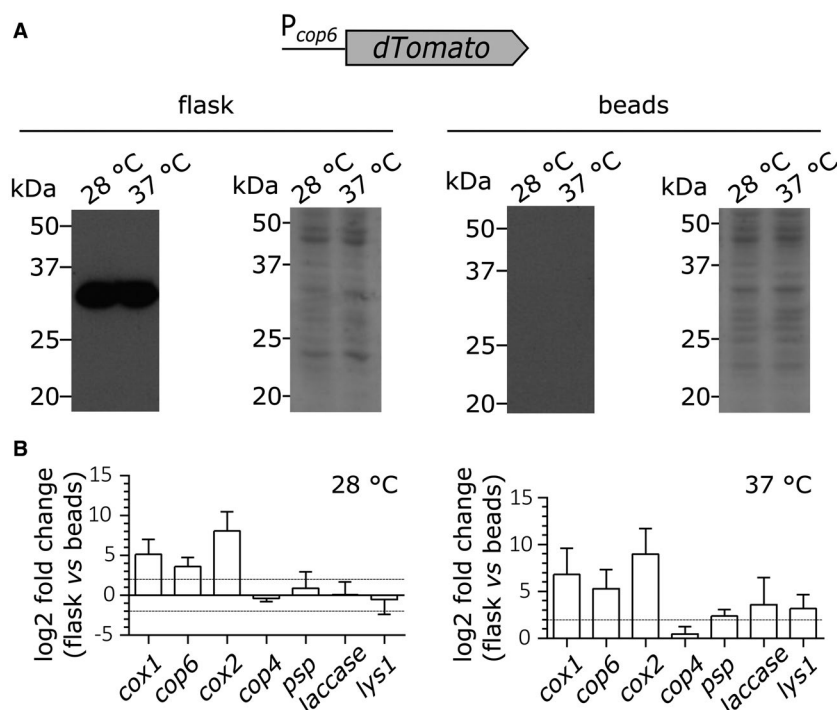
of the SM lagopodin B (Agger *et al.*, 2009). To date, however, no interdependence between expression of the *cop6* gene cluster, lagopodin B production and antibacterial activity by *C. cinerea* was demonstrated. Based on the bacterial induction of the *cop6* gene cluster, we set out to determine how the fungal metabolite profile would change in co-culture with bacteria compared to an axenic fungal culture and if a mass corresponding to lagopodin B with the molecular formula of  $C_{15}H_{18}O_4$  or other oxygenated  $\alpha$ -cuprenene compounds would be induced. For this purpose, *C. cinerea* was cultivated on glass beads in the presence and absence of *B. subtilis* NCIB 3610. The supernatant of both cultures was extracted with ethyl acetate (EtOAc) and the crude extract was subjected to liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). In the crude extract of the co-culture, a compound (**1**) corresponding to the exact mass of lagopodin B (obs.  $[M + H]^+ = 263.1278$ , calcd. for 263.1278), that was absent in the axenic fungal culture, eluted at 16.3 min (Fig. S4). In order to link the activity of the *cop6* gene product to the production of compound **1**, a *C. cinerea cop6* deletion strain was prepared by homologous recombination. Since homologous recombination is a rare event in the *C. cinerea* AmutBmut wt strain, a  $\Delta ku70$  variant of this strain, which is deficient in non-homologous end-joining (Nakazawa and Honda, 2015), was used for this purpose. The *cop6* gene was replaced by the heterologous *Pcpab* cassette (Stöckli *et al.*, 2017) (Fig. S5). LC-MS/MS analysis revealed the absence of the mass peak corresponding to compound **1** in the crude extract of the *cop6* deletion strain, whereas the peak was present and induced in the crude extract of *C. cinerea* AmutBmut  $\Delta ku70$  upon co-cultivation with *B. subtilis* NCIB 3610 (Fig. 3A and B). In order to confirm the transcriptional induction of the *cop6* gene cluster in these strains upon co-cultivation with *B. subtilis* NCIB 3610, mycelial RNA was isolated, cDNA synthesized and quantitative real-time PCR performed. Another sesquiterpene synthase gene, *cop4* (COP4, JGI protein ID 30510), which is not induced in the presence of bacteria (Kombrink *et al.*, 2019), served as a negative control. As positive control, the above-mentioned *psp* gene coding for a secreted protein (Kombrink *et al.*, 2019) was used. The qRT-PCR results revealed that the genes of the putative *cop6* gene cluster were induced in the presence of bacteria in all strains tested (Figs 3C and S4B). The correlation between production of compound **1** and induction of *cop6* upon co-cultivation with bacteria suggested that the gene product of *cop6* is involved in the production of compound **1**. As a first test for the antibacterial activity of compound **1**, a disk diffusion assay with the crude EtOAc extracts of the different mycelial culture supernatants was performed. However, none of the crude extracts showed antibacterial activity in this assay (Fig. S4C).

