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EVALUACIÓN DE LOS MECANISMOS DE BIOCONTROL DE LA CEPA BACTERIANA SER3 CONTRA PATÓGENOS POSTCOSECHA: UN ENFOQUE GENÓMICO-FUNCIONAL

Tesis que presenta

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‘‘Cada uno de nosotros existe durante un tiempo muy breve, y en dicho intervalo tan sólo explora una parte diminuta del conjunto del universo. Pero los humanos somos una especie marcada por la curiosidad. Nos preguntamos, buscamos respuestas…

…La filosofía no se ha mantenido al corriente de los desarrollos modernos de la ciencia. Los científicos se han convertido en los portadores de la antorcha del descubrimiento en nuestra búsqueda del conocimiento’’.

Stephen Hawking

A mis abuelos, que con su ejemplo me enseñaron que el trabajo y ser constante son herramientas indispensables para alcanzar las metas.

Agradecimientos

Me gustaría mencionar algunos de los momentos vividos durante todo mi trayecto en el Instituto de Investigaciones Químico-Biológicas, institución que es muy querida para mí por los elementos que ha brindado en mi formación, primero como maestra y después en mi formación como doctora. En estos años en el Instituto, he tenido la fortuna de encontrar excelentes personas, como profesionales, compañeros, y estudiantes (porque también tuve la experiencia de transmitir y compartir los conocimientos obtenidos en el laboratorio con los compañeros algunas veces más inexpertos). Pero, sobre todo, encontré compañeros, es decir, personas que compartieron espacio y tiempo. El tiempo, que es una de las cosas más valiosas y preciadas. Pienso que el tiempo que pasé formando parte de este programa y de esta institución fue una muy buena inversión, me llevo muchas experiencias, algunas buenas y otras no tanto, pero sobre todo me llevo algo invaluable, conocimiento. Y bueno, creo que sería apropiado empezar los agradecimientos por mi director de tesis, el doctor Gustavo, ya que fue la persona que me abrió las puertas de su laboratorio y me dio la bienvenida a su equipo de trabajo, con este hecho pude conocer a las personas y vivir las experiencias que he mencionado previamente. Siempre agradeceré la confianza y las oportunidades dadas, porque eso me permitió crecer, dar los primeros pasos en la importante profesión de la investigación, no me veo en otro lugar haciendo lo que logré y aprendiendo lo que ahora sé. Como segundo agradecimiento, a los compañeros de trabajo, que fueron cambiando con el paso del tiempo, naturalmente al graduarse se marchaban, pero siempre llegaban nuevos. Fueron muy importantes y también aprendí algo de cada uno de ellos, por ejemplo, de las discusiones sobre algunos protocolos de experimentos, que muchas veces ayudaron a ver las cosas de diferentes ángulos, detectar fallas que podrían ocurrir y plantear soluciones. Pero sobre todo les agradezco por los momentos de apoyo en las decepciones y frustraciones, en mi caso, muchas veces aliviadas con alguna broma o comentario chistoso que me hacía reír (parecía que podía estar triste y feliz al mismo tiempo), por las palabras de ánimo y su solidaridad, por las ocasiones en las que no tenía oportunidad de salir a comer y me llevaron comida. También por los momentos de relajo y convivencias, aquí entran también los compañeros del laboratorio de Ecología Microbiana, a quienes agradezco empezando por el doctor Valencia, que, en conjunto con los compañeros, imaginábamos posibles soluciones, muchas veces graciosas, a ciertos conflictos o situaciones, también por sus buenas recomendaciones y consejos.

