Development of quantitative high-throughput screening methods for identification of antifungal biocontrol strains

Bodil Kjeldgaard^{1,2}, Ana Rute Neves^{1,3}, César Fonseca¹, Ákos T. Kovács², Patricia Domínguez- Cuevas^{1*}

¹Discovery, R&D, Chr. Hansen A/S, Denmark

²Bacterial Interactions and Evolution Group, DTU Bioengineering, Technical University of Denmark, Denmark

³Current affiliation: Arla Foods Ingredients, Denmark

*For correspondence. E-mail: dkpacu@chr-hansen.com, Bøge Alle 10, 2970 Hørsholm, Denmark, Tel. +4552180826

Abstract

Large screens of bacterial strain collections to identify potential biocontrol agents are often time consuming, costly, and fail to provide quantitative results. In this study, we present two quantitative and high-throughput methods to assess the inhibitory capacity of bacterial biocontrol candidates against fungal phytopathogens. One method measures the inhibitory effect of bacterial culture supernatant components on the fungal growth, while the other accounts for direct interaction between growing bacteria and the fungus by co-cultivating the two organisms. The antagonistic supernatant method quantifies the culture components' antifungal activity by calculating the cumulative impact of supernatant addition relative to a non-treated fungal control, while the antagonistic co-cultivation method identifies the minimal bacterial cell concentration required to inhibit fungal growth by co-inoculating fungal spores with bacterial culture dilution series. Thereby, both methods provide quantitative measures of biocontrol efficiency and allow prominent fungal inhibitors to be distinguished

from less effective strains. The combination of the two methods shed light on the type of inhibition mechanisms and provide the basis for further mode of action studies. We demonstrate the efficacy of the methods using *Bacillus spp.* with different levels of antifungal activities as model antagonists and quantify their inhibitory potency against classic plant pathogens.

Importance: Fungal phytopathogens are responsible for tremendous agricultural losses on annual basis. While microbial biocontrol agents represent a promising solution to the problem, there is a growing need for high-throughput methods to evaluate and quantify inhibitory properties of new potential biocontrol agents for agricultural application. In this study, we present two high-throughput and quantitative fungal inhibition methods that are suitable for commercial biocontrol screening.

Keywords: *Bacillus*, *Fusarium culmorum*, *Fusarium graminearum*, *Botrytis cinerea*, fungal growth inhibition method, quantification of antifungal properties, biocontrol agents, bacterial-fungal co-inoculation, bioactive compounds

Introduction

On annual basis, it is estimated that the global crop production suffers losses between 20 to 40 percent due to pests and plant diseases [1]. Plant diseases alone are predicted to cost the global economy a staggering \$220 billion per year [2]. Among other plant pathogens, fungal phytopathogens contribute to considerable losses in agriculture and greatly impact food security in developing countries [3–5]. Not only do fungal pathogens affect the yield, but fungal crop infections also lead to severe reductions of

43 post-harvest crop quality. For instance, accumulation of high levels of mycotoxins render crops unsafe
 44 for human consumption and for animal forage [6,7]. In modern intensified agriculture, fungal diseases
 45 are commonly fought using fungicides [8], but the rising fungicide resistance and chemical pollution
 46 represent a challenge to the sustainable use of these chemicals in agriculture [9–12]. In addition, many
 47 fungicides are hazardous to humans and infer developmental or reproductive toxicity [13]. Application
 48 of microbial biocontrol agents represent a safe alternative to the intensive use of agrochemicals [11,14].
 49 Biocontrol agents derived from plant growth promoting rhizobacteria (PGPR) reside in close association
 50 with plant roots, and protect the plant from phytopathogens by priming the plant defense response,
 51 competing for nutrients and/or directly antagonizing growth and development of the pathogenic
 52 intruders [15,16]. Strains from *Pseudomonas*, *Burkholderia*, *Streptomyces* and the *Bacillus* genera are
 53 well known for their antifungal capacity and for the production of a large variety of bioactive
 54 metabolites [14,17–22]. Although, the inhibitory effect of specific soil bacteria is well documented and
 55 recognized, there is a lack of quantitative and HTP screening procedures to identify competent
 56 biocontrol agents. Consequently, many potential biocontrol agents eventually fail to suppress plant
 57 diseases in field trials [23,24]. Classic antagonistic screens assess the impact of the biocontrol
 58 candidates on the phytopathogen after co-inoculation on solid media, which are referred to as dual-
 59 culture, plate confrontation or inhibition zone assays [23,25]. Such methods account for numerous
 60 factors including nutrients or space competition, cell surface components and the induced or
 61 constitutive secretion of volatile or soluble metabolites [19,25–27]. Other antagonistic assays evaluate
 62 the effect of individual inhibitory components on the phytopathogens' growth like volatiles,
 63 polyketides, lipopeptides, siderophores and lytic enzymes including, chitinases, glucanases, and

proteases [19,25]. More complex antagonistic assays, such as leaf disc or seedlings assays [28,29], investigate the tripartite interaction between biocontrol candidate, phytopathogen and plant host, while non-antagonistic assays assess the importance of complementary inhibitory mechanisms including niche colonization and priming of the plant immune response [25]. Nevertheless, most screening systems are low throughput and provide only semi-quantitative measurements of the inhibition potential against the fungus. Therefore, there is a need to develop more efficient screening methods combining quantitative measurements of antimicrobial activity with automation to increase speed and reduce resources required for the identification of good candidates.

Here, we describe two fungal inhibition methods to evaluate antifungal potency of potential biocontrol agents. Both methods accommodate high-throughput (HTP) screening of bacterial biocontrol candidates and provide accurate quantification of their inhibitory capacity. The major difference between the two methods is represented by the use of growing bacterial cells as opposed to (cell-inactive) culture supernatants. We demonstrate the efficacy of the methods using bacterial strains with different antifungal performance. Using the two novel methods, antifungal properties of bacteria were compared, and prominent fungal inhibitors were distinguished from less effective bacterial strains. Both methods were developed utilizing *Fusarium culmorum* as model plant pathogen and *Bacillus* spp. as model antagonists. In addition, the methods were further applied for screening *Bacillus* spp. against other important phytopathogens, i.e. *Fusarium graminearum* and *Botrytis cinerea*, proving that our method can readily be adjusted to other fungal species.

Results

Co-inoculation of fungal spores with bacterial dilution series facilitates quantification of inhibition potency

The so-called dual-culture assay is among the most common screening methods to identify potent fungal inhibitors from microbial collections [17,21,30–32]. Typically, the assay is performed by inoculating potential biocontrol agents at a fixed distance from the pathogenic fungal inoculum on a petri dish as illustrated in Fig. 1A. Subsequently, the biocontrol agent's ability to suppress fungal growth is manually assessed by measuring the radius of the mycelial growth relative to the control or the size of the inhibition zone [33–36]. However, accurate comparison and subsequent ranking of large numbers of strains is difficult with this assay due to the format of the readout. To improve the evaluation and accurate quantification of antifungal potency, we developed a HTP fungal inhibition assay based on direct co-inoculation of bacterial cultures and fungal plant pathogenic spores. First, bacterial overnight cultures were normalized to the same optical density at 600 nm (OD_{600}) and 5-fold serial dilutions were prepared (down to an amount of approximately 3-5 bacterial cells inoculated). The OD_{600} of each strain used in the study was correlated to viable cell counts (colony forming units (CFUs)). A fungal spore suspension was prepared and mixed thoroughly to ensure a homogeneous spore distribution. Then, the bacterial dilution series were co-inoculated with a fixed quantity of fungal spores to determine the minimal inhibitory cell concentration (MICC) that abolishes fungal growth (Fig. 1B). With this setup, a low MICC indicates a higher antifungal potency for a given bacterial strain. The assay was prepared on appropriate agar medium for fungal cultivation in 48-well microtiter plates and incubated 5 days at room temperature before assessing the fungal growth.