#### The *cop6* gene cluster is also induced by abiotic stress

Although co-cultivation of *C. cinerea* with *B. subtilis* NCIB 3610 on glass beads led to induction of the expression of the *cop6* gene cluster and the production of compound **1**, its crude extract did not show any antibacterial activity. We reasoned that the lack of antibacterial activity could be due to low concentrations found in the 18 times concentrated crude extracts. Since up-scaling of the used co-cultivation method was not practicable and induction of many fungal SM gene clusters had been reported for both biotic and abiotic triggers (Scherlach and Hertweck, 2009; Gressler *et al.*, 2015), we tested different axenic culture conditions to produce higher amounts of compound **1**. In these trials, the expression of the sesquiterpene synthase gene *cop6* was monitored using the *C. cinerea* reporter strain *pcop6-dTomato*. We observed that the *cop6* gene was highly expressed by culturing the mycelium in Erlenmeyer flasks without agitation. Under these conditions, *C. cinerea* was growing partly as a surface mat and partly as submersed mycelium (Fig. 4A). The expression of the *cop6* gene was independent of the two growth temperatures tested, as dTomato production was detected in the WCP extract of *C. cinerea* in the flask at 28°C and 37°C. As observed in the previous experiments, the expression of the *cop6* gene was very low when *C. cinerea* was cultivated on glass beads (Fig. 4A). To test if this represents a general expression pattern of bacteria-induced genes, we cultivated *C. cinerea* AmutBmut (wt) under both above described conditions, extracted RNA from the mycelium, synthesized cDNA and performed qRT-PCR analysis on the genes of the *cop6* gene cluster, the sesquiterpene synthase-encoding gene *cop4*, and the bacterially induced genes coding for LYS1, the above-mentioned secreted protein PSP and a laccase (JGI protein ID 502564) (Fig. 4B). All three genes from the *cop6* gene cluster were induced in the flask cultures relative to the glass bead cultures at both growth temperatures tested. However, the standard deviation of the three biological replicates were quite high due to high variation of the expression in the flask culture. The other three bacteria-induced genes were not induced at the lower growth temperature and only slightly induced at 37°C. In summary, these results suggest that the *cop6* gene cluster, in contrast to other bacterially induced genes from *C. cinerea*, respond to both biotic and abiotic environmental cues.

#### Compound **1** is antibacterial

To produce more of compound **1**, we cultured the different *C. cinerea* strains in Erlenmeyer flasks and prepared crude extracts by EtOAc extraction of 40 ml culture supernatant. LC-MS/MS analysis confirmed the



**Fig. 4.** Expression of the *cop6* gene cluster cultivated in static flask cultures of *C. cinerea*.

A. *C. cinerea* *pcop6-dTomato* was cultivated either on bead plates (beads) or in Erlenmeyer flasks (flask) and either at 37°C or 28°C.

Expression of *cop6* was assessed indirectly by presence of the reporter protein dTomato. About 50 µg of the extracted WCPs were separated on 12% SDS-PAGE, then either stained with Comassie or transferred to nitrocellulose membrane and probed with α-dTomato antibody.

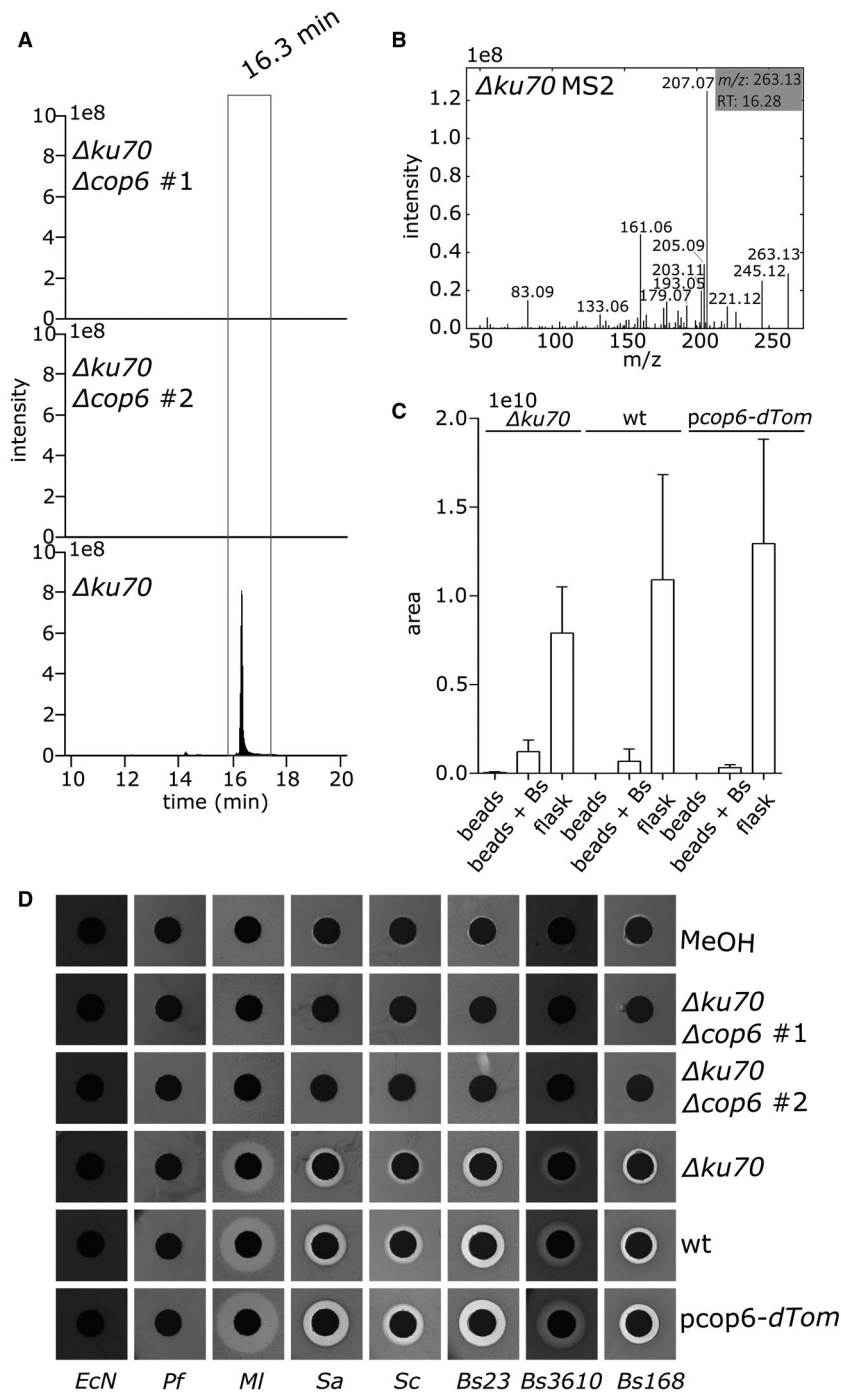
B. *C. cinerea* AmutBmut wt was cultivated the same way as described in (A) and RNA was extracted for gene expression analysis by qRT-PCR. Mean and standard deviation of three biological replicates are represented. Dotted lines are depicted at a log<sub>2</sub>-fold change of 2 and -2, which were considered as the threshold for significant expression induction and repression respectively.

