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Evaluation of Biocontrol Potential of *Bacillus* spp. and *Pseudomonas fluorescens* UM270 against Postharvest Fungal Pathogens

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Abstract: Fungal pathogens are the main causal agents of postharvest diseases of fruits and vegetables. To prevent this problem and avoid the use of harmful chemical fungicides, safer and greener alternatives have been sought. One of these alternatives is the use of plant-growth-promoting bacteria (PGPB). In this study, we evaluated in vitro four well-known PGPB strains (*Pseudomonas fluorescens* UM270, *Bacillus toyonensis* COPE52, *Bacillus* sp. E25, and *Bacillus thuringiensis* CR71) for their biocontrol potential against nineteen postharvest fungal pathogens. In vivo assays were also performed, and bacterial cells were inoculated on harvested strawberries and grapes with the pathogens *Botrytis cinerea*, *Alternaria alternata*, and *Fusarium brachygibbosum* to evaluate loss of firmness and disease incidence. Our results show that the four strains antagonized fungi in direct and indirect confrontation assays. Stronger antagonism was observed by the action of diffusible metabolites (DMs) compared to volatile organic compound (VOC) activity. All PGPB significantly improved the fruit firmness and reduced disease incidence caused by the fungal pathogens tested. However, strain UM270 showed excellent biocontrol activity, reducing the disease incidence of *Fusarium brachygibbosum*, *Botrytis cinerea*, and *Alternaria alternata* on strawberry fruits by 60%, 55%, and 65%, respectively. Diffusible antifungals and VOCs such as 2,4-diacetyl phloroglucinol, siderophores, auxins, fengycins, and N, N-dimethyl-hexadecyl amine, among others, might be responsible for the beneficial activities observed. These results suggest excellent biocontrol activities to inhibit postharvest pathogenic fungi and improve harvested fruit quality.

Keywords: PGPB; biocontrol; postharvest pathogens

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1. Introduction

Postharvest diseases cause considerable losses of fruits and vegetables during handling, transportation, and storage [1]. Spoilage caused by fungi is primarily responsible for significant losses during storage. Fruit infections caused by fungal pathogens both in the field and after harvest result in postharvest deterioration or decay [2]. High levels of losses due to fungal pathogens are related to high levels of moisture, nutrients, low pH values, and a decrease in intrinsic resistance after harvest [3]. Postharvest spoilage caused by fungi represents a concern not only to producers and traders but also to consumers, due

to the presence of mycotoxins. Indeed, some species of postharvest genera, i.e., *Botrytis*, *Aspergillus*, *Penicillium*, and *Alternaria*, produce toxic secondary metabolites, which pose a health risk to humans and animals [4].

Synthetic fungicides were the first products to be used to control postharvest decay; however, they are not the most appropriate option since they pollute the atmosphere, damage the environment, leave harmful residues, and can lead to the development of resistant strains, with the potential to harm consumer health [5]. According to reports from the World Health Organization (WHO), there are about 20,000 unintentional deaths and 2 million poisonings each year, mostly caused by the mishandling of synthetic fungicides in third-world countries. The use of synthetic fungicides in the storage of food products has had numerous adverse effects on human health, such as carcinogenicity, teratogenicity, and hormonal imbalances, among others [6].

Mexico is the world's fourth-largest producer of berries. At the national level, 248,512 tons of blackberries are produced, of which Michoacán contributes more than 90% (238,832 tons). Strawberry production nationwide is 468,000 tons and Michoacan contributes more than 60% (341,130 tons). The blueberry production in Michoacan is positioned in second place nationally, with an annual production of 6000 tons (SEDRUA, 2017). Therefore, searching for agroecological alternatives to reduce postharvest pathogens is imperative and urgent for producers.

A viable, effective, and economical alternative to synthetic fungicides is the use of microbial antagonists, also called biocontrol agents. These are microorganisms that decrease the damaging effects of pathogens. Glick [7] described plant-growth-promoting bacteria (PGPB), and, as their name suggests, they stimulate plant growth, either through direct mechanisms, such as facilitating the acquisition of resources or modulating the levels of plant hormones, or through indirect mechanisms, therefore reducing the effects of pathogens acting as biocontrol bacteria. Due to this biocontrol activity, these microorganisms have the potential to control or reduce the effects of pathogens that affect postharvest fruits and vegetables, without the harmful side effects associated with fungicides. There are several modes of action of microbial antagonists. Some of these include competition for space and nutrients, antibiosis, induced resistance, and direct parasitism [1,8,9]. Other authors have also described mechanisms of action such as the production of biofilms and quorum sensing [10].

