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To cite this article: Mohamed A. Embaby, Tamer Elsakhawy, Sally I. Abd- Elfatah & Tapani Yli-Mattila (2024) Biological control of grey mould in strawberry fruits by soil Rhizosphere bacterial isolates, Archives of Phytopathology and Plant Protection, 57:10, 758-775, DOI: [10.1080/03235408.2024.2404320](https://doi.org/10.1080/03235408.2024.2404320)

To link to this article: <https://doi.org/10.1080/03235408.2024.2404320>



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Biological control of grey mould in strawberry fruits by soil Rhizosphere bacterial isolates

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ABSTRACT

The goal of this study was to find effective bacterial isolates possessing antagonistic effects against *Botrytis cinerea* in strawberry in field conditions and to evaluate the antifungal properties of the extracellular metabolites produced by these isolates. Four bacterial isolates with considerable antifungal activity against *B. cinerea* were isolated from strawberries collected from local markets in Giza, Egypt. The molecular identification of bacterial isolates indicated that two of the isolates belong to the *Bacillus subtilis*, while the other two isolates belong to *Pseudomonas stutzeri*. The supernatants of the isolates were extracted and subjected to GC/MS analysis, which revealed that the supernatants of the isolates contained different types of fatty acids with expected antifungal activity. The most potent *B. subtilis* strain was further applied as a foliar spray over heavily infected strawberry fields, which led to significant reduction of *B. cinerea*. Therefore, this strain should be investigated further.

ARTICLE HISTORY

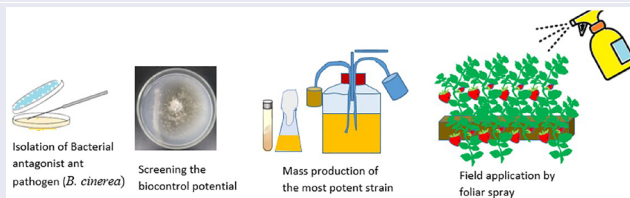
Received 29 September 2023



Accepted 7 September 2024


KEYWORDS

Antifungal activity; *Botrytis cinerea*; *Bacillus subtilis*

GRAPHICAL ABSTRACT



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 Supplemental data for this article can be accessed online at <https://doi.org/10.1080/03235408.2024.2404320>.

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Introduction

Strawberry is grown worldwide more than 370000 hectares (FAO STAT 2014). According to the Food and Agricultural Organisation, roughly 45% of produced fruits, vegetables, roots, and tubers suffer economic losses due to illness between harvest and consumption, which leads to significant food waste (FAO 2015). Beyond its economic significance, strawberries are valued for their nutritional contributions, offering essential macronutrients, micronutrients, vitamins, and health-promoting antioxidants (Giampieri et al. 2015).

Botrytis cinerea is the most important pathogen affecting strawberries worldwide. The pathogen is classified as necrotroph and it prefers to infect and grow on damaged or senescing tissues causing tissue death. Pesticide applications are the most common control against *B. cinerea* (Petrasch et al. 2019), while biocontrol products are mostly *Bacillus subtilis*-based (Pertot et al. 2017). The use of chemical pesticides in agriculture has reduced crop losses brought on by microbial phytopathogens, but it is also linked to environmental pollution, the establishment and development of pathogens with heightened resistance, and risks to human health (Prabhukarthikeyan et al. 2017). Alternative strategies to synthetic fungicides have garnered interest, particularly the utilisation of naturally occurring microorganisms as biological control agents. This approach offers promising prospects for safeguarding plant health and productivity while aligning with the growing demand for environmentally friendly agricultural practices.

Studies by Spadaro et al. (2004) and Droby et al. (2009) have stimulated interest in finding potential synthetic fungicide alternatives. Utilising naturally occurring microbes as biological control agents is a possible substitute that could preserve plants' health and productivity while satisfying the growing demand for environmentally acceptable techniques. In addressing the negative ramifications of agrochemicals and providing alternative solutions to pathogenic microorganisms, several studies have advocated for harnessing beneficial rhizobacteria as biocontrol agents (Abdallah et al. 2016; Egamberdieva et al. 2017). Rhizosphere-associated microorganisms play a pivotal role in the quest for effective biological agents, as they secrete diverse substances capable of suppressing pathogens.

Numerous antagonistic microorganisms, including *Metschnikowia fructicola* (Arrebola et al. 2010), *Bacillus amyloliquefaciens* (Banani et al. 2015), and *Aureobasidium pullulans* (Mari et al. 2012), display biological control activity that can inhibit the development of postharvest fungal pathogens on fruit. Among the noteworthy biocontrol microorganisms, *Bacillus subtilis* stands out for its widespread application in agricultural

biocontrol and plant growth promotion (Ma et al. 2015). Although *B. subtilis*-based biocontrol products are predominantly used, their efficacy in commercial strawberry production is constrained due to limitations in field applicability or supply chain integration (Pertot et al. 2017). *B. subtilis*, which is a Gram-positive, rod-shaped, endospore-forming bacterium found in soil, water and in association with plants and the gastrointestinal tract of humans and animals (Earl et al. 2008). This common species is found all across nature, including the upper layers of the soil and the gastrointestinal tracts of animals and human (Earl et al. 2008).