presence of compound **1** in the crude extracts of the *C. cinerea* strains containing an intact *cop6* gene ( $\Delta ku70$ , AmutBmut, and the reporter strain *pcop6-dTomato*) (Figs 5A,B and S6A,B). These crude extracts were loaded onto blank disks and the antibacterial activity tested on the disk diffusion assay against *B. subtilis* 168 (Fig. 5D first lane). The crude extracts of the strains with an intact *cop6* gene exhibited antibacterial activity against *B. subtilis* 168 and a clear inhibition zone was observed around the disks. In contrast, the crude extracts of the  $\Delta cop6$  strains were not active, suggesting that *cop6* is involved in the production of an antibacterial compound. As mentioned above, the crude extracts prepared from the bacteria-induced glass bead cultures did not show any activity (Fig. S4C). We compared the amount of compound **1** in the different crude extracts by comparing the peak area of the extracted ion chromatography for *m/z* 263.1265–263.1291 (Fig. 5C). The amount of compound **1** was 6.4×, 15.9× and 39× higher for the crude extracts of the flask cultures prepared from  $\Delta ku70$ , the wt and the *pcop6-dTomato*, respectively, compared to the crude extracts prepared from the induced glass bead cultures of these strains, which is in agreement with the biological activity of the

different extracts (Fig. 5D compared to Fig. S4C). In our previous study, we showed that both the Gram-positive bacterium *B. subtilis* NCIB 3610 and the Gram-negative bacterium *E. coli* Nissle 1917 induced the expression of the *cop6* gene cluster (Kombrink *et al.*, 2019). However, the crude extracts showed antibacterial activity against *B. subtilis* NCIB 3610 but not against *E. coli* Nissle 1917. For a more detailed analysis of the specificity of the antibacterial activity of compound **1**, the crude extracts were tested for growth-inhibitory activity against a set of Gram-positive and Gram-negative bacteria. The extracts showed clear activity against all tested Gram-positive bacteria but no detectable activity against any tested Gram-negative bacteria (Fig. 5D).

#### Antibacterial compound **1** is lagopodin B

To determine the structure of antibacterial compound **1**, the crude extract was fractionated in a semi-preparative scale and antibacterial activity of the fractions was determined. The active fraction contained compound **1** which could be described, according to high-resolution mass determination (Fig. S7B) by the molecular formula of  $C_{15}H_{18}O_4$  (obs.  $[M + H]^+ = 263.1269$ , calcd. for



