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# Actinobacteria Warfare Against the Plant Pathogen *Sclerotinia sclerotiorum*: 2,4,6-Trimethylpyridine Identified as a Bacterial Derived Volatile With Antifungal Activity

Katharina Belt[1] | Gavin R. Flematti[2] | Björn Bohman[2,3,4] | Heng Chooi[2] | Margaret M. Roper[1] | Lachlan Dow[5,6] | Andrew W. Truman[7] | Barrie Wilkinson[7] | Karam B. Singh[1] | Louise F. Thatcher[5,6,8]

[1]Commonwealth Scientific and Industrial Research Organisation (CSIRO) Agriculture and Food, Floreat, Western Australia, Australia | [2]School of Molecular Sciences, The University of Western Australia, Crawley, Western Australia, Australia | [3]Department of Plant Protection Biology, The Swedish University of Agricultural Sciences, Lomma, Sweden | [4]Research School of Biology, The Australian National University, Acton, Australian Capital Territory, Australia | [5]Commonwealth Scientific and Industrial Research Organisation (CSIRO) Agriculture and Food, Acton, Australian Capital Territory, Australia | [6]CSIRO Microbiomes for One Systems Health Future Science Platform, Acton, Australian Capital Territory, Australia | [7]Department of Molecular Microbiology, John Innes Centre, Norwich Research Park, Norwich, UK | [8]CSIRO Advanced Engineering Biology Future Science Platform, Acton, Australian Capital Territory, Australia

**Correspondence:** Katharina Belt (katharina.belt@uwa.edu.au)

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## ABSTRACT

Bacteria and fungi produce a wide range of specialised metabolites, including volatile organic compounds (VOCs) that can act as signals or act directly to inhibit niche-competing microbes. Despite their ecological importance, most VOCs involved as signalling compounds remain uncharacterised. We have previously screened a collection of Actinobacteria strains sourced from Western Australia for their ability in vitro to suppress the growth of plant fungal pathogens. Here we explored the potential of four of the most active strains to produce antifungal metabolites by growing the strains on a range of nutrient-containing media. A casein-based (CYPS) culture medium was found to induce the production of antifungal compounds with high activity against *Sclerotinia sclerotiorum*, a major necrotrophic fungal pathogen of crops such as canola. We further observed that VOCs were produced that influenced pH and affected the bacterium-fungus interaction. The presence of *Sclerotinia* induced further VOC production in the Actinobacteria. Solid-phase microextraction (SPME) coupled with gas chromatography–mass spectrometry (GC–MS) analysis identified 2,4,6-trimethylpyridine, a compound not identified previously from Actinobacteria, which showed antifungal activity against different isolates of *S. sclerotiorum* and increased the pH of the medium. Overall, this study showed that Actinobacteria or their volatile products have the potential to be used in the protection of crops against *S. sclerotiorum*.

## 1 | Introduction

Fungal diseases of crops are severely threatening yield productivity in agriculture. Today, fungal disease control is built on breeding and selecting resistant crop varieties, cultural practices, and the use of fungicides (Russell 2005). However, many fungal pathogens overcome plant host resistance (Rimbaud et al. 2018) and broad-spectrum fungicides over time (Hawkins and Fraaije 2018; Lucas, Hawkins, and Fraaije 2015), leading to a requirement for new formulations and modes of action.

Human food supplies as well as animal feeds depend on crops such as canola, pulses, rice, wheat and maize. Canola (*Brassica napus*, oilseed rape) is the second most important oilseed crop after soybean, with a production of approximately 70 million tonnes globally in 2019/20 (Mobsby 2016; Sadras and Calderini 2021). Fungal diseases such as Sclerotinia stem rot, caused by the fungus *Sclerotinia sclerotiorum*, are major threats to canola production (Derbyshire and Denton Giles 2016). Cultural and management practices, in particular the heavy use of synthetic fungicides, are currently the main approach to control the disease (O'Sullivan, Belt, et al. 2021).

Suppression of plant pathogen populations by living organisms, known as biological control, offers many possibilities for crop protection (Heimpel and Mills 2017). Microbial biological control agents (MBCAs) can protect crops from pathogens via multiple modes of action (Aggarwal, Thind, and Sharma 2016; Köhl, Kolnaar, and Ravensberg 2019), including through competition, antibiosis, or directly inducing or priming plant resistance response without direct interaction with the pathogen (Conrath et al. 2015; Pieterse et al. 2014). One group of MBCAs is Actinobacteria, a group of Gram-positive soil bacteria well known for their production of specialised metabolites. *Streptomyces* are particularly well-known producers of antibiotic compounds, such as hygromycin and streptomycin (de Lima Procópio et al. 2012; Dow et al. 2023; Hutchings, Truman, and Wilkinson 2019). The mechanisms by which *Streptomyces* and other Actinobacteria inhibit plant fungal pathogens are broad, with many aspects, including mechanistic details, still unexplored (Barka et al. 2015; Rey and Dumas 2017; Zhang et al. 2020).

Amongst these diverse specialised metabolites are volatile organic compounds (VOCs), defined by their relatively low boiling point (Choudoir et al. 2019). VOCs produced by microorganisms in nature play important ecological roles, including the suppression of diseases in plants (Avalos et al. 2020). One famous example is geosmin, an earthy-smelling compound occurring on wet soil after rain (Becher et al. 2020; Dickschat et al. 2005; Gust et al. 2003) that was recently found to attract soil-dwelling invertebrates that helped the geosmin-producing *Streptomyces* spp. disperse its spores (Becher et al. 2020). Such volatiles can have diverse effects on different organisms. For example, sulphur-containing compounds such as dimethyldisulfide and dimethyltrisulfide have been shown to strongly inhibit fungal growth and to promote the growth of bacteria and plants (Garbeva et al. 2014a, 2014b; Meldau et al. 2013). In other cases, plant exposure to microbial volatiles can trigger defence responses and systemic resistance, thus protecting the plant from pathogen infection (Ditengou et al. 2015; Pieterse et al. 2014). Microbial consortia can have synergistic effects on plant health when inoculated together with the host plant in vivo (Prigigallo et al. 2023). The diverse effects induced by VOCs may lead to many new opportunities for the application of volatile-producing microorganisms such as Actinobacteria and their secondary metabolites in crop protection.

About 300 Actinobacteria strains have previously been isolated from the south of Western Australia (O'Sullivan, Roper, et al. 2021; Roper 2004). In this study, four strains from this collection were selected (three *Streptomyces* and one *Micromonospora*) and screened for antifungal activity against *S. sclerotiorum*, based on previous data, which detected antifungal activity against *Sclerotinia* and other necrotrophic pathogens in vitro (Belt et al. 2021; Roper et al. 2020). Our aims were to gain insight into possible mechanisms underlying this cross-kingdom chemical communication and to identify new antifungal compounds with the potential for application as future biofungicides against *Sclerotinia*. We identified nutrient media compositions that greatly enhanced the induced antifungal activity of the Actinobacteria in vitro, identified a pyridine antifungal volatile, and demonstrated that pH can be a major factor involved in the Actinobacteria-*Sclerotinia* interaction.

## 2 | Experimental Procedures

### 2.1 | Microbial Strains

Actinobacteria isolates were sourced from two CSIRO culture collections (O'Sullivan, Roper, et al. 2021; Roper 2004). Strains were cultured and maintained on plates containing half-strength potato dextrose agar (PDA) at 26°C in the dark. Spores were gently scraped off the plates once the Actinobacteria started to sporulate (1–2 weeks). They were resuspended in sterile water and filtered through miracloth to remove mycelial fragments. The concentration of spore stocks (colony-forming units, cfu) was calculated from a decimal dilution series on PDA plates. A solution of $10^6$ cfu/mL was prepared in sterile water for use in all assays. Actinobacteria used in this study included a *Streptomyces griseoaurantiacus* MH191 (whole genome sequencing deposited at DDBJ/ENA/GenBank under the accession number CP150879-CP150880), *Streptomyces tendae* 9a, *Streptomyces tendae* KB001, and *Micromonospora sp*. MH33 (whole genome sequencing deposited at DDBJ/ENA/GenBank under the accession number MUYZ00000000) (Table S1). DNA extractions and whole genome sequencing for MH191 and MH33 were performed as described previously (Thatcher et al. 2018). The strain MH191 genome was assembled into 7.09 Mbp (296 scaffolds; N50: 47), and MH33 into 7.68 Mbp (662 scaffolds; N50: 107 scaffolds).