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Resumen

Los patógenos postcosecha causan graves pérdidas económicas en cereales, verduras y frutas, ya que su calidad se puede ver mermada. Para inhibir las enfermedades causadas por patógenos, tales como hongos, se usan agroquímicos con diversos efectos tóxicos en la salud humana y en el ambiente. Por lo tanto, es necesario tener alternativas inocuas y sustentables que reduzcan la incidencia de potenciales patógenos. En el presente trabajo se caracterizó taxonómica y funcionalmente la cepa bacteriana SER3, aislada de la superficie de fresas, como un nuevo agente control biológico contra diversos hongos patógenos postcosecha. Los resultados mostraron que la cepa SER en co-inoculación directa inhibe el crecimiento de varios de estos hongos por compuestos difusibles, principalmente. Al secuenciar su genoma completo, se encontró que tiene identidad del 100% con *Rouxiella badensis* DSM 10043^{T} cuando comparamos el gen ARNr *16S,* así como porcentajes de similitud del 99.6% con el algoritmo ANI (Identidad Promedio de Nucleótidos) y 98.2% con el algoritmo GGDC (Calculadora Distancia Genoma-Genoma). De forma interesante, el programa anti-SMASH arrojó que la cepa posee grupos de genes para la producción de metabolitos secundarios como: sideróforos, enzima péptido no ribosomal sintetasa (NRPS), tiopéptidos, aril polienos, policétido sintetasa tipo I (T1PKS) y tipo III (T3PKS), homoserilactonas, entre otros. Al realizar la comparación de estos grupos de genes con los de las bacterias filogenéticamente cercanas a la cepa SER3, observamos que hay una similitud en el tipo de metabolitos que pueden producir, así como en los porcentajes de semejanza de los grupos de genes, resaltando los del género *Rahnella*, el cual ha sido reportado como agente de control biológico, debido a esta similitud en los grupos de genes, se sugiere que el género *Rouxiella* también tiene actividad de control biológico. Cuando se corroboraron los resultados de anti-SMASH en lo que corresponde a la síntesis de sideróforos, realizamos ensayos en medio sólido cromo azurol y en confrontación con el patógeno *Fusarium brachygibbosum* 4BF en diferentes tiempos de inoculación con la cepa SER3. Los resultados mostraron que hay una relación entre el tiempo de inoculación de la cepa con la concentración de sideróforos que produce, y que esta afecta el crecimiento y la producción de sideróforos del patógeno. Además, al suministrar una solución de $FeCl_3$ en el medio CAS el hongo restablece su crecimiento, lo cual comprueba que los sideróforos son los responsables de la inhibición del crecimiento de *F. brachygibbosum.* El espectro obtenido con espectrofotometría UV-Vis de nanodrop nos indica que el sobrenadante de la cepa en medio rico en nutrimentos, así como en medio pobre, contiene un sideróforo tipo hidroxamato, el cual se sugiere que se trata de la desferroxamina E (nocardamina). Los experimentos *in vivo* realizados en fresas, mostraron que la cepa SER3 y su sobrenadante tienen actividad de control biológico contra *Botrytis cinerea* y *F. brachygibbosum*, y que la cepa afecta la morfología de las hifas de estos patógenos cuando se realizan co-cultivos. En conclusion, nuestros resultados muestran que la cepa SER3 de *Rouxiella badensis* posee rasgos genómicos-funcionales asociados al antagonismo de patógenos fúngicos postcosecha, siendo un excelente agente de bicoontrol.

Palabras clave: patógenos, postcosecha, control biológico, metabolitos secundarios, bioinformática.

Abstract

Postharvest pathogens cause significant economic losses in cereals, vegetables, and fruits as their quality can be compromised. To inhibit diseases caused by pathogens such as fungi, agrochemicals with various toxic effects on human health and the environment are used. Therefore, it is necessary to have safe and sustainable alternatives that reduce the incidence of potential pathogens. In this study, the bacterial strain SER3, isolated from the surface of strawberries, was taxonomically and functionally characterized as a new biological control agent against various postharvest fungal pathogens. The results showed that the SER strain in direct co-inoculation mainly inhibits the growth of several of these fungi through diffusible compounds. By sequencing its complete genome, it was found to have 100% identity with *Rouxiella badensis* DSM 10043T when comparing the *16S* rRNA gene, as well as similarity percentages of 99.6% with the ANI (Average Nucleotide Identity) algorithm and 98.2% with the GGDC (Genome-to-Genome Distance Calculator) algorithm. Interestingly, the anti-SMASH program revealed that the strain possesses gene clusters to produce secondary metabolites such as siderophores, non-ribosomal peptide synthetase (NRPS) enzyme, thiopeptides, aryl polyenes, type I polyketide synthase (T1PKS) and type III (T3PKS), homoserine lactones, among others. When comparing these gene clusters with those of bacteria phylogenetically close to the SER3 strain, we observed a similarity in the type of metabolites they can produce, as well as in the percentages of gene cluster similarity, highlighting those of the *Rahnella* genus, which has been reported as a biological control agent. Due to this similarity in gene clusters, it is suggested that the *Rouxiella* genus also has biological control activity. When the anti-SMASH results regarding siderophore synthesis were corroborated, we conducted assays in CAS medium and in confrontation with the pathogen *Fusarium brachygibbosum* 4BF at different inoculation times with the SER3 strain. The results showed that there is a relationship between the inoculation time of the strain and the concentration of siderophores it produces, and that this affects the growth and siderophore production of the pathogen. Additionally, supplying an $FeCl_3$ solution in the CAS medium restored the growth of the fungus, confirming that siderophores are responsible for the inhibition of *F. brachygibbosum* growth. The spectrum obtained with UV-Vis nanodrop spectrophotometry indicates that the supernatant of the strain in nutrient-rich as well as nutrient-poor medium contains a hydroxamate-type siderophore, which is suggested to be desferrioxamine E (Nocardamine). *In vivo* experiments conducted on strawberries showed that the SER3 strain and its supernatant have biological control activity against *Botrytis cinerea* and *F. brachygibbosum*, and that the strain affects the morphology of these pathogens hyphae when co-cultured. In conclusion, our results show that the SER3 strain of *Rouxiella badensis* possesses genomic-functional traits associated with the antagonism of postharvest fungal pathogens, making it an excellent biocontrol agent.