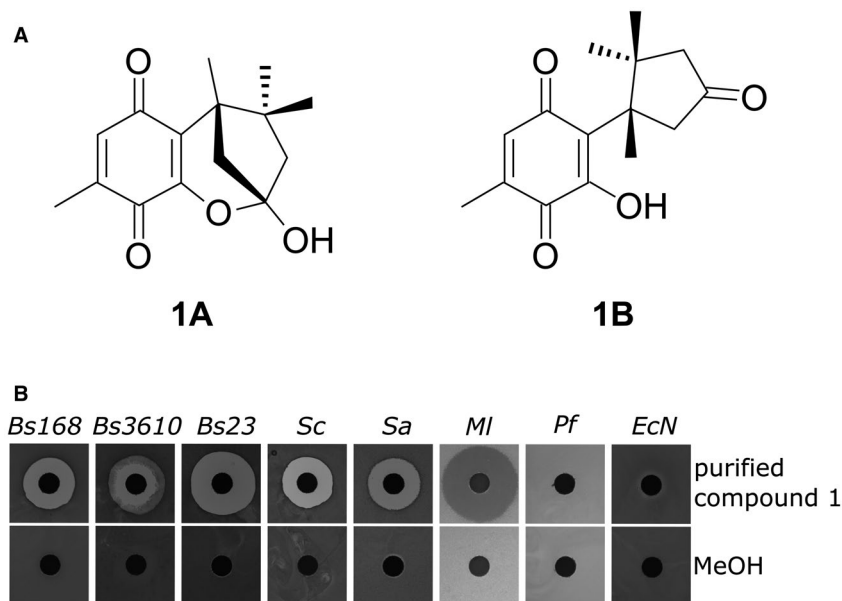
**Fig. 5.** Antibacterial activity of *C. cinerea* crude culture broth extracts against different Gram-positive bacteria.

A. The different *C. cinerea* strains were grown in Erlenmeyer flasks without shaking. The culture broth was extracted with EtOAc and applied for LC-MS/MS analysis. Extracted ion chromatography for  $m/z$  263.1265–263.1291 for compound 1 with the formula  $C_{15}H_{18}O_4$  and its theoretical  $m/z$  263.1278 is represented. This mass was not observed in the crude extract of the *cop6* deletion strain.

B. The MS2 spectrum showed the same fragment ions as the MS2 spectrum from the *B. subtilis* NCIB 3610-induced crude extracts in Figure 3B.

C. The peak area of the extracted ion chromatography for  $m/z$  263.1265–263.1291 for compound 1 of the different culture methods is depicted. Peak area was integrated by XCalibur 3.0 Qual Browser. Crude extracts were prepared from 40 ml of culture broth of the flask cultures (40 times concentrated) and 12 ml of the bead plate cultures (18 times concentrated).

D. Antibacterial activity was assayed by applying the crude extract on blank disks and putting them on a bacterial lawn. Bs 168 stands for *B. subtilis* 168, Bs 3610 for *B. subtilis* NCIB 3610, Bs W23 for *B. subtilis* W23, Sc for *Staphylococcus carnosus* 361, Sa for *Staphylococcus aureus* 113, MI for *Micrococcus luteus*, Pf for *Pseudomonas fluorescens* Pf-5 and EcN for *E. coli* Nissle 1917.



**Fig. 6.** Structure determination and antibacterial activity of *C. cinerea* compound 1.

A. The two possible tautomeric forms of lagopodin B (compound 1) are depicted.

B. Antibacterial activity of the purified compound 1 was assayed by applying 100 µg on blank disks and placing them on a bacterial lawn.

*Bs 168* stands for *B. subtilis* 168, *Bs 3610* for *B. subtilis* NCIB 3610, *Bs W23* for *B. subtilis* W23, *Sc* for *Staphylococcus carnosus* 361, *Sa* for *Staphylococcus aureus* 113, *MI* for *Micrococcus luteus*, *Pf* for *Pseudomonas fluorescens* Pf-5 and *EcN* for *E. coli* Nissle 1917. Blank disks containing only MeOH were used as negative control.

263.1278).  $^1\text{H}$ NMR of the purified compound was nearly identical to that reported for lagopodin B (Bollinger, 1965; Bottom and Siehr, 1975; Bu'Lock and Darbyshire 1976) (Table S4). Two tautomeric forms are possible for lagopodin B, either A or tautomer B (Fig. 6A). Long-range  $^1\text{H}$  to  $^{13}\text{C}$  correlations observed in the gHMBC spectrum were used to differentiate between the two tautomers. Namely, H-9 and Me-11a both showed correlations to a quaternary carbon C10 with a  $^{13}\text{C}$  chemical shift of  $\delta$  108.8 ppm (Fig. S7A and Table S4). Therefore, the material purified under these conditions shows the dominant presence of the hemi-ketal tautomer A. We propose the C7S configuration as reported for lagopodin B. The antibacterial activity of the purified compound 1 was tested using the disk diffusion assay (Fig. 6B) and the same antibacterial activity profile as for the crude extracts was observed.

## Discussion

The increasing number of available fungal genome sequences reveal the potential of fungi to produce SMs (Grigoriev *et al.*, 2011; Lackner *et al.*, 2012; Bills *et al.*, 2013; Sandargo *et al.*, 2019). In basidiomycete genomes, mainly terpene synthase encoding genes are found (Wawrzyn *et al.*, 2012). However, under laboratory conditions, many of these SM biosynthetic genes are