Three isolates of *S. sclerotiorum* were used in this study. Model strain 12.11 (Thatcher et al. 2017) and two isolates, 11.19 and 6.1, that were kindly provided from the Centre for Crop and Disease Management (CCDM) at Curtin University (Denton-Giles et al. 2018). For *Sclerotinia* growth, a single sclerotia was placed onto half-strength PDA plates for 3–4 days at room temperature in the dark to allow mycelial growth.

### 2.2 | Anti-*Sclerotinia* Bioactivity Assays on Agar Plates

An aliquot (50 μL) of Actinobacteria spore solution ($10^6$ cfu/mL) was spread over the top quarter of each agar plate containing different nutrient media (CYPS, ISP2, MEP, YEG, Table S2) and incubated for 14 days at 26°C in the dark to facilitate the production of potential antifungal compounds (Siupka et al. 2020). At the end of the 14 days, a *Sclerotinia* agar plug was taken from the outer edge of the pathogen mycelial growth and placed on the opposite end (to the Actinobacteria) on each agar plate.

Plates were incubated for another 7 days at 26°C in the dark. The growth inhibition zone of *Sclerotinia* was used to determine antifungal activity. To test for volatiles, divider plates with a strict separation between the two ends of the plate were used. Commercial pH strips (Merck, pH 0–14, p/n 1.09535) were used to measure pH change in the plates. The pH strips were placed face down on the media plates and incubated for 2 min before the colour change was analysed for the corresponding pH range based on the manufacturer's directions. To measure inhibition zones, *Sclerotinia* growth was observed over 10 days, and inhibition zones were measured after three and 10 days on CYPS media and 3, 5 and 10 days on MEP media, as *Sclerotinia* was growing much faster on these plates.

### 2.3 | *S. sclerotiorum* Growth on pH-Adjusted CYPS and PDA Plates

Agar plates with either CYPS or half-strength PDA (Table S2), adjusted to pH 5 (acetic acid) or pH 7 (1 M NaOH), were used to test the effect of pH on *Sclerotinia* growth following the transfer of mycelia agar plugs of *S. sclerotiorum* to the agar surface. Two isolates were tested: (1) *S. sclerotinia* strain 11.19, which has shown high production of oxalic acid in previous studies and is highly pathogenic to canola (Denton-Giles et al. 2018), and (2) our model strain *S. sclerotinia* 12.11. Growth on plates and change of pH in the medium were observed over 7 days using pH strips as described above. Assays were carried out with three biological replicates consisting of new batches of media, fungal and bacterial culture, and the average of inhibition was calculated based on these replicates. The length of each petri dish was set to 100, meaning if *Sclerotinia* would grow over the whole length of the plate, it would grow 100%, and 0% inhibition would be observed. The length of mycelia growth was measured using Image J, and inhibition was calculated relative to the length of the petri dish.

### 2.4 | Identification of Bacterial Compounds Using Solid-Phase Microextraction (SPME) and GC–MS

Actinobacteria isolates (Table S1) were grown for 2 weeks at 26°C under sterile conditions on CYPS and MEP media in 50-mL beakers, covered with aluminium foil. A solid-phase microextraction (SPME) fibre (divinylbenzene/carboxen/polydimethylsiloxane, p/n 57348-U, needle size 24 ga, StableFlex; Sigma, USA) was used to capture volatiles from the headspace of the cultures. The fibre was activated at 280°C for 5 min between volatile collections of 24 h to purge any volatiles from the previous 24 h collection. The fibres were incubated in the beakers for 24 h to capture volatiles that were emitted by Actinobacteria on the different media types.

Actinobacteria volatiles were trapped by SPME and analysed by GC–MS. The Actinobacteria strains were grown for 14 days before SPME sampling for 24 h occurred. For co-cultures, one *Sclerotinia* mycelia plug was added after 14 days of preculturing the Actinobacteria, and SPME sampling was conducted for two subsequent days. As references, CYPS and MEP media-only plates were also analysed. All assays were carried out with three biological replicates, and only compounds that were detected in all replicates were included in this study.

Structural characterisation of compounds was carried out by (a) comparison of mass spectra with MS databases (Wiley Registry 12th edition and NIST mass spectral library 2020), (b) retention indices with those present in the Wiley and NIST libraries (Stein 2012) or Flavornet (Arn and Acree 1998), and (c) obtaining standards for compounds of special interest.

GC–MS total ion chromatograms were recorded on an Agilent 5973 mass detector connected to an Agilent 6890 GC equipped with a DB-5 ms (50 m × 0.2 mm id. × 0.33 μm film thickness; J&W Scientific, USA) column with UHP helium as the carrier gas (1 mL/min). Splitless injections with a purge time of 1 min were applied. The initial oven temperature was set to 40°C before increasing at 5°C/min to 200°C, then 10°C/min to 280°C which was held for 5 min (inlet temperature 280°C; transfer line 280°C). The ion source was set to 200°C, and the mass range to 45–400 amu. Gas chromatography with high-resolution mass spectrometry (GC–HRMS) was performed on a Waters GCT Premier TOF-MS using a DB-5 ms column (30 m × 0.25 mm id. × 0.25 μm film thickness; J&W Scientific, USA) and identical method conditions as described above.

### 2.5 | Bioactivity Assays of Identified Compounds

All compounds tested were purchased from Sigma Aldrich. Assays were performed as described in previous studies (Fernando et al. 2005) with slight modifications. To test antifungal activity, 50 μL of neat 2,4,6-trimethylpyridine (~46 mg), 2,3-dimethylpyrazine (~51 mg), 2,5-dimethylpyrazine (~50 mg), and 6-methyl-2-heptanone (~41 mg) were assayed on half-strength PDA plates against different *Sclerotinia* isolates by addition of the compounds onto sterile Whatman paper discs. A *Sclerotinia* mycelia plug was added to the end opposite to the Whatman paper, and growth of the pathogen was observed over 7 days. The pH of the medium was measured using a pH strip (Merck). Combinations of compounds were tested with a total volume of 100 μL (50 μL compound 1 and 50 μL compound 2, both neat) on Whatman paper discs on half-strength PDA plates. All assays were carried out in triplicate.

### 2.6 | Test for Ammonia Production by Actinobacteria

To test for ammonia production of MH191, MH33, 9a, and KB001 on CYPS plates, bacteria were grown on CYPS divider plates for 14 days before ammonia test strips (Hach, Lane Cove, Australia) were placed on both sites of the divider. Strips were placed on the media for 1 min. To test the cross-reactivity of these strips, 1% and 0.1% (v/v) aqueous solutions of 2,4,6-trimethylpyridine were prepared and the test strips dipped in.

### 2.7 | Buffered Plate Assays

Split agar plates were prepared, with one side containing regular CYPS media, while the other side (the 'fungi' side) contained CYPS media or CYPS media buffered to pH 6.3 using 0.2 M HEPES/HCl. Bioassays were conducted as described above, with one side of the plate either empty or containing 25 μL of 2,4,6-trimethylpyridine

or MH191 culture. Each treatment was conducted with at least five replicates, and the entire experiment was repeated. The pH and presence of ammonia were measured using pH strips and ammonia test strips (Hach) at the termination of the experiment.

### 2.8 | Headspace SPME Analysis

One millilitre of CYP liquid media was inoculated with one of 20 different Actinobacteria isolates in 10 mL GC–MS vials, sealed with screw-cap lids with septa, and incubated for 24 h at 28°C. Three biological replicates were prepared for each isolate, along with uninoculated media as controls. After incubation, cultures were cooled to 8°C and placed on the autosampler stage of a Shimadzu GC2010 gas chromatograph equipped with a GCMS-QP2010 mass spectrometer and a PAT HTX headspace autosampler.

The samples were incubated at 30°C with shaking for 5 min prior to SPME needle insertion. The volatiles were extracted using a StableFlex DVB/CAR/PDMS fibre (Supelco) by equilibrating the needle for 30 min at 30°C, followed by a 5-min desorption. Splitless injection was performed onto an SH-Stabilwax column (0.25 μm thickness, 0.25 mm ID), with helium as the carrier gas at a linear velocity of 30 cm/s. The oven temperature was initially held at 55°C for 5.5 min, then ramped to 170°C over 8 min, followed by an increase to 250°C over 12 min with a 5.5-min hold at 250°C.

Electron impact ionisation was conducted at 200°C, with the interface temperature set to 250°C. Compounds were identified by comparing mass spectra to the NIST database. Extracted ion counts of specific compounds, including 2-ethyl-3,5-dimethylpyridine (EMP) and trimethylpyridine (TMP), were analysed using GraphPad Prism software.