Several studies have been carried out in which PGPB have been effective in controlling the growth of pathogenic fungi, in both in vitro and in vivo experiments, demonstrating their ability as biocontrol agents. *Bacillus* sp. strain E25 is an endophytic strain isolated from husk tomato roots in Michoacán, México, that has displayed excellent biocontrol and plant-growth-promoting activities. In silico analysis showed that this strain has 17 gene clusters to produce active antagonistic compounds, including bacteriocins, siderophores, lanthipeptides, lipopeptides, ladderanes, and terpenes [11]. Rojas-Solis et al., 2018 [12] evaluated the antagonistic capacity of the bacterial strains *B. thuringiensis* CR71 and *Bacillus* sp. E25 against *B. cinerea*. They found that both bacterial strains inhibit the growth of *B. cinerea*. Through the production of volatile organic compounds (VOCs), E25 inhibits mycelial growth by 40%, while strain CR71 inhibits it by 52%. However, the diffusible compounds produced by E25 inhibit the growth of the fungus by 12%, while CR71 inhibits it by 24%.

Pseudomonas fluorescens strain UM270 was isolated from the rhizosphere of wild *Medicago*. Hernandez-Salmeron et al. [13] reported the draft genome and at first analysis revealed the presence of multiple genes participating in the synthesis of diffusible metabolites and volatile organic compounds. Hernandez-Leon et. al. [14] analyzed the antifungal and plant-growth-promoting effects of diffusible compounds and VOCs produced by *P. fluorescens* UM270. This strain showed a high degree of antagonism against the phytopathogen *B. cinerea* during in vitro confrontation assays. Furthermore, during in vivo biocontrol experiments, *P. fluorescens* UM270 was able to protect *M. truncatula* plants from *B. cinerea* infection by reducing stem disease symptoms and root browning. Furthermore, this strain

can produce dimethyl-hexadecyl-amine, a compound with antifungal and plant-growth-promoting activities [15].

Contreras-Perez et. al. [16] evaluated the bacterium *Bacillus toyonensis* strain COPE52, an endophytic bacterium isolated from the roots of blackberry plants (*Rubus fruticosus*), to demonstrate plant growth promotion activity; they also reported the draft genome to detect the genes involved in this activity. COPE52 was able to produce IAA and showed protease activity. Furthermore, this strain restricted the mycelial growth of *Botrytis cinerea* via diffusible compound and VOC emission.

Due to the importance of berries in the state of Michoacan and the postharvest problems caused by fungi, as well as the problems caused by the use of fungicides and agrochemicals, it is important to look for safer and more ecological alternatives for the protection of berries. Thus, the main objective of this work was to evaluate four plant-growth-promoting bacteria in respect of their biocontrol activity against postharvest fungal pathogens.

2. Materials and Methods

2.1. Biological Material

Fungal pathogens were previously isolated and identified by Morales-Cedeno et al., 2020 [17]. Briefly, *Botrytis cinerea* 62BCV and *Fusarium brachygibbosum* 4BF were isolated from decaying strawberry fruits and *Alternaria alternata* 1A was isolated from decaying blueberry fruits. Genomic DNA of the fungal strains was extracted using Mahuku's 2004 protocol [18] followed by PCR analysis to amplify the internal transcribed spacer (ITS) regions with the primers ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). The amplified regions were sequenced at Macrogen, Seoul, Korea. Homology blast analysis was performed and sequences were deposited in GenBank (accession numbers: MN365049.1, MN365015.1, MK881030.1).

The plant-growth-promoting bacteria used in this study belong to our endophytic and rhizospheric bacterial collection. UM270 strain was isolated and characterized by Hernandez-Leon et al., 2015 [14], COPE52 by Contreras-Perez et al., 2019 [16], and E25 and CR71 by Rojas-Solis et al., 2018 [12]. DNA of all the bacterial strains was extracted, and PCR analysis was performed to amplify the RNAr *16S* gene using the primers FD1 (5′-CAGAGTTTGATCCTGGCTCAG-3′) and RD1 (5′-AAGGAGGTGATCCAGCC-3′). To determine the taxonomic affiliation, BLAST analysis was performed and sequences were deposited in GenBank (accession numbers: KJ801568.1, CP031292.1, CP031749.1, CP031748.1).

2.2. In Vitro Evaluation of the Antagonistic Effects of Diffusible and Volatile Compounds Produced by Bacteria

The antagonism of compounds produced by plant-growth-promoting bacteria against the fungal pathogens was evaluated in bioassays performed in Petri dishes as previously reported [12]. In brief, the bacterial strains (*Pseudomonas fluorescens* UM270, *Bacillus toyonensis* COPE52, *Bacillus* sp. E25, *B. thuringiensis* CR71) were streaked in a cross shape on PDA plates; then, a 6 mm portion of the mycelium was deposited in the center of each formed quadrant on the plates. Subsequently, the mycelium growth was measured, and the inhibition percentage was calculated with the formula used by Hernández-León et al., 2015 [14], and described as follows: % of growth inhibition = $[(Ac - Ab)/Ac] \times 100$, where Ac is the control mycelial area and Ab is the mycelial area with treatment. To evaluate the antifungal effect of VOCs emitted by plant-growth-promoting bacteria, divided Petri dishes with PDA were used. A bacterial inoculum of each strain (100 µL O.D. 1) was deposited on one side of the Petri dish, and the 6 mm plug of the pathogenic fungi mycelium was inoculated on the other side of the plate. Subsequently, the mycelium growth was measured [14]. Both experiments (diffusible and volatile compounds) were independently performed three times.