Previous studies have demonstrated the inhibitory effects of *B. subtilis* against various fungal pathogens, including *Penicillium expansum*, *Monilinia fructicola*, and *Botrytis cinerea*, owing to its production of anti-fungal compounds such as lipopeptides (e.g. surfactin, iturin, and fengycin) and antifungal enzymes (e.g. chitinase and chitosanase) (Wang and Yeh 2008; Liu et al. 2011; Torres et al. 2016).

Bacillus subtilis has a long-standing history of safe use in the food industry, particularly in the commercial production of food enzymes, owing to its fermentation capabilities and high protein secretion efficiency (Harwood 1992; van Dijn and Hecker 2013). Furthermore, risk assessments conducted by the U.S. Environmental Protection Agency and the Center for Veterinary Medicine of the FDA have confirmed the safety of *B. subtilis* as a probiotic Direct-Fed.

The primary objective of this study is to identify rhizosphere bacterial isolates demonstrating antagonistic activity against *Botrytis cinerea*, a prominent pathogen affecting strawberry crops. Unlike previous studies that predominantly focused on *in vitro* assessments, this research underscores the significance of field trials in evaluating the efficacy of bacterial isolates under real-world growing conditions.

Material and methods

Isolation of fungal isolates

Strawberry fruits of cultivar Polka that appeared to be infected with *B. cinerea* were sampled from greenhouses and transferred in sterile polyethylene bags to the laboratory. The infected fruits were sliced using a sterile cutter and inoculated in sterile potato dextrose agar petri dishes (PDA) with 100 mg/L streptomycin sulphate, then incubated at $23 \pm 2^\circ\text{C}$ to stimulate the growth of the fungi. After 3 days, a 5 mm-diameter disc was cut from the active growing edge and transferred to the surface of the PDA plate, which was then incubated at the same conditions to be ready for further use.

DNA extraction, PCR and identification of fungal isolates

The obtained 11 fungal isolates were molecularly identified by amplifying and sequencing their Internal Transcribed Spacers (ITS) region (White et al. 1990; Abbas et al. 2020). The isolates were refreshed on PDA at 25°C for seven days. Mycelia from this seven days old culture were inoculated on 0.5 mL malt peptone broth in a 1.5 mL Eppendorf tube at 25°C for three days. The grown mycelium was scrapped off to a new Eppendorf and frozen in liquid N₂. DNA was extracted from 100 mg mycelia tissue using GenElute™ Plant Genomic DNA Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's recommendations. The forward (ITS-1) (5'-TCCGTAGGTGAACCTGCCG-3') and reverse (ITS-4) (5'-TCCTCCGCTTATTGATATGC-3'') primers were used in the polymerase chain reactions (PCR) to amplify the ITS region of the rRNA operon. Each PCR mixture contained PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 0.25 mM dNTPs, 50 μM of each primer, 2 U Taq DNA polymerase (F-501L, Thermo Scientific) and 25 ng genomic DNA. Amplification was carried out in a PTC-200 DNA Engine Thermal cycler (MJ Research, Inc.). The cycling program used was an initial denaturation (94°C for 3 min), 35 cycles of denaturation (94°C for 30 s), annealing (55°C for 45 s) and elongation (72°C for 1 min), then final elongation (72°C for 7 min). The amplified products were purified using Amicon® Ultra-0.5 Centrifugal Filter Devices (Merck, Darmstadt, Germany) according to the user guide. After separation on 1% agarose gel, size of the bands was estimated using 100 bp DNA Marker (150–2100 bp). The amplified ITS products from 11 isolates were sequenced by the FIMM Technology Centre, Helsinki, Finland. Phylogenetic analysis was performed by EMBL-EBI Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>). NJ consensus trees were prepared by using SEQBOOT (100 bootstraps, random seed 81), DNADIST (Kimura), NEIGHBOR, CONSENSE and DRAWGRAM of PHYLIP program as described by Yli-Mattila et al. (2002, 2018).

Isolation of bacterial antagonists

Soil samples collected from the rhizosphere of strawberry plants (cultivar Polka obtained from the farm used in the field experiment in Kafr Al-Maraziqah, Qullin, Kafr El-sheikh, Egypt (31°01'43"N 30°53'56")) was suspended in sterile saline at a ratio of 1:9 w/v under aseptic conditions, then diluted serially to 10⁻⁷. From dilutions, one loop was streaked on nutrient agar plates, and incubated at 30°C ± 2°C for 24 h. Morphologically different colonies were picked and maintained on nutrient agar slants at 4°C for further study (Petatán-Sagahón et al. 2011; Wang et al. 2019).