Four *Bacillus* strains of different species were selected based on their differential properties for fungal inhibition. The inhibitory capacity of the selected strains, *Bacillus subtilis* BCF001, *Bacillus amyloliquefaciens* BCF007, *Bacillus paralicheniformis* BCF009 and *Bacillus velezensis* BCF015, was quantified against *F. culmorum* DSM1094 using the developed method (Fig. 2B). The assay was conducted as three biological independent replicates, rendering very similar results, which indicates that the method is highly reproducible.

Fungal growth inhibition was scored as *no growth* (1), *weak growth* (2) and *(positive) growth* (3) (Fig. 2B). Fungal growth was defined by presence of visible hyphae in the well, including those that were half-covered by fungal mycelia. No fungal growth was defined by complete absence of visible fungal mycelia. Weak growth was defined by the presence of barely visible hyphae in all replicates, or by the absence of growth in half of the replicates. The weak growth category included the wells where *F. culmorum* produced (orange) pigmentation, even in the absence of visible hyphae.

Comparison of bacterial MICC allowed ranking of the strains in accordance to their fungal inhibition properties (Fig. 3A, Table S1). The strains *B. amyloliquefaciens* BCF007 and *B. velezensis* BCF015 showed the most potent inhibition properties, and even at the highest dilution step (corresponding to an initial OD₆₀₀ of 6.4×10^{-6} , equivalent to 3-5 CFUs inoculated), the two strains were able to inhibit *F. culmorum* growth. Determination of the minimal cell concentration that inhibits fungal growth even more accurately would require to conduct the assay using serial dilutions at smaller dilution factors. Nevertheless, the experimental setup is optimized for HTP screening of large strain collections.

B. subtilis BCF001 also displayed potent fungal inhibition properties, and abolished fungal growth up to an OD₆₀₀ of 3.2×10^{-5} , corresponding to 19 CFU inoculated. *B. paralicheniformis* BCF009, however,

did not affect fungal growth even at the lowest dilution step, corresponding to an OD₆₀₀ of 4×10^{-3} , 3.89×10^4 CFUs inoculated.

To generate visual and directly comparable plots of the inhibition results, we calculated a numerical inhibition score that reflects the inhibitory capacity of each strain. In brief, the lowest cell concentration that caused fungal growth inhibition was identified for each strain, and a numerical inhibition score was calculated based on the natural logarithm to the MICC, by applying the empirical formula #1 (see material and methods section) (Fig. 3A, Table S1). Plotting the inhibition scores clearly indicated that *B. amyloliquefaciens* BCF007 and *B. velezensis* BCF015 were the most efficient strains inhibiting growth of *F. culmorum*. *B. subtilis* BCF001 also showed a high inhibition score although smaller than the ones calculated for the two former strains. Scoring results for *B. paralicheniformis* BCF009 matched the low inhibitory activity of this strain against *F. culmorum*.

Evaluation of inhibition method with additional fungal phytopathogens

Using the newly developed method, the antifungal activity of *B. subtilis* BCF001, *B. amyloliquefaciens* BCF007, *B. paralicheniformis* BCF009 and *B. velezensis* BCF015 was also quantified against other phytopathogenic filamentous fungi, specifically against *F. graminearum* and *B. cinerea* strains. Images acquired to record the experimental results, the calculated MICCs and inhibition scores can be found in Fig. S1, Table S1. Interestingly, our inhibition assay proved applicable to these additional species of plant pathogenic fungi. The initial spore concentration was the only parameter that required adjustment when testing growth inhibition against the new fungal strains.

The previously described scoring system was applied to the results obtained for the three fungal species (Fig. 3). While *B. amyloliquefaciens* BCF007 and *B. velezensis* BCF015 displayed the most efficient growth inhibition of *F. culmorum*, differences between them and *B. subtilis* BCF001 were minimal when assayed against *F. graminearum* DSM4528. Furthermore, the strain *B. subtilis* BCF001 proved more efficient against *B. cinerea* Kern B2 compared to *B. amyloliquefaciens* BCF007 and *B. velezensis* BCF015. Inhibition scoring results obtained for *B. paralicheniformis* BCF009 were in accordance with the poor inhibitory properties of this strain, regardless of the fungal pathogen tested.

Inoculation of fungal spores with bacterial culture supernatants validates the inhibitory importance of supernatant components

While the antagonistic co-inoculation method assesses several inhibitory mechanisms, the use of cell-inactive supernatants accounts for the bioactivity of secreted metabolites, like enzymes, lipopeptides and polyketides, on the fungus. In the antagonistic supernatant method, fungal growth was estimated by OD₆₀₀ measurements in liquid cultures and therefore, reduced fungal growth in the presence of bacterial metabolites reflects greater inhibition potency of a given strain. For this method, bacterial cultures were grown overnight and normalized to the same OD₆₀₀ value prior to collection of the culture supernatants by centrifugation (Fig. 4). A range of increasing supernatant volumes (10-80 µl) were inoculated into fungal spore suspensions prepared in liquid medium in 48-well microtiter plates. To avoid any possibility of misreads arising from remaining bacterial cells present in the supernatants, bacteriostatic antibiotics were added to the fungal spore suspension to prevent bacterial growth. The

microtiter plates were incubated statically at 25°C in darkness. Subsequently, the fungal growth was quantified by spectrophotometric measurement.

The inhibition assay demonstrated a reverse correlation between the added volume of bacterial cell-inactive supernatant and growth of *F. culmorum*, *F. graminearum* or *B. cinerea* (Fig. 5A-C). For potent fungal inhibitory strains, including *B. subtilis* BCF001, *B. amyloliquefaciens* BCF007 and *B. velezensis* BCF015, increasing volumes of bacterial supernatant correlated with a progressive decrease of fungal growth. Addition of *B. paralicheniformis* BCF009 supernatant impacted fungal growth to a much lesser extent, which is in accordance with the results obtained with the co-inoculation method. In some cases, a slight increase in fungal growth was observed in response to addition of the largest supernatant volume (80 µl culture supernatant), suggesting an effect of supplemented nutrients (not consumed by the bacteria), which outweighed the antifungal bioactive metabolites.

Scoring of supernatant inhibition potency

A numerical inhibition score was assigned to each strain by quantifying the fungal growth in response to supernatant addition (5 volumes). The inhibition scores were calculated by subtracting the accumulated relative fungal growth from total potential fungal growth in the 5 conditions tested (Equation #2, see material and methods section).

The inhibition scores can be found in Table S1. Culture supernatants of *B. subtilis* BCF001 and *B. amyloliquefaciens* BCF007 showed the most effective growth inhibition of *F. culmorum* (Fig. 5A and D). Against *F. graminearum*, *B. subtilis* BCF001 culture supernatants proved most inhibitory, while *B. subtilis* BCF001, *B. amyloliquefaciens* BCF007 and *B. velezensis* BCF015 all displayed similar inhibition

potency of *B. cinerea*. *B. paralicheniformis* BCF009 culture supernatants poorly inhibited *F. culmorum* growth.

It is worth mentioning that assays prepared from different fungal spore stock solutions showed small variations in final fungal growth (Fig. S2), possibly due to variations in the initial spore count or spore viability. These variations could be minimized either by prolonging the incubation time until readout or by preparing assays from a unique spore stock. For this reason, biological replicates were prepared with the same fungal spore solution. Nevertheless, the overall conclusions remained unchanged despite variations in the final fungal growth.