not expressed and, thus, the exact encoded SM often remains unclear (Scherlach and Hertweck, 2009). The six *C. cinerea* sesquiterpene synthases, named COP1 to COP6, were functionally characterized by heterologous expression in yeast by (Agger *et al.*, 2009). The six genes are expressed at low level during axenic vegetative growth, with between 100 and 300 reads per kilo base per million of mapped reads (RPKM) for *cop1*, *cop2*, *cop4* and *cop5* and even below 100 RPKM for *cop2* and *cop6* (Kombrink *et al.*, 2019). The expression of only one of these genes, *cop6*, together with its two neighboring genes *cox1* and *cox2* was found to be induced in co-cultivation with bacteria (Kombrink *et al.*, 2019). These three genes were suggested to be involved in the biosynthesis of the antibacterial sesquiterpene lagopodin B. However, in the heterologous co-expression of *cop6* with the two monooxygenases *cox1* and *cox2* only oxygenated compounds other than lagopodin B were identified (Agger *et al.*, 2009). Unfortunately, no expression data of the *C. cinerea* genes in the heterologous yeast system were presented in this study and it is, therefore, unclear if these three genes are really involved and sufficient for lagopodin B production. In our study, we provide evidence that the *cop6* gene product is required for lagopodin B production in the laboratory strain of *C. cinerea* and confirmation that lagopodin B shows antibacterial activity. Former evidence was recently confirmed by an independent study (Masuya *et al.*, 2019). In accordance with the

previous report on the antibacterial activity of lagopodins (Bastian, 1985), lagopodin B was not active against the tested Gram-negative bacteria, but inhibited the growth of all tested Gram-positive bacterial species, despite the variations in their cell wall structure. This activity spectrum differs from the ones of the *C. cinerea* lysozymes (Kombrink *et al.*, 2019) and cysteine-stabilized  $\alpha\beta$ -defensins (Essig *et al.*, 2014; Kombrink *et al.*, 2019) which both target the cell wall or biosynthesis intermediates thereof. As a benzoquinone derivative, lagopodin B is likely to act as a Michael acceptor of functionally relevant thiol groups in proteins (Nakagawa *et al.*, 2017; König *et al.*, 2019). Such a mechanism is supported by abolishment of the antibacterial activity of lagopodin B by the addition of L-cysteine (Bastian, 1985). Thus, the difference between the susceptibilities of Gram-positive and Gram-negative bacteria might be due to the different accessibility of target proteins in the two types of bacteria due to the absence and presence of an outer membrane respectively.

Lagopodin B was originally purified from axenic shake flask cultures of different *Coprinopsis* species (Bollinger, 1965; Bastian, 1985). We purified lagopodin B from the supernatant of an axenic static flask culture of the *C. cinerea* laboratory strain AmutBmut where only part of the mycelium grew submerged. In this culture setup, the expression of the *cop6* gene cluster was induced as compared to the axenic glass bead culture. It is known that fungal metabolite profiles can vary using different culturing conditions (Scherlach and Hertweck, 2009). As an example, starvation induced the expression of a *C. cinerea* gene coding for a nematotoxic lectin (Bertossa *et al.*, 2004). This was, however, not the case for the *cop6* gene under the starvation conditions used in this study. We conclude that in addition to the biotic trigger, i.e. the co-cultivation with bacteria, also abiotic triggers can lead to lagopodin B production. However, this was not a general feature of bacteria-induced genes, as *lys1*, *psp* and a laccase gene, all induced in co-culture with bacteria (Kombrink *et al.*, 2019), were not expressed in the static flask culture. This finding could be an indication for a function of lagopodin B in addition or alternatively to its role in antibiosis. For example, the compound could act as an auto- or paracrine signaling molecule under axenic conditions.

Intriguingly, soluble compounds present in the supernatant of *B. subtilis* cultures were sufficient to induce the expression of the *cop6* gene and the other tested bacteria-induced genes (*lys1* and *psp*) of *C. cinerea*. The isolation and identification of the inducing compound(s) is the main goal of a current follow-up study. Bacteria-derived molecules known to elicit a response in fungi and, thus, candidates for the induction of antibacterial defense in *C. cinerea*, are peptidoglycan fragments (Xu *et al.*, 2008; Svahn *et al.*, 2014), lipoteichoic acids, lipopolysaccharide (Svahn *et al.*, 2014), lipo-oligosaccharides (Ipcho *et al.*,

2016) and bacterial quorum sensing molecules (Hogan *et al.*, 2004; Wang *et al.*, 2004). Our results differ from the ones described by Schroeckh *et al.* (2009) where physical contact between the ascomycete *Aspergillus nidulans* and specific *Streptomyces* strains was necessary to induce the expression of SM gene clusters in the fungus.

Interestingly, lagopodin B, as well as the previously characterized *C. cinerea* lysozymes and cysteine-stabilized  $\alpha\beta$ -defensins (Essig *et al.*, 2014; Kombrink *et al.*, 2019), were mainly active against Gram-positive bacteria although both Gram-positive and Gram-negative bacteria were able to induce the *C. cinerea* antibacterial defense response. We, therefore, consider the observed response in *C. cinerea*, a general antibacterial defense response rather than a specific response against Gram-positive bacteria. It remains to be seen whether any of the other bacterially induced *C. cinerea* genes may contribute to the defense of this fungus against Gram-negative bacteria. In this regard, we did not experimentally assess a possible selective advantage of the *C. cinerea* wt strain compared to the *cop6* deletion strain in its competitiveness toward Gram-positive bacteria since *C. cinerea* produces additional defense molecules against this type of bacteria under these cultivation conditions (Essig *et al.*, 2014; Kombrink *et al.*, 2019).