2.3. Strawberry and Grape Assay

Bacterial strains *Pseudomonas fluorescens* UM270, *Bacillus toyonensis* COPE52, *Bacillus* sp. E25, and *B. thuringiensis* CR71 were used to inoculate strawberries and grapes to further evaluate their antagonism and biocontrol activity against three selected pathogens: *Fusarium brachygibbosum*, *Botrytis cinerea*, and *Alternaria alternata.* Similar assays were previously reported by Shi and Sun et al., 2017 [19]; Tsalgatidou et al., 2022 [20]; and Heo et al., 2022 [21]. We followed their protocol with some modifications. Briefly, fruits were washed with running water and subsequently placed in a container with 70% ethanol for 1 min. The ethanol was decanted and the berries were then washed with 2.5% sodium hypochlorite for 1 min. This process was repeated three times. Finally, the fruits were rinsed thrice with sterile deionized water. Following this procedure, the fruits were allowed to dry in a laminar flow hood, and a cross incision was made on each fruit with the tip of a sterile scalpel. A 20 µL aliquot of every one of the four bacterial strains used in this study was cultured until a reading of 1×10^8 colony-forming units (CFUs)/mL was obtained. In addition, 10 µL of each bacterial cell-free supernatant of the same cultures and 10 µL of sterilized distilled water as a control treatment were inoculated on the surface of each fruit wound and incubated at room temperature for 1 h, before applying the fungi (*F. brachygibbosum*, *B. cinerea*, or *A. alternata*). The conidial suspension was prepared by flooding PDA plates of a 10-day-old solid culture with sterilized dH_2O to gently remove the conidia and adjust the concentration to approximately 1×10^4 spores/mL. Finally, 10 µL of fungal spore suspension of each fungus was injected into each wound. Inoculated strawberries and grapes were placed into plastic boxes to maintain high relative humidity (approximately 60–80%) and incubated in a dark growth chamber at 25 °C for 5 days. The experiment was conducted in triplicate (15 fruit/replicate). The percentage of infected fruits was calculated to assess disease incidence (DI) as follows: % disease incidence, (DI) = number of infected fruits/total number of fruits × 100 [19–21].

2.4. Comparison of Secondary Metabolite Biosynthesis Gene Clusters and VOCs

The genome sequences of the four bacterial strains were downloaded from GenBank according to the accession numbers reported in previous works in our lab by Hernandez-Salmeron et al. [13], Flores et al. [22], Perez-Equihua et al. [11], and Contreras-Perez et al. [16]. Then, biosynthetic gene clusters (BGCs) for antibiotic and secondary metabolite production were identified using the antiSMASH 4.0 pipeline. A comparison of the BGCs produced by the four strains was performed. Volatile organic compounds produced by the four strains were analyzed using SPME-GC-MS on PDMS/DVB fibers as previously reported by Hernandez-Leon et al., 2015 [14]. The GC-MS was equipped with a DB-23 capillary column (30 m × 0.32 mm × 0.25 µm) and was operated under the following conditions: helium was used as the carrier gas (1 mL/min) and the detector temperature was 250 °C. The column was held for 1 min at 40 °C, then programmed to increase at a rate of 3 °C per minute to a final temperature of 180 °C, which was maintained for 1 min. The source pressure was 7 Pa, the filament voltage was 70 eV, and the scan rate was 1.9 scan S^{-1}. Using the Mass Spectra Library (NIST/EPA/NIH, 'Chem Station' Agilent Technologies Rev. D.04.00 2002), the compounds were identified by comparison. Three independent determinations were made for each bacterial strain. Information with respect to volatile organic compounds produced by the four bacterial strains was compared according to the results reported by Rojas-Solis et al. [12], Hernandez-Leon et al., 2015 [14], and Contreras-Perez et al. [12,14,16,23].

2.5. Statistical Analysis

The results were analyzed using Statistica 8.0 software. An analysis of variance and Duncan's test were performed for the comparison of means in bioassays (p-value < 0.05). For the biocontrol of pathogens in fruits, a Tukey analysis (p-value < 0.05) was carried out using Microsoft Excel 2010.

3. Results

3.1. Effect of PGPB Diffusible Compounds on Fungal Mycelial Growth

The diffusible compounds of the antagonist bacteria showed inhibition of the growth of the fungi that were isolated from strawberries, blackberries, and blueberries; the results are shown in Table 1. Most of the bacterial strains exhibited significant differences in the growth inhibition of the fungi. The strain causing the highest percentages of inhibition was Pseudomonas fluorescens UM270, except with respect to Mucor circinelloides, where the best percentage of inhibition was obtained with the bacterial strain E25, and strain CR71 exhibited the best results for Mucor fragilis.

