



Volatiles produced by *Streptomyces* spp. delay rot in apples caused by *Colletotrichum acutatum*

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ABSTRACT

Volatile organic compounds (VOCs) produced by microorganisms may prevent postharvest rot in fruits. Here, it was examined if VOCs from different species of *Streptomyces* can control infection in apples caused by the fungal pathogen *Colletotrichum acutatum*. Incubation of *C. acutatum*-infected apples in semi-closed boxes with actively growing strains of three *Streptomyces* (*S. coelicolor*, *S. diastatochromogenes* and *Streptomyces* strain 2R) showed that VOCs reduced rot areas of the apples by 45-66% after 8 days and 39-57% after 10 days, relative to infected apples incubated without *Streptomyces*. No differences in inhibition among the three strains were seen. In contrast, a mutant strain of *Streptomyces* that lacks major genes involved in biosynthesis of secondary metabolites, did not reduce development of rot in the apples. Furthermore, *Streptomyces* VOCs reduced radial hyphal growth of *C. acutatum* on agar. Several of the VOCs produced by three *Streptomyces* strains have previously shown fungicidal properties. Although the specific VOCs being active in inhibition of *C. acutatum* remain to be determined, VOCs may have a great potential as biofumigants to minimize postharvest diseases in fruits.

1. Introduction

In most studies on microbial interactions, cell-to-cell contact or dispersal of soluble compounds or substances dissolved in cellular extracts have been examined, while little attention has been paid to volatile compounds (Audrain et al., 2015; Garbeva and Weiskopf, 2020). Microbially active volatile compounds include both organic molecules, e.g., terpenes, hydrocarbons and acids, as well as inorganic substances, e.g., NO, H₂S and HCN (Audrain et al., 2015). Among bacteria, several species are known to produce volatile organic compounds (VOCs), but in particular species of *Streptomyces* have been recognized as producers of a wide spectrum of VOCs, especially of terpenoids, but several other chemical groups of VOCs have also been identified (Pollak and Berger, 1996; Salwan and Sharma, 2020).

VOCs produced by strains of *Streptomyces* have shown efficient in “biofumigation” for a general modulation of fungal infections in plants and hereby stimulating the plant growth (Garbeva and Weiskopf, 2020), but also for inhibition of specific fungal diseases, e.g., leaf spot disease in cabbage (Wonglom et al., 2019), and for reduction of common storage fungi (Wang et al., 2013), e.g., fungal growth on soybean seeds (Boukaew and Prasertsan, 2020). Profiles of VOCs in these studies

showed that up to 39 different VOCs were produced by different strains of *Streptomyces*, with geosmin (Boukaew and Prasertsan, 2020) and 2-methylisoborneol (2-MIB) (Wang et al., 2013) being among the most abundant VOCs. Yet, it remains to be determined which specific VOCs that are most active in affecting fungal growth.

Inhibitory effects of fungi by VOCs appear caused by various mechanisms. After fumigation by volatiles from *S. globisporus* JK-1, cells of the plant pathogenic fungus *Botrytis cinerea* showed alternations of the plasma membrane, affecting permeability, osmotic balance and loss of organelles that finally caused plasmolysis of the cells (Li et al., 2012). Furthermore, morphological changes of hyphae and conidia occurred (*ibid*). Likewise, Cho et al. (2017) found damages to cell membranes of *B. cinerea* when exposed to VOCs produced by *Streptomyces* sp. S4-7. Disruption of the endomembrane system and cell wall changes were also shown for *Peronophythora litchii* after fumigation by VOCs produced by *S. fimicarius* BWL-H1 (Xing et al., 2018). Another mode of action in inhibition of fungi is blocking of the glycolysis. Thus, the volatile pentalenol acetone, produced by *S. roseogriseus* and other *Streptomyces*, was found to inhibit glyceraldehyde-3-phosphate dehydrogenase and hereby blocking glycolysis in a variety of fungi (Tetzlaff et al., 2006).