## 3 | Results

### 3.1 | Casein-Based Medium Triggers High Production of Antifungal Volatiles in Actinobacteria

Strain MH191 from our collection of Actinobacteria isolates showed moderate to high in vitro activity against a range of plant fungal pathogens in previous screens when grown on Potato Dextrose Agar (PDA), a common culturing media (O'Sullivan, Roper, et al. 2021; Roper et al. 2016; Roper et al. 2020). As shown in previous reports, inhibition of fungal growth ranged from 49% to 91%, with growth of *S. sclerotiorum* inhibited by 78% (Roper et al. 2016). Isolate MH191 was identified as *Streptomyces griseoaurantiacus* based on whole genome sequencing analysis and was selected as a representative Actinobacterium with potential for further antifungal activity enhancement and functional analysis. To identify culture conditions that would induce higher antifungal compound production in MH191, different culture media were used (Pan et al. 2019), and the inhibition of *S. sclerotiorum* was monitored (Figure 1A). Strain MH191 showed strong (>99%) antifungal activity against *Sclerotinia* when grown on casein-based media (CYPS; casein and yeast extract) (Figure 1A). Culturing on MEP (malt extract, peptone) had the opposite effect, and *Sclerotinia* completely outcompeted MH191. ISP2 (yeast extract, malt extract) plates showed a small inhibition zone between bacteria and fungi. Neutral co-existence was observed on YEG (yeast extract, glucose) plates where *Sclerotinia* mycelia was able to develop on the plate except for where MH191 was present. A comparison of the *Sclerotinia*-only control plates (Figure 1A) showed that MEP and ISP2 supported better fungal growth than CYPS and YEG, indicating that culture conditions also influenced fungal behaviour. Overall, *Sclerotinia* was able to grow and develop sclerotes on all media control plates, confirming that the observed inhibition of growth was caused by MH191.

Based on these results, we hypothesised that antifungal compounds produced by MH191 on CYPS plates were secreted into the agar and caused inhibition of fungal growth. To confirm this hypothesis, we repeated the assay on divider plates (Figure 1B–E; Figure S1). If antifungal compounds were produced and secreted into the medium, they should not be able to pass the divider, and *Sclerotinia* growth should not be inhibited. We used MEP as a negative reference to CYPS, as it showed the least antifungal effect (Figure 1A), and we expected a significant difference in compound production by bacteria grown on these two media. As expected, fungal growth was not inhibited on the MEP divider plates (Figure 1C; Figure S1B). In contrast, and against our hypothesis, fungal growth was completely inhibited on CYPS medium (Figure 1C; Figure S1C). These results indicated that compounds produced on CYPS medium were volatile and able to pass the divider, rather than only diffusing through the medium. Previous studies have shown that volatiles produced by *Streptomyces* species can alter pH and ion availability, making the environment less favourable for the growth of competing microorganisms (Jones et al. 2019; Netzker et al. 2020). To investigate if the volatile compounds produced by MH191 grown on CYPS medium would change the pH of the medium, pH was measured before and after the growth of MH191 (Figure 1B,C; Figure S1D,E). MH191 growth altered the pH of the agar medium from pH 5 to 7 on MEP plates and from pH 6 to 8 on CYPS (Figure 1C; Figure S1D,E) close to where MH191 grew. In contrast to the MEP plates, the CYPS plates also increased the pH of the medium from 6 to 8 on the other side of the divider where *Sclerotinia* was inoculated (Figure 1C; Figure S1E), implying that compounds produced by MH191 on CYPS medium were volatile and increased the pH on both sides of the plate.

To test if other Actinobacteria grown on the casein-based medium could produce antifungal volatiles against *Sclerotinia*, additional Actinobacteria strains from the CSIRO collection were tested on CYPS and MEP in divider plates (Figure S2A,B). These included two other *Streptomyces* strains, *Streptomyces tendae* 9a and *Streptomyces tendae* KB001, and a *Micromonospora* strain, *Micromonospora* sp. MH33 (Table S1). All these strains were selected based on high antifungal activity observed in in vitro bioassays against *S. sclerotiorum* and/or other necrotrophic fungal pathogens (Belt et al. 2021; O'Sullivan, Roper, et al. 2021; Roper et al. 2020).

The growth of all isolates on CYPS medium showed 80%–100% inhibition of *Sclerotinia* growth (Figure S2A,C) and increased the pH of the medium from pH 5 to 7, much like the results observed for MH191. This further confirmed that culturing Actinobacteria on casein-based CYPS medium generally activated the production of specialised volatile metabolites leading to high antifungal activity. All strains failed to show strong

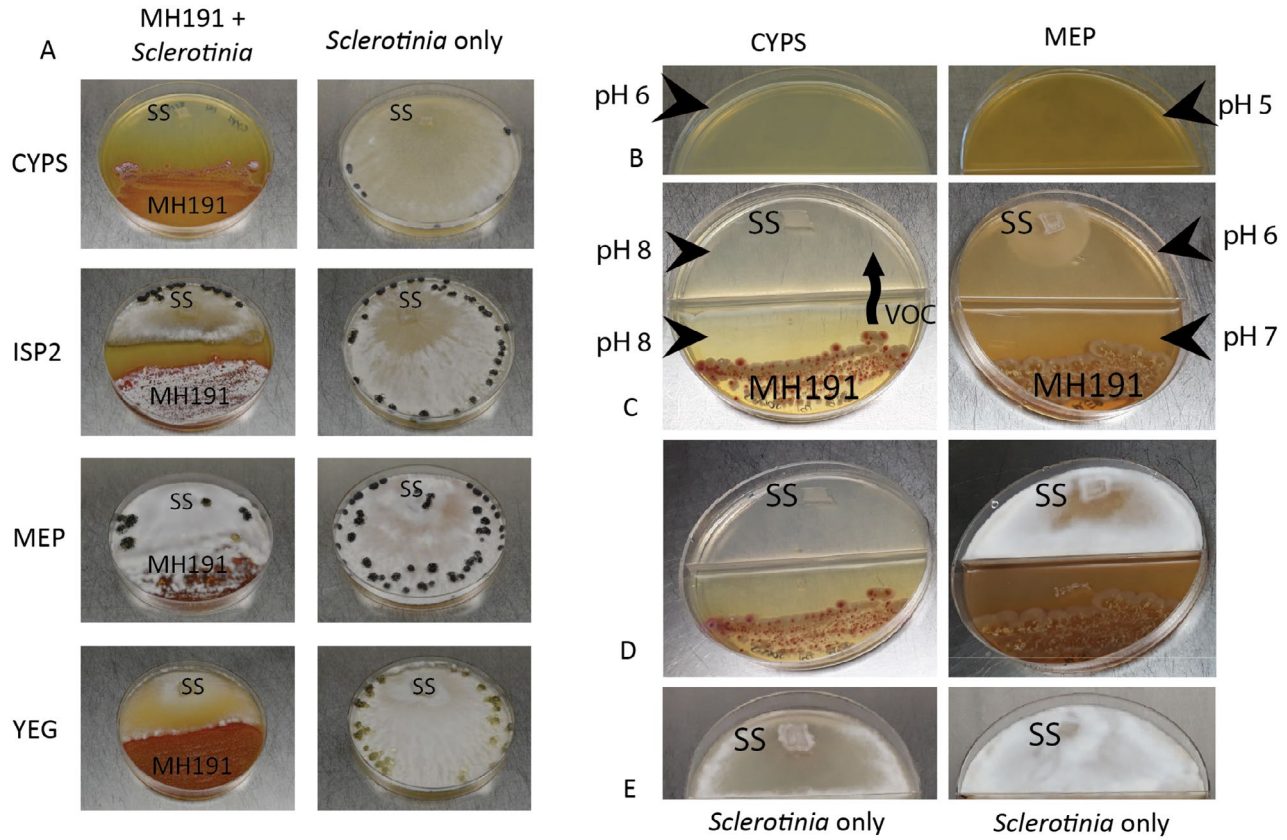


**FIGURE 1** | MH191 produces volatiles that increase pH on casein-based medium and inhibit *Sclerotinia* growth. (A) Four different media types were tested (CYPS, ISP2, MEP, YEG) to identify culture conditions that would trigger antifungal compound production in MH191. MH191 was grown for 14 days before *S. sclerotiorum* (SS) was added to the plate. Control plates containing SS only were included for all media types. (B) CYPS and MEP media only, showing an initial pH of 5 for MEP and 6 for CYPS. (C) MH191 grown on CYPS media triggers the production of VOCs that alter pH in the media from 6 to 8 after 14 days of growth on divider plates and inhibit SS growth opposite the divider. Pictures are taken 2 days after *Sclerotinia* agar plug was added to the plates. As a reference, MH191 was also grown on MEP media, but when grown on these plates, no inhibition of fungal growth was observed. (D) Inhibition of SS after 7 days. Growth was still inhibited on CYPS plates, whereas MEP plates showed full SS mycelium development. (E) *Sclerotinia* only control plates taken 7 days after SS was added to the plate showed mycelia growth over the whole half of the plate for both media, but it did occur to be stronger on MEP media.

antifungal activity when cultured on MEP (Figure S2B,C), showing 0% inhibition of *Sclerotinia* after a growth period of 10 days and without an increase of pH in the media, demonstrating the importance of identifying the optimal culture conditions to screen for desired antifungal activity and potential antifungal compounds.