Table 1. Inhibition percentages of postharvest fungal pathogens in dual confrontation assays (direct contact).

	Fungal Growth Inhibition by Diffusible Compounds of Bacterial Strains (%)			
Fungal Strain	***Bacillus toyonensis* COPE52**	***Bacillus* sp. E25**	***B. thuringiensis* CR71**	***Pseudomonas fluorescens* UM270**
Alternaria alternata 1A	28.3 ± 4.9 b	40.7 ± 5.8 c	40.4 ± 11.2 c	43.6 ± 4.8 c
Alternaria alternata 2Z	-	-	-	22.4 ± 6.8 b
Alternaria alternata 4A	16.4 ± 9.3 c	27.6 ± 6.8 bc	32.0 ± 10.9 bc	40.9 ± 12.0 b
Alternaria alternata 6A	1.9 ± 0.6 a	5.5 ± 8.5 ab	16.6 ± 7.0 b	34.1 ± 7.8 c
Alternaria sp. 3A	4.9 ± 11.2 a	14.0 ± 9.6 a	13.8 ± 10.4 a	36.6 ± 9.8 b
Botryosphaeria rhodina 5A	9.2 ± 5.7 ab	8.4 ± 1.5 ab	12.6 ± 3.8 b	12.5 ± 8.9 b
Botrytis cinerea 62BCV	11.5 ± 5.3 ab	17.2 ± 7.3 ab	35.0 ± 7.2 b	62.6 ± 25.9 c
Botrytis sp. 62C	13.6 ± 21.7 ab	28.5 ± 9.6 bc	48.4 ± 6.0 c	78.6 ± 0.4 d
Cladosporium sp. 1BOA	39.2 ± 11.7 c	48.6 ± 6.2 bc	45.5 ± 8.9 bc	59.5 ± 5.3 b
Fusarium brachygibbosum 4BF	14.0 ± 8.6 ab	28.0 ± 7.9 bc	38.1 ± 19.6 c	45.1 ± 6.8 c
Fusarium brachygibbosum HBF	29.5 ± 8.3 c	34.3 ± 2.8 bc	29.8 ± 4.8 c	45.0 ± 12.2 b
Geotrichum candidum FRB	12.2 ± 9.3 ab	21.2 ± 9.5 ab	26.4 ± 13.9 b	60.7 ± 23.1 c
Geotrichum phurueaensis 7Z	22.7 ± 4.1 b	37.7 ± 7.3 d	35.4 ± 8.8 d	52.6 ± 8.3 c
Mucor circinelloides 1BF	7.0 ± 3.0 ab	14.6 ± 4.1 b	14.2 ± 5.3 b	12.1 ± 7.8 b
Mucor fragilis 22	15.8 ± 6.4 c	18.8 ± 5.4 c	30.7 ± 10.4 b	12.8 ± 4.4 c
Mucor fragilis FRA	-	9.1 ± 21.2 a	8.4 ± 29.8 a	-
Penicillium crustosum 1F	5.8 ± 28.2 a	10.3 ± 14.4 a	17.3 ± 9.2 ab	40.8 ± 10.1 b
Penicillium expansum 230	9.7 ± 19.5 a	6.9 ± 4.9 a	20.8 ± 10.7 a	45.4 ± 8.3 b
Penicillium expansum 5F	13.3 ± 14.4 a	7.0 ± 10.3 a	24.2 ± 13.3 ab	41.4 ± 20.7 b

±SD values. Letters indicate that the means differed significantly after Duncan's multiple range test ($p < 0.05$).

3.2. Effect of VOCs on Fungal Mycelial Growth

The results of the tests carried out to evaluate the antagonistic capacity of the bacterial strains on the growth of studied pathogenic fungi via the production of volatile organic compounds are shown in Table 2. Strain UM270 caused significant inhibition of the pathogen *Alternaria alternata*, one of the main pathogens causing disease in blueberry fruits.

Table 2. Inhibition percentages of postharvest fungal pathogens by employing divided Petri plate assays (VOCs emission).