Finally, we also studied the spatial distribution of *cop6* induction within the *C. cinerea* mycelium and found that the gene is only expressed in hyphae that were directly exposed to the bacterial culture supernatant. This is in marked contrast to the propagation of the induction of antinematode genes upon challenge of the same organism with fungivorous nematodes (Schmieder *et al.*, 2019). The difference might reflect the higher motility of nematodes compared to bacteria necessitating a systemic fungal defense in order to avoid death of the entire fungal colony. In contrast, bacteria are not evenly distributed in the substrate but mainly occur within water-filled gaps (Young and Crawford, 2004; Crawford *et al.*, 2005). Therefore, it might suffice and be economically advantageous for the fungus if the SM gene cluster is expressed only when and where its product is needed. We speculate that the local concentration of the antibiotic at these induction sites is sufficient to kill bacteria, whereas the concentration of the antibiotic in the supernatant of a bacterially induced mycelium is not. The difference between the propagation of the antinematode and antibacterial defense response might also have to do with the fact that antibiosis molecules against predatory nematodes are usually kept intracellular, whereas antibacterials are usually secreted and thus spread in the immediate environment providing another type of systemic defense (Künzler, 2018).

In summary, our results suggest that fungi possess sophisticated molecular mechanisms to adjust their chemical defense to specific antagonists. Insight into these mechanisms will tell us about the evolution of this

defense system in comparison to the innate immune systems of plants and animals and will allow us to characterize more fungal defense chemicals and their applications in fighting plant and animal pathogens and parasites.

## Experimental procedures

### Strains and cultivation conditions

Fungal and bacterial strains used in this study are summarized in Table S1. *C. cinerea* strains were cultivated on solid yeast extract-maltose-glucose (YMG) medium at 37°C in aerated, dark and humid boxes. *Saccharomyces cerevisiae* W303a was cultivated on yeast extract-peptone-dextrose (YPD) medium and was used for homologous recombination of plasmids in which case it was selected on synthetic complete dextrose without uracil (SD Ura-) medium. *E. coli* DH5 $\alpha$  was used for cloning and maintenance of plasmids. Preparation of competent cells and transformations were carried out as described by Inoue *et al.* (1990). *E. coli* DH5 $\alpha$  containing plasmids was cultivated on Luria Bertani (LB) medium containing 100  $\mu\text{g ml}^{-1}$  ampicillin at 37°C. The sequences of all plasmids that were PCR-generated were confirmed by DNA sequencing (Microsynth). Bacterial strains used for disk diffusion assays were cultivated on LB medium except for *Micrococcus luteus* which was cultivated on nutrient broth (DifcoTM, Becton Dickinson).

### Construction of *C. cinerea* promoter-reporter fusion strains

The promoter regions of the following four bacterially induced genes were fused to the reporter gene dTomato: the terpene synthase encoding gene *cop6* (JGI protein ID 394772; Broad gene number CC1G\_03563), the lysozyme-encoding gene *lys1* (JGI protein ID 432813; Broad gene number CC1G\_03076) and a gene encoding a secreted protein *psp* (JGI protein ID 417969; Broad gene number CC1G\_08057). The region between the start codon and the 3'-UTR of the upstream gene was considered as 'promoter' region. The promoter regions for *cop6*, *lys1* and *psp* were PCR-amplified from *C. cinerea* AmutBmut gDNA using the primer pairs P03563\_f/P03563\_r, P03076\_f/P03076\_r and P08057\_f/P08057\_r respectively. The primers contained homology regions for recombination into plasmid pMA412 (Stanley *et al.*, 2014) to exchange pABgpdII with the promoter regions of the bacteria-induced genes. pMA412 was linearized using the restriction enzyme *Clal*. Homologous recombination was carried out in *S. cerevisiae* W303a as described previously (Wälti *et al.*, 2006) and resulted in plasmid pMA1069 for the *cop6* construct, pMA1070 for the *lys1* construct and pMA1074 for the *psp* construct. The plasmids were transformed into *C. cinerea* AmutBmut by protoplasting of mononucleate asexual spores as described in (Granado *et al.*, 1997) for ectopic integration into the *C. cinerea* genome.

### Construction of a *C. cinerea* knockout strain in *cop6*

The deletion of the terpene synthase *cop6* gene was carried out in the *C. cinerea* AmutBmut  $\Delta ku70$  strain ku3-24 27 in an analogous manner to that described in (Stöckli *et al.*, 2017). A detailed description of the procedure can be found in the Supporting Information.

### Total RNA extraction and qRT-PCR analysis

To monitor the expression of the terpene gene cluster under various growth conditions, RNA was extracted from the fungal mycelium and cDNA was prepared as described previously (Plaza *et al.*, 2014). qRT-PCR and data analysis was performed as described by Stöckli *et al.* (2017) using the primers listed in Table S2.

