Journal of Plant Diseases and Protection (2024) 131:1271–1277
https://doi.org/10.1007/s41348-024-00905-1

T.C. : REINHARDSBRUNN SYMPOSIUM COLLECTION

Metabolic re-programming in confrontations of *Colletotrichum graminicola* and *Aspergillus nidulans* with *Bacillus* biocontrol agents

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Received: 14 September 2023 / Accepted: 5 March 2024 / Published online: 5 April 2024

Abstract

We established confrontations between two different fungi, i.e., the maize anthracnose and stalk rot pathogen *Colletotrichum graminicola*, and the ubiquitous fungus *Aspergillus nidulans*, and different biocontrol species, i.e., *Bacillus subtilis*, *Bacillus velezensis*, and *Bacillus amyloliquefaciens*. In all fungus–bacterium confrontations tested, growth arrest and, thus, distance inhibition was observed on solid substrata. LC–MS/MS analyses of culture filtrates suggested formation of several metabolites only synthesized in confrontations. Interestingly, microscopy of fungal hyphae grown in liquid medium showed protrusions and color changes occurred only in media harboring fungus-bacterium confrontations. These observations indicate metabolic re-programming and suggest formation of putative secondary metabolites in interactions involving microbial biocontrol agents.

Keywords Antagonistic microorganisms · *Aspergillus nidulans* · *Bacillus* spp. · *Colletotrichum graminicola* · Microbial biological control agents · Secondary metabolism

Introduction

The extensive use of synthetic fungicides in agriculture has since long raised concerns regarding environmental and consumer safety (Bruhn et al. 1992). As a consequence, presumably eco-friendly microbial biological control agents (MBCAs) have received considerable global attention (Barratt et al. 2018). MBCAs suppress plant pathogens by various indirect or direct modes of action (Köhl et al. 2019), including induction of resistance and priming (Pieterse et al. 2014; Conrath et al. 2015), competition for space and nutrients (Spadaro and Droby 2016), and formation of chemically diverse antimicrobial secondary metabolites (SMs) such as polyketides, nonribosomal peptides, indole alkaloids, and/or terpenes (Künzler 2018; Brakhage 2013; Keller 2019).

In microorganisms, genes encoding SM-synthesizing enzymes are usually organized in clusters (Lind et al. 2017). In many cases, these SM clusters are silent under laboratory conditions, and neither the chemical structure nor the toxicity of the respective products are known. In confrontations, however, metabolic re-programming may lead to activation of an unknown number of SM gene clusters (Schroeckh et al. 2009; Netzker et al. 2015) and, increasing the complexity of the compounds synthesized, metabolites produced by one microorganism may be taken up by another, further modified, and secreted again (Deising et al. 2017). Indeed, numerous experiments demonstrated that chemistries hitherto unreported in databases are produced in co-cultures (Zuck et al. 2011; Sun et al. 2021; Shang et al. 2017). Following this line of arguments, co-cultivations of two

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biocontrol species, i.e., *Trichoderma asperellum* and *Bacillus amyloliquefaciens*, were employed to enhance metabolite formation and improvement of biocontrol activity (Karuppiah et al. 2019). However, the species complexity on leaves significantly exceeds that of these defined experiments, with an estimate of 10^6–10^7 bacteria residing on one cm^2 of leaf surface (Vorholt 2012) and a lower but yet significant number of fungal propagules (Lindow and Brandl 2003). Moreover, environmental samples collected from cherry fruits, from apple leaves and flowers, and from soil from different apple and cherry orchards in Switzerland revealed a microbial complexity of 47 taxa in 376 isolates (Magoye et al. 2020). The large number of microorganisms occurring in natural and agricultural environments clearly highlights the unpredictable numbers of chemistries formed, and this complexity would be fostered by employing antagonistic microorganisms in crop protection. It may be considered naïve to assume that formation of putatively toxic microbial SMs might not have the potential to harm consumers and/or the environment. Therefore, it is important to analyze changes in microbial metabolism in confrontations.

Here, we describe confrontations between the filamentous fungi *Aspergillus nidulans* and *Colletotrichum graminicola* with the biocontrol bacteria *Bacillus amyloliquefaciens*, *Bacillus velezensis*, and *Bacillus subtilis*. *Bacillus* species caused growth arrest, and swellings of hyphae of the maize pathogen *Colletotrichum graminicola*, but not of those of *A. nidulans*. Color changes in media harboring both confrontation partners strongly suggested SM formation. Indeed, color changes in and LC–MS/MS analyses of culture filtrates revealed dramatic metabolic re-programming in confrontations and strongly suggest SM formation.