	Inhibition by Volatile Compounds of Bacterial Strains (%)			
Fungal Species/Strain	***Bacillus toyonensis* COPE52**	***Bacillus* sp. E25**	***B. thuringiensis* CR71**	***Pseudomonas fluorescens* UM270**
Alternaria alternata 1A	6.4 ± 6.9 ab	8.4 ± 8.7 ab	7.3 ± 6.4 ab	17.4 ± 8.1 b
Alternaria alternata 2Z	-	-	-	-
Alternaria alternata 4A	13.6 ± 14.9 a	11.3 ± 3.6 a	22.0 ± 12.9 a	27.8 ± 28.0 a
Alternaria alternata 6A	-	-	-	5.4 ± 2.1 b
Alternaria sp. 3A	0.5 ± 5.2 a	2.4 ± 6.2 a	4.1 ± 2.1 a	4.3 ± 5.9 a
Botryosphaeria rhodina 5A	3.5 ± 6.1 a	1.2 ± 9.5 a	2.8 ± 1.3 a	6.1 ± 11.6 a
Botrytis cinerea 62BCV	8.7 ± 6.7 a	14.5 ± 17.2 a	19.7 ± 14.1 a	4.1 ± 3.5 a
Botrytis sp. 62C	33.1 ± 35.6 a	36.4 ± 27.2 a	15.5 ± 46.5 a	44.6 ± 37.8 a
Cladosporium sp. 1BOA	-	1.3 ± 15.0 a	5.8 ± 5.7 a	-
Fusarium brachygibbosum 4BF	-	2.3 ± 2.5 a	1.7 ± 6.7 a	-
Fusarium brachygibbosum HBF	-	-	-	-
Geotrichum candidum FRB	0.4 ± 2.3 a	2.8 ± 6.9 a	-	5.8 ± 7.5 a
Geotrichum phurueaensis 7Z	4.3 ± 16.2 a	5.7 ± 19.2 a	3.4 ± 20.3 a	-
Mucor circinelloides 1BF	-	-	-	-
Mucor fragilis 22	-	-	-	-
Mucor fragilis FRA	12.4 ± 14.4 a	5.8 ± 10.6 a	9.6 ± 8.3 a	-
Penicillium crustosum 1F	-	2.4 ± 36.8 a	-	6.5 ± 38.8 a
Penicillium expansum 230	-	-	-	-
Penicillium expansum 5F	-	-	-	-

±SD values. Letters indicate that the means differed significantly after Duncan's multiple range test ($p < 0.05$).

3.3. Biocontrol Assay on Strawberries and Grapes

The assays realized on strawberry fruits show that the four strains significantly avoided the loss of firmness caused by *Botrytis cinerea* and *Alternaria alternata*. When the fruits were infected by *Fusarium brachygibbosum*, the strains UM270 and CR71 maintained firmness by 87% and 94%, respectively, compared with the control. In addition, the disease incidence due to the three phytopathogens was reduced significantly when each of the bacterial strains was inoculated on the fruits; strain UM270 reduced the disease incidence of *Fusarium brachygibbosum*, *Botrytis cinerea*, and *Alternaria alternata* by 60%, 55%, and 65%, respectively.

On grapes, the four bacterial strains helped to maintain firmness when they were infected by the studied phytopathogens. When grapes were infected with *Fusarium brachygibbosum*, strain UM270 reduced the disease incidence by 47%, strain E25 reduced the disease incidence by 40%, and CR71 reduced the disease incidence by 53%. When the fruits were infected with *Botrytis cinerea* and *Alternaria alternata*, the four strains reduced the disease incidence significantly compared with the control (Figure 1).