Comparison of methods to remove or inactivate cells from culture supernatants

Typically, bacterial cell-inactive supernatants are generated by filtration of the cell cultures [37–40]. In contrast, in the proposed setup cell-inactive supernatants were generated by centrifugation to pellet bacterial cells. To avoid cell growth of remaining bacteria in suspension, we added the bacteriostatic antibiotics chloramphenicol and tetracycline. Plating of the fungal growth suspension on bacteria-selective medium after the end point measurement produced no bacterial growth, indicating that the antibiotics effectively inhibited growth of any putative remaining bacterial cells (data not shown). Final fungal growth measurements reached by *F. culmorum*, *F. graminearum* and *B. cinerea* were somewhat affected by addition of the respective antibiotics (Fig. S3). The results obtained with filtered supernatants with and without antibiotics revealed that the relative inhibition results remained unaltered, although the addition of antibiotics slightly but significantly reduced the fungal growth (Fig.

S4, Table S2). Both filtration and antibiotic addition had generally no effect on the fungal inhibitory potency, suggesting that bioactive metabolites remain active after both procedures (Fig. S5, Table S2).

Comparison of the two methods proposed in the present study and the dual-culture assay

The two proposed quantitative HTP methods were compared to the common dual-culture assay using plates inoculated with fungus and *Bacilli* (Fig. 1A). In accordance with results from the two methods, zones of fungal growth inhibition were observed around the bacterial colonies of *B. subtilis* BCF001, *B. amyloliquefaciens* BCF007 and *B. velezensis* BCF0015, whereas growth of *F. culmorum*, *F. graminearum* and *B. cinerea* was nearly unaffected by *B. parlicheniformis* BCF009. Although, the traditional dual-culture assay allowed assessment of fungal inhibitory capacity, the results lack the accurate quantification of the bacterial inhibition potency provided by the proposed methods. In addition, the simple numerical scoring systems allow easy comparison between a large number of strains.

While the antagonistic supernatant method specifically evaluates the inhibition potency of secreted metabolites, like enzymes, lipopeptides and polyketides, the antagonistic co-inoculation method quantifies the inhibitory effect of actively growing bacteria, and thereby accounts for additional factors like competition for nutrients and space. Therefore, differences in results obtained by the two methods could provide insights on the mode of action and serve as the starting point for in-depth characterization of their molecular inhibitory mechanism.

Discussion

Despite the steadily growing market share of biocontrol products compared to the use of conventional pesticides [15,41], plant pathogen management continues to heavily rely on chemical substances with associated risks to human health and the environment [42]. Regardless of the increased (research) efforts on microbial biocontrol product development, their deployment into market is difficult due to the absence of a global harmonized framework, public misinformation, complex regulation and registration procedures as well as expensive field trials [43–46]. The application of reliable methods to identify and select potent biocontrol strains is of critical importance to reduce product development costs and accelerate their market implementation.

Large screens of strain collections are often laborious and expensive, where the complexity of the screening system is proportional to the cost [47]. Simple HTP screens are cost-effective, however they often fail to provide quantitative results for accurate comparison of the biocontrol strains. Furthermore, following the HTP screening and candidate selection, many strains eventually show low efficacy in field trials, demonstrating a discrepancy between *in vitro* laboratory conditions and *in planta* application experiments. To reduce costs and minimize failed tests, it is therefore crucial to identify biocontrol candidates in primary screens before moving on to complex experimental systems such as greenhouses or field trials. Accordingly, development of simple, reliable and quantitative HTP screening methods is crucial for ranking and selection of the best biocontrol candidates.

In this study, bacterial strains were ranked according to their bioactivity against fungal plant pathogens using two novel HTP antagonistic methods: i) an antagonistic co-cultivation method based on the inoculation of a constant number of fungal spores with bacterial dilutions series on a solid media to generate a quantitative measurement of the minimal inhibitory (bacterial) cell concentration (MICC) of

251 fungal growth and, ii) an antagonistic cell-inactive supernatant method based on the inoculation of a
 252 constant number of fungal spores with different volumes of bacterial culture supernatants in a liquid
 253 media to provide a quantitative measurement of fungal growth inhibition by secreted metabolites.
 254 Although classic antagonistic methods that assess adjacent growth of two species on agar plates allow
 255 assessment of fungal inhibition potential [17,30–32,48], their accuracy is limited. Factors such as
 256 inoculum size, diffusion rate of metabolites, and differential conditions between agar plates contribute
 257 to the inaccuracy of classic antagonistic assays and impairs ranking of biocontrol candidates. The
 258 quantification provided by the proposed antagonistic co-cultivation method allowed ranking of
 259 biocontrol candidates according to their inhibitory strength by means of MICC values. In addition, the
 260 direct co-inoculation of fungal spores with bacterial cells do not only assesses the impact on mycelial
 261 growth, but also on fungal spore germination, contrary to the classic agar plate methods. These type of
 262 antagonistic methods, employing direct co-inoculation of biocontrol candidate and pathogen have
 263 been developed for other applications, for instance lactic acid biocontrol bacteria co-inoculated with
 264 food spoilage fungi in dairy industry [49,50]. However, these assays lack precise quantification of
 265 inhibition potency, regardless of the similarities with the co-inoculation method described here.
 266 Compared to simple dual-culture assays like ours, the more complex *in planta* assays include the
 267 tripartite interaction of biocontrol candidate, phytopathogen and plant host [28,29,51], and thereby
 268 attempt to mimic field settings. Despite the closer resemblance to natural conditions, the complexity
 269 of these screening systems limits the throughput (Haidar et al., 2016) and leads to additional variation
 270 [52], which complicates the interpretation of results compared to our method. However, screens that

271 consider the interaction between three biological systems may be advantageous to apply to a subset
 272 of biocontrol candidates following an initial HTP screen [53,54].

273 While the antagonistic co-cultivation methods investigate the direct interaction between pathogen and
 274 biocontrol candidate, the supernatant antagonistic methods estimate the inhibition potency of
 275 secreted bioactive metabolites from the biocontrol candidate on the pathogen. The experimental setup
 276 is either agar- or liquid-based, like the proposed antagonistic cell-inactive supernatant method
 277 described in this study. Agar-based screens rely on evaluation of fungal colony growth or inhibition
 278 zones in response to addition of supernatant from the biocontrol candidates [32,38,55]. Some assays
 279 allow a quantitative comparison of inhibition capacity between strains by calculating the reciprocal to
 280 the highest supernatant dilution that exhibits a clear zone of inhibition [38,55]. Even so, the scoring is
 281 notably laborious and low-throughput compared to the proposed antagonistic supernatant method
 282 described in this study.

283 Liquid-based supernatant assays depend on the assessment of fungal morphology by microscopy
 284 [55,56], evaluation of fungal growth by dry weight [57], or spectrophotometry in response to the
 285 biocontrol supernatants [34,37]. The latter share great resemblance to the proposed cell-inactive
 286 supernatant method and allows HTP and reproducible assessment of the antifungal metabolite
 287 potency. However, the accurate quantification of antifungal capacity by determination of supernatant
 288 inhibition scores in the proposed method allows easy benchmarking and comparison between
 289 biocontrol supernatants, which represents a major advantage compared to previously reported
 290 methods.

Both assays were initially developed utilizing *F. culmorum* as model plant pathogen, and different *Bacillus* species as model antagonists. Subsequently, the assays were validated with the relevant plant pathogenic fungi *B. cinerea* and *F. graminearum*. The results demonstrated that the methods are robust and can readily be adapted for different fungal species. Moreover, the methods can be adapted to assess the potential for inhibiting bacterial plant pathogens. In agriculture, not only fungal plant pathogens, but also bacterial plant pathogens contribute to significant yield losses [58]. The adaptation of the proposed methods to quantify inhibition potency against plant pathogenic bacteria simply requires a fluorophore-labelled bacterial pathogen, a bacterial pathogen with a selective marker (i.e. antibiotic resistance) or a pathogen-selective growth medium in order to make it applicable for HTP screening. For discrimination between bacterial strains with similar inhibition potency, the antagonistic co-cultivation method can be adjusted by using a smaller dilution factor. Furthermore, the dilution factors can be adjusted to fit more or less potent biocontrol candidates. Finally, the applicability of the methods may even be extended to other areas such as biocontrol screens against human or animal pathogens or screens against food spoilage microorganisms.