Screening the antifungal activities of the bacterial isolates against *B. cinerea*

The antifungal potential of the 30 bacterial isolates against *B. cinerea* was evaluated using the *in vitro* dual culture technique as described by Kilani-Feki et al. (2016). A single 5-mm-diameter mycelial disc from the growing edge of a 5-day-old agar plate of the test fungus was transferred to the center of potato dextrose agar (PDA) plates. The bacterial isolates were inoculated using a stab method in agar so that they were distributed around the circumference of a circle with a radius of 3 cm, the center of which was the fungal disc, and incubated for 5 days at 30 °C.

Fungal growth inhibition was calculated by the formula: $GI = (R_1 - R_2) / R_1 \times 100$, where GI is the growth inhibition percentage, R_1 is the distance between the center of the plate and the growing edge of the mycelium as a control, and R_2 is the distance between the center of the plate and the growing edge of the mycelium that faces the bacterial growth. The most potent isolates in preventing the radial growth of fungal pathogen in a dual culture technique (four morphologically different isolates) were further screened.

DNA extraction and molecular identification of bacterial isolates

The genomic DNA of bacterial isolates was isolated by a modified method of Sambrook and Russell (2001). The bacterial isolate (0.25 g) from overnight culture was resuspended in 1 mL of TE buffer pH (8.0) and centrifuged at 4500 rpm for 5 min at 4 °C. The pellet was resuspended in 450 μ L of 50 mM Tris-HCl pH 8.0, 0.7 M sucrose and 50 μ L Lysozyme solution (20 mg/mL) and stored on ice for 5 min. 60 μ L of 0.5 M EDTA pH 8.0 and 75 μ L of 10% SDS were added to the mixture, which was mixed and stored on ice for 5 min. The sample was warmed to room temperature, 200 μ L of 3 M potassium acetate solution was added and the tube was vortexed for 20 s. After centrifugation at 12000 rpm for 3 min at 10 °C, the supernatant was transferred to a new tube containing 600 μ L isopropanol. The solution was mixed and the DNA precipitate was recovered by centrifuging at 12000 rpm for 10 min at room temperature. DNA was precipitated by 70% ethanol and the supernatant was removed after centrifugation. The DNA pellet was dried and dissolved in 50 μ L of TE (pH 7.6).

PCR was used for amplification of the 16S rDNA gene from the genome of isolated strains using conserved primers designed to amplify the full length (1500 bp) of the 16S rDNA gene according to the *E. coli* genomic DNA sequence. The forward primer was 5'-AGAGTTTGAT CMTGGCTCAG-3' and the reverse primer was 5'-TACGGYTACCTTGTT ACGACTT-3'. Gel electrophoresis was used for the detection of

extracted DNA from purified isolates as well as for the detection of PCR products. The PCR product was purified and sequenced using the dideoxy chain termination method according to Sanger et al. (1977), and the isolates were identified to species level by comparing sequences with known sequences in GenBank using BLAST searches (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Phylogenetic analysis was performed by EMBL-EBI Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>). NJ consensus trees were prepared by using SEQBOOT (100 bootstraps, random seed 81), DNADIST (Kimura), NEIGHBOR, CONSENSE and DRAWGRAM of PHYLIP program as described by Yli-Mattila et al. (2002, 2018).

Gas chromatography/mass spectrometry analysis (GC/MS) of ethyl acetate bacterial extract

Bacterial cultures grown in nutrient broth medium at 30°C for 72 h on a rotary shaker were centrifuged at 10000 × g for 15 min under cooling, and the supernatant of each culture was extracted by ethyl acetate (1:3), then the organic phase was concentrated using a rotary evaporator at 40°C. Then subjected to GC/MS analysis after being re-suspended in 50 µL of BSTFA and incubated in a Dry Block Heater at 70°C for 30 min. The GC-MS system (Agilent Technologies) was equipped with a gas chromatograph (7890B) and mass spectrometer detector (5977 A) at the Central Laboratories Network, National Research Centre, Cairo, Egypt. The identification of different constituents was determined by comparing the spectrum fragmentation pattern with those stored in Wiley and NIST Mass Spectral Library data.

Field experiment

Experiments were initiated in the growing season at a strawberry farm located in Kafr Al-Maraziqah, Qullin, Kafr El-Sheikh, Egypt, 31°01'43"N 30°53'56". The strawberry plants were sown using plastic mulch system. The treatments were distributed in rows of the same size (0.7×10m) with a vegetation density of six plants per cubic meter, marked in a randomized block design. Each treatment was conducted in five replicates distributed randomly.