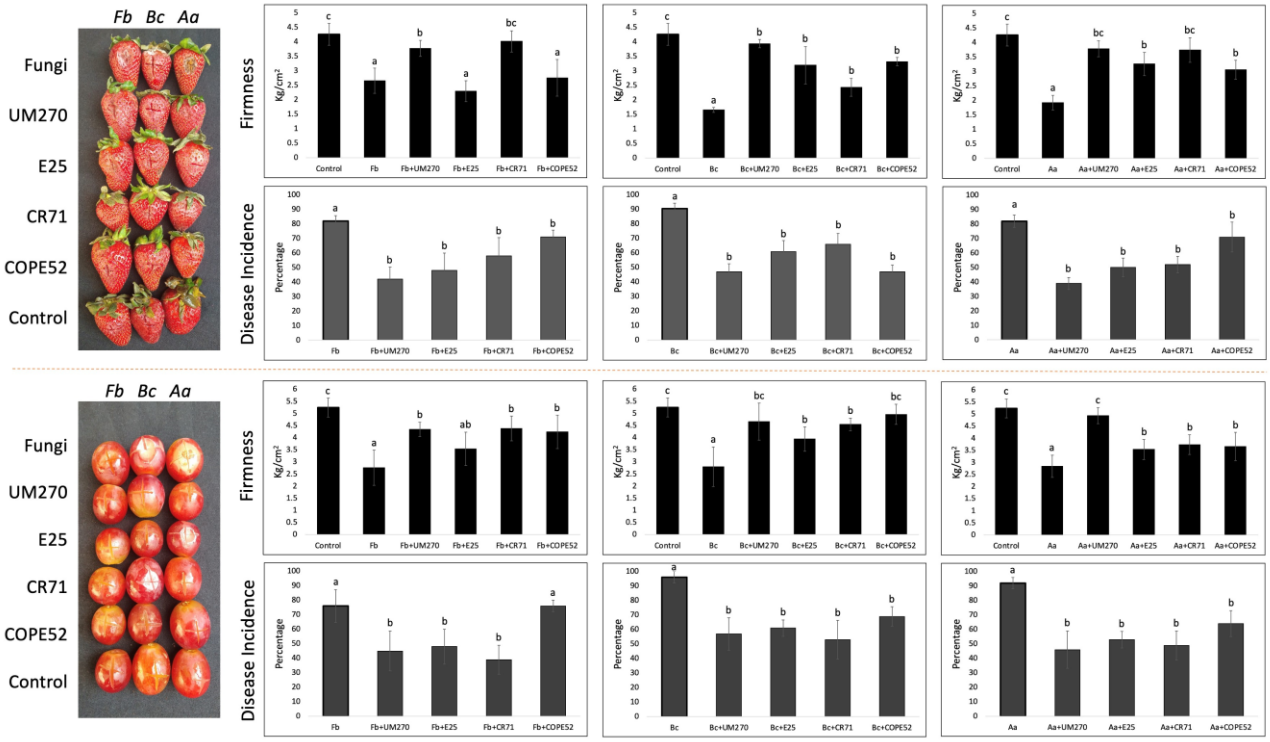

Figure 1. Biocontrol assay for strawberries and grapes. The top panel shows firmness and disease incidence for strawberries. The bottom panel shows firmness and disease incidence for grapes. The first column of the graphics shows results with *Fusarium brachygibbosum*, the second column with *Botrytis cinerea*, and the third column with *Alternaria alternata.* Bars represent the mean ± SE values; letters indicate that the means differed significantly after Duncan's multiple range test ($p < 0.05$).

3.4. Comparative Analysis of the Secondary Metabolite Biosynthesis Gene Clusters and Produced VOCs

In previous works in our lab, we reported some biosynthesis gene clusters of the four bacterial strains [11,13,22]. However, in this work, we decided to perform a new analysis and compared the biosynthesis gene clusters between the four strains [12,14,16]. The results are shown in Table 3. The studied strains from the genus *Bacillus* share gene clusters for petrobactin, bacillibactin, and molybdenum cofactor. *Bacillus toyonensis* COPE52 also has a gene cluster for bacitracin and paeninodin. The four strains have a gene cluster for fengycin, a nonribosomal peptide synthetase cluster (NRPS), and unspecified ribosomally synthesized and post-translationally modified peptide product (RiPP) cluster. *Pseudomonas fluorescens* UM270 has a gene cluster for the antibiotic 2,4-diacetyl phloroglucinol, fragin, and serobactin, among others.

Table 3. Gene clusters predicted by antiSMASH pipeline by the four PGPR, *Bacillus* spp., strains COPE52, E25, CR71, and *Pseudomonas fluorescens* strain UM270.

Gene Cluster	*Bacillus* sp. COPE52	*Bacillus* sp. E25	*B. thuringiensis* CR71	*Pseudomonas fluorescens* UM270
Bacitracin	55%	-	-	-
Petrobactin	100%	100%	100%	-
Bacillibactin	46%	46%	46%	-
Fengycin	40%	40%	40%	13%
Molybdenum cofactor	17%	17%	17%	-
Paeninodin	80%	-	-	-

Table 3. *Cont.*

Gene Cluster	*Bacillus* sp. COPE52	*Bacillus* sp. E25	*B. thuringiensis* CR71	*Pseudomonas fluorescens* UM270
NRPS	+	+	+	+
LAP	+	-	+	-
RiPP-like	+	+	+	+
NRPS-like	-	+	+	-
Anabaenopeptin NZ857/nostamide A	-	100%	100%	-
Lassopeptide	-	+	+	-
transAT-PKS	-	+	+	-
S-layer glycan	-	26%	26%	-
Thusin	-	100%	100%	-
Serobactin C/B/A	-	-	-	15%
Pyoverdin	-	-	-	3%
Crochelin A	-	-	-	7%
Lankacidin C	-	-	-	13%
Fragin	-	-	-	37%
N-acetyl glutaminylglutamine amide	-	-	-	+
Siderophore	-	-	-	+
Butyrolactone	-	-	-	+
2,4-diacetylphloroglucinol	-	-	-	100%
APE Vf	-	-	-	40%

Biosynthetic gene cluster similarity.

Volatile organic compounds (VOCs) of the plant-growth-promoting bacterial strains are shown in Table 4. The four strains can produce dimethyl disulfide, which has been reported to exhibit antimicrobial activity. *P. fluorescens* UM270 produces several other sulfur compounds and dimethylhexadecilamide, a compound that promotes plant growth and has antifungal activity [24].

Table 4. Comparison of the VOCs produced by the four bacterial strains, *Bacillus* spp., strains COPE52, E25, CR71, and *Pseudomonas fluorescens* strain UM270.