In this study, we tested if VOCs produced by different species of

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Streptomyces can inhibit or reduce rot in apples caused by the fungus *Colletotrichum acutatum*. Species of *Colletotrichum* are among the most common fungi causing postharvest rot in fruits, especially in pome fruit (Wenneker and Thomma, 2020). Antagonistic activity of *S. hygroscopicus* against *Colletotrichum* (isolated from apples and grown on agar media) has previously been observed (Grahovac et al., 2014; Tadijan et al., 2016), but effect of VOCs for modulation of apple rot has so far only been confirmed in one study, in which VOCs from yeast led to an 88% reduction of growth of the post-harvest pathogen *Botrytis cinerea* (Di

Francesco et al., 2015). In our study, apples were inoculated with *C. acutatum* and incubated in semi-closed boxes with actively growing cultures of four strains of *Streptomyces*. One of the *Streptomyces* strains was a mutant in which genes encoding major metabolic pathways were silenced. In addition to study *in vitro* inhibitory interactions between *Streptomyces* strains and *C. acutatum*, the aim of the study was also to determine if exposure with VOCs from *Streptomyces* have a potential for extending storage of apples and other fruits.

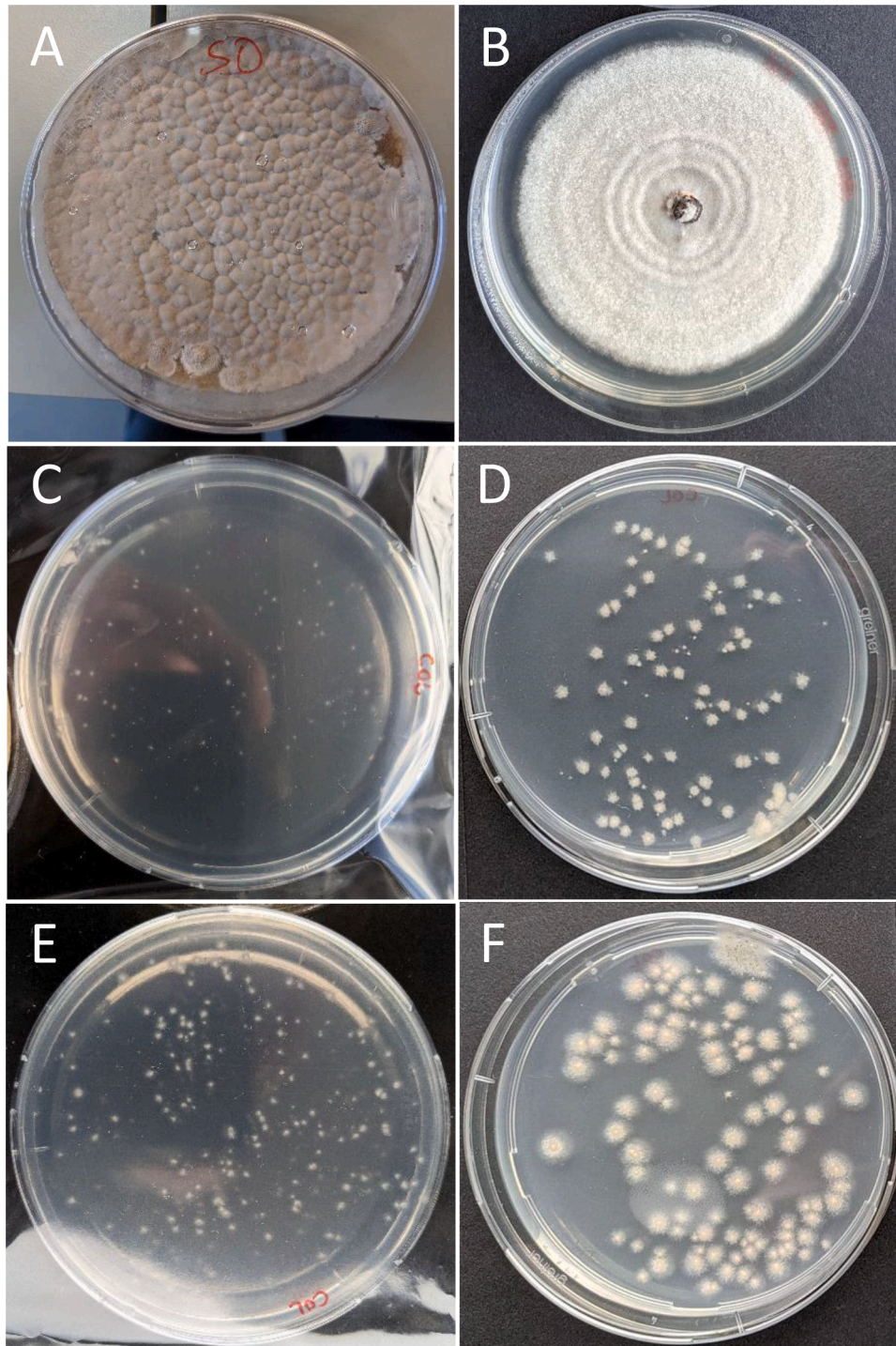


Fig. 1. Pure culture of *Streptomyces diastatochromogenes* on OMA (A) and *Colletotrichum acutatum* on PDA (B). Co-incubation of *C. acutatum* and *S. diastatochromogenes*: colonies of *C. acutatum* at Day 1 (C) and Day 3 (D) after sporulation. Incubation of *C. acutatum* without *S. diastatochromogenes*: colonies of *C. acutatum* at Day 1 (E) and Day 3 (F) after sporulation. Triplicate incubations were used in C, D, E and F.

2. Material and methods

2.1. Strains and cultivation

Four strains of *Streptomyces* were cultured at room temperature on commercial oatmeal agar (OMA) medium (Difco™ Oatmeal Agar, product 255210; Becton-Dickinson, New Jersey, USA). The strains were *S. coelicolor* (identical to *S. albidoflavus*, DSM Strain 40233), *S. diastatochromogenes* (DSM Type Strain 40449), *S. avermitilis* SUKA17 (transformant in which several genes in the biosynthesis of secondary metabolites were deleted (Komatsu et al., 2013), and *Streptomyces* 2R (GenBank Accession No. AY295794; isolated from an aquatic environment; Klausen et al. (2005)). For illustration of the morphology of *Streptomyces* strains, a pure culture of *S. diastatochromogenes* is shown in Fig. 1A. For incubation with apples, 9 cm petri dishes with OMA were inoculated with about 5 million *Streptomyces* conidia per dish.