Buffering zones, consisting of three untreated cultivated rows, separated the treated plots. The treatments were administered during fruit development at the onset of the rainy season, usually at the end of December. The bacterial treatment was repeated four times every 10 days, while fungicide treatment was applied only once, as it needed to be halted 14 days before harvesting, according to the manufacturer's

instructions. One of the treatments involved the application of commercial copper oxychloride sprays (Q-copper 50% WP, Kafr el Zayat Pesticides and Chemicals Company) at a rate of 2.5 kg per hectare in 500 liters of water. Ripe picked strawberry fruits were classified morphologically, and the infection percentage was calculated.

$$\text{Infection \%} = \frac{\text{Weight of infected fruits}}{\text{Weight of total harvested fruits}} \times 100$$

Preparation of *B. subtilis* spray suspension

Mass cell production of *B. subtilis* was achieved by inoculating 250 mL Erlenmeyer flasks containing 100 mL of nutrient broth with *B. subtilis*, flasks were incubated at 30 °C for 48 h at 200 rpm on orbital shaker then the resulted culture was used to inoculate 20-liter glass bottle fitted with filtered air supply system and external heating element to keep temperature around 30 °C during 48h of incubation. After incubation cells were harvested using CEPA continuous centrifuge at 20000 rpm and 10 C°. The resulting bacterial pellets were washed with ringer solution then re-suspended in isotonic ringer solution to obtain cell concentration of 10⁹ CFU. The antagonist formulation was mixed into 100 liters of water in a spray tank to obtain a final concentration of 10⁷ CFU mL⁻¹. Each raw was sprayed using high volume ground sprayers (100 liter) till runoff to obtain full coverage of leaves, fruit, and branches.

Data analysis

Data were analyzed using the Statistical Analysis System (COSTAT) and one-way ANOVA was performed. Significant differences were determined using Duncan's multiple range test at $p \leq 0.05$.

Results

Fungal identification

All ITS1 sequences of the 11 fungal isolates found in the strawberry fruits in the current study matched with the reported sequences in GenBank® (Table 1, Supplementary figure 1). Six ITS sequences were sent to GenBank (MZ488969-MZ488974). The results of one of them showed 99% similarity to the sequence of *Botrytis cinerea* with GenBank accession number MN077161.1.

Table 1. Molecular identification of fungal isolates based on ITS sequences.

Sample name	NCBI identity	GenBank Accession number	Identification name
2w(1)	498/498(100%)	MT267811.1	<i>Penicillium chrysogenum</i>
2w(2)	458/458(100%)	MT609916.1	<i>Aspergillus niger</i>
3w(3)	540/541(99%)	MT530123.1	<i>Trichoderma harzianum</i>
3w(4)	478/478(100%)	MT090021.1	<i>Aspergillus flavus</i>
3	481/483(99%)	MT240540.1	<i>Neopetalotiopsis clavispora</i>
9	445/445(100%)	MG827243.1	<i>Alternaria alternata</i>
25	352/354(99%)	MT267809.1	<i>Penicillium chrysogenum</i>
26	492/496(99%)	MK646007.1	<i>Mucor fragilis</i>
48	480/490(98%)	MK530069.1	<i>Lasiodiplodia theobromae</i>
50	510/510(100%)	MK534877.1	<i>Alternaria alternata</i>
61	472/476(99%)	MN077161.1	<i>Botrytis cinerea</i>

Table 2. Effect of bacterial isolates on *B. cinerea* strain 61 by dual culture method. All changes were statistically significant.

Bacterial isolate code	Radial growth of pathogen in (mm)	Reduction %
Control	35 ± 0.2 ^a	0 ± 0 ^e
S	15 ± 1 ^b	57 ± 1 ^a
W	19 ± 1.2 ^b	45.7 ± 1.2 ^b
P	21 ± 0.9 ^b	40 ± 1.4 ^c
B	22 ± 0.8 ^c	37.1 ± 1.1 ^d
LSD 0.01	2.58	0.22

Table 3. Molecular identification of bacterial isolates with biological activity based on 16S rDNA gene sequences.

Bacterial Isolate Code	Percent homology with GeneBank database	GeneBank Accession Number	Identified species with clone in GeneBank
P	97.77%	MT588731.1	<i>Bacillus subtilis</i>
W	92.59%	KP236290.1	<i>Pseudomonas stutzeri</i>
B	96.30%	MT052342.1	<i>Bacillus subtilis</i>
S	98.70	KU236477	<i>Pseudomonas stutzeri</i>

Screening of antagonistic activity of bacterial isolates against *Botrytis cinerea*

B. cinerea was sensitive to four different bacterial isolates, with growth inhibition ranging from 37.1 to 57% (Table 2). As a result, the inhibitory zone seen in the dual culture assays could be the result of diffusible lipopeptide compounds and fatty acid production by the investigated bacterial isolates. The other 26 strains did not show any antagonistic activity against *B. cinerea*.