Several previous studies employ combinatory screens with two or more assays for identification of potent pathogen inhibitors [31,33,51,56,59,60]. In this study, we propose the combination of two HTP antagonistic methods to improve confident selection of potent biocontrol bacterial strains. While the antagonistic co-cultivation method accounts for the entire repertoire of direct inhibitory mechanisms displayed by a biocontrol strain, such as bioactive compounds and competition for nutrients and space, the antagonistic supernatant method pinpoints the inhibitory effect of secreted metabolites such as

enzymes, lipopeptides and polyketides. Thus, the two methods provide different results that in combination may aid further mechanistic elucidation. In addition, while the antagonistic co-inoculation method may facilitate the identification of potent candidates ideal for in furrow application or seed coating, where pathogens and biocontrol agents actively compete, the antagonistic supernatant method identifies high producers of bioactive metabolites, which would be advantageous to develop a liquid formulation for foliar product application. In light of the results obtained and considering the different mechanistic aspects involved in pathogenic inhibition, we argue that the combination of our novel antagonistic co-cultivation and supernatant methods constitutes an improved strategy for biocontrol strain identification. The combination of the two presented methods: 1) confidently reflects the fungal inhibition capacity of biocontrol candidates, 2) facilitates HTP screening of large strain collections, 3) can provide valuable insights into type of inhibition mechanisms for further studies and, 4) allows easy comparison of strains by accurate quantification of their inhibition potency.

Materials and Methods

Microbial species and growth conditions. The fungal and bacterial strains used in the inhibition assays are shown in Table 1. Fungal species were cultivated on potato dextrose agar (PDA) medium ([Carl Roth]; potato infusion 4 g/l, glucose 20 g/l, agar 15 g/l, pH value 5.6 ± 0.2). For inhibition assays on PDA, cultures were incubated at room temperature with natural light. For inhibition assays in broth, fungal spores were inoculated in potato dextrose broth (PDB) medium ([Carl Roth]; potato infusion 6.5 g/l, glucose 20 g/l, pH value 5.6 ± 0.2) and incubated at 25°C in darkness. *Bacilli* were grown overnight in

332 Lysogeny Broth ([Difco]; Bacto tryptone 10 g/l, [Oxoid]; yeast extract 5 g/l, [Merck]; NaCl 10 g/l, pH
333 value 7.2 ± 0.2) at 37°C with agitation at 250 rpm.

334

335 **Fungal spore harvest.** Spores were harvested as described by Benoit et al., 2015 and Kjeldgaard et al.,
336 2019. In brief, PDA plates were inoculated with fungi and incubated at 22°C with 16h light/8h dark
337 cycles for at least 2 weeks. Fungal spores were harvested from PDA plates using saline tween solution
338 (8 g/l NaCl, 0.05 ml/l Tween 80) and gentle scraping with an L-spreader. The spore solution was filtered
339 through double layered Miracloth [Millipore] and pelleted by centrifugation at 5000 rpm for 10 min.
340 The supernatant was discarded and the spore pellet resuspended in saline tween. Spore stocks were
341 prepared by adding an appropriate concentration of glycerol.

342

343 **Quantification of fungal growth inhibition by co-inoculation with bacterial dilution series.** The fungal
344 inhibition assay was prepared in 48-microtiter plates with 0.5 ml PDA in each well. *Bacillus* cultures
345 were adjusted to 2×10^{-2} or 8×10^{-4} at OD₆₀₀ and 5-fold dilution series were prepared with 6 steps using
346 peptone saline as diluent ([Millipore]; Maximum Recovery Diluent 9.5 g/L). 15 µl of each bacterial
347 dilution was inoculated in consecutive wells by spotting the solution in the center of the wells. One
348 strain was assigned per column. Following, a fungal spore solution was prepared with peptone saline
349 and vortexed vigorously to disperse clumps of spores. 15 µl fungal spore solution was co-inoculated
350 with the bacterial dilutions in each well. The approximate final fungal spore concentration of
351 *F. culmorum*, *F. graminearum* and *B. cinerea* was 5.5×10^4 spores/ml, 3.1×10^3 spores/ml and 7.5×10^5
352 spores/ml, respectively. Co-inoculated plates were sealed with 3M tape ([Millipore] 0.5 cm) to reduce

growth differences between inner and peripheric wells, and incubated at room temperature under natural light conditions. After 5 days, the fungal inhibition was evaluated first by visual inspection and later scored by scanning the plates. The minimal inhibitory cell concentration (MICC) was identified for each strain and averaged between technical duplicates, then averaged between biological triplicates. The MICC (CFU) against each fungal species was converted to an inhibition score by Equation #1 and statistical significance was calculated by two-tailed student's T-test.

$$\text{\#1} \quad \text{Inhibition score} = 5 - \frac{\text{Ln}(\text{MICC})}{2.5}$$

Quantification of fungal inhibition potency by metabolites in bacterial (cell-inactive) supernatants.

The fungal inhibition assay was prepared in 48-microtiter plates with 0.5 ml PDB medium. Spores of *F. culmorum*, *F. graminearum* or *B. cinerea* were added to a final concentration of 1.1×10^4 spores/ml, 6.1×10^2 spores/ml and 7.5×10^4 spores/ml respectively. The spore suspensions were mixed thoroughly to ensure a homogeneous distribution. *Bacillus* spp. cultures were adjusted to OD₆₀₀ of 2 and centrifuged to pellet the cells. The supernatants were collected and the cell pellets discarded. Increasing bacterial supernatant volumes (ranging from 10-80 µl) were inoculated into the fungal spore suspension. Two approaches were implemented for sterilization of the supernatant to omit bacterial growth. Either the bacteriostatic antibiotics chloramphenicol and tetracycline were added to the fungal spore suspension to a final concentration of 50 µg/ml and 10 µg/ml, respectively, or the supernatants were sterilized by filtration ([Sartorius] Minisart Syringe Filter 0.2 µm) prior to co-inoculation with the fungal spores. The plates were sealed with 3M tape and incubated at 25°C without shaking in the darkness. The fungal growth was quantified by spectrophotometric measurements (OD₆₀₀) after 48h

for *F. graminearum* and *B. cinerea* inhibition assays and after 67-72h for *F. culmorum* inhibition assays. For more accurate fungal growth estimation, the OD₆₀₀ was measured using a 5x5 well-scanning matrix. Following the evaluation of fungal growth, the supernatants were collected and plated on LB with fungicides (50 mg/L nystatin) to check for unwanted bacterial growth. Statistical analyses were done to compare to the effect of 1) bacterial culture supernatants on fungal growth, 2) filtration and antibiotics on the bacterial supernatants potency, and 3) prokaryotic antibiotics on fungal growth. Statistical significance was calculated by students T-test (two-tailed). A numerical inhibition score was assigned to each strain by quantifying the fungal growth in response to supernatant addition (5 volumes). The inhibition scores were calculated by subtracting the accumulated relative fungal growth from total potential fungal growth (5 conditions tested) as shown in Equation #2:

$$\#2 \quad 5 - \text{SUM}(\text{relative fungal growth})$$

References

- [1] Food and Agriculture Organization of the United Nations. Plant health and food security. Int Plant Prot Conv 2017.
- [2] FAO. New standards to curb the global spread of plant pests and diseases 2019. <http://www.fao.org/news/story/en/item/1187738/icode/> (accessed March 28, 2020).
- [3] Savary S, Ficke A, Aubertot JN, Hollier C. Crop losses due to diseases and their implications for global food production losses and food security. Food Secur 2012;4:519–37. <https://doi.org/10.1007/s12571-012-0200-5>.
- [4] Almeida F, Rodrigues ML, Coelho C. The Still Underestimated Problem of Fungal Diseases