Results

Confrontations between *C. graminicola* and different *Bacillus* species on minimal medium cause formation of hyphal conglobations

On minimal medium agar (MMA), *C. graminicola* grows radially and develops filamentous hyphae, as shown by Differential Interference Contrast (DIC) and fluorescence microscopy after Calcofluor White (CFW) staining (Fig. 1A). Fungal colonies exhibit only background auto-fluorescence under UV light (Fig. 1A). By contrast, all *Bacillus* species tested exhibited massive fluorescence (Fig. 1B, green arrow). The fact that fluorescence surrounded the bacterial colony suggested that fluorescing compounds are constitutively secreted into the medium and that *C. graminicola* encounters these and other compounds at confrontation sites. Indeed, microscopy of fungal hyphae confronting colonies of *B. amyloliquefaciens*, *B. subtilis*, or *B. velezensis* revealed severe swellings, similar as those known to be induced by cyclic lipopeptides such as iturin A or plipastatin A (Gong et al. 2015), which are produced by *Bacillus* species (Fig. 1C–E, arrowheads). Fluorescence surrounding *Bacillus* colonies and occurrence of hyphal conglobations at confrontation sites revealed that secondary metabolites are present in the inhibition zone. Intriguingly, in interactions with *B. amyloliquefaciens* and *B. velezensis*, but not in the interaction with *B. subtilis*, a red pigment was formed (Fig. 1E, red arrow), suggesting differential secretion of SM(s) at specific confrontations.

Not only on solid substrata, but also in liquid medium confronting interactions between *C. graminicola* and *A. nidulans* with the biocontrol bacterium *B. amyloliquefaciens* were observed (Fig. 2). In co-cultures, both fungi formed smaller ball-like colonies, in comparison to large-diameter colonies formed in solo-cultures (Fig. 2; compare mono- and co-cultures, left panel). Moreover, like on solid substrata, hyphae of the maize anthracnose fungus *C. graminicola* exhibited large protrusions in co-cultures in liquid potato dextrose broth (PDB), rather than hyphal filaments, as revealed by DIC and fluorescence microscopy after CFW staining (Fig. 2; *C. graminicola* + *B. amyloliquefaciens*, blue arrowheads; compare with Fig. 1; *C. graminicola* vs *B. amyloliquefaciens*, DIC and CFW, white arrowheads). Intriguingly, by contrast, hyphal morphology of *A. nidulans* appeared to be not affected by the biocontrol bacterium (Fig. 2; *A. nidulans* + *B. amyloliquefaciens*, DIC and CFW).

Confrontational metabolome of liquid co-cultures of *C. graminicola* and *B. amyloliquefaciens* indicated major metabolic re-programming

Changes in pigmentation, presumably indicative of altered SM, were clearly seen in culture filtrates obtained from the confrontations, but not or to a lesser extent in those of solo-cultures (Fig. 3A). In order to detect metabolic differences of mono-cultures of *C. graminicola* and co-cultures of this fungus with *B. amyloliquefaciens*, the culture filtrates were analyzed by LC–MS/MS, and the data were compared using the GNPS platform (Allard et al. 2016). The resulting molecular network of the co-cultivated *C. graminicola* + *B. amyloliquefaciens* samples consisted of 417 nodes representing compound ions, as compared with 310 and 315 ions detected in the mono-cultures of *C. graminicola* and *B. amyloliquefaciens*, respectively (Fig. 3B). The global molecular networking-based Venn diagram displays the ion distribution obtained from culture filtrates of mono- and co-cultures (Fig. 2C). Importantly, 96 ions, many of which belong to the fatty acyls according to analysis with the software SIRIUS (https://bio.informatik.uni-jena.de/software/sirius/), were only present in the co-culture. Several ions indicative of highly unsaturated compounds as well