Conidia of *C. acutatum* strain BJ93 (isolated from an apple rot symptom; mycelium is shown in Fig. 1B) were acquired by cultivation of conidia on potato dextrose agar (PDA) (Becton, Dickinson & Company, product 8801441) in alternating 12:12 h dark-UV cycles. Emerging conidia were harvested by adding 1-2 ml sterile water onto the agar plates and scraping off the conidia with a spatula. After filtration through a 1 mm mesh, numbers of conidia were quantified in a counting chamber by microscopy and stored at -70°C in 70% glycerol until start of the incubations.

2.2. Co-incubation of *Colletotrichum acutatum* and *Streptomyces diastatochromogenes* in polypropylene bags

Possible effects of *Streptomyces* VOCs on radial growth of *C. acutatum* colonies were tested *in vitro* using Petri dishes with vents (94 mm x 16 mm, Greiner Bio-One GmbH, Austria). Fungal conidia were evenly spread on PDA (approximately 100 conidia per dish) and at the same time a culture of *S. diastatochromogenes* (5×10^6 spores/conidia per dish) was spread on OMA. The two cultures were placed in a O₂ and CO₂ permeable, clear polypropylene bag (16 cm x 26 cm, 50 µm thickness; Sealed Air®; Svenska AB, Sweden) that was subsequently heat-sealed to avoid escape of VOCs (Fig. S1). The “vents” between the lid and the dish allowed transport of VOCs between the two petri dishes inside the bags. Empty OMA dishes incubated with *C. acutatum* served as controls. All Petri dishes were incubated in triplicate in the dark at room temperature. At Day 1, areas of fungal colonies appearing on the PDA dishes were measured from diameter of the colonies by microscopy at 200 x magnification and using an ocular micrometer. At Day 3, areas were determined from photographs using a pixel-based software (see below for information on pixel calculation). Only colonies without a nearby colony were measured to minimize the risk of inter-colony effects on the colony development. The total number of measured colonies on the triplicate agar plates ranged from 44 to 60.