- Worldwide. *Front Microbiol* 2019;10:214. <https://doi.org/10.3389/fmicb.2019.00214>.
- [5] Fisher MC, Henk DA, Briggs CJ, Brownstein JS, Madoff LC, Mccraw SL, et al. Emerging fungal threats to animal, plant and ecosystem health. *Nature* 2012;484. <https://doi.org/10.1038/nature10947>.
- [6] Hoffmann V, Jones K, Leroy JL. The impact of reducing dietary aflatoxin exposure on child linear growth: A cluster randomised controlled trial in Kenya. *BMJ Glob Heal* 2018;3:e000983. <https://doi.org/10.1136/bmjgh-2018-000983>.
- [7] De Lucca AJ. Harmful fungi in both agriculture and medicine. *Rev Iberoam Micol* 2007;24:3–13. [https://doi.org/10.1016/s1130-1406\(07\)70002-5](https://doi.org/10.1016/s1130-1406(07)70002-5).
- [8] Lucas JA, Hawkins NJ, Fraaije BA. The Evolution of Fungicide Resistance. *Adv Appl Microbiol* 2015;90:29–92. <https://doi.org/10.1016/bs.aambs.2014.09.001>.
- [9] Hahn M. The rising threat of fungicide resistance in plant pathogenic fungi: Botrytis as a case study. *J Chem Biol* 2014;7:133–41. <https://doi.org/10.1007/s12154-014-0113-1>.
- [10] Hellin P, King R, Urban M, Hammond-Kosack KE, Legrève A. The adaptation of *Fusarium culmorum* to DMI Fungicides Is Mediated by Major Transcriptome Modifications in Response to Azole Fungicide, Including the Overexpression of a PDR Transporter (FcABC1). *Front Microbiol* 2018;9:1385. <https://doi.org/10.3389/fmicb.2018.01385>.
- [11] Brauer VS, Rezende CP, Pessoni AM, De Paula RG, Rangappa KS, Nayaka SC, et al. Antifungal agents in agriculture: Friends and foes of public health. *Biomolecules* 2019;9:521. <https://doi.org/10.3390/biom9100521>.
- [12] Zubrod JP, Bundschuh M, Arts G, Brühl CA, Imfeld G, Knäbel A, et al. Fungicides: An Overlooked

- 416 Pesticide Class? Environ Sci Technol 2019;53:3347–65.
- 417 <https://doi.org/10.1021/acs.est.8b04392>.
- 418 [13] Gupta PK. Herbicides and Fungicides. Reprod. Dev. Toxicol., 2011, p. 503–21.
- 419 <https://doi.org/10.1016/B978-0-12-382032-7.10039-6>.
- 420 [14] Cawoy H, Bettiol W, Fickers P, Ongena M. Bacillus-Based Biological Control of Plant Diseases.
- 421 Pestic Mod World - Pestic Use Manag 2011;1849:273–303. [https://doi.org/DOI:](https://doi.org/DOI:10.5772/17184)
- 422 [10.5772/17184](https://doi.org/DOI:10.5772/17184).
- 423 [15] Prashar P, Kapoor N, Sachdeva S. Biocontrol of Plant Pathogens Using Plant Growth Promoting
- 424 Bacteria. In: Lichtfouse E, editor. Sustain. Agric. Rev., vol. 12, 2013, p. 319–60.
- 425 https://doi.org/10.1007/978-94-007-5961-9_10.
- 426 [16] Blake C, Christensen MN, Kovács ÁT. Molecular Aspects of Plant Growth Promotion and
- 427 Protection by Bacillus subtilis. Mol Plant Microbe Interact 2021;34:15–25.
- 428 <https://doi.org/10.1094/MPMI-08-20-0225-CR>.
- 429 [17] Durairaj K, Velmurugan P, Park JH, Chang WS, Park YJ, Senthilkumar P, et al. An investigation of
- 430 biocontrol activity Pseudomonas and Bacillus strains against Panax ginseng root rot fungal
- 431 phytopathogens. Biol Control 2018;125:138–46.
- 432 <https://doi.org/10.1016/j.biocontrol.2018.05.021>.
- 433 [18] Fira D, Dimkić I, Berić T, Lozo J, Stanković S. Biological control of plant pathogens by Bacillus
- 434 species. J Biotechnol 2018;285:44–55. <https://doi.org/10.1016/j.jbiotec.2018.07.044>.
- 435 [19] Cesa-Luna C, Baez A, Quintero-Hernández V, De La Cruz-Enríquez J, Castañeda-Antonio MD,
- 436 Muñoz-Rojas J. The importance of antimicrobial compounds produced by beneficial bacteria on

- 437 the biocontrol of phytopathogens. *Acta Biol Colomb* 2020;25:140–54.
- 438 <https://doi.org/10.15446/abc.v25n1.76867>.
- 439 [20] Penha RO, Vandenberghe LPS, Faulds C, Soccol VT, Soccol CR. *Bacillus* lipopeptides as powerful
- 440 pest control agents for a more sustainable and healthy agriculture: recent studies and
- 441 innovations. *Planta* 2020;251:1–15. <https://doi.org/10.1007/s00425-020-03357-7>.
- 442 [21] Kieseewalter HT, Lozano-Andrade CN, Wibowo M, Strube ML, Maróti G, Snyder D, et al. Genomic
- 443 and chemical diversity of *Bacillus subtilis* secondary metabolites against plant pathogenic fungi.
- 444 *MSystems* 2020;6. <https://doi.org/10.1101/2020.08.05.238063>.
- 445 [22] Steinke K, Mohite OS, Weber T, Kovács ÁT. Phylogenetic distribution of secondary metabolites
- 446 in the *Bacillus subtilis* species complex. *MSystems* 2020;6.
- 447 <https://doi.org/10.1101/2020.10.28.358507>.
- 448 [23] Pliego C, Ramos C, de Vicente A, Cazorla FM. Screening for candidate bacterial biocontrol
- 449 agents against soilborne fungal plant pathogens. *Plant Soil* 2011;340:505–20.
- 450 <https://doi.org/10.1007/s11104-010-0615-8>.
- 451 [24] Folman LB, Postma J, Van Veen JA. Inability to find consistent bacterial biocontrol agents of
- 452 *Pythium aphanidermatum* in cucumber using screens based on ecophysiological traits. *Microb*
- 453 *Ecol* 2003;45:72–87. <https://doi.org/10.1007/s00248-002-2013-0>.
- 454 [25] Raymaekers K, Ponet L, Holtappels D, Berckmans B, Cammue BPA. Screening for novel
- 455 biocontrol agents applicable in plant disease management – A review. *Biol Control* 2020.
- 456 <https://doi.org/10.1016/j.biocontrol.2020.104240>.
- 457 [26] Deketelaere S, Tyvaert L, França SC, Hofte M. Desirable traits of a good biocontrol agent against