Molecular identification of the most potent bacterial isolates

Table 3 and supplementary Figure 2 show the molecular identification of selected bacterial isolates with antifungal activity against plant pathogenic fungus (*Botrytis cinerea* strain 61). The findings revealed that the bacterial isolates P and B belonged to the Gram-positive *Bacillus* genus, with homology percentages with gene bank databases of 97.77% and 96.30% for *B. subtilis*, respectively. The other two isolates W and S were discovered to

belong to the Gram-negative genus *Pseudomonas*, with homology percent of 92.59% and 99.70% with *Pseudomonas stutzeri*, respectively.

Chemical profile of ethyl acetate fractions of the four bacterial isolates using Gas chromatography/mass spectrometry (GC/MS)

As shown in Figure 1 and Table 4(a,b,c,d), the chemical components detected in the ethyl acetate fractions (EAFs) of the four bacterial isolates are phenols, ketones, alcohols, esters and carboxylic acids. In *Pseudomonas stutzeri* (CP062162.1), the major component was 2,3-Butanediol (23.88%), followed by Oleic Acid (11.32%), L-(+)-Lactic acid (8.07%), 2-Pentanol (6.64%), 3-Hydroxy-2-butanone (4.86%) and Palmitic Acid. In *Pseudomonas stutzeri* (KP236290.1), Tyrosol (27.99%) was also the highest produced metabolite, followed by 1-Docosene (11.63%), 10-Heneicosene (c,t) (7.97%), Phenol, 3-(2-trimethylsilyloxyethyl) (7.49%), Decamethyltetrasiloxane (6.52%) and 1-Hexadecanol (5.32%). In *B. subtilis* (MT588731.1), 2-Hexanol (20.47.99%) was also the highest produced metabolite, followed by 3-Methylbutanoic acid (12.3%), 2,3-Butanediol (12.2%), Tyrosol (3.93%), and 1-Hexadecanol (3.78%). Also, Heptadecanoic acid and Palmitic Acid was detected with low percent (1.86%) and (1%), respectively. In *B. subtilis* (MT052342.1), Oleic Acid (21.58%) was also the highest produced metabolite, followed by 1-(2S/R,3R/S)-1-Methylidene-1,2,3,4-tetrahydro-2-methyl-3-phenyl-7-methoxy-9H-xanthen-9-one (12.83%), D-(-)-Citramalic acid (9.83%), Tyrosol (9.78%), 3-Phenyllactic acid (3.78%) and Palmitic Acid (2.76%). There was an overlap of some compounds between these four strains indicating that the four strains share certain metabolic pathways.

Effect of spraying a suspension of *B. subtilis* on strawberry in the infected field with *Botrytis cinerea*

The effectiveness of the *Bacillus* isolate with strong antifungal activity *in vitro* against *Botrytis cinerea* was evaluated *in vivo*, to confirm its potential use as a biocontrol agent of this important strawberry disease. The effect of foliar spray with *B. subtilis* was clearly observed compared with the traditional treatment or control, which did not deliver any treatment. The strawberry field was naturally suffering from severe infection with *Botrytis cinerea*, and the ribbed fruits were infected when still attached to the plant in the field. Results indicated that the treatment with *B. subtilis* suspension was promising and achieved good protection against fungal infection compared with commercial fungicide (Table 5). The protection rate increased as the spraying times increased, which may be attributed to the good colonisation and adaptation of the microbe with time.