### 3.2 | *Sclerotinia* Acidifies the Environment to Optimise Growth

Our results indicated that strains MH191, MH33, 9a and KB001 produce volatile antifungal compounds when grown on CYPS medium, and we hypothesised that this inhibition is associated with an increase in pH. *Sclerotinia* virulence depends on fungal enzymes secreted during plant invasion, which are most active at a low pH (Cessna et al. 2000). Oxalic acid is secreted by *Sclerotinia* to lower the pH and to suppress defence responses such as the oxidative burst in the host plant, thereby facilitating pathogenesis (Bateman and Beer 1965; Cessna et al. 2000; Xu et al. 2018). It is therefore possible that our previous observations of impaired *Sclerotinia* growth are associated with changes in the media pH when Actinobacteria are grown. To test whether pH was a driving factor in fungal growth inhibition, *Sclerotinia* was grown on half-strength PDA and CYPS media that were adjusted to pH 5 or 7 (Figures S3 and S4). We tested a *Sclerotinia* isolate highly pathogenic in canola and a high producer of oxalic acid (isolate 11.19) next to our model *Sclerotinia* strain (isolate 12.11). When grown on PDA medium (Figure S3B), both *Sclerotinia* isolates grew at a slightly faster rate on plates with pH initially adjusted to 5 than on plates with pH initially adjusted to 7, but after 4–5 days, all plates showed close to 100% mycelium growth over the plate (Figure S3C), demonstrating that *Sclerotinia* can grow in less acidic environments. We measured changes in pH after 4 days, and both *Sclerotinia* isolates had reduced the pH to 2 on the initial pH 5 plates (Figure S3B). When grown on pH 7 adjusted plates, the pH was reduced to 5 by isolate 11.19 and to 3 by isolate 12.11. When grown on CYPS medium, however, both *Sclerotinia* isolates grew more slowly (Figure S4). Both isolates showed reduced growth at pH 7 for the first 5 days, and only grew about half as fast compared to pH 5 (Figure S4B,C). Over 7 days the pH of plates remained constant (isolate 11.19) or became more acidic (isolate 12.11).

To further analyse the effect of pH on *Sclerotinia* growth on CYPS plates, pH was adjusted to 8, and *Sclerotinia* growth was monitored (Figure S5). Similar to our observation at pH 7 on CYPS plates, growth of isolate 11.19 was more sensitive to higher pH than isolate 12.11. The 11.19 mycelia growth was aberrant compared to control plates grown at pH 5, and pH did not decrease, but still measured pH 8 after 7 days (Figure S5). Mycelial growth for isolate 12.11 on pH 8 CYPS plates was similar to that observed at pH 7 (Figure S4). Combined, these results indicated that pH impacts *Sclerotinia* growth, and the degree of effect may be isolate specific. Altogether, the results indicated that *Sclerotinia* actively acidifies its environment and that the CYPS medium supports *Sclerotinia* growth, but it is not as optimal for growth as PDA. Our results further indicated that *Sclerotinia* growth is not solely inhibited by a higher pH, implying *Sclerotinia* inhibition on divider plates in the presence of MH191 is likely due to a combination of antifungal volatiles and pH manipulation.

### 3.3 | The pH of the Media Is a Driving Factor in MH191-*Sclerotinia* Interaction

To investigate whether *Streptomyces* MH191 could still inhibit *Sclerotinia* when grown in an acidic environment, divider plates with one half containing CYPS medium adjusted to pH 6 to grow MH191 and the other half adjusted to pH 5 to favour *Sclerotinia* growth were prepared, and bioactivity assays were performed as described before. As shown in Figure 2, after 14 days of MH191 preculture, the pH increased to 6 on the medium that was adjusted to pH 5 (Figure 2A,B), indicating the production of volatiles that increase pH in the absence of *Sclerotinia*. Once *Sclerotinia* was added to the plate, pH increased further, from pH 5 to 8 over 2 days (Figure 2C; Figure S6), indicating that the presence of *Sclerotinia* and the potential detection of its volatile signals may have further activated compound production in MH191 to increase the pH. After 7 days, fungal growth was still inhibited (~85%–90%), and *Sclerotinia* turned dark and initiated sclerote formation (Figure 2C; Figure S6), whereas *Sclerotinia* covered the agar without any change in colour on control plates (Figure 2C). These results indicate that pH and volatile detection are driving factors in the MH191-*Sclerotinia* interaction.

### 3.4 | Identification of Volatile Metabolites Using SPME and GC–MS

To identify compounds responsible for the observed antifungal activity and/or pH changes, analyses of the headspace volatiles were performed. VOCs were analysed from MH191, MH33, 9a and KB001 separately and for each isolate in the presence or absence of *Sclerotinia*. To investigate the effect of different culture media on VOC production, Actinobacteria (MH191) were cultured on CYPS and MEP media. Volatiles were adsorbed with solid-phase microextraction (SPME) and analysed by GC–MS. We detected 17 VOCs, some of which were tentatively identified based on mass spectral matches with MS database spectra and retention indices. (Figure 3; Table 1).

*Sclerotinia* alone produced four specific VOCs not detected in the presence of Actinobacteria. These included 1-octen-3-ol (Table 1) on MEP medium, tentatively identified by mass spectra and retention index (Figure 3), and a compound on CYPS medium, suggested by the NIST MS database to be 2,4-dithiopentane.

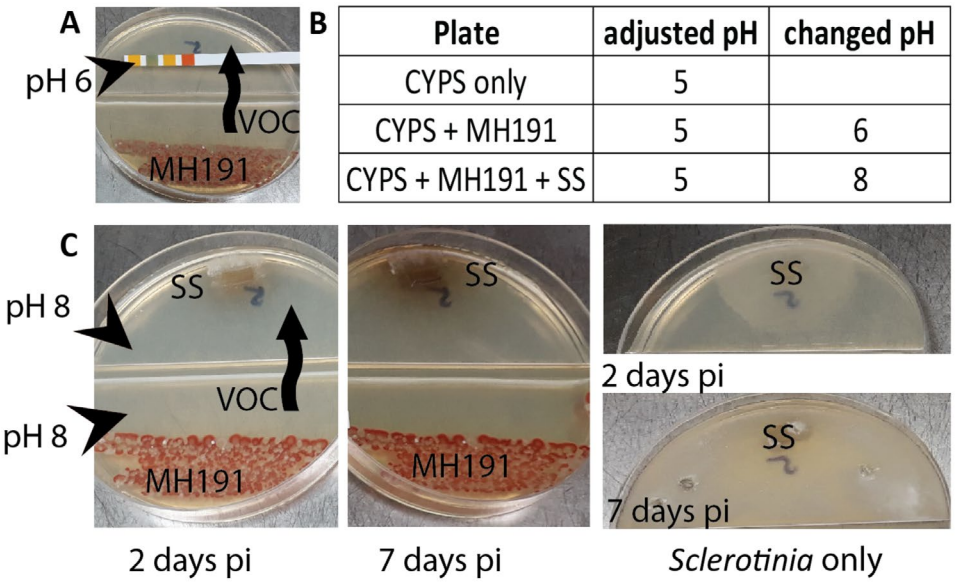


**FIGURE 2** | Actinobacteria MH191 manipulates pH upon sensing *Sclerotinia sclerotiorum* (SS). MH191 senses the introduction of *Sclerotinia* to its environment and produces VOCs that increase the pH. (A) MH191 was grown for 14 days on CYPS medium at pH 6 with the alternate top half adjusted to pH 5. On MH191 only plates, pH was increased from 5 to 6. (B) Table illustrating the changes of pH on the different plates. (C) A *Sclerotinia* mycelia agar plug, was transferred onto the top half of the CYPS medium adjusted to pH 5 to optimise the environment for fungal growth. After 2 days of *Sclerotinia* incubation, the pH on both halves of the divider had increased to pH 8, and *Sclerotinia* growth was arrested compared to the *Sclerotinia*-only control plate shown for 2 and 7 days in the absence of MH191.