- 458 Verticillium wilt. Front Microbiol 2017;8:1186. <https://doi.org/10.3389/fmicb.2017.01186>.
- 459 [27] Andrić S, Meyer T, Ongena M. Bacillus Responses to Plant-Associated Fungal and Bacterial
460 Communities. Front Microbiol 2020;11:1350. <https://doi.org/10.3389/fmicb.2020.01350>.
- 461 [28] Zhang X, Zhou Y, Li Y, Fu X, Wang Q. Screening and characterization of endophytic Bacillus for
462 biocontrol of grapevine downy mildew. Crop Prot 2017;96:173–9.
463 <https://doi.org/10.1016/j.cropro.2017.02.018>.
- 464 [29] Latz MAC, Jensen B, Collinge DB, Lyngs Jørgensen HJ. Identification of two endophytic fungi
465 that control Septoria tritici blotch in the field, using a structured screening approach. Biol
466 Control 2020;141:104128. <https://doi.org/10.1016/j.biocontrol.2019.104128>.
- 467 [30] Petatán-Sagahón I, Anducho-Reyes MA, Silva-Rojas HV, Arana-Cuenca A, Tellez-Jurado A,
468 Cárdenas-Álvarez IO, et al. Isolation of bacteria with antifungal activity against the
469 phytopathogenic fungi Stenocarpella maydis and Stenocarpella macrospora. Int J Mol Sci
470 2011;12:5522–37. <https://doi.org/10.3390/ijms12095522>.
- 471 [31] Wang LY, Xie YS, Cui YY, Xu J, He W, Chen HG, et al. Conjunctively screening of biocontrol
472 agents (BCAs) against fusarium root rot and fusarium head blight caused by Fusarium
473 graminearum. Microbiol Res 2015;177:34–42. <https://doi.org/10.1016/j.micres.2015.05.005>.
- 474 [32] Jung SJ, Kim NK, Lee DH, Hong S Il, Lee JK. Screening and evaluation of Streptomyces species as
475 a potential biocontrol agent against a wood decay fungus, Gloeophyllum trabeum.
476 Mycobiology 2018;46:138–46. <https://doi.org/10.1080/12298093.2018.1468056>.
- 477 [33] Khan N, Martínez-Hidalgo P, Ice TA, Maymon M, Humm EA, Nejat N, et al. Antifungal activity of
478 bacillus species against fusarium and analysis of the potential mechanisms used in biocontrol.

- 479 Front Microbiol 2018;9:2363. <https://doi.org/10.3389/fmicb.2018.02363>.
- 480 [34] Besset-Manzoni Y, Joly P, Brutel A, Gerin F, Soudière O, Langin T, et al. Does in vitro selection of
481 biocontrol agents guarantee success in planta? A study case of wheat protection against
482 Fusarium seedling blight by soil bacteria. PLoS One 2019;14:e0225655.
483 <https://doi.org/10.1371/journal.pone.0225655>.
- 484 [35] Dai Y, Wu XQ, Wang YH, Zhu ML. Biocontrol potential of *Bacillus pumilus* HR10 against
485 Sphaeropsis shoot blight disease of pine. Biol Control 2021;152:104458.
486 <https://doi.org/10.1016/j.biocontrol.2020.104458>.
- 487 [36] Kim YS, Lee Y, Cheon W, Park J, Kwon HT, Balaraju K, et al. Characterization of *Bacillus*
488 *velezensis* AK-0 as a biocontrol agent against apple bitter rot caused by *Colletotrichum*
489 *gloeosporioides*. Sci Rep 2021;11:626. <https://doi.org/10.1038/s41598-020-80231-2>.
- 490 [37] Kousser C, Clark C, Sherrington S, Voelz K, Hall RA. *Pseudomonas aeruginosa* inhibits *Rhizopus*
491 *microsporus* germination through sequestration of free environmental iron. Sci Rep 2019;9:1–
492 14. <https://doi.org/10.1038/s41598-019-42175-0>.
- 493 [38] Shehata MG, Badr AN, El Sohaimy SA, Asker D, Awad TS. Characterization of antifungal
494 metabolites produced by novel lactic acid bacterium and their potential application as food
495 biopreservatives. Ann Agric Sci 2019;64:71–8. <https://doi.org/10.1016/j.aosas.2019.05.002>.
- 496 [39] Li Y, Héloir MC, Zhang X, Geissler M, Trouvelot S, Jacquens L, et al. Surfactin and fengycin
497 contribute to the protection of a *Bacillus subtilis* strain against grape downy mildew by both
498 direct effect and defence stimulation. Mol Plant Pathol 2019;20:1037–50.
499 <https://doi.org/10.1111/mpp.12809>.

- 500 [40] Arroyave-Toro JJ, Mosquera S, Villegas-Escobar V. Biocontrol activity of *Bacillus subtilis* EA-
501 CB0015 cells and lipopeptides against postharvest fungal pathogens. *Biol Control*
502 2017;114:195–200. <https://doi.org/10.1016/j.biocontrol.2017.08.014>.
- 503 [41] Robin DC, Marchand PA. Evolution of the biocontrol active substances in the framework of the
504 European Pesticide Regulation (EC) No. 1107/2009. *Pest Manag Sci* 2019;75:950–8.
505 <https://doi.org/10.1002/ps.5199>.
- 506 [42] Barzman M, Bàrberi P, Birch ANE, Boonekamp P, Dachbrodt-Saaydeh S, Graf B, et al. Eight
507 principles of integrated pest management. *Agron Sustain Dev* 2015;35:1199–215.
508 <https://doi.org/10.1007/s13593-015-0327-9>.
- 509 [43] Droby S, Wisniewski M, Teixidó N, Spadaro D, Jijakli MH. The science, development, and
510 commercialization of postharvest biocontrol products. *Postharvest Biol Technol* 2016;122:22–9.
511 <https://doi.org/10.1016/j.postharvbio.2016.04.006>.
- 512 [44] Backer R, Rokem JS, Ilangumaran G, Lamont J, Praslickova D, Ricci E, et al. Plant growth-
513 promoting rhizobacteria: Context, mechanisms of action, and roadmap to commercialization of
514 biostimulants for sustainable agriculture. *Front Plant Sci* 2018;871:1473.
515 <https://doi.org/10.3389/fpls.2018.01473>.
- 516 [45] Barratt BIP, Moran VC, Bigler F, van Lenteren JC. The status of biological control and
517 recommendations for improving uptake for the future. *BioControl* 2018;63:155–67.
518 <https://doi.org/10.1007/s10526-017-9831-y>.
- 519 [46] Köhl J, Booij K, Kolnaar R, Ravensberg WJ. Ecological arguments to reconsider data
520 requirements regarding the environmental fate of microbial biocontrol agents in the

- 521 registration procedure in the European Union. *BioControl* 2019;64:469–87.
- 522 <https://doi.org/10.1007/s10526-019-09964-y>.
- 523 [47] Köhl J, Postma J, Nicot P, Ruocco M, Blum B. Stepwise screening of microorganisms for
- 524 commercial use in biological control of plant-pathogenic fungi and bacteria. *Biol Control*
- 525 2011;57:1–12. <https://doi.org/10.1016/j.biocontrol.2010.12.004>.
- 526 [48] Leelasuphakul W, Hemmanee P, Chuenchitt S. Growth inhibitory properties of *Bacillus subtilis*
- 527 strains and their metabolites against the green mold pathogen (*Penicillium digitatum* Sacc.) of
- 528 citrus fruit. *Postharvest Biol Technol* 2008;48:113–21.
- 529 <https://doi.org/10.1016/j.postharvbio.2007.09.024>.
- 530 [49] Salas ML, Thierry A, Lemaître M, Garric G, Harel-Oger M, Chatel M, et al. Antifungal activity of
- 531 lactic acid bacteria combinations in dairy mimicking models and their potential as bioprotective
- 532 cultures in pilot scale applications. *Front Microbiol* 2018;9:1787.
- 533 <https://doi.org/10.3389/fmicb.2018.01787>.
- 534 [50] Garnier L, Salas ML, Pinon N, Wiernasz N, Pawtowski A, Coton E, et al. Technical note: High-
- 535 throughput method for antifungal activity screening in a cheese-mimicking model. *J Dairy Sci*
- 536 2018;101:4971–6. <https://doi.org/10.3168/jds.2017-13518>.
- 537 [51] Han JH, Shim H, Shin JH, Kim KS. Antagonistic activities of *Bacillus* spp. Strains isolated from
- 538 tidal flat sediment towards anthracnose pathogens *Colletotrichum acutatum* and *C.*
- 539 *Gloeosporioides* in South Korea. *Plant Pathol J* 2015;31:165–75.
- 540 <https://doi.org/10.5423/PPJ.OA.03.2015.0036>.
- 541 [52] Porter AS, Evans-FitzGerald C, McElwain JC, Yiotis C, Elliott-Kingston C. How well do you know