Morphology of the hyphae in the incubations was examined by fluorescence microscopy after staining with Calcofluor White (Product 18909; Sigma-Aldrich, St. Louis, Missouri, USA;) that binds to cellulose and chitin in cell walls.

2.3. Co-incubation of *Colletotrichum acutatum* infected apples and *Streptomyces* in boxes

To examine if volatiles produced by *Streptomyces* can have practical applications for reduction of fungal growth, pilot experiments were conducted with wounded apples inoculated with *C. acutatum* spores and placed in sterile polypropylene boxes (Sterivent 107 x 94 x 96 mm, Duchefa Biochemie BV, The Netherlands). Two OMA plates with *S. coelicolor* were then placed next to the apple in each of three boxes and incubated for 17 days (6 days at 25°C and 11 days at 6°C) (Fig. S2). The pilot experiments showed that development of rot was reduced in apples co-incubated with *S. coelicolor*, as compared with rot development (up to

3.5 cm rot) in apples without *S. coelicolor* in the boxes after 17 days. No tissue browning was observed in the controls (apples inoculated with sterile water and incubated in boxes without *S. coelicolor*).

In the final experimental set-up, organic Royal Gala apples at 65–70 mm diameter were wounded on one side with a sterile toothpick to a depth of 3 mm and inoculated with a 10 µl suspension containing 2×10^3 *C. acutatum* conidia, or sterile water for controls. The apples were placed individually in the plastic boxes as described above on folded aluminum foil to minimize movements. In each box, two petri dishes with actively growing *Streptomyces* cultures (*S. coelicolor*, *S. diastatochromogenes*, *S. avermitilis* SUKA17 and *Streptomyces* 2R) on OMA, or empty OMA dishes for controls, were placed. Lids were removed from the petri dishes and open surfaces with the *Streptomyces* cultures were facing the apple. For each *Streptomyces* strain, five apples were incubated in individual boxes. The boxes were closed with lids, but the boxes were not airtight. To exclude build-up of CO₂ from apple respiration or *Streptomyces* growth in the boxes, 10 ml 0.5 M NaOH in a 20 ml glass vial was placed in each box and replaced with fresh NaOH every second day. The apples were incubated at 25°C in the dark in an incubator.

Preliminary experiments showed that incubation of apples with *Streptomyces* on OMA led to formation of condensed water on walls and bottom of the boxes, except for cultures of *S. avermitilis* SUKA17. To ensure that a high humidity did not interfere with potential effects of the VOCs, 20 g of 3 mm size silica gel beads with indicator (Product 101969; Merck, Kenilworth, New Jersey, USA) were placed in a 55 mm petri dish on top of each apple in the boxes and replaced daily. No effects of the silica gel on degree of *Colletotrichum* infection of the apples were seen in an initial, week-long incubation with and without the silica gel beads. The experimental set-up is displayed in Fig. 3, showing the infection status at Day 6 and 10.

2.4. Calculation of areas of rot in the apples

Areas of rot of the apples, i.e., brown areas on the surface of the apples caused by fungal infection, were monitored by measuring numbers of pixels in the infected areas on photographs of the apples, using the GIMP-2.10 software (www.gimp.org). The software detects deviant colors on pictures by defining a suitable threshold of selectivity of colors. The number of pixels were converted to areas from a 1 x 1 cm square placed as a reference on one of the apple boxes for each treatment (Fig. 3) and on petri dishes lids in the *in vitro* co-incubation experiment in the bags.

3. Results

3.1. Effect of *Streptomyces* VOCs on colony size of germinating *C. acutatum* spores

The size of emerging colonies at Day 1 and 3 after inoculation of *C. acutatum* spores on PDA plates were significantly reduced when *C. acutatum* was co-incubated with an actively growing *Streptomyces* culture in sealed polypropylene bags. When incubated alone, mean colony areas of *C. acutatum* were 0.69 ± 0.01 mm² (Day 1) and 21.8 ± 6.30 mm² (Day 3), but was reduced to 0.30 ± 0.02 mm² (Day 1) and 5.06 ± 0.06 mm² (Day 3), when incubated with *S. diastatochromogenes* (Fig. 1). This corresponds to a reduction of the colony areas by 56% at Day 1 and 77% at Day 3.