Volatile Compound	UM270	E25	CR71	COPE52
	%	%	%	%
Methanethiol	15.13	n.d.	n.d.	n.d.
Dimethyl sulfide	23.4	n.d.	n.d.	n.d.
2-Butanone	n.d.	2.32	2.24	0.99
1-Nonene	2.02	n.d.	n.d.	n.d.
Methyl thiolacetate	1.17	n.d.	n.d.	n.d.
Dimethyl disulfide	5.62	2.11	2.65	2.63
1-Decene	0.53	n.d.	n.d.	n.d.
1-Undecanol	50.01	n.d.	n.d.	n.d.
2,4-Dithiapentane	n.d.	n.d.	n.d.	n.d.
1-Dodecene	n.d.	n.d.	n.d.	n.d.

Table 4. *Cont.*

Volatile Compound	UM270	E25	CR71	COPE52
	%	%	%	%
Dimethyl trisulfide	0.57	n.d.	n.d.	n.d.
S,S-Dimethyl dithiocarbonate	n.d.	n.d.	n.d.	n.d.
2-Nonanone	n.d.	n.d.	n.d.	n.d.
Decyl oxirane	n.d.	n.d.	n.d.	n.d.
Methyl methylthiomethyl disulfide	n.d.	n.d.	n.d.	n.d.
2-Amino-5-methyl benzoic acid	n.d.	n.d.	n.d.	n.d.
Thiazole	0.41	n.d.	n.d.	n.d.
Butylated hydroxytoluene	0.49	n.d.	n.d.	n.d.
Dimethylhexadecilamine	0.64	n.d.	n.d.	n.d.
Acetone	n.d.	10.71	n.d.	n.d.
Isopropyl alcohol	n.d.	0.74	n.d.	n.d.
Ethyl propionate	n.d.	1.14	3.17	n.d.
Ethyl isobutyrate	n.d.	0.82	6.14	6.78
3-Methyl-2-pentanone	n.d.	6.86	n.d.	n.d.
Trichloromethane	n.d.	38.85	n.d.	n.d.
Ethyl-2-methylbutanoate	n.d.	n.d.	3.49	6.45
Ethyl isovalerate	n.d.	n.d.	1.95	5.19
3-Methylbutanenitrile	n.d.	12.93	n.d.	n.d.
S-Methyl thio butyrate	n.d.	n.d.	5.91	3.36
1-Butanol	n.d.	n.d.	0.93	n.d.
1,3-Diazine	n.d.	11.3	3.24	n.d.
Ethyl tiglate	n.d.	1.92	4.94	5.16
Methyl pyrazine	n.d.	1.18	n.d.	1.04
Acetoin	n.d.	n.d.	8.11	3.8
Isobutyl isothiocyanate	n.d.	10.47	25.86	n.d.
Acetic acid	n.d.	n.d.	5.4	6.03
Ethyl-3-hydroxybutanoate	n.d.	0.48	6.24	n.d.
2-(Methylthio)ethanol	n.d.	2.1	2.74	2.75
Propionic acid	n.d.	n.d.	1.16	n.d.
2-Methylpropanoic acid	n.d.	n.d.	3.72	n.d.
Phenyloxirane	n.d.	2.43	2.14	1.65
Butanoic acid	n.d.	n.d.	1.37	1.11
3-Methylbutanoic acid	n.d.	n.d.	2.32	4.28
Methyl salicylate	n.d.	n.d.	0.29	0.75
2-Butenoic acid	n.d.	n.d.	6.07	n.d.
Acetamide	n.d.	1.24	0.31	n.d.
Benzyl alcohol	n.d.	0.45	1.15	1.75
Ethyl propanoate	n.d.	n.d.	n.d.	1.45
Ethyl butanoate	n.d.	n.d.	n.d.	6.55
Isobutane	n.d.	n.d.	n.d.	4.6

Table 4. *Cont.*

Volatile Compound	UM270	E25	CR71	COPE52
	%	%	%	%
S-Methyl 3-methylbutanethioate	n.d.	n.d.	n.d.	7.84
3-Hydroxy-2-butanone	n.d.	n.d.	n.d.	3.49
Ethyl 3-hydroxybutanoate	n.d.	n.d.	n.d.	16.21
Propanoic acid	n.d.	n.d.	n.d.	0.97
2,3-Butanediol	n.d.	n.d.	n.d.	2.61
Menthol	n.d.	n.d.	n.d.	0.78
Ethyl phenylacetate	n.d.	n.d.	n.d.	1.44
Butyl butanoate	n.d.	n.d.	n.d.	0.33

Analysis of volatile organic compounds produced by UM270, E25, CR71, and COPE52 strains, detected by GC/MS analysis (n.d. means not detected).