- 542 your growth chambers? Testing for chamber effect using plant traits. Plant Methods
543 2015;11:44. <https://doi.org/10.1186/s13007-015-0088-0>.
- 544 [53] Haidar R, Roudet J, Bonnard O, Dufour MC, Corio-Costet MF, Fert M, et al. Screening and
545 modes of action of antagonistic bacteria to control the fungal pathogen *Phaeomoniella*
546 *chlamydospora* involved in grapevine trunk diseases. Microbiol Res 2016;192:172–84.
547 <https://doi.org/10.1016/j.micres.2016.07.003>.
- 548 [54] Wang T, Liang Y, Wu M, Chen Z, Lin J, Yang L. Natural products from *Bacillus subtilis* with
549 antimicrobial properties. Chinese J Chem Eng 2015;23:744–54.
550 <https://doi.org/10.1016/j.cjche.2014.05.020>.
- 551 [55] Kilani-Feki O, Ben Khedher S, Dammak M, Kamoun A, Jabnoun-Khiareddine H, Daami-Remadi
552 M, et al. Improvement of antifungal metabolites production by *Bacillus subtilis* V26 for
553 biocontrol of tomato postharvest disease. Biol Control 2016;95:73–82.
554 <https://doi.org/10.1016/j.biocontrol.2016.01.005>.
- 555 [56] Oh HS, Lee YH. A target-site-specific screening system for antifungal compounds on
556 appressorium formation in *Magnaporthe grisea*. Phytopathology 2000;90:1162–8.
557 <https://doi.org/10.1094/PHYTO.2000.90.10.1162>.
- 558 [57] Kilani-Feki O, Culioli G, Ortalo-Magné A, Zouari N, Blache Y, Jaoua S. Environmental
559 *Burkholderia cepacia* strain Cs5 acting by two analogous alkyl-quinolones and a didecyl-
560 phthalate against a broad spectrum of phytopathogens fungi. Curr Microbiol 2011;62:1490–5.
561 <https://doi.org/10.1007/s00284-011-9892-6>.
- 562 [58] Mansfield J, Genin S, Magori S, Citovsky V, Sriariyanum M, Ronald P, et al. Top 10 plant

pathogenic bacteria in molecular plant pathology. Mol Plant Pathol 2012;13:614–29.

<https://doi.org/10.1111/j.1364-3703.2012.00804.x>.

[59] Chen X, Wang Y, Gao Y, Gao T, Zhang D. Inhibitory abilities of *Bacillus* isolates and their culture filtrates against the gray mold caused by *Botrytis cinerea* on postharvest fruit. Plant Pathol J 2019;35:425–36. <https://doi.org/10.5423/PPJ.OA.03.2019.0064>.

[60] Xu W, Wang K, Wang H, Liu Z, Shi Y, Gao Z, et al. Evaluation of the biocontrol potential of *Bacillus* sp. WB against *Fusarium oxysporum* f. sp. *niveum*. Biol Control 2020;147. <https://doi.org/10.1016/j.biocontrol.2020.104288>.

[61] Benoit I, van den Esker MH, Patyshakuliyeva A, Mattern DJ, Blei F, Zhou M, et al. *Bacillus subtilis* attachment to *Aspergillus niger* hyphae results in mutually altered metabolism. Environ Microbiol 2015;17:2099–113. <https://doi.org/10.1111/1462-2920.12564>.

[62] Kjeldgaard B, Listian SA, Ramaswamhi V, Richter A, Kieseewalter HT, Kovács ÁT. Fungal hyphae colonization by *Bacillus subtilis* relies on biofilm matrix components. Biofilm 2019;1:100007. <https://doi.org/10.1016/j.bioflm.2019.100007>.

578 Table 1: Fungal and bacterial strains used in the present study.

Species and strains	Number	Source
Mold fungi		
<i>F. culmorum</i>	DSM1094	DSMZ German Collection
<i>F. graminearum</i>	DSM4528	DSMZ German Collection
<i>B. cinerea</i>	Kern B2	University of California, Davis
Bacteria		
<i>B. subtilis</i>	BCF001	Chr. Hansen A/S
<i>B. amyloliquefaciens</i>	BCF007	Chr. Hansen A/S
<i>B. paralicheniformis</i>	BCF009	Chr. Hansen A/S
<i>B. velezensis</i>	BCF015	Chr. Hansen A/S

579

580

A

B

Fig. 1: Fungal inhibition assays. (A) *B. subtilis* BCF001, *B. amyloliquefaciens* BCF007, *B. paralicheniformis* BCF009 and *B. velezensis* BCF015 were spotted around central inocula of *F. culmorum*, *F. graminearum* and *B. cinerea* on agar medium. Inhibition zones were observed 4-5 days post inoculation. (B) In each well of a 48-well microtiter plate, molten PDA medium was aliquoted and let to solidify. 5-fold dilution series of *Bacillus* strains were prepared from cultures normalized to the same OD₆₀₀. In each column, consecutive wells were co-inoculated with the *Bacillus* dilution series and a fixed quantity of fungal spores (constant volume of fungal spore suspension) on the agar surface. The spore suspensions were mixed thoroughly before aliquoting. Assay results were evaluated by visual inspection after 5 days incubation at room temperature.

Fig. 2: Comparison of *Bacillus* spp. inhibitory properties against *F. culmorum*. (A) *F. culmorum* growth was scored following a three steps scale illustrated with three image examples per category. (B) Dilution series of *B. subtilis* BCF001, *B. amyloliquefaciens* BCF007, *B. paralicheniformis* BCF009 and *B. velezensis* BCF015 were prepared and inoculated in consecutive columns of a 48-well microtiter plate. A constant spore concentration of *F. culmorum* was inoculated in each well. ODs of serial dilutions are indicated on the left panel (OD₆₀₀).

Fig. 3: Inhibition potency of *Bacilli* against fungal phytopathogens. The inhibition potency of *B. subtilis* BCF001, *B. amyloliquefaciens* BCF007, *B. paralicheniformis* BCF009 and *B. velezensis* BCF015 was assigned a numerical score based on the minimal inhibitory cell concentration (MICC) against (A) *F. culmorum*, (B) *F. graminearum* and (C) *B. cinerea*. Inhibition scores were calculated by applying the formula #1.

607

608 Fig. 4 Supernatant inhibition method. A fungal spore suspension was prepared with PDB medium and
 609 the bacteriostatics tetracycline (TET) and chloramphenicol (CM). The suspension was mixed well and
 610 aliquoted into each well of a 48-well microtiter plate. *Bacillus* overnight cultures were adjusted to 2
 611 OD₆₀₀ and were subsequently centrifuged to collect the supernatants. In each column, *Bacillus*
 612 supernatant volumes (10-80 µl) were added to consecutive wells. Fungal growth was evaluated by
 613 spectrophotometric measurements after 5 days incubation at 25°C in darkness.

614

Fig. 5: Fungal Growth inhibition by *Bacillus* spp. culture supernatants. *Bacillus* spp. cultures were grown overnight in LB broth and normalized to OD₆₀₀ 2. Different volumes of cell-inactive supernatants (10, 20, 40, 60 and 80 µl) were added to 48-well microtiter plates containing PDB medium and a fixed fungal spore concentration. Bacterial growth was inhibited by presence of bacteriostatic antibiotics in the culture medium (50 µg/ml chloramphenicol and tetracycline 10 µg/ml, respectively). Plates were incubated at 25°C without shaking for 60 h (in darkness) and fungal growth was measured by spectrophotometry at 600 nm. Plots correspond to inhibition results for (A) *F. culmorum*, (B) *F. graminearum* and (C) *B. cinerea*. Statistical significance for each supernatant volume compared to the control was calculated based on biological triplicates. *P<0.05, **P<0.005, ***P<0.0005. For quantification of the culture supernatant inhibition potency, an inhibition score was assigned to each *Bacillus* strain by subtracting the accumulated relative fungal growth of (D) *F. culmorum*, (E) *F. graminearum* and (F) *B. cinerea* in response to all supernatant volumes from the total potential growth according to Equation #2. Standard deviations were calculated based on biological triplicates.