Introducción

Los microorganismos constituyen una parte integral de frutas y vegetales, pueden encontrarse en las superficies como epífitos o dentro de los tejidos como endófitos. La mayoría de ellos no causan enfermedad y están relacionados con la fisiología de las frutas y vegetales en etapa postcosecha y con la respuesta de defensa hacia patógenos (Zhang *et al*., 2021).

El darse cuenta de que en las frutas y vegetales se encuentran microorganismos benéficos fomentó el campo del control biológico utilizando estos microorganismos para desarrollar productos comerciales (Droby & Wisniewski, 2018). Generalmente nos referimos a estos microorganismos como agentes de control biológico o antagonistas microbianos. Estos agentes pueden definirse como microorganismos que impiden la proliferación y desarrollo de microorganismos patógenos a través de diferentes mecanismos de acción. Sin embargo, la aplicación práctica de estos agentes en productos comerciales se ve obstaculizada por su eficacia comparada con los productos químicos sintéticos. Por lo tanto, es importante entender cómo actúan los microorganismos benéficos, cuáles son los mecanismos de acción que utilizan para mejorar su eficacia y seleccionar las mejores cepas (Massart *et al*., 2015).

Cabe resaltar que los agentes de control biológico no solo pueden encontrarse en la superficie de frutas y vegetales, también pueden ser aislados de distintas fuentes, comúnmente son aislados de alguna parte vegetal o del suelo en relación con la planta, por ejemplo, de la rizosfera, filosfera o como endófitos (dentro de los tejidos de la planta) y pueden ser evaluados por su capacidad de control biológico en frutas y vegetales en etapa postcosecha con el objetivo de evitar el deterioro ocasionado por hongos (Morales-Cedeño *et al*., 2023).

Algunas de las características con las que debe contar un agente de control biológico son que debe ser genéticamente estable, no fastidioso de acuerdo a sus requerimientos nutricionales, capaz de sobrevivir a diferentes condiciones ambientales, no debe producir metabolitos dañinos para humanos, no debe ser patógeno del hospedero, que pueda ser formulado para almacenarse y dispersarse adecuadamente, que sea compatible con otros tratamientos físicos y químicos que se aplican a frutas y vegetales en etapa postcosecha, entre otros (Morales-Cedeño *et al*., 2021).

Para conocer las características de un agente de control biológico, así como los mecanismos de acción que utiliza, se pueden emplear distintas metodologías. Los métodos microbiológicos suelen ser los primeros en utilizarse para evaluar la capacidad de antagonizar el crecimiento de ciertos microorganismos patógenos haciendo cultivos duales. Los métodos bioquímicos son utilizados para identificar proteínas o diversos metabolitos, por ejemplo, aquellos relacionados con la resistencia del hospedero en confrontación con el patógeno.

Las técnicas moleculares como la secuenciación, la metabolómica, transcriptómica, proteómica, etc. Son otra metodología que permite estudiar de una manera holística los mecanismos de acción de un agente de control biológico (Massart *et al*., 2015).

La era de la genómica ha proporcionado herramientas para identificar en el genoma de los agentes de control biológico grupos de genes biosintéticos para la producción de antibióticos y metabolitos secundarios. Los algoritmos que se han desarrollado para para buscar estos grupos de genes incluyen: anti-SMASH, BAGEL, NP.searcher y PRISM. Muchos de estos algoritmos se basan en patrones derivados de grupos de genes de antibióticos conocidos y motivos proteicos asociados.