### LC-MS/MS analysis

Samples were analyzed on a calibrated Q Exactive mass spectrometer (Thermo Fischer Scientific) coupled to a Thermo Dionex Ultimate 3000 UHPLC system (Thermo Fischer Scientific). A Kinetex XB-C18 core-shell column with 2.6  $\mu\text{m}$  particle size, 100 Ångström pore size and dimensions of 150  $\times$  4.6 mm (Phenomenex) was used as the stationary phase. Two buffers, buffer A (H<sub>2</sub>O containing 0.1% FA) and buffer B (ACN containing 0.1% FA), used as the mobile phase. The samples were eluted with a flow rate of 0.3 ml/min, using a gradient from 0% to 5% buffer B in 2 min, from 5% to 99% buffer B in 18 min and then 99% buffer B for 5 min. The column oven was set to 50°C. Both LC and MS were controlled by XCalibur 2.2 SP1 software with the following parameters for MS1 (*m/z* range: 113–1700, resolution was set to 70,000 at 200 *m/z*) and MS2 (resolution was set to 17,500 at 200 *m/z* Top10, HCD 35, 40, 45, with an isolation window 4 *m/z*).

### Disk diffusion assay

To assess the antibacterial activity (bioactivity) of the crude extracts and purified compound **1**, disk diffusion assays were performed. A single colony of the test organism was inoculated in 10 ml LB medium (37°C, 6 h, 180 rpm) except for *P. fluorescens* and *M. luteus* (28°C, 18 h, 180 rpm). The bacterial culture was diluted in 8 ml prewarmed (42°C) water agar (1%) to optical density at 600 nm (OD<sub>600</sub>) 0.1, poured on a LB agar plate and solidified. A sterile paper filter disk (Oxoid) was loaded with 40  $\mu\text{l}$  in case of the crude extracts of the bead plates and 20  $\mu\text{l}$  in case of the crude extracts of the liquid cultures or 100  $\mu\text{g}$  of the purified compound dissolved in MeOH, air dried and placed on the bacterial lawn. The plates were incubated overnight at 28°C.

### Assessment of gene expression using reporter strains: Immunoblotting of protein extracts

*C. cinerea* reporter strains were grown on glass bead plates as described previously (Essig *et al.*, 2014) with some modifications. Briefly, an agar plug containing *C.*

*cinerea* mycelium grown on YMG agar (37°C, 3 days) was inoculated in a Petri dish (55 mm in diameter) containing 13 g borosilicate glass beads (5 mm in diameter, Sigma-Aldrich) and 5 ml of CCMM pH 6.4. After incubation of the plates (28°C, 2.5 days in the dark) either a treatment solution or bacteria were added to the plates and the plates were further incubated (28°C, 12 h in the dark). Bacteria were grown in CCMM pH 6.4 to an OD<sub>600</sub> of 0.3, pelleted and resuspended in the fungal medium to an end OD<sub>600</sub> of 0.1 for *E. coli* Nissle 1917 and 0.2 for *B. subtilis* NCIB 3610. Bacteria were killed by exposure to ultra violet (UV) light as described by Stanley *et al.* (2014). Cell-free supernatant was prepared from a bacterial culture grown in CCMM pH 6.4 in non-buffed Erlenmeyer flasks (28°C, 24 h, 180 rpm) with a start OD<sub>600</sub> of 0.1. The bacteria were pelleted (4°C, 10 min, 5000 rpm), the supernatant sterilized by filtration through a syringe filter (TPP, Switzerland) with a pore size of 0.22 µm and dried by lyophilization. The dried cell-free supernatant was extracted with one volume of MeOH by vortexing (4°C, 12 h, 2000 rpm) and the insoluble part precipitated by centrifugation (4°C, 15 min, 5000 rpm). MeOH from the soluble part was evaporated under reduced pressure and the dried film resuspended in CCMM pH 6.4 15× concentrated. About 400 µl of this suspension was added to the bead plates. The whole cell proteins (WCPs) of the fungal mycelium were extracted as described previously (Stöckli *et al.*, 2017). WCP samples were boiled in Lämmli Buffer, run on a 12% polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was probed with a 1:5000 dilution of the primary dTomato antibody (anti-Red Fluorescent Protein AA 234 antibody, antibodies-online GmbH) and a 1:3000 dilution of the secondary antibody (horseradish peroxidase conjugated goat anti-rabbit immunoglobulin G, Santa Cruz Biotechnology). Immunoblots were developed using SuperSignal West Dura Extended Duration Substrate (Perbio Science) and light-sensitive films (Super RX, Fujifilm).