Supplementary

Table S1: The minimal inhibitory cell concentration (MICC) was determined for *B. subtilis* BCF001, *B. amyloliquefaciens* BCF007, *B. velezensis* BCF015 and *B. paralicheniformis* BCF009, as the lower number of cells (CFUs) that inhibit growth of *F. culmorum*, *F. graminearum* and *B. cinerea* by co-cultivation. The MICC against each fungal species was converted to an inhibition score by formula #1. For quantification of the culture supernatant inhibition potency, an inhibition score was calculated by formula #2 for each bacterial strain and for each experimental cell-inactivation method.

	BCF001	BCF007	BCF009	BCF015
MICC (CFU)				
<i>F. culmorum</i>	19	<3	>3.89*10 ⁴	<5
<i>F. graminearum</i>	57	60	>1.09*10 ⁵	74
<i>B. cinerea</i>	67	150	>1.10*10 ⁵	153
Co-cultivation inhibition score				
<i>F. culmorum</i>	3.8	4.5	0.8	4.4
<i>F. graminearum</i>	3.4	3.4	0.4	3.3
<i>B. cinerea</i>	3.3	3.0	0.4	3.0
Supernatant inhibition score (antibiotics)				
<i>F. culmorum</i>	2.63	2.81	0.62	2.06
<i>F. graminearum</i>	2.14	1.57	-0.43	1.81
<i>B. cinerea</i>	3.53	3.43	1.75	3.50
Supernatant inhibition score (filtration)				
<i>F. culmorum</i>	2.52	2.64	0.46	2.13
<i>F. graminearum</i>	2.04	1.22	-0.50	1.81
<i>B. cinerea</i>	3.55	3.42	1.32	3.53
Supernatant inhibition score (filtration and antibiotics)				
<i>F. culmorum</i>	2.47	2.56	0.26	1.81
<i>F. graminearum</i>	2.35	1.37	-0.40	1.72
<i>B. cinerea</i>	3.50	3.35	0.81	3.50

Fig. S1: Fungal inhibition assay with *Bacillus* dilution series. Dilution series of *B. subtilis* BCF001, *B. amyloliquefaciens* BCF007, *B. paralicheniformis* BCF009 and *B. velezensis* BCF015 were prepared and inoculated in consecutive columns of a 48-well microtiter plate. In each well, a constant spore concentration was inoculated of (A) *F. graminearum* and (B) *B. cinerea*. Pictures are representative of triplicates.

Fig. S2: Variation of *F. culmorum* growth with *Bacillus* supernatants. Inhibition assays with *F. culmorum* were prepared from different spore solutions and reached different final growth after 67h (A) and 72h (B) cultivation in PDB medium both with and without bacterial culture supernatants. Growth was

650 evaluated by spectrophotometric measurements (OD₆₀₀). Standard deviations were calculated from
651 biological triplicates.

652

653

654 Fig. S3: Growth of *F. culmorum*, *F. graminearum* and *B. cinerea* without and without addition of
655 antibiotics (50 µg/ml chloramphenicol and 10 µg/ml tetracycline). ***P<0.0005

656

657 Table S2: Statistical significance of different *Bacillus* supernatant sterilization methods on fungal
658 inhibition results. Statistical significance was calculated using student's t-test. Statistical significant
659 values (p<0.05) are indicated in red.

Filtered supernatants with antibiotics compared to supernatants with antibiotics						
Supernatant volume (µl)		10	20	40	60	80
<i>F. culmorum</i>	BCF001	0.43	0.55	0.69	0.18	0.12
	BCF007	0.21	0.01	0.24	0.82	0.11
	BFC009	0.97	0.2	0.05	0.08	0.01
	BFC015	0.77	0.31	0.09	0.01	0.04
<i>F. graminearum</i>	BCF001	0.88	0.09	0.01	0	0.94
	BCF007	0.31	0.14	0.66	0.85	0.06
	BFC009	0.3	0.44	0.45	0.61	0.78
	BFC015	0.28	0.01	0.6	0.34	0.32

<i>B. cinerea</i>	BCF001	0.11	0.61	0.4	0.89	0.71
	BCF007	0.81	0.51	0.08	0.34	0.34
	BFC009	0.07	0.1	0.02	0.1	0.22
	BFC015	0.46	0.51	0.06	0.82	1
Filtered supernatants with antibiotics compared to filtered supernatants						
Supernatant volume (μl)		10	20	40	60	80
<i>F. culmorum</i>	BCF001	0.77	0.04	0.97	0.52	0.84
	BCF007	0.02	0	0.06	0.02	0.87
	BFC009	0.07	0.13	0.62	0.97	0.09
	BFC015	0.42	0	0.18	0.09	0.04
<i>F. graminearum</i>	BCF001	0.39	0.2	0.01	0.03	0.32
	BCF007	0.42	0.75	0.08	0.05	0.98
	BFC009	0.29	0.8	0.04	0.91	0.37
	BFC015	0.09	0.93	0.71	0.63	0.72
<i>B. cinerea</i>	BCF001	0.55	0.71	0.52	0.35	0.27
	BCF007	0.46	0.82	0	0.11	0
	BFC009	0.24	0.01	0.47	0.22	0.33
	BFC015	0.15	0.89	0.03	0.35	0
Filtered supernatants compared to supernatants with antibiotics						
Supernatant volume (μl)		10	20	40	60	80
<i>F. culmorum</i>	BCF001	0.38	0.29	0.7	0.04	0.16
	BCF007	0.31	0	0.23	0.01	0.41
	BFC009	0	0.71	0.07	0.07	0.13
	BFC015	0.58	0.61	0.47	0.69	0.27
<i>F. graminearum</i>	BCF001	0.42	0.82	0.16	0.81	0.37
	BCF007	0.19	0.12	0.18	0.03	0.07
	BFC009	0.19	0.54	0.14	0.42	0.24
	BFC015	0.2	0.19	0.53	0.81	0.16
<i>B. cinerea</i>	BCF001	0.2	0.32	0.35	0.5	0.44
	BCF007	0.03	0.47	0.04	0.36	0
	BFC009	0.29	0.47	0.06	0.13	0.56
	BFC015	0.32	0.7	0.06	0.19	0.01

660

661

Fig. S4: Comparison of methods to circumvent bacterial cell growth in fungal inhibition assays with bacterial supernatants. Fungal spore suspensions of *F. culmorum* (A,B), *F. graminearum* (C,D) and *B. cinerea* (E,F) were inoculated with filtered bacterial culture with antibiotics (A, C, and E) or without addition of antibiotics (B, D, and F). *P<0.05, **P<0.005, ***P<0.0005.

667

668 Fig. S5: Comparison of supernatant inhibition potencies using *Bacillus* cell inactivation by filtration and
669 antibiotic addition or by filtration only. The culture supernatant inhibition potencies of *B. subtilis*
670 BCF001, *B. amyloliquefaciens* BCF007, *B. paralicheniformis* BCF009 and *B. velezensis* BCF015 against
671 *F. culmorum* (A,D), *F. graminearum* (B,E) and *B. cinerea* (C,F) were calculated by applying the formula
672 #2. The effect of bacterial cell inactivation by filtration and antibiotic addition (A-C) was compared to
673 bacterial cell inactivation by filtration (D-F).