Within the Actinobacteria samples, when cultured alone, a similar number of VOCs were detected on both CYPS and MEP media. These included common antifungal compounds such as dimethyldisulfide and dimethyltrisulfide (Tyagi et al. 2020; Wang et al. 2013), together with several pyrazine derivatives and short-chained oxygenated hydrocarbons (Figure 3; Table 1). In all four Actinobacteria isolates cultured on CYPS medium, 2,4,6-trimethylpyridine (TMP) was an abundant compound with the highest relative abundances, based on peak area, for KB001 and 9a (Figures 3 and 4). This compound was not detectable in any isolate sample grown on MEP, making it a key candidate to investigate for antifungal activity. In addition, independent surveys for pyridine-producing isolates in liquid CYPS media identified three further Actinobacteria isolates producing both TMP and 2-ethyl-3,5-dimethylpyridine (EMP), along with KB001 and 9a (Figure S7), further indicating that TMP might be specifically produced by Actinobacteria when cultured in CYPS (solid or liquid). Besides 2,4,6-trimethylpyridine, two other candidate compounds only found in isolates grown on CYPS media were tentatively identified as 2,5-diisopropyl pyrazine and 5-methyl-2-heptanone, respectively (Figure 3). Within the CYPS control samples, benzaldehyde (tentatively identified) was the most abundant compound (Table S3). Other compounds identified in the CYPS-only samples were 2,5-dimethylpyrazine (identified by standard), 2-ethyl-1-hexanol (tentatively identified), undecane and dodecane (tentatively identified, Table S3).

Very few additional compounds were detected when co-culturing Actinobacteria and *Sclerotinia*, indicating that most compounds were also produced in monocultures. However, some

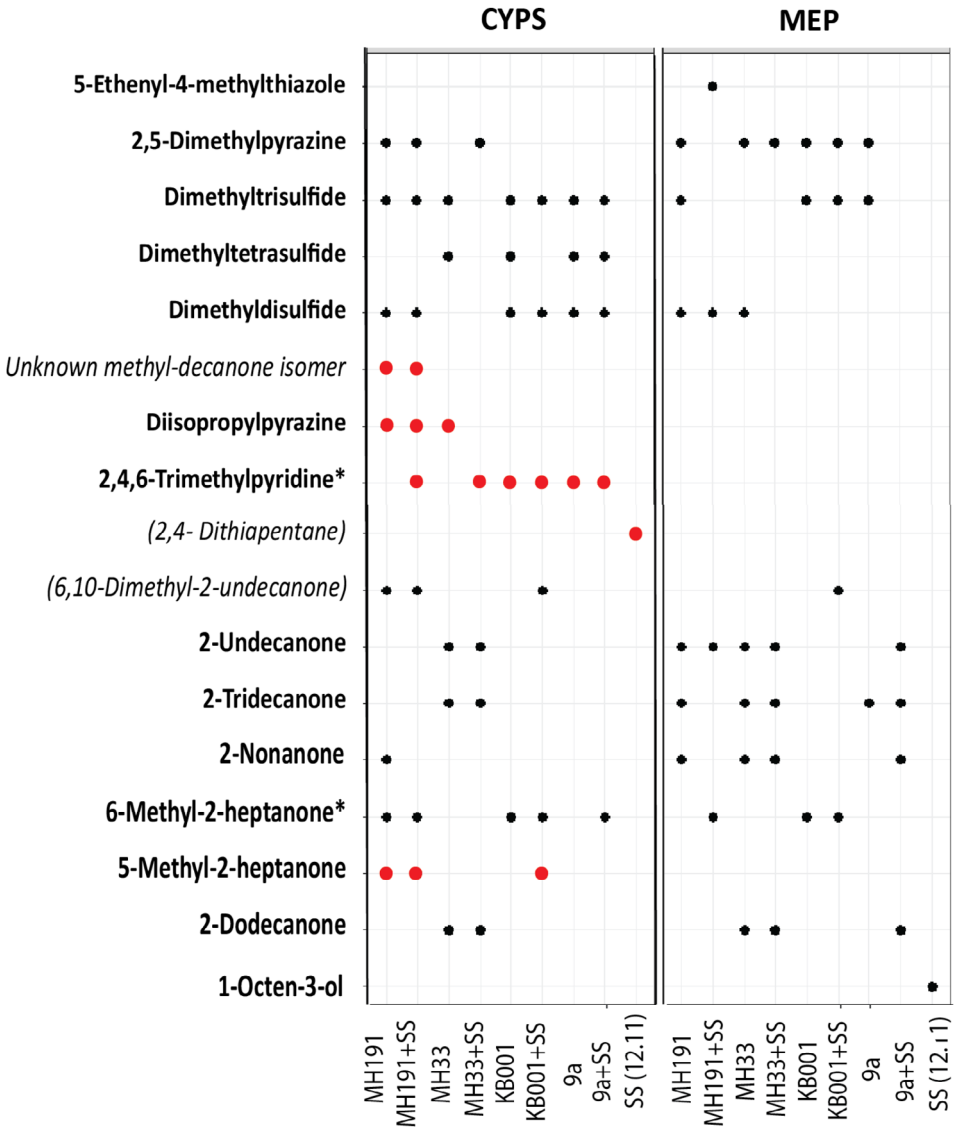


**FIGURE 3** | Volatile compounds are produced by Actinobacteria and *Sclerotinia sclerotiorum* (SS) when cultured together or independently. Volatile compounds of *Streptomyces griseoaurantiacus* MH191, *Micromonospora* sp. MH33, *Streptomyces tendae* 9a, and *Streptomyces tendae* KB001 grown on either CYPS or MEP media were identified by SPME GC–MS. Isolates were incubated with (+SS) or without *Sclerotinia* strain 12.11. *Sclerotinia* 12.11-only samples were also analysed. Unknown compounds or tentatively identified compounds identified based on MS are given in italics. Closest compound matches based on the NIST database are given in brackets. Compounds confirmed by retention index are illustrated in bold (see Table 1 for details). Key compounds were additionally identified by synthetic standards (marked with *). Compounds exclusively identified in isolates grown on CYPS medium are in red.

**TABLE 1** | Tentatively identified compounds, mass spectra and retention index (RI) data from GC–MS analysis.

| RT (min) | Compound | Identified in | Experimental RI | Database/ Lit. RI | Mass of ions (rel. abundance) | Amount CYPS | Amount MEP |
|---|---|---|---|---|---|---|---|
| 8.9 | Dimethyldisulfide | MH191, KB001, 9a | 786 | 785 | 94 (100), 79 (60), 45 (60) | 4 | 0 |
| 13.52 | (2,4-Dithiapentane,[b] 83% similarity) | SS 12.11 | 926 | 862 | 108 (75), 61 (100), 45 (50) | 4 | 0 |
| 14.11 | 2,5-Dimethylpyrazine[a] | MH191, KB001, MH33, 9a | 939 | 925 | 108 (100), 81 (20), 42 (100), 39 (45) | 3 | 3 |
| 15.34 | 6-Methyl-2-heptanone[a] | MH191, KB001, 9a | 966 | 965 | 71 (20), 58 (60), 43 (100) | 3 | 0 |
| 15.7 | 5-Methyl-2-heptanone | MH191, KB001 | 974 | 974 | 71 (30), 58 (30), 43 (100) | 2 | 3 |
| 16.25 | 1-Octen-3-ol | SS 12.11 | 986 | 980 | 72 (25), 57 (100), 43 (35) | 1 | 2 |
| 16.35 | Dimethyltrisulfide | MH191, MH33, KB001, 9a | 988 | 977 | 126 (100), 79 (50), 45 (55) | 3 | 0 |
| 16.66 | 2,4,6-Trimethylpyridine[a] | MH191, MH33, KB001, 9a | 993 | 996 | 121 (100), 106 (25), 79 (30) | 2 | 0 |
| 18.03 | 5-Ethenyl-4-methylthiazole | MH191 | 1035 | 1031 | 125 (100), 97 (75), 58 (55), 39 (25) | 0 | 2 |
| 19.93 | 2-Nonanone | MH191 | 1093 | 1093 | 71 (25), 58 (90), 43 (100) | 2 | 0 |
| 22.13 | Diisopropylpyrazine isomer | MH191, MH33 | 1163 | | 164 (30), 149 (100), 136 (55) | 2 | 0 |
| 24.4 | Unknown methyl-decanone isomer | MH191 | 1238 | | 109 (60), 86 (45), 71 (70), 58 (55), 43 (100) | 1 | 0 |
| 24.55 | Dimethyltetrasulfide | MH33, KB001, 9a | 1243 | 1237 | 158 (95), 79 (100), 64 (50), 45 (77) | 1 | 0 |
| 26.09 | 2-Undecanone | MH33, KB001, MH191, 9a | 1296 | 1302 | 71 (30), 58 (80), 43 (100) | 1 | 0 |
| 28.91 | 2-Dodecanone | MH33, 9a | 1396 | 1404 | 143 (100), 71 (30), 57 (75) | 1 | 0 |
| 29.13 | (6,10-Dimethyl-2-undecanone,[b] 84% similarity) | MH191, KB001 | 1404 | 1391 | 71 (30), 58 (75), 43 (100) | 2 | 0 |
| 31.6 | 2-Tridecanone | MH33, MH191, 9a | 1499 | 1496 | 71 (30), 58 (100), 43 (90) | 2 | 2 |