Las estrategias de genómica comparativa también pueden ser útiles para identificar nuevos grupos de genes biosintéticos. Esta técnica se basa en la comparación de secuencias de genes candidatos con los que se encuentran en el genoma de alguna cepa utilizando la herramienta tblastn (Williams *et al*., 2020).

En lo que respecta a los mecanismos de control biológico que puede utilizar un antagonista microbiano, se han descrito distintos, los principales son: competencia con el patógeno por espacio y nutrientes, antibiosis, parasitismo directo y resistencia inducida (Sharma et al., 2009). Sin embrago, diversos autores han descrito algunos más, por ejemplo: formación de biopelículas, quorum sensing, producción de compuestos orgánicos volátiles (VOCs) y producción de sideróforos (Dukare *et al*., 2019),(Carmona-Hernandez *et al*., 2019).

Algunos mecanismos de acción pueden estar relacionados entre sí, por ejemplo, el quorum sensing, la comunicación y la densidad de la población bacteriana tiene relación con la competencia por espacio y nutrientes y también se relaciona con la producción de biofilm y ésta a su vez puede ligarse con la resistencia del hospedero. La producción de sideróforos también se relaciona con la competencia por espacio y nutrientes ya que los sideróforos son compuestos quelantes del hierro, de esta manera queda poco hierro disponible para lo patógenos y así se limita su crecimiento y proliferación (Morales-Cedeño *et al*., 2020). Los mecanismos no solo pueden estar relacionados, también puede ser que el agente antagonista utilice más de un mecanismo para biocontrolar un patógeno, también se debe considerar que se implica una interacción compleja entre el huésped, el patógeno, el agente antagonista y el ambiente (Nunes, 2012).

La competencia por espacio y nutrientes es el mecanismo que se define como uno de los principales, es utilizado por muchos agentes de control biológico. Se refiere a la capacidad que tienen los antagonistas de adaptarse y utilizar los recursos o nutrimentos de una manera más eficiente que el patógeno. Para lograr esto el agente de control biológico debe estar presente en cantidades suficientes en el momento y sitio correcto (Spadaro & Droby, 2016). Por ejemplo, si hay una lesión en la fruta y ésta se encuentra colonizada por algún microorganismo antagonista en una concentración importante, será difícil que un patógeno pueda ocupar ese espacio y proliferar.

La competencia por espacio y nutrientes está relacionada con la producción de sideróforos, el hierro es un micronutriente muy importante para el crecimiento y proliferación de los microorganismos, así como un elemento necesario para el crecimiento y virulencia de los patógenos. En la competencia por el hierro, los sideróforos producidos por los agentes de control

biológico captan el hierro presente, limitando la ingesta de los patógenos y de esta manera impiden su crecimiento germinación y patogénesis. (Dukare *et al*., 2019).

Los sideróforos son compuestos quelantes del hierro, forman un complejo fuerte y estable con los iones de hierro con la finalidad de solubilizarlo y facilitar su toma a través de selectivos receptores de membrana que realizan la importación del complejo hierro-sideróforo. Producir sideróforos también tiene un costo de energía por lo que los microorganismos no productores de sideróforos tienden a incentivar la absorción de sideróforos que no producen, para lograrlo necesitan de vías de absorción que pueden surgir por mutaciones de transportadores para captar sideróforos exógenos, o por transferencia genética horizontal de la vía de captación desde los productores de sideróforos (Boiteau *et al*., 2019).

La prevalencia de esta ‘‘piratería’’ de sideróforos induce una presión selectiva para quienes los producen, es decir, deben sintetizar nuevos sideróforos que no puedan ser tomados por los competidores. Lo que se ve reflejado en cientos de sideróforos únicos que se han esclarecido estructuralmente. Pueden surgir nuevos sideróforos a partir de la evolución divergente de las vías de biosíntesis, lo que da como resultado sideróforos estructuralmente similares con ligeras diferencias en las propiedades químicas (Fischbach *et al*., 2008).

Los sideróforos también se relacionan con otro mecanismo de control biológico ya que pueden activar la resistencia sistémica inducida en el hospedero, sin embargo, el mecanismo por el cual disparan esta respuesta es poco entendido (Orozco-Mosqueda *et al*., 2023).

Los cientos de sideróforos que se han descrito pueden clasificarse por su naturaleza química principalmente en 4 tipos: carboxilatos, hidroxamatos, catecolatos y tipo mixto (Ghosh *et al*., 2020).