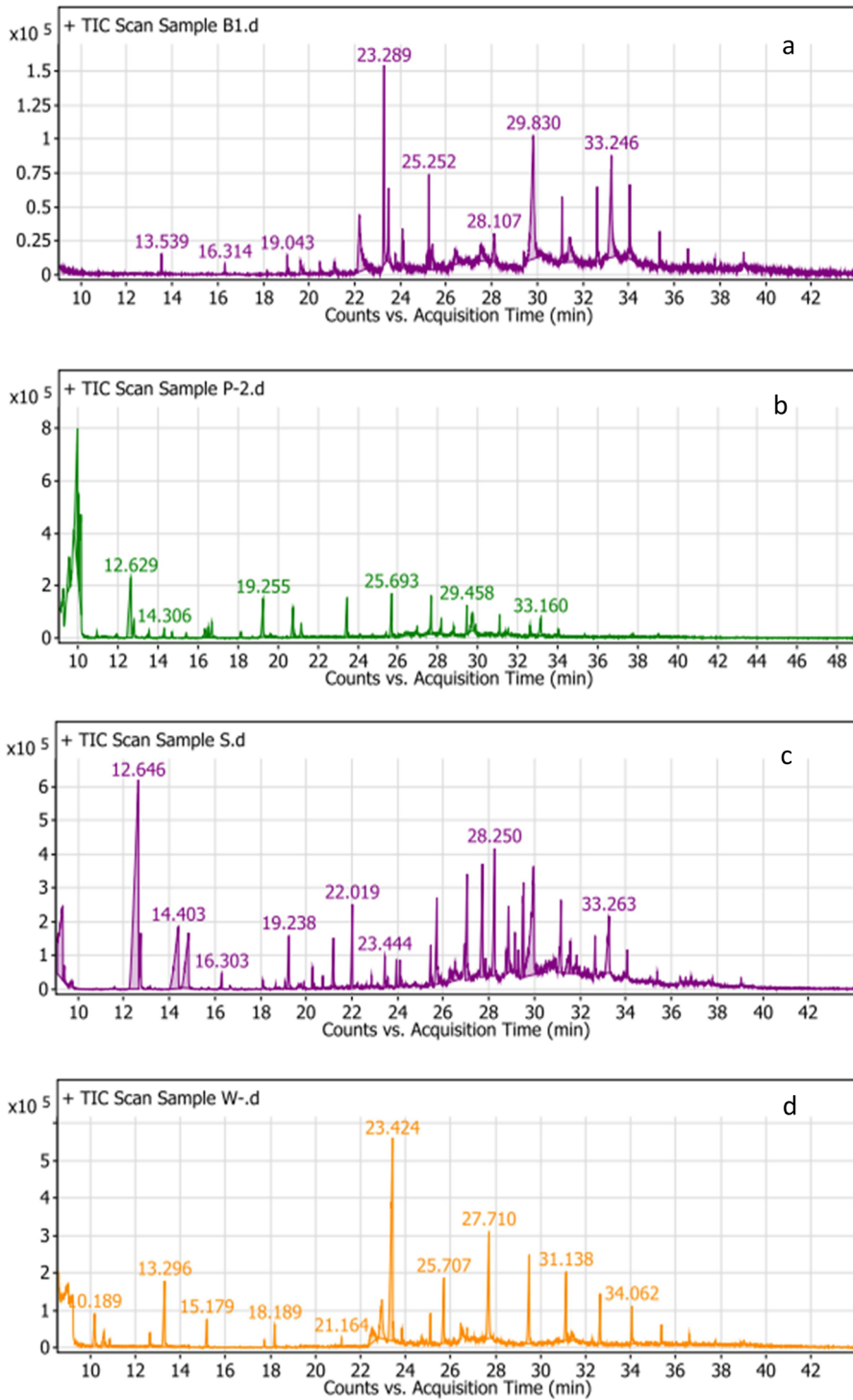


Figure 1. GC/MS chromatogram of the ethyl acetate fraction from (a) *B. subtilis* (MT052342.1), (b) *B. subtilis* (MT588731.1), (c) *P. stutzeri* (CP062162.1) and (d) *P. stutzeri* (KP236290.1).

Table 4. The chemical components detected in the ethyl acetate fractions (EAFs) of the four bacterial isolates using GC/MS.

Peak	Compound Name	RT	RA
a. <i>Bacillus subtilis</i> (MT052342.1)			
7	D-(-)-Citramalic acid	22.202	9.83
8	Tyrosol	23.289	9.78
9	3-Phenyllactic acid	23.484	3.78
20	Palmitic Acid	28.107	2.76
21	Oleic Acid	29.83	21.58
25	(2S/R,3R/S)-1-Methylidene-1,2,3,4-tetrahydro-2-methyl-3-phenyl-7-methoxy-9H-xanthen-9-one	33.246	12.83
Peak	Compound Name	RT	RA
b. <i>Bacillus subtilis</i> (MT588731.1)			
3	2-Hexanol	9.963	20.47
5	3-Methylbutanoic acid	10.129	12.3
8	2,3-Butanediol	12.629	12.2
20	Tyrosol	23.461	3.93
21	1-Hexadecanol	25.693	3.78
25	Palmitic Acid	28.182	1.32
26	Heptadecanoic acid	28.8	1
Peak	Compound Name	RT	RA
c. <i>Pseudomonas stutzeri</i> (CP062162.1)			
1	L-(+)-Lactic acid	9.339	8.07
4	2,3-Butanediol	12.646	23.88
5	2-Pentanol	14.403	6.64
6	3-Hydroxy-2-butanone	14.844	4.86
19	Pentadecanoic acid	27.043	3.76
21	Palmitic Acid	28.25	4.82
26	Oleic Acid	29.95	11.32
27	Alpha.-Linolenic acid	30.493	0.53
Peak	Compound Name	RT	RA
d. <i>Pseudomonas stutzeri</i> (KP236290.1)			
4	Dcamethyl tetrasiloxane,	13.296	6.52
9	Phenol, 3-(2-trimethylsilyloxyethyl)-	22.955	7.49
10	Tyrosol	23.424	27.99
14	1-Hexadecanol	25.707	5.32
19	1-Docosene	27.71	11.63
20	10-Heneicosene (c,t)	29.495	7.97

Table 5. Effect of spraying a suspension of *B. subtilis* strain P on strawberry in the infected field with *B. cinerea*.