*Note:* Relative amounts represent peak integrals based on compounds found in bacteria or fungi samples. Shown is the average out of three biological replicates. They are classified as a percentage of total peak area: 1: 0%–1%, 2: 1%–3%, 3: 3%–6% and 4: > 6%. 0: Not detectable. Relative peak area for CYPS and MEP samples is shown for SS 12.11 and MH191.

[a]Confirmed with authentic standards.

[b]Closest library match to the NIST 2020 mass spectral library.

compounds, such as 2,4,6-trimethylpyridine, were only detected in co-cultured samples of MH191 and MH33 (MH191 + SS and MH33 + SS). The addition of these compounds in the media after *Sclerotinia* is added indicates that the presence of *Sclerotinia* is potentially detected by Actinobacteria, which induced further VOC production. Increased VOC production would also

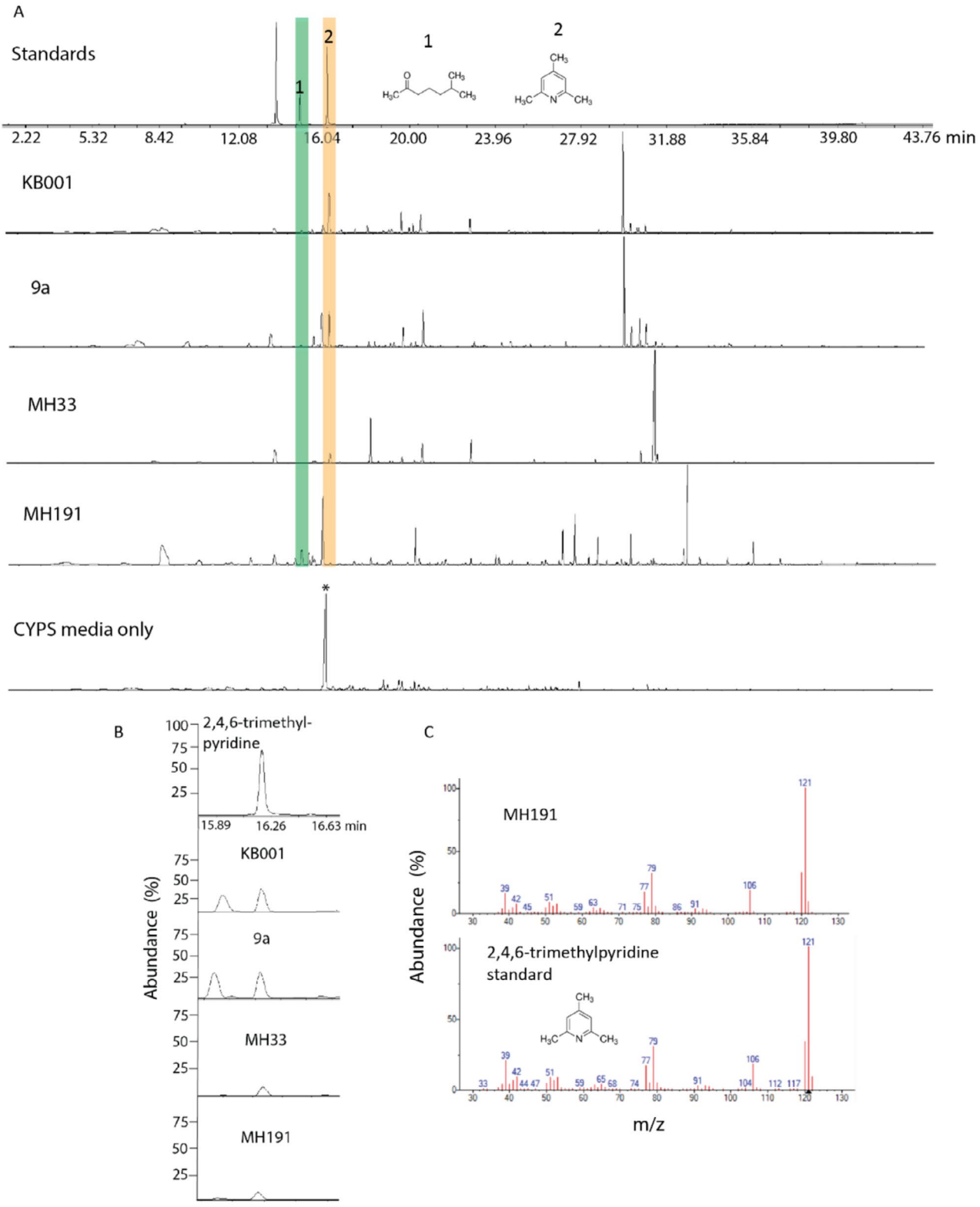


**FIGURE 4** | GC–MS Total Ion Chromatogram (TIC) of standards versus Actinobacteria isolates. (A) TICs of 6-methyl-2-heptanone (1) and 2,4,6-trimethylpyridine (2) compared to the Actinobacteria samples. Coloured areas mark the corresponding peaks. Retention time is given on the *x*-axis. A CYPS media-only reference sample that did not show corresponding peaks was also included. A highly abundant compound with a similar RT was detected in CYPS and identified as benzaldehyde (marked with *, Table S3). (B) Magnified peak area of 2,4,6-trimethylpyridine standard and Actinobacteria samples, showed overlapping peaks at the same retention time. (C) Mass spectrum of MH191 sample identified from *Streptomyces griseoaurantiacus* as 2,4,6-trimethylpyridine compared with mass spectrum of authentic standard.

support our finding that the pH of the media increases following co-incubation of *Sclerotinia* with MH191 (Figure 2). The three VOCs, 2,4,6-trimethylpyridine, 2,5-diisopropylpyrazine and 5-methyl-2-heptanone, were only found from isolates grown on CYPS media and were therefore selected as candidate antifungal compounds for follow-up in vitro bioassays against *S. sclerotiorum*. While we could only tentatively identify 5-methyl-2-heptanone, we did confirm 6-methyl-2-heptanone and 2,4,6-trimethylpyridine by injection of authentic standards (Figure S8; Figure 4B).

### 3.5 | Antifungal Activity of Candidate Volatiles

To evaluate the antifungal activity of 2,4,6-trimethylpyridine, 2,5-diisopropylpyrazine and 5-methyl-2-heptanone, a series of in vitro antifungal bioassays were performed. Tests with 2,4,6-trimethylpyridine and 2,3-dimethylpyrazine inhibited the fungal growth of all *Sclerotinia* isolates by 40% to 60%, respectively (Figure 5; Figure S9), while 6-methyl-2-heptanone showed significantly lower antifungal activity. Neither 5-methyl-2-heptanone nor the tentatively identified 2,5-diisopropylpyrazine were commercially available. The structurally related 2,5-dimethylpyrazine was available, but no antifungal activity could be observed (data not shown), and this compound was also present in the CYPS media. The structural analogue 2,3-dimethylpyrazine was also available, did show antifungal activity, and was used in lieu of 2,5-diisopropylpyrazine in our experiments.

To test the autotoxicity of each compound, MH191 was grown on PDA plates containing the same amounts of test compound (Figure S9). MH191 grew in the presence of all three compounds, indicating that the activity of the compounds is not toxic for MH191.

To determine if any of these compounds would affect the pH of the medium, pH was measured before and 24 h after the addition of compounds on PDA plates (Figure S9). Only 2,4,6-trimethylpyridine increased the pH, similar to that observed with MH191 (from pH 5 to 8, Figure 1). This result is in alignment with the relative basicity of the three compounds, with pyridines (pKaH = 5.2, Zhao, Cao, and Xiao 2020) being more basic than pyrazines (pKaH = 0.7, Zhao, Cao, and Xiao 2020), and suggests that 2,4,6-trimethylpyridine could be one of the compounds produced by Actinobacteria and that is associated with an increased pH as well as *Sclerotinia* inhibition.