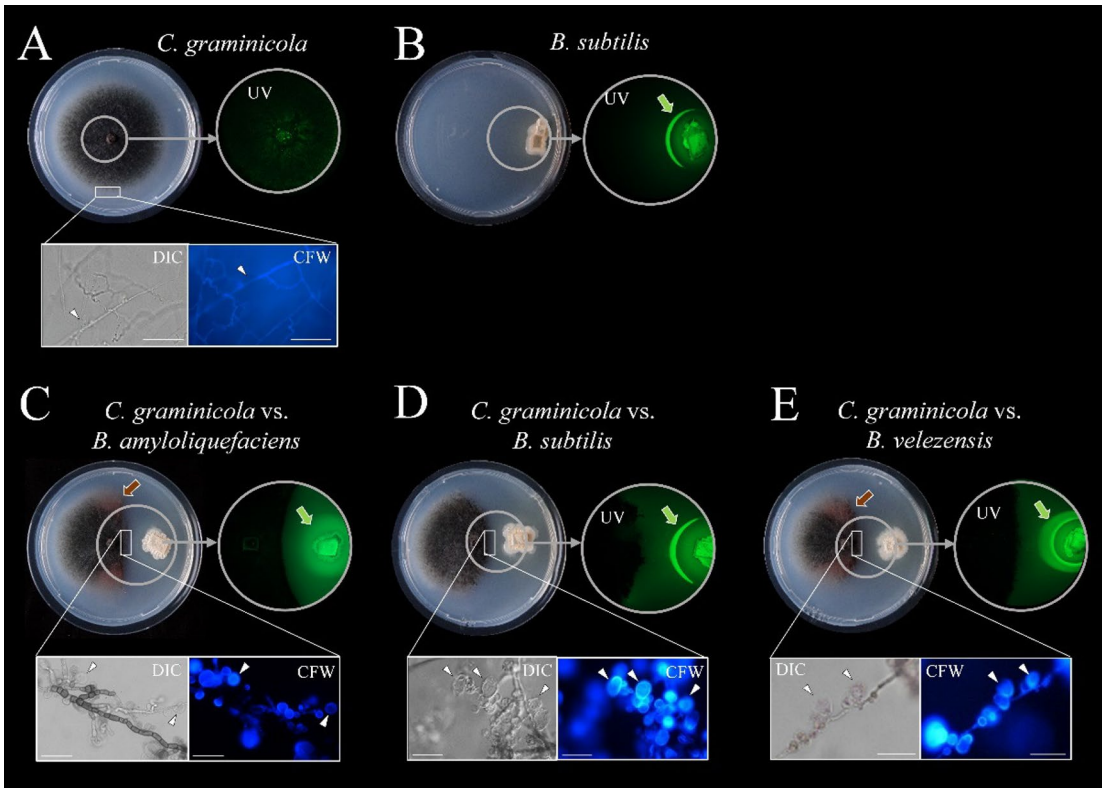

Fig. 1 In vitro microbial confrontations of the maize pathogen *Colletotrichum graminicola* and biocontrol *Bacillus* spp. on minimal medium agar at 12 dpi. **A**, **B** show solo-cultures of *C. graminicola* and *B. subtilis*. **C**–**E** show confrontations between *C. graminicola* and *B. amyloliquefaciens*, *B. subtilis*, and *B. velezensis*. Circled areas of plates are shown under UV light excitation; DIC and fluorescence microscopy after CFW staining of hyphae in rectangles are shown below the petri dishes. Arrowheads in **A** indicate smooth hyphae of the fungus without and arrowheads in **C**–**E** indicate protrusions of hyphae confronting biocontrol bacteria. Green arrows indicate fluorescing chemistries secreted by *Bacillus* strains, and red arrows point at red discoloration of fungal mycelia. Size bars are 20 µm

as α-amino acids and their derivatives were formed by *B. amyloliquefaciens* monocultures (Fig. 2B and C). Collectively, LC–MS/MS analyses showed the enormous plasticity of metabolic responses in this microbial confrontation.