4. Discussion

Postharvest fruit has a certain shelf life and undergoes a normal process of deterioration or decay due to respiration, ethylene production, the presence of fungi, and storage conditions (humidity, temperature, atmosphere, etc.). During this process, the fruit loses weight and firmness, and quality decreases. The main postharvest pathogens of a variety of fruits and vegetables have been reported to be the genera *Aspergillus*, *Botrytis*, *Fusarium*, *Geotrichum*, *Gloeosporium*, *Monilia*, *Mucor*, *Penicillium*, *Alternaria*, and *Rhizopus* [2]. *Botrytis*, *Fusarium*, and *Alternaria* are frequently found and are the major causative agents of postharvest disease in berries [25–32]. In a previous work, we isolated 20 fungi from berries in postharvest decay [17]. These isolates were characterized to belong mainly to the genera *Botrytis*, *Fusarium*, *Geotrichum*, *Mucor*, *Penicillium*, and *Alternaria*, which belong to the most common postharvest pathogens in fruits and vegetables [2]. In this work, we performed antagonistic essays against these postharvest fungal pathogens using four different plant-growth-promoting bacterial strains. We also selected three fungal pathogens (*Fusarium brachygibbosum* 4BF, *Botrytis cinerea* 62BCV, and *Alternaria alternata* 1A) to evaluate the bacterial strains for their biocontrol potential against the pathogens on strawberry and grape fruits.

PGPB can be used to prevent fungal pathogen growth via their biocontrol properties [21]. Tsalgatidou et al., 2023 [33] evaluated the biocontrol and plant-growth-promoting activities of two distinct *Bacillus halotolerans* strains (Cal.l.30 and Cal.f.4). The application of the two strains individually and as a mixture significantly enhanced the growth parameters of *Arabidopsis* and tomato plants. The two strains also significantly inhibited the growth of *Botrytis cinerea*. In a previous work, the authors isolated the strain Cal.1.30 from the medicinal plant *Calendula officinalis*, and it was selected for its strong biological potential against *Botrytis cinerea*. The *Bacillus halotolerans* strain and its cell-free supernatant reduce the gray mold disease severity index and disease incidence on harvested grapes and cherry tomato fruits. It has also been shown via HPLC-HRMS analysis that this strain synthesizes and secretes metabolites with antimicrobial activity, including the lipopeptides fengycin, surfactin, and mojavensin A, bacillaene isoforms, L-dihydroanticapsin, and bacillibactin, among others [20].

In Ref. [19] in this study, beneficial bacilli and pseudomonad strains showed significant percentages of mycelial growth inhibition against these previously characterized pathogens. Regarding the results of inhibition in vitro by diffusible and volatile compounds, it was observed that the inhibition of several isolates was greater by diffusible compounds than by volatile organic compounds. A probable explanation for this result could be that the compounds produced by bacteria that diffuse in the medium affect the growth of the fungus more directly since they are in direct contact with the vegetative mycelium, and perhaps harm the fungus in its ability to acquire nutrients and develop.

The bacterial strain that caused the highest percentages of growth inhibition of the isolated fungi in vitro was *Pseudomonas fluorescens* UM270. The results of trials carried out on fruits demonstrate the importance of developing experiments further, i.e., in vivo, where there is tripartite interaction between the bacteria, the pathogen, and the host (fruit). Therefore, there are other factors that could change the results that were observed in in vitro tests. All the analyzed bacterial strains significantly reduced the disease incidence caused by *Fusarium brachygibbosum*, *Botrytis cinerea*, and *Alternaria alternata* in strawberries and grapes. In addition, most bacterial strains analyzed helped to maintain fruit firmness.

A comparison of the secondary metabolite biosynthesis gene clusters between the four bacterial strains was performed. This can help us to compare the characteristics that the plant-growth-promoting bacteria have in common, and determine which of them are related or are important for biocontrol activity. The UM270 genome has been sequenced and analyzed [13]. The results of that analysis showed that UM270 can produce various antifungal compounds, including phenazine (*phzFABCD*), pyocyanin (*pcnCDE*), pyoverdine (*pvdPD*), 2,4-diacetyl phloroglucinol (*phlACBD*), and the volatile hydrogen cyanide (*hcnCB*), which are important for the biological control of several plant diseases caused by phytopathogenic fungi, oomycetes, and bacteria. Further, the E25 strain contains gene clusters to produce bacteriocins, siderophores, lanthipeptides, lipopeptides, ladderanes, and terpenes [11]. CR71 shares similar gene clusters to produce siderophores and peptide antibiotics [22]. COPE52 has gene clusters to produce bacitracin and paeninodin, a family lasso peptide; some of these lasso peptides exhibit antimicrobial activity [34].

We also compared the volatile organic compounds produced by the four bacterial strains. We observed that bacilli strains have in common several volatile organic compounds; however, all the strains assayed can produce dimethyl disulfide, which has antimicrobial activity.

5. Conclusions

The biocontrol activity of PGPB such as *Bacillus toyonensis* COPE52, *B. thuringiensis* CR71, *Bacillus* sp. E25, and *Pseudomonas fluorescens* UM270 can be used to inhibit postharvest fungal pathogens. It is necessary to determine the complete characteristics of a biocontrol agent microorganism through the application of microbiological, biochemical, bioinformatics, and molecular tools, and to improve or provide optimal conditions for proliferation.

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