Ammonia emissions from certain bacteria, observed in previous studies, can lead to an increase in pH (Weise, Kai, and Piechulla 2013). Previous studies have shown that bacterial-derived ammonia can inhibit fungal growth (Caulier et al. 2019; Liu et al. 2021). We examined the potential production of ammonia ($NH_3$) by MH191 and three other *Streptomyces* strains using $NH_3$-sensitive test strips (Figure S10). All strains showed positive results for $NH_3$ production when grown on CYPS plates. This suggests that the observed pH change could be attributed to $NH_3$ production. However, ammonia tests also returned positive results upon exposure to 1% or 0.1% (v/v) solutions of 2,4,6-trimethylpyridine, indicating the ammonia test may also detect Actinobacteria-derived 2,4,6-trimethylpyridine and possibly other related compounds.

To further investigate the effect of MH191 and TMP on pH, we repeated *Sclerotinia* growth suppression assays on CYPS divider plates and compared pH-buffered media (HEPES buffer, pH 6.3) against non-buffered (Figure S11). Interestingly, from

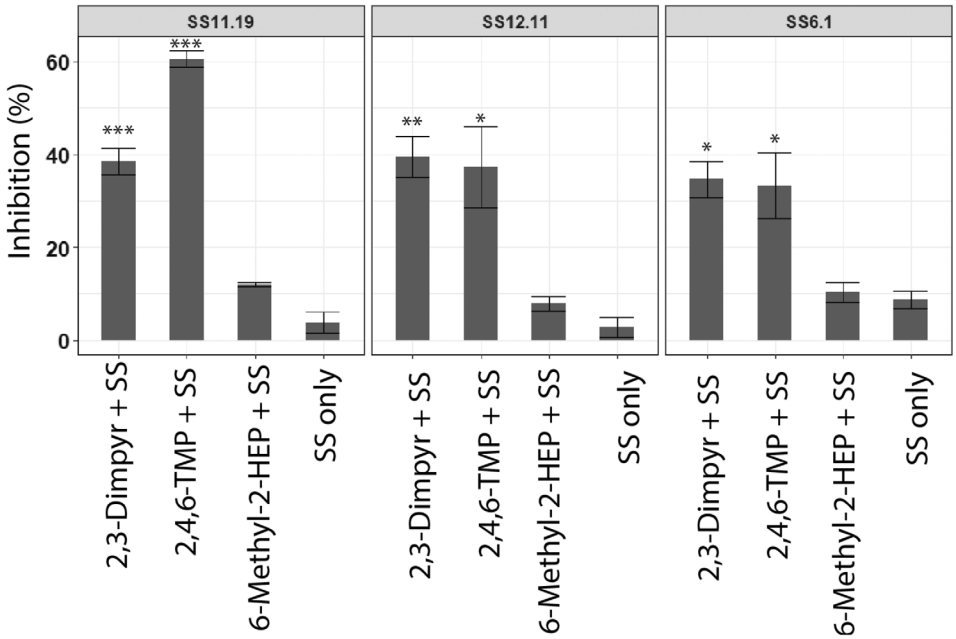


**FIGURE 5** | *Sclerotinia sclerotiorum* (SS) growth is inhibited in the presence of identified SPME compounds. Inhibition of SS illustrated as a percentage. The full length of the plates was set to 100%, and inhibition was calculated based on the length of the SS growing margin. Shown is the average based on three biological replicates. Statistical differences between SS only and SS+ neat compounds were calculated using Student's *t*-test with $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$. 2,3-Dimepyr, 2,3-dimethylpyrazine; 2,4,6-TMP, 2,4,6-trimethylpyridine; 6-Methyl-2-HEP, 6-methyl-2-heptanone.

these experiments, when pH was maintained below pH 7 on the buffered plates, it was shown that TMP and live cultures of MH191 still inhibited *Sclerotinia* growth, indicating the antifungal mechanisms of these agents alone are sufficient to inhibit *Sclerotinia* growth (Figure S11).

### 3.6 | 2,4,6-Trimethylpyridine Affects *Sclerotinia* Growth in a Dose Dependent Manner

To determine a dose effect, 2,4,6-trimethylpyridine was added in increasing amounts and tested against *S. sclerotiorum* on plates with and without a divider. An increase in fungal growth inhibition was observed with increased amounts of 2,4,6-trimethylpyridine (Figure 6; Figure S12), indicating that there is a concentration-dependent effect. On plates without a divider, over 50% of fungal inhibition could be observed when 75 μL (~69 mg) or more were applied. Around 75% inhibition could be achieved with amounts of 100 μL or higher (Figure 6).

On plates with a divider, larger amounts were needed to achieve a similar inhibitory effect; 100 μL of compound only achieved about 30% inhibition (Figure 6; Figure S12). Nevertheless, an increase in inhibition was observed with an increase in compound amounts. These results are consistent with the hypothesis that MH191 and other Actinobacteria produce 2,4,6-trimethylpyridine and this compound contributes to antifungal activity.

To further increase the antifungal activity, combinations of VOCs were tested on three SS isolates (12.11, 11.19 and 6.1) Isolate 6.1 was included as an additional highly pathogenic strain (Figure S13). Similar to the individual compounds (Figure 5), *Sclerotinia* inhibitions ranged from 40% to 60% (Figure S13B), indicating no increased inhibitory effect of combinations of VOCs compared with individual VOCs. Interestingly, a decrease in response was observed for the combination of 2,4,6-trimethylpyridine and 6-methyl-2-heptanone towards isolates 12.11 and 6.1, indicating that combinations of compounds could also negate bioactivity. As shown before, combinations with 2,4,6-trimethylpyridine showed an increase in media pH. Overall, 2,4,6-trimethylpyridine was identified as a key biologically active compound with the highest antifungal activity amongst the compounds tested against *Sclerotinia*.

## 4 | Discussion

This study provides new insights into the roles of VOCs in the interaction between *S. griseoaurantiacus* MH191 and the plant fungal pathogen *Sclerotinia sclerotiorum*. The growth of *Sclerotinia* was strongly inhibited by volatiles produced by MH191 when grown on a casein-based medium but not on MEP medium, demonstrating that production of compounds by MH191 is dependent on culture conditions. This was further validated through bioassays with three additional Actinobacteria strains, indicating the nutrient composition of the CYPS medium (casein and yeast extract) is a potent inducer of antifungal compound production under laboratory conditions. Low concentrations of sugar and high contents of amino acids coupled with casein (a complex organic N source) in CYPS likely caused the differential production of VOCs. Sugar depletion or other localised nutrient depletion is commonly associated with the activation of microbial secondary metabolite production, such as antibiotics, as a means of sensing microbial competition in the environment and is an important survival mechanism (Hoskisson and Fernández-Martínez 2018; van Bergeijk et al. 2020). It is also known that bacteria and fungi have differential production of VOCs on nutrient-rich versus nutrient-poor media (Weisskopf, Schulz, and Garbeva 2021). Although not explored in our study, other abiotic factors such as temperature, humidity and oxygenation are also known to influence microbial VOC production (Weisskopf, Schulz, and Garbeva 2021).