Antecedentes

Para conocer los mecanismos de acción que posee un agente de control biológico es importante utilizar una combinación de diferentes métodos, es decir, utilizar tanto métodos microbiológicos y bioquímicos como moleculares, esto nos permite tener un panorama más amplio, ya que algunas veces, aunque un agente de control biológico tenga las características que le permiten desarrollar un mecanismo de acción, este no lo realiza bajo determinadas condiciones. Un ejemplo de esto es mencionado en el trabajo de Tian *et al*., 2020, en donde observaron mediante ensayos microbiológicos (haciendo cultivos duales) que tanto la cepa de *Bacillus* sp. W176 como su sobrenadante inhiben el crecimiento de *Penicillium digitatum* (figura 1), dados estos resultados decidieron analizar el sobrenadante utilizando cromatografía liquida acoplada a espectrometría de masas (LC-MS), en donde encontraron varios compuestos con actividad antimicrobiana como: macrolactina, bacilaeno, micosubtilina y surfactina. También utilizaron métodos moleculares como PCR para amplificar y detectar genes relacionados con la síntesis de compuestos antimicrobianos, mediante este último análisis encontraron genes necesarios para la producción de iturina, sin embargo, la iturina no fue encontrada en sobrenadante.

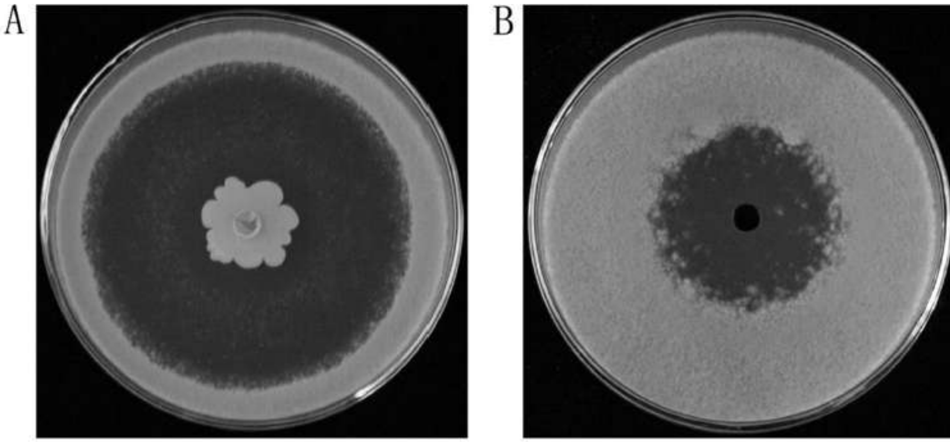


Figura 1. Cultivos duales de *Bacillus* sp. W176 (panel A) y su sobrenadante (panel B) con *Penicillium digitatum*.

Cuando se trata de evaluar la capacidad antagonista de un agente de control biológico también es importante comprobar que el efecto que se observa en los experimentos *in vitro* se observe en los experimentos *in vivo*. Por lo cual también realizaron ensayos utilizando como modelo frutas de mandarina (figura 2). Los resultados mostraron que cuando se utiliza la cepa w176 la incidencia de enfermedad provocada por *P. digitatum* disminuye 90%, mientras que al utilizar el sobrenadante la incidencia de enfermedad disminuye 57.5%.

Figura 2. Efecto de la cepa w176 y su sobrenadante en el deterioro provocado por *P. digitatum* inoculado en mandarinas. Panel (A): Frutas con 20µL de agua estéril. Panel (B): frutas con 10 µL de agua estéril y 10 µL de una suspensión de conidios de *P. digitatum*. Panel (C): frutas 10 µL del sobrenadante libre de células de la cepa w176 y 10 µL de una suspensión de conidios de *P. digitatum*. Panel (D): 10 µL de cultivo de la cepa w176 en PDB y 10 µL de una suspensión de conidios de *P. digitatum.* La concentración de la cepa w176 fue de $2X10^8$ esporas/mL y la concentración de la suspensión de conidios fue de $1X10^5$ esporas/mL. Las frutas fueron incubadas en cámaras climáticas a 25°C y 90% de humedad relativa por 5 días. Se utilizaron 20 frutas por cada replica, los ensayos se repitieron 3 veces.