The reduced colony size appeared to be mainly due to a shorter hyphal length, but a reduced density of hyphae within the colonies was also observed. Staining with Calcofluor White indicated that hyphae morphology changed from “feather” or branching-like structures when grown without *S. diastatochromogenes*, to thicker hyphae with fewer side branches when co-cultured with *S. diastatochromogenes* (Fig. S3). The number of emerging colonies appeared not to be affected by the co-cultivation, indicating that the *Streptomyces* strains did not affect the germination of *C. acutatum* conidia, but this was not tested for statistical

significance.

3.2. Effects of VOCs on *C. acutatum* rot in apples

All apples infected with *C. acutatum* developed emerging brown rot symptoms and after 6 days of incubation, there was no significant differences between co-incubation with or without any of the four different *Streptomyces* strains tested (ANOVA; $p > 0.05$). However, at Day 8 and 10, reduction of rot in the apples was seen for both *S. coelicolor*, *Streptomyces* sp. 2R and *S. diastatochromogenes* with mean rot areas at Day 8 of 1.66–2.67 cm², as compared to 4.87 cm² in the *C. acutatum* controls (infected apples incubated without *Streptomyces*), and mean rot areas at Day 10 of 3.57–5.01 cm² (8.27 cm² in the controls) (Fig. 2). The lower rot areas at Day 8 and 10 correspond to a significant reduction of 45–66% and 39–57% at Day 8 and 10, respectively (ANOVA; $p < 0.05$). There was no significant difference in inhibition between the three *Streptomyces* strains at Day 8 and 10 (ANOVA; $p > 0.05$). Furthermore, co-

incubations with *S. avermitilis* SUKA17 had no influence on rot development (ANOVA; $p > 0.05$). The degree of infection of the apples due to the *C. acutatum* infection is illustrated in Fig. 3, showing the infection status at Day 6 and 10. No infections were observed in the apples inoculated with sterile water.

After Day 10, areas of rot in the apples continued to increase in all the infected apples, and at Day 17 no differences in rot areas between controls and infected apples were seen, irrespective of *Streptomyces* species in the co-incubations (data not shown).

4. Discussion

Co-cultivation of the fungus *C. acutatum* with the three *Streptomyces* stains (*S. coelicolor* strain 40233, *S. diastatochromogenes* strain 40449 and *Streptomyces* sp. 2R) had a clear inhibitory effect on the fungus, but no inhibition was seen for the mutant *S. avermitilis* SUKA17. An inhibition occurred whether *Streptomyces* and the fungus were co-incubated as