Discussion

Consumer-safe plant protection measures are of utmost importance. This is accomplished by strict legislation of synthetic pesticides to be employed in pathogen, pest or weed control (Regulation (EC) 1107/2009 on the Placing of Plant Protection Products on the Market; https://www.europarl.europa.eu/RegData/etudes/STUD/2018/615668/EPRS_STU(2018)615668_EN.pdf). While testing consumer and environmental toxicity of defined chemistries is easily accomplishable, evaluation of antagonistic microorganisms is difficult, as it is unclear which SM clusters are activated under confrontations with numerous distinct partners. The difficulty is particularly evident in multi-membered confrontations, e.g., on densely inhabited plant surfaces (Lindow and Brandl 2003; Vorholt 2012). The exchange and further modification of SMs has been demonstrated with melanin biosynthesis mutants of two distinct *Colletotrichum* species exhibiting distinct melanin biosynthesis defects (Deising et al. 2017). In a multi-membered consortium, however, one may expect an enormous increase in SM complexity, as compared with co-cultures consisting of two members only. Indeed, in a microcosm experiment employing three rhizosphere bacteria, i.e., *Bacillus cereus*, *Flavobacterium johnsoniae*, and *Pseudomonas koreensis*, Handelsman and co-workers showed that the dynamics of secondary metabolism depends on community species composition and interspecies interactions, indicating that microbiome interactions may shape the SM composition in nature (Chrevette et al. 2022). These experiments also suggest that introducing biocontrol agents into a pre-shaped complex microbiome would significantly impact SM formation.

In order to evaluate whether or not SMs are formed in consortia consisting of two or several partners, a simple method yielding a clear read-out would be beneficial. Intriguingly, as SM formation is epigenetically controlled in fungi (Connolly et al. 2013), it was not surprising that histone deacetylase inhibitors strongly modified