Treatments	Infection % in strawberry fruits during successive 5 harvest cycles					Mean of infection % in 5 harvest cycles
	1 st harvesting	2 nd harvesting	3 rd harvesting	4 th harvesting	5 th harvesting	
Foliar spray with <i>B. subtilis</i>	25	23	18	18	15	19.8
Foliar spray with fungicide	50	55	48	57	55	53
Control (without treatment)	74	75	78	75	73	75
L.S.D. 0.05	4.75**	2.83**	4.32**	6.21**	2.83**	

Discussion

Antagonistic activity of Bacillus subtilis and Pseudomonas stutzeri

The inhibition zone observed in our dual culture assays may be due to the secretion of diffusible lipopeptide compounds and fatty acids by the tested bacterial isolates. Several studies indicate that, in *B. subtilis* species

complex, the antagonism is related to the secretion of antibiotic lipopeptides and fatty acids. Cawoy et al. (2015) showed that, in dual culture tests, *B. subtilis*/*B. amyloliquefaciens* secreted lipopeptides such as iturin and fengycin, which inhibited the growth of *Fusarium oxysporum*. Mnif et al. (2015) confirmed the antifungal activity of bacterial lipopeptides in an *in vitro* assay, using an extract of lipopeptides produced by *B. subtilis* SPB1. The authors observed that the bacterial extract generated mycelial lysis, polynucleation, spore destruction and inhibition of mycelial growth in *Fusarium solani*, which is phylogenetically closely related to *F. euwallaceae* (O'Donnell et al. 2015). The fungal membrane has the fundamental role of maintaining cell order and integrity and hence antifungal treatment mostly target the fungal membrane (Avis 2007). Avis and Bélanger (2001) determined the general mechanism with which antifungal fatty acids directly interacts with the fungal cell membrane. The antifungal fatty acids naturally insert themselves into the lipid bi-layer of the fungal membranes and physically disturb the membrane, resulting in increased fluidity of the membrane. These elevations in membrane fluidity will cause a generalised disorganisation of the cell membrane that leads to conformational changes in membrane proteins, the release of intracellular components, cytoplasmic disorder and eventually cell disintegration.

The use of fatty acids as antifungal agents offers some advantages. Liu et al. (2008) proposed that antifungal fatty acids could replace chemicals in use to control plant diseases worldwide, which negatively affect the environment by affecting non-target organisms. *Bacillus* species are considered as good candidates to develop bio-pesticide formulations due to their ability to produce a wide range of antibiotics and antifungal volatile compounds and to form heat and UV resistant spores (Ji et al. 2013).

Pseudomonas is a genus of non-fermentative gram-negative *Proteobacteria* that have been isolated from soil and fresh and salt water. This genus consists of species that are diverse genetically (Anzai et al. 2000). Uzair et al. (2008) reported a novel compound designated zafrin [4 β -methyl-5, 6, 7, 8 tetrahydro-1 (4 β -H)-phenanthrenone], which isolated from a crude extract of a marine bacterium identified as *Pseudomonas stutzeri*. Zafrin showed strong antibacterial activity against both Gram-positive and Gram-negative bacteria. Kumar et al. (2022) concluded that *Pseudomonas stutzeri* isolated from *Withania somnifera* seed extract demonstrated significant potential to inhibit *Rhizoctonia solani* and *Fusarium oxysporum* var. *ciceri* pathogens, which causes sheath blight and wilt in paddy and chickpea, respectively. Also; two *Pseudomonas stutzeri* strains showed antifungal activity against phytopathogenic fungus *Stemphylium botryosum*, which was isolated from *Phaseolus vulgaris* L. Antifungal activities of these two *Pseudomonas* strains against *S. botryosum* revealed very highly significant

inhibition percentages of $38.46 \pm 3.85\%$ and $56.56 \pm 2.22\%$ for each strain, respectively as reported by Mokrani et al. (2019).