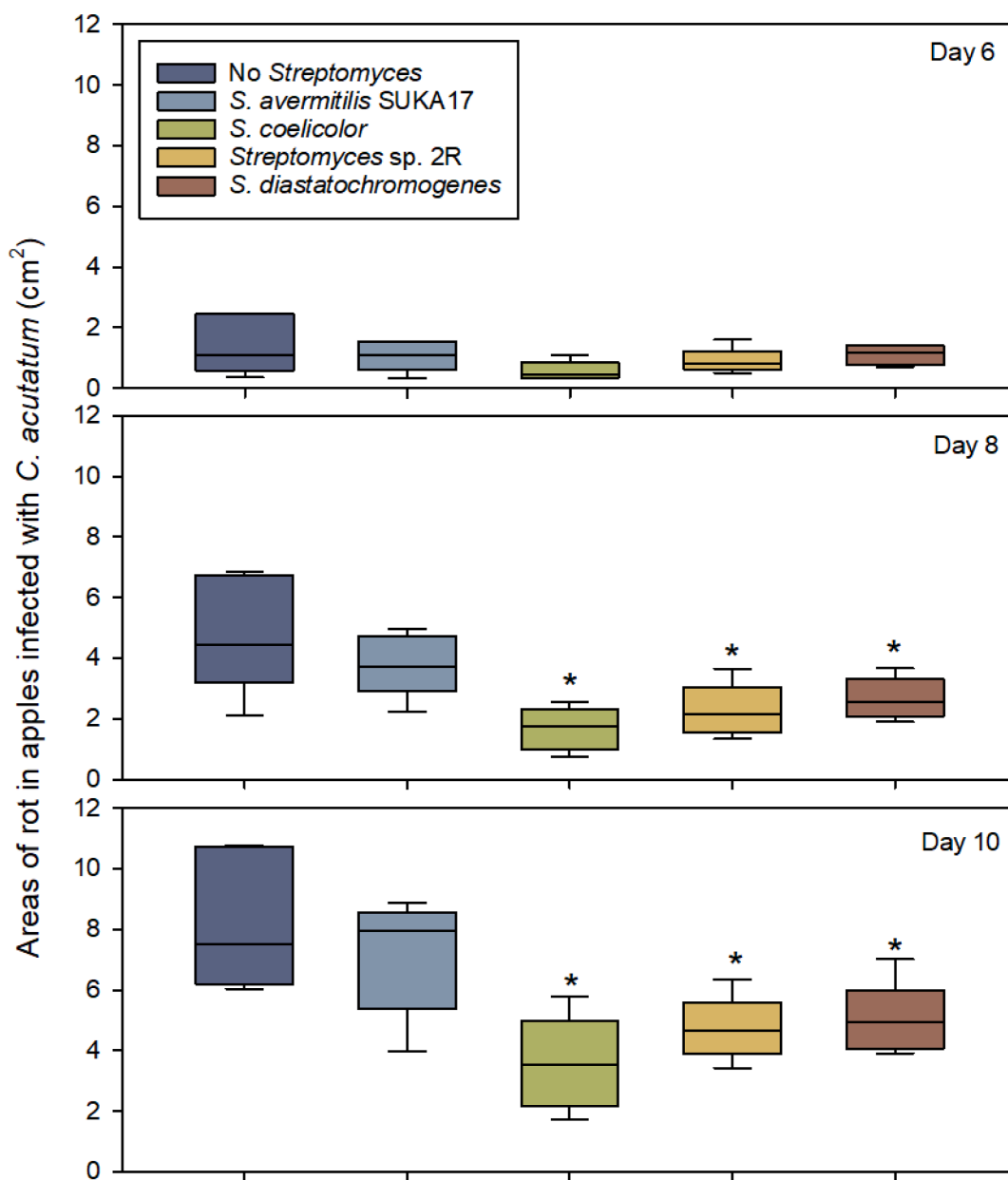


Fig. 2. Areas of rot in apples infected with *C. acutatum* spores and incubated without *Streptomyces* or co-incubated with one of four different *Streptomyces* cultures. The box plots show medians of 5 boxes (each with one apple) and 25th and 75th percentiles. Error bars indicate 10th and 90th percentiles. Asterisks (*) indicate rot areas that were significantly smaller than rot areas in apples incubated without *Streptomyces* (t-test; $p < 0.05$).



Fig. 3. Incubation of apples in semi-tight boxes to test for effects of VOCs produced by four strains of *Streptomyces* on rot caused by *C. acutatum*. The panels show development of rot at Day 6 (upper panels) and Day 10 (lower panels). The incubations were: (A) Apples inoculated with sterile water; no *Streptomyces* in boxes; (B) Apples infected with *C. acutatum* and incubated with two empty petri dishes with OMA media in each box; (C to F) Apples infected with *C. acutatum* and incubated with either *S. avermitilis* SUKA17, *S. coelicolor*, *Streptomyces* 2R or *S. diastatochromogenes* on two OMA petri dishes in each box. All treatments included five apples in individual boxes.

pure cultures on agar media in sealed bags (only tested for *S. diastatochromogenes*), or the fungus was inoculated in apples and co-incubated with *Streptomyces* in semi-closed boxes. Due to the absence of physical contact between the fungus and the bacteria, we assume that the inhibition was due to VOCs produced by *Streptomyces*. None of the three *Streptomyces* strains were capable of completely suppressing fungal growth.