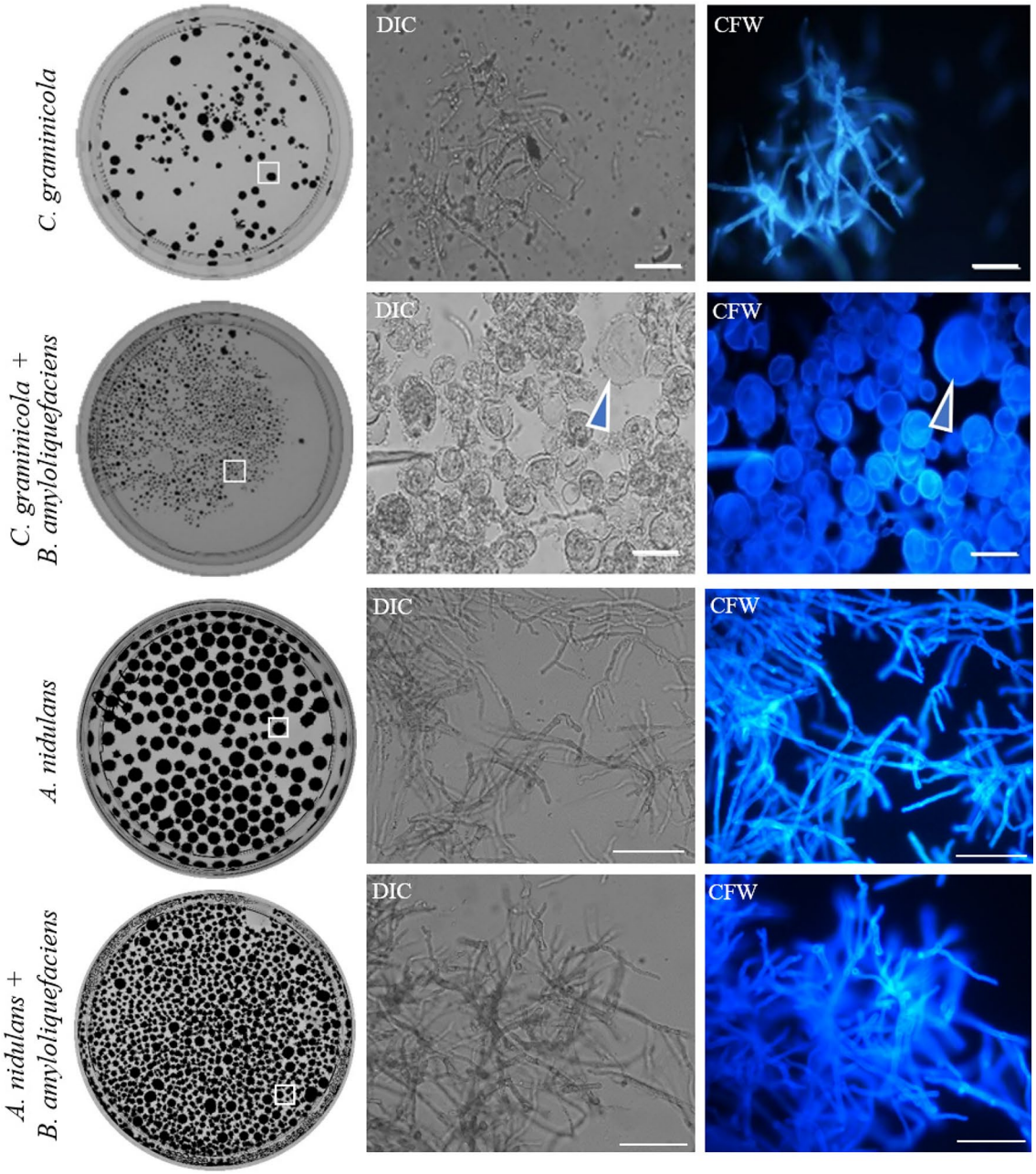

Fig. 2 Phenotypical and microscopical observation of micro-organismic interaction between *C. graminicola* and *A. nidulans* with *B. amyloliquefaciens* in potato dextrose broth. Photographs were taken at 12 dpi. Mycelial balls from the controls and confrontations are located on the left panel (squares on left panel); microscopy of fungal hyphae by DIC and fluorescence microscopy after CFW staining is shown on the right panel. Arrowhead indicates large hyphal swelling. Size bars are 20 µm

SM formation and that these modifications were readily indicated by the color of the culture filtrates of *Aspergillus nidulans* (Albright et al. 2015). The assumption that microbial confrontations would likewise modify SM production led to the discovery in the work described here that SM formation can easily be visualized in liquid media. The method described here would be applicable to two- or multi-membered consortia and may allow preliminary evaluation of consumers' risks imposed by biological control agents at early stages of legislation.