Secondary metabolites and antifungal activity

The presence of the broad range of secondary metabolites such as fatty acids supports the antifungal activities of these strains. The difference in the anti-fungal activity of the four isolated stains could be explained by the difference in the fatty acid and other compounds composition of the four stains. *Bacillus* species are known for their capacity to produce a great variety of antifungal compounds to suppress or kill fungal pathogens (Chaurasia et al. 2005). One well-characterised volatile is 2,3-butanediol, which was examined in multiple Gram-negative and Gram-positive bacterial species such as *Bacillus* spp., *Aerobacter* spp., *Serratia* spp., *Enterobacter* spp., and *Klebsiella* spp. (Ryu et al. 2004; Han et al. 2006). The bacterial secretion of 2,3-butanediol activated plant defenses in the roots, mainly *via* salicylic acid and ethylene signaling pathways. This is supported by recent research in which direct soil application of 2,3-butanediol stimulated defense responses against foliar pathogenic anthracnose fungus and *Pseudomonas syringae* (Han et al. 2006; Cortes-Barco et al. 2010a, 2010b). Also, it has been reported that 10-Heneicosene (c,t) has an antifungal activity (Mahamuni 2015). Indeed, the antimicrobial properties of certain of characterised fatty acids in the four bacterial strains have been reported by many scientists. Linolenic acid was identified as an antimicrobial agent in *Spirulina* extract (Matsue et al. 2019). The antibacterial and antifungal activities of palmitic acid against many species such as *Streptococcus mutants*, *Streptococcus gordonii*, *Streptococcus sanguis*, *Candida albicans*, *Aggregatibacter actinomycetemcomitans* were reported by Huang et al.

Antifungal mechanisms of *B. subtilis* lipopeptides against *B. cinerea* in strawberry

Torres-García et al. (2024) employed a biofilm containing *B. subtilis* incorporated into a mixture of guar gum and glycerol to shield strawberries against fungal contamination during postharvest. Their findings underscore that integrating *B. subtilis* into edible films marks a notable progress in prolonging the freshness of strawberries, outperforming traditional techniques. Also; In the study conducted by Hang et al. (2005), *B. subtilis* S1-0210 was utilised in a formulation to combat *B. cinerea* in strawberries. Their findings revealed that a wettable powder preparation of *B. subtilis* S1-0210 led to a substantial decrease in infection rates, with less than 5% compared to over 70% in untreated controls. The

formulation exhibited control efficacies ranging from 85% to 89% in reducing incidences of gray mold on strawberry fruits grown in pots.

Lipopeptides from *Bacillus* are classified in three families of cyclic compounds: surfactin, iturin and fengycin. These lipopeptides, made of amino acids and a fatty acid, are amphiphilic membrane-active biosurfactants and peptide antibiotics with potent antifungal activities. Each family contains variants with the same peptide length but with different residues at specific positions. Moreover, each variant can have several homologues of different length and isomer of the fatty acid chain, leading to a remarkable structural heterogeneity.

Fengycins are produced by *B. subtilis* and other *Bacillus* strains such as *Bacillus amyloliquefaciens* and *Bacillus thuringiensis* (Yuan et al. 2012). Fengycins are less haemolytic than iturins and have strong antifungal activity, specifically against filamentous fungi (Steller et al. 1999). Fengycins and the strains producing them are useful in protecting plants against fungal pathogens including *Fusarium graminearum*, *Rhizoctonia solani*, *Pythium irregulare* and *Cladosporium fulvum*, phytopathogenic fungi responsible for severe agricultural losses. Similar to other lipopeptides, fengycins act in a synergistic manner making them more effective, when iturin and fengycin are used together (Romero et al. 2006).

Many researchers use *B. subtilis* metabolites as an alternative to chemical substances to control the plant infections by phytopathogenic fungi. Mounia et al. demonstrated that *B. subtilis* and *B. mojavensis* have a strong antifungal activity especially against *C. albicans*. The mechanism of such antifungal activities is probably due to the production of cell wall-degrading enzymes and different categories of lipopeptides, including fengycins, surfactins, and iturins.

Conclusion

The current study aimed to control the fungal infection of strawberries using biocontrol agents in order to reduce the use of chemical antifungal compounds. Isolation and identification resulted in four bacterial isolates with considerable antagonistic effects against *Botrytis cinerea*, which causes grey mould on strawberries. The four bacterial isolates belonged to *Bacillus subtilis* and *Pseudomonas stutzeri*. Spraying a suspension of *B. subtilis* on strawberries in the infected field with *B. cinerea* was promising and achieved good protection against fungal infection as compared with commercial fungicides.

Acknowledgements

The authors thank the National Research Centre (NRC), Cairo, Egypt, for technical and financial support. Also, the authors are thankful to Dr. Taha Hussien and PhD student

Asmaa Abbas for their help in sequencing the ITS sequences. Asmaa Abbas and Dr. Tatiana Gagkaeva gave valuable comments on the manuscript.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was funded by the National Research Centre (NRC), Cairo, Egypt, under research project no. 12050304.

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