#### *Assessment of gene expression using reporter strains: fluorescence microscopy*

Microfluidic devices were prepared and inoculated with fungal mycelium as described previously (Stanley *et al.*, 2014) with some minor modifications. Briefly, inlet and outlet holes were punched into the poly(dimethylsiloxane) (PDMS) using a precision cutter (Syneo) having a cutting edge diameter of 2.49 mm. Bonding of the PDMS top layer, containing the microchannels, was sealed to the glass-bottomed petri dish using a Diener ZEPTO plasma cleaner (Diener electronic) under the following conditions: power 50%; coating time, 1 min. The device with the fungal inoculum was incubated at 28°C until hyphae blocked the constriction channels of the exchange box, which took approximately 24 to 30 h. For the exchange of the fluid in the microfluidic platform, the medium in the inlet and outlet holes was removed and 10 µl of the new fluid was introduced into the inlet, incubated for 1 min, removed and again 10 µl of the new fluid added. Small differences in hydrostatic pressure, therefore, drive the flow and enable

the media surrounding the hyphae to be exchanged with the new fluid. Live-cell imaging of hyphae was performed as described by Stanley *et al.* (2014) except that an exposure time of 300 ms was used during epifluorescence microscopy time-lapse experiments with TRITC filters. Time frame between time points was 30 min. NIS-Elements Advanced Research imaging software (Nikon) with autofocus was used to synchronize long-term, multi-position, time-lapse imaging experiments. The images in the ND document file were exported to Tagged Image File Format (TIFF) files using NIS-Elements Viewer (version 4.20). The images were stitched using custom software and analyzed using Fiji (Schindelin *et al.*, 2012). Detailed description of image analysis can be found in the supplementary data files.

#### *Bacterial challenge of C. cinerea for qRT-PCR and metabolite analysis*

To record the difference of the metabolite profile produced by *C. cinerea* in presence and absence of bacteria *C. cinerea* was grown on glass bead plates (diameter 92 mm) as described previously (Essig *et al.*, 2014) filled with 15 ml CCMM pH 6.4. Three mycelial plugs were inoculated per glass bead plate and the plates were incubated (28°C, 3 days in a dark humid environment). *B. subtilis* NCIB3610 was grown to an OD<sub>600</sub> of 0.3 in CCMM pH 6.4 and inoculated to an OD<sub>600</sub> of 0.1 in the glass bead plates as described above. The co-culture and the control plates without bacteria were further incubated (28°C, 12 h in the dark). To monitor the expression of the terpene gene cluster in the presence and absence of bacteria, RNA was extracted from the fungal mycelium and cDNA was prepared for qRT-PCR analysis as described above.

To analyze the metabolite profile produced by the fungus in the presence and absence of *B. subtilis* NCIB 3610, the culture supernatant (12 ml) was extracted with EtOAc. For this purpose, the culture supernatant was centrifuged (4°C, 10 min, 5000 rpm) to pellet the bacteria and sterilized by filtration through a syringe filter (TPP) with a pore size of 0.22 µm and the supernatant extracted (EtOAc, 2× 12 ml). The residue obtained after evaporation under reduced pressure was taken up in MeOH and subjected to LC-MS/MS analysis or its antibacterial activity tested on the disk diffusion assay.

#### *Test of different C. cinerea cultivation conditions for induction of bacteria-induced genes by qRT-PCR analysis*

To assess the different expression of the putative defense genes under different cultivation conditions, one mycelial plug of *C. cinerea* AmutBmut was inoculated either on the glass bead plate (55 cm diameter) with 5 ml CCMM pH 6.4 or in a 100 ml Erlenmeyer flask containing 5 ml CCMM pH 6.4 and incubated (37°C or 28°C, 3 days in a dark humid environment, without shaking). The fungal mycelium was collected, shock frozen and RNA was extracted for qRT-PCR analysis as described above.

### Production of compound 1 in static Erlenmeyer flasks

To produce higher quantities of compound 1, four mycelial plugs of *C. cinerea* were inoculated in 20 ml CCMM pH 6.4 in a 500 ml Erlenmeyer flask and incubated (37°C without shaking, in the dark) to allow mycelial mats form. Then 25 ml of CCMM pH 6.4 was added to the flasks and they were shifted to 28°C until the culture broth appeared colored. The fungal mycelium was separated from the culture broth by filtration and extracted with EtOAc (2 × 40 ml). The crude extract was dried by evaporation under reduced pressure as described above.

### Isolation of compound 1

For the purification of compound 1, 3 L of culture was grown as described for the liquid culture assay. The culture broth was extracted with EtOAc (2 × 1 L), and the solvent was evaporated under reduced pressure to give the crude extract (48 mg). The crude extract was subjected to two successive HPLC runs using two buffer solutions: buffer A (H<sub>2</sub>O with 0.1% formic acid) and B (CH<sub>3</sub>CN with 0.1% formic acid). The first run was performed on a preparative HPLC using a Phenomenex Luna C18(2) column (5 µm particle size, 100 Å pore size, and dimensions of 250 × 21.2 mm), a flow rate of 21.2 ml/min, and a gradient starting at 25% buffer B for 1 min and then ramped to 100% buffer B over 30 min. UV detection was set at 270 nm. The major peak was collected and the solvent evaporated under reduced pressure. The activity was confirmed by disk diffusion assay using *B. subtilis* 168 as the test organism. The second purification step was carried out by HPLC using a Phenomenex Luna C18(2) column (5 µm particle size, 100 Å pore size and dimensions of 250 × 10 mm), a flow rate of 2 ml/min and a mobile phase of 46% buffer A and 54% buffer B for 30 min. The major peak was collected and the solvent evaporated under reduced pressure to give compound 1 (4.0 mg). Activity was confirmed by disk diffusion assay and the remaining sample was used for NMR analysis.

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### Data availability

Data are available on request from the authors.

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### Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.