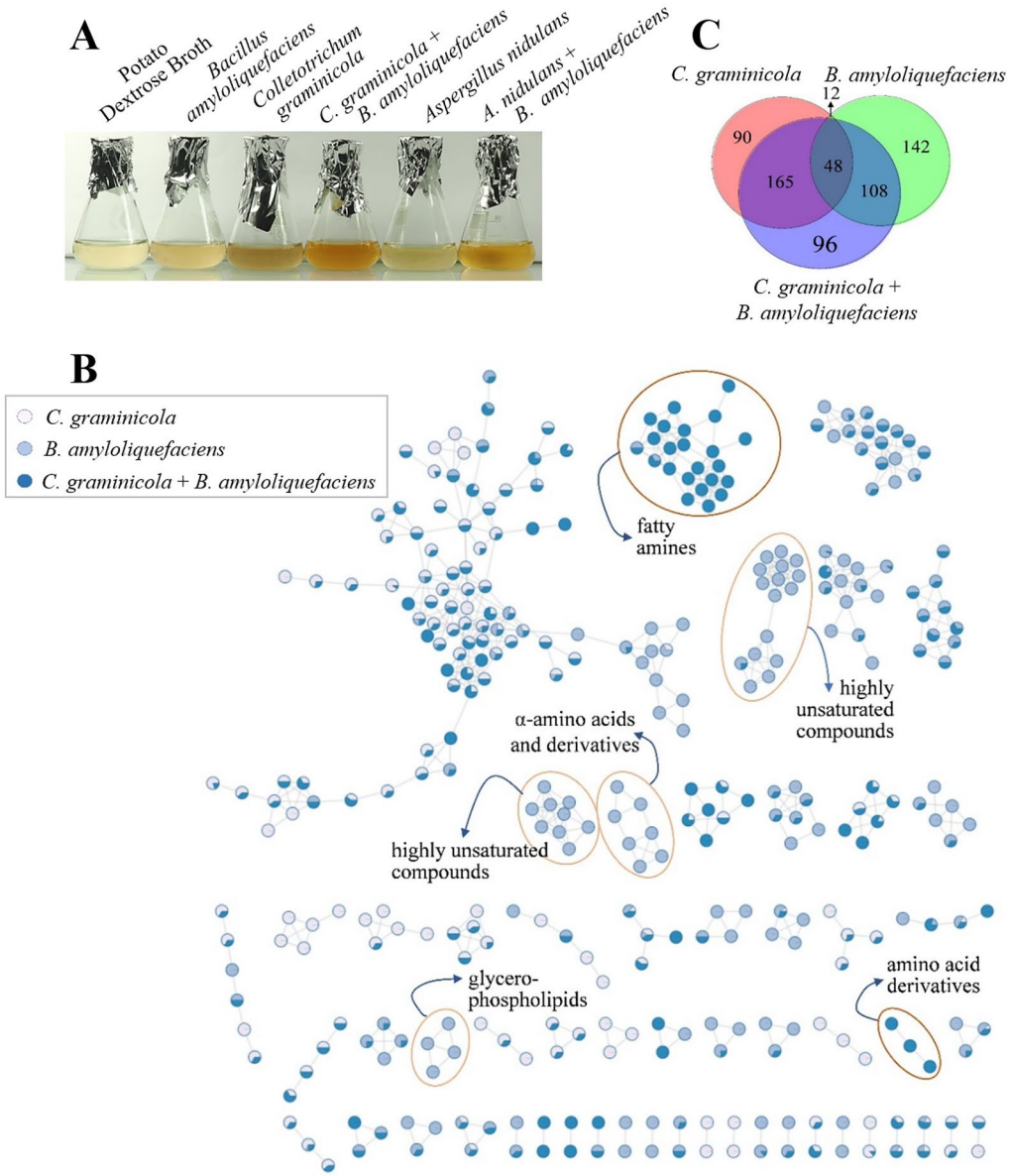

Fig. 3 Metabolomic re-programming in liquid cultures under confrontations at 12 dpi. **A** Induction of secondary metabolite formation in *C. graminicola* and *A. nidulans* co-cultivated with *B. amyloliquefaciens*, in comparison with the respective solo cultures. **B** Global molecular network of co-culture between *C. graminicola* and *B. amyloliquefaciens.* White nodes, solo-cultures from *C. graminicola*; light-blue nodes, solo-cultures from *B. amyloliquefaciens*; dark-blue nodes, co-cultures. Two-clusters (fatty amines and amino acid derivatives) are detected only under co-cultivation of fungus and bacterium. **C** Venn diagram based on the molecular network. Red, ions obtained from fungal solo-culture; green, ions obtained from bacterial solo-culture; purple, ions obtained from co-culture (96 unique ions for co-cultures)

Materials and methods

In vitro confrontation assay

Antagonism studies were performed on agar plates containing Minimal Medium Agar (MMA; Oliveira-Garcia and Deising 2013). *Bacillus amyloliquefaciens* (JKI-BI-7332/2) and *Bacillus subtilis* (JKI-BI 7325/1) provided by Ada Linkies (Julius-Kühn-Institute, Institute for Biological Control, Dossenheim, Germany), and *Bacillus velezensis* (NCCB 100737) obtained from Westerdijk Fungal biodiversity Institute (Utrecht, The Netherlands) were used as biocontrol strains. To study the antagonistic activity, a 4 mm mycelial disk of a 14-day-old *Colletotrichum graminicola* M2 culture growing on Oat Meal Agar (OMA) was used to inoculate MMA plates, and the bacterial strains were placed 2.5 cm away from the mycelial disk. All combinations were done in

triplicates; plates were incubated at 23 °C and photographed 12-day post-inoculation (dpi).

Co-cultivation of microbes in liquid minimal medium and secondary metabolite extraction

Three mycelial plugs (4 mm diameter) from *Aspergillus nidulans* RMS011 or *Colletotrichum graminicola* M2 were excised from 2-week-old potato dextrose agar (PDA) plates and transferred into sterile potato dextrose broth (PDB). After 4 days of incubation at 28 °C, a single plug (4 mm^2) from a one-week-old PDA plate with *B. amyloliquefaciens* was excised and co-cultivated with the fungus. The incubation was carried out for 8 days. After the designated time period of 12 days, the samples were filtered through sterile syringe filters (ø 0.2 µm, Sartorius Stedim Biotech GmbH, Göttingen, Germany), and the obtained culture filtrates were photographed and used for metabolite extraction. SMs were enriched from culture filtrates using solid phase chromatography extraction (SPE) on a Chromabond C18 column (Macherey–Nagel, Düren, Germany). The column was conditioned with 1 ml of methanol (MeOH), followed by 1 ml of 2% (v/v) formic acid. The culture filtrate was loaded onto the column; the column was washed twice with 1 ml of water and eluted twice with 1 ml of MeOH containing 2% (v/v) formic acid. The eluates were dried under vacuum (Concentrator Plus, Eppendorf AG, Hamburg, Germany) for 6 h at 40 °C and subsequently dissolved in 70% (v/v) MeOH in an ultrasonic bath (Elmasonic S 100H, SK Sonic, Mörfelden-Walldorf, Germany) for 30 min. The eluates were then centrifuged at 13,000 × *g* for 10 min at 20 °C.