The reduced *C. acutatum* colony areas in the co-incubations with *S. diastatochromogenes* agree with suppression of fungal pathogens by VOCs observed in other studies. In peanut kernels, VOCs produced by *S. yanglinensis* 3–10 were shown to reduce disease severity and inhibit aflatoxin production by two *Aspergillus* strains under storage (Lyu et al., 2020). Likewise, efficacy of volatile compounds from *S. philanthei* RL-1-178 as biofumigant for controlling growth and aflatoxin

production by two aflatoxin-producing fungi on soybean seeds was demonstrated by Boukaew and Prasertsan (2020). The partial inhibition of *C. acutatum* observed in our co-inoculation assays may reflect that VOC production and *Streptomyces* biomass on the OMA agar plates were insufficient for a complete suppression of the fungal growth. Besides, a portion of the produced VOCs probably escaped during the incubation and when opening the boxes for replacement of CO₂ and humidity traps, but we cannot quantify this. The high inhibitory effect observed by Lyu et al. (2020) and Boukaew and Prasertsan (2020) may reflect that a denser *Streptomyces* biomass was obtained on wheat grains used in their study than on the OMA media used in our assay.

The mode of suppression of *C. acutatum* in this study was not determined. We conducted microscopic examination of *C. acutatum* hyphae from the *C. acutatum* - *S. diastatochromogenes* co-incubation assays using the FUN-1 Cell Stain kit (ThermoFisher product F7030) to search for cellular damages, as observed by Li et al. (2012), Cho et al. (2017) and Xing et al. (2018), and causing a changed viability. However, we did not find similar morphological alterations (data not shown). In other studies, SEM analysis showed distortion of hyphae, curling of tips and reduced apical growth after exposure to VOCs (Boukaew and Prasertsan, 2020). Supporting an effect of VOCs on the fungal growth, in the bag co-cultures with *S. diastatochromogenes*, we observed a reduced fungal biomass (smaller colony areas) and a tendency to thicker hyphae with fewer side branches. This may speculatively reflect a reduced metabolic fitness, e.g. due to a suppressed glycolysis (Tetzlaff et al., 2006), but more work is needed to confirm this. The finding that the number of emerging colonies appeared unaffected by the *Streptomyces* strains in our experimental set-up suggests that VOCs of these strains did not inhibit germination of *C. acutatum* conidia.

In the boxes with *C. acutatum*-infected apples, the inhibitory effect by the three *Streptomyces* strains was similar at Day 8 and 10, whereas no inhibition was found for the mutant *S. avermitilis* SUKA17 in which genes encoding major pathways in the production of secondary metabolites have been impaired (Komatsu et al., 2013). The three inhibitory *Streptomyces* strains do all produce several VOCs, but the actual composition of volatiles in the boxes with apples could not be determined due to presence of volatile compounds produced by the apples and that interfered with GC-MS identification of the VOCs (R. Podduturi, personal communication). In cultures of *Streptomyces* 2R and *S. diastatochromogenes* grown on agar plates, more than hundred different VOCs have been identified by each strain, and more than 50 terpenoids were detected among these VOCs (Podduturi et al., 2018). Similarly, *S. coelicolor* strain 40233 has been shown to produce a large number of secondary, volatile metabolites (Wilkins and Schöller, 2009). Some of the VOCs identified by the three *Streptomyces* strains are also components of fungicidal essential oils produced by plants. This applies to β -pinene (Alvarez-Castellanos et al., 2001; Asili et al., 2009), β -myrcene (KpadonouKpoviessi et al., 2012) and humulene (Malik et al., 2019). Furthermore, dimethyl disulfide, which is produced by *S. coelicolor* and *S. diastatochromogenes* (Wilkins and Schöller, 2009) has been shown to effectively inhibit plant pathogenic fungi (Papazlatani et al., 2016; Tyagi et al., 2020). Supporting an inhibitory effect by dimethyl disulfide, Wang et al. (2013) observed that *S. albobiflavus* produced this compound and that it had antifungal activity against *F. moniliforme*. Similarly, Li et al. (2010) found that disulfide and trisulfide compounds, but also acetophenone, produced by *S. globisporus* strain JK-1, were important in suppression of a *Penicillium* strain. Another compound produced by *S. coelicolor*, cyclopentanone, has also been shown to inhibit both spore germination and growth in fungi (Lin et al., 1993).