In addition to the abiotic factors mentioned above, biotic factors such as microbe-microbe interactions are also known to influence the production of VOCs (Weisskopf, Schulz, and Garbeva 2021). We presented evidence that *S. griseoaurantiacus* strain MH191 actively increased the pH in its environment when *Sclerotinia* was introduced. This suggests MH191 could recognise the intruding *Sclerotinia* at a distance. Apart from passive diffusion of VOCs through cell membranes, little is known about how microbes perceive VOCs including whether volatile-specific receptors exist (Weisskopf, Schulz, and Garbeva 2021). The strong pH-dependent interaction between *Streptomyces* and *Sclerotinia* has not been shown before and gives new insights into how *Streptomyces* compete with fungi. Previous studies observed that pH in agar media affects the growth and interaction of other fungi with their environment, further demonstrating

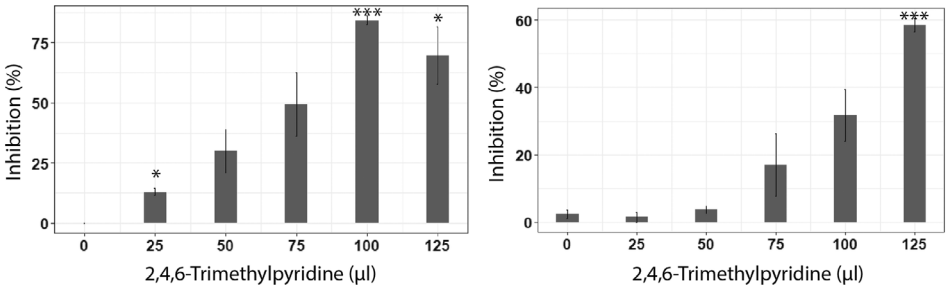


**FIGURE 6** | Inhibition of *S. sclerotiorum* (SS) in the presence of different amounts of neat 2,4,6-trimethylpyridine. Illustrated is the percentage inhibition of SS in the presence of 2,4,6-trimethylpyridine. Assays were performed on plates without a divider (left) and with a divider (right). Inhibition of SS was compared to SS-only plates after 7 days. The full length of the plate was set to 100 (%) and inhibition of SS was calculated based on the length of growth on the plates. Statistical differences between SS only (0) and SS+ 2,4,6-trimethylpyridine amounts were calculated based on three biological replicates using Student's *t*-test with $*p < 0.05$, and $***p < 0.001$.

that pH has an important role in fungal behaviour and development (Bousset et al. 2019). How these interactions play out in plant–soil environments with other microorganisms will be intriguing to uncover.

We found the volatile 2,4,6-trimethylpyridine (TMP) was detectable as a key VOC produced by MH191 and was also produced by the Actinobacteria strains, *Micromonospora* sp. MH33, *Streptomyces tendae* 9a, and *Streptomyces tendae* KB001, when grown on CYPS medium. Interestingly, while all four Actinobacteria strains tested had similar inhibitory effects on *Sclerotinia*, the relative abundance of 2,4,6-trimethylpyridine was lower in MH191 samples. This suggests a small amount of the compound might be sufficient to achieve *Sclerotinia* inhibition. Other compounds with antifungal activity may also contribute, possibly in an additive or synergistic manner. Exposure of 2,4,6-trimethylpyridine to *Sclerotinia* isolates severely impaired their growth, indicating the efficacy of this compound. The quantities of neat 2,4,6-trimethylpyridine applied exogenously to inhibit fungal growth are likely much higher than those biologically produced but were comparable to other studies that tested volatile compounds against *Sclerotinia* and other plant fungal pathogens like *Fusarium* and *Rhizoctonia* (Chen et al. 2020; Fernando et al. 2005).

The identified 2,4,6-trimethylpyridine increased the pH of the media from 5 to 8 within 24 h of the addition of the pure compound to the media plate. Another *Streptomyces*-derived volatile compound that has been shown to increase the pH of its environment is trimethylamine (TMA) (Jones et al. 2019). This alkaline volatile produced by *Streptomyces venezuelae* increases the environmental pH, thereby altering iron availability to competing microbes, triggering cell exploration in *Streptomyces* (Jones et al. 2017, 2019). *S. venezuelae* can overcome an iron-limiting environment by secreting iron-chelating siderophores. Within our study we were not able to identify TMA, possibly due to differences in culture conditions of bacteria and the use of different bacterial isolates. Our identification of 2,4,6-trimethylpyridine as another Actinobacteria-derived alkaline VOC reveals further roles for these basic compounds in microbial community behaviour. Interestingly, further experiments using divided plates and media buffered to a pH of 6.3 indicated that TMP and live cultures of MH191 still inhibit *Sclerotinia* growth, even when pH is kept acidic. This suggests that the VOCs produced by MH191 act independently of their pH altering effects, or condense directly onto fungal tissue, circumventing the buffered media. In line with the effects observed by TMA, a tertiary amine, and the 2,4,6-trimethylpyridine (a nitrogen heterocycle with no nitrogen-hydrogen bonds), ammonia emissions from certain bacteria have also been observed in prior studies, elevating pH (Weise, Kai, and Piechulla 2013). MH191 exhibited a positive result for $NH_3$ when grown on CYPS plates, as did the other three strains. However, neat solutions of 2,4,6-trimethylpyridine also yielded a positive result for $NH_3$. Therefore, at this stage, it cannot be ruled out whether MH191 produces ammonia, and did not produce TMA, but 2,4,6-trimethylpyridine acts in an analogous manner, adding to the repertoire of basic nitrogen-containing VOCs produced by soil bacteria.

This study confirmed 2,4,6-trimethylpyridine as an antifungal compound produced by Actinobacteria. It was previously only tentatively identified in the volatile metabolome of clinical isolates of *Pseudomonas aeruginosa* (Bean, Rees, and Hill 2016). Furthermore, 2,4,6-trimethylpyridine has also been identified in *Collimonas*, a fungus-associated bacterium, but no association to antifungal activity was demonstrated (Garbeva et al. 2014b). One study has shown that 3,5-diacetyl-1,4-dihydro-2,4,6-trim ethylpyridine has antimicrobial activity against Gram-positive bacteria (Abu-Melha 2013), but this dihydropyridine derivative is not aromatic and consequently rather different from 2,4,6-trimethylpyridine isolated from our Actinobacteria strains.

We also included 2,5-dimethylpyrazine and 2,3-dimethylpyrazine as structurally similar compounds to 2,5-diisopropylpyrazine, with 2,3-dimethylpyrazine showing moderate antifungal activity. It has been shown in previous studies that several bacteria produce pyrazines that are known to exhibit antifungal activity (Agisha et al. 2019; Janssens et al. 2019). Pyrazines, as a group of VOCs, are commonly found in many bacteria (Weisskopf, Schulz, and Garbeva 2021). 2,5-Dimethylpyrazine is considered a common VOC, which agrees with our results with this compound identified within all our Actinobacteria strains. Other commonly known antifungal VOCs we identified in Actinobacteria included dimethyldisulfide and dimethyltrisulfide. These two sulphur-derived compounds are commonly reported in microbial VOC studies; for example, dimethyldisulfide has been proposed as an agricultural soil fumigant (Mao et al. 2019; Yan et al. 2019). While many other studies have identified bacterial VOCs linked to fungal inhibition, very few compounds have been individually validated.

Some VOCs were also produced specifically by *Sclerotinia*. These include 1-octen-3-ol, a common compound in fungi (Miyamoto et al. 2014). A volatile found exclusively in *Sclerotinia* cultured on CYPS could not be confirmed by retention data, but based on mass spectral library matches, it was suggested to be 2,4-dithiopentane, a flavouring odour known in truffles (Vahdatzadeh, Deveau, and Splivallo 2015).

In conclusion, our results show that *S. griseoaurantiacus* strain MH191 and *Sclerotinia* interactions have a strong pH dependence, which impacts the production of metabolites. We propose that volatiles specifically produced by MH191 on a casein-based medium, such as the identified 2,4,6-trimethylpyridine, inhibit fungal growth, in part due to its basicity, and to unidentified other mechanisms. Furthermore, our data indicates that MH191 detected the presence of *Sclerotinia* through volatiles produced by the fungal pathogen, and this further induced metabolite production by the actinobacterium. Our experiments strengthen the case that VOCs are key interkingdom signal molecules used by Actinobacteria to defend themselves and to perceive their environment but also highlight the potential of Actinobacteria, such as the *S. griseoaurantiacus* strain MH191 and its VOCs, for application in crop protection as more selective and environmentally friendly alternatives to the currently used broad-spectrum synthetic pesticides.

### Author Contributions

**Katharina Belt:** conceptualization, data curation, formal analysis, investigation, methodology, project administration, validation,

visualization, writing – original draft, writing – review and editing. **Gavin R. Flematti:** methodology, resources, validation, writing – review and editing. **Björn Bohman:** methodology, resources, validation, writing – review and editing. **Heng Chooi:** investigation, methodology, resources, validation, writing – review and editing. **Lachlan Dow:** investigation, methodology, validation, visualization, writing – review and editing. **Andrew W. Truman:** methodology, validation, writing – review and editing. **Barrie Wilkinson:** methodology, validation, writing – review and editing. **Karam B. Singh:** conceptualization, investigation, methodology, validation, visualization, writing – review and editing. **Louise F. Thatcher:** conceptualization, investigation, methodology, project administration, supervision, validation, visualization, writing – original draft, writing – review and editing. **Margaret M. Roper:** methodology, validation, writing – review and editing.


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### Conflicts of Interest

The authors declare no conflicts of interest.

### Data Availability Statement

The data that supports the findings of this study are available in the Supporting Information of this article.

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**Supporting Information**

Additional supporting information can be found online in the Supporting Information section.