LC–MS/MS analysis

For compound comparisons, the samples were analyzed using a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Germany) equipped with a heated electrospray ionization (pos. mode) interface coupled to an Ultimate 3000 HPLC system (Thermo Fischer Scientific, Germany). The capillary temperature was set to 350 °C, and a mass range of 150 to 1950 m/*z* was selected. Chromatography was performed on a Kinetex® C18 column (50 × 2.1 mm i.d., 2.6 µm; Phenomenex, USA). Separation was performed with 0.1% aqueous formic acid (A) and 0.1% formic acid in acetonitrile (B), applying a gradient of 5 to 100% B in A (0–16 min), followed by 100% B (16–20 min). The column temperature was set to 50 °C, the flow rate to 0.4 ml/min.

Molecular networking and compound classification

LC–MS/MS data were uploaded to Global Natural Products Social Molecular Networking (GNPS) v1.3.16 using the mzML format for positive mode ionization. A molecular network was created with a cosine score above 0.7, and the minimum number of matched fragment ions was adjusted to 6. Further edges between two nodes were kept in the network only if each of the nodes appeared in each other's respective top 10 most similar nodes. Additionally, before networking, the filter "Spectra from G6" was set as "Blank". The MS/MS spectra were searched against GNPS' spectral libraries, using a minimum of 6 matched fragments for spectral matching. Cytoscape v3.9.0 was used for molecular network visualization. Manual classification of compound clusters was achieved by submitting the mzML output files from MSConvertGUI (v3.0.19330, ProteoWizard, USA) to Sirius 5.7.2 + CSI: FingerID + CANOPUS for prediction of the elemental composition (C, H, N, O, S, P) (Dührkop et al. 2015, 2019, 2021; Shen et al. 2014) and molecular structure database search with *m/z* tolerance set to 5 ppm.

Microscopy

Structural alterations were examined in fungal hyphae at the confrontation interface at 12 dpi. Differential interference (DIC) and fluorescence microscopy were performed using a Nikon Eclipse 600 microscope (Nikon, Düsseldorf, Germany). Calcofluor White (CFW; Calcofluor White-M2R, Sigma-Aldrich, Steinheim, Germany) was mixed with 10% (w/v) KOH at a ratio of 1:1 and applied to the sample, incubated for 15 min at room temperature and analyzed by fluorescence microscopy (UV-2A filter, Nikon Eclipse 600 microscope, Düsseldorf, Germany). An excitation wavelength of 350 nm was used, and images were taken with a Nikon microscopic camera DS-Ri2. Image processing was performed with NIS-Elements imaging software (Nikon, Düsseldorf, Germany).

Acknowledgements Skilled technical assistance of Diana Gottschling and Otto Naumann, and critical reading of and comments on the manuscript by Lala-Aliyeva Schnorr (all Martin-Luther-Universität Halle-Wittenberg) are acknowledged.

Authors contribution BRFD and HBD developed the concept of this study; experiments were conducted by BRFD, DABA, and HU; data analyses were done by all authors; the manuscript was written by BRFD and HBD and revised by DABA, HU, YP-G, and THJN. All authors have read and approved the manuscript. DABA, THJN and HBD acquired funding.

Funding Open Access funding enabled and organized by Projekt DEAL. HBD acknowledges funding of the project 'Epigenetic factors as molecular drivers for microbial diversity' by the German Centre for Integrative Biodiversity Research Halle-Jena-Leipzig (iDiv). DABA thanks the DAAD for financial support (91726521). THJN acknowledges funding from the DFG (INST 271/388–1).

Data availability All data are provided in this manuscript.

Declarations

Conflict of interest The authors have no competing interests to declare.

Humans and animals rights Studies on humans and/or animals have not been conducted.

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