The large variety of VOCs produced by the present three strains complicates identification of the most inhibitory compound(s). In other studies, attempts have been made to detect the dominant fungal inhibitory VOCs produced by *Streptomyces*. Lyu et al. (2020) found that the VOCs methyl 2-methylbutyrate, 2-phenylethanol and β -caryophyllene, produced by *S. yanglinensis* 3-10, all had antifungal properties and inhibited germination of *Aspergillus* spores. A similar effect was not

observed for 2-MIB which was the dominant VOC produced by *S. yanglinensis* 3-10. In another study, geosmin, L-linalool and 2-mercaptoethanol were the most abundant VOCs produced by *S. philanthi* RL-1-178 (Boukaew and Prasertsan, 2020). These three VOCs also controlled growth and aflatoxin production in two *Aspergillus* strains. Recently, Ayed et al. (2021) showed that VOCs, e.g., the terpenoids 3-carene 2,5-dione, geosmin and β -cubebene, produced by *Streptomyces* sp. strain S97, significantly inhibited growth of grey mold caused by *Botrytis cinerea*, but the most effective VOC in this study could not be identified. Largely all studies have focused on organic compounds as the inhibiting substances, but the inorganic compound ammonia, produced by *Streptomyces* symbionts in nests of the leaf-cutting ant *Acromyrmex*, was recently also been found as a strong inhibitor of the parasitic fungus *Escovopsis* (Dhodary and Spiteller, 2021).

When comparing effects of VOCs on fungal growth, the experimental set-up should avoid artifacts and biased results. Accumulation of gasses other VOCs, e.g., ammonia and CO₂, as well as formation of metabolic water, might affect the fungal growth. Here, we observed that humidity from the actively growing *Streptomyces* cultures on the petri dishes led to condensation of water in the boxes with apples, and tests revealed that the high humidity stimulated the fungal infection of the apples. Therefore, the water content was stabilized by silica, and we further added CO₂-trapping material to the boxes to eliminate build-up of CO₂. Also, to ensure that oxygen was not limiting, air transparent bags and semi-closed boxes were used in the incubations.

For practical biofumigation purposes, exposure time is also relevant. Typically, a continuous co-incubation of *Streptomyces* and fungus for several days has been performed (Li et al., 2012, 2010; Boukaew and Prasertsan, 2020), as in the present study. However, a shorter exposure period might also be efficient as observed by Boukaew et al. (2021) who exposed *Colletotrichum*-infected chili fruits to a dense biomass of *Streptomyces* for 24 h and found a complete inhibition of the fungus.

The present observation of a reduced growth of *Colletotrichum* by VOCs produced by the three *Streptomyces* strains is the first observation of volatiles causing inhibition of rot in apples. Analysis of inhibitory effects of VOCs on fungal growth has mainly focused on experimental assays, rather than practically applicable approaches that might be developed into large-scale suppression of postharvest rot in stored fruits. Other than this study, the prevention of rot in chilis by Boukaew et al. (2021), and reduction of *Penicillium italicum* growth in infected mandarins (*Citrus microcarpa*) by exposure to VOCs produced by *S. globisporus* JK-1 (Li et al., 2010), are among assays that might be developed into practical antifungal treatments of postharvest crops. Application of biofumigation by VOCs for control of fungal diseases has potential for being a future “green” treatment, but practical issues, e.g., with respect to selection of efficiently VOC-producing species or strains, condition for incubation, as well as possible side effects on the crops, must be considered and optimized.

In future studies of fungal inhibition by *Streptomyces* metabolites, the spectrum of examined compounds should not focus only on terpenoids, as in most of the studies cited above. Including other microbial compounds with antifungal properties, e.g., dimethyl disulfide and ammonia, is probably also of interest. It might also be relevant to examine if an inhibitory effect is merely related a cocktail effect (low concentrations of many compounds) rather than a high concentration of a single compound.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at ...

CRedit authorship contribution statement

Thea Jepsen: Conceptualization, Data curation, Formal analysis, Writing – original draft. **Birgit Jensen:** Conceptualization, Writing – original draft. **Niels O G Jørgensen:** Conceptualization, Data curation, Writing – original draft.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Niels O. G. Jørgensen reports financial support was provided by The Danish Nature Agency.

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Supplementary materials

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