La secuenciación de alto rendimiento ha permitido explorar los genomas bacterianos completos y poder compararlos, esta técnica ha sido de gran ayuda en la taxonomía, pudiendo definir características de diferentes géneros bacterianos. También ha permitido hacer comparaciones entre cepas de la misma especie, esto es útil porque nos permite definir porque algunas cepas pueden ser potenciales agentes de control biológico y que es lo que las distingue de otras.

El trabajo realizado por Contreras-Pérez *et al*., 2019 es un ejemplo de la vasta información que puede ser obtenida al secuenciar el genoma completo de una bacteria. Ellos aislaron a la cepa COPE52, endófita de raíces de *Rubus fruticosus*, y utilizando diferentes herramientas bioinformáticas, como la Identidad Promedio de Nucleótidos por sus siglas en inglés (ANI) y la Calculadora de Distancia Genoma-Genoma (GGDC) pudieron calcular el Índice General de Relación del Genoma, el cual ayudó a identificar taxonómicamente a la cepa COPE52 como *Bacillus toyonensis* COPE52. Con el servidor RAST, pudieron identificar 5978 secuencias codificantes, incluyendo secuencias que son importantes para la promoción directa e indirecta del crecimiento vegetal (fig.3). Este estudio fue complementado con ensayos microbiológicos y bioquímicos, realizando cultivos duales confrontando a la cepa COPE52 con el patógeno *Botrytis cinerea* de manera directa y también indirecta, para evaluar el efecto de los compuestos orgánicos volátiles (VOCs), al observar que estos tienen un efecto inhibiendo el crecimiento del hongo,

decidieron hacer cromatografía de gases acoplada a espectrometría de masas (GC-MS), los resultados de este análisis mostraron que la cepa COPE52 produce compuestos con actividad antimicrobiana como el dimetildisulfuro, así como acetoína y 2,3-butanodiol que son compuestos que inducen resistencia sistémica en las plantas (tabla 1). Otros ensayos bioquímicos que realizaron mostraron que esta cepa produce ácido indol acético (IAA), y proteasas (tabla 2).

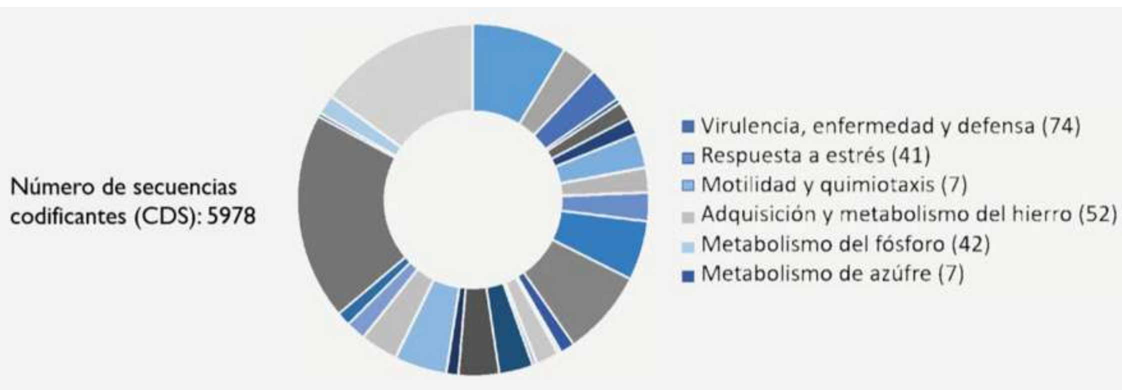


Figura 3. Distribución de secuencias codificantes agrupadas en categorías de *B. toyonensis* COPE52. Del lado derecho se muestran el número de secuencias que son importantes para antagonismo, colonización rizosférica y endosférica.

Tabla 1. Compuestos orgánicos volátiles producidos por *B. toyonensis* COPE52 detectados con GC/MS.

Compuestos Orgánicos Volátiles	Tr (min)	*Bacillus toyonensis* COPE52 (%)
Acetoína	1.4	3.8
2-Butanona	1.95	0.99
Propanoato de etilo	2.65	1.45
Isobutirato de etilo	2.76	6.78
Butanoato de etilo	4.28	6.55
2-metilbutanoato de etilo	4.72	6.45
Isovalerato de etilo	5.24	5.19
Dimetil disulfuro	5.35	2.63
Isobutano	6.43	4.6
S-Metil-tiobutirato	7.56	3.36
S-Metil 3-metilbutanotioato	11.55	7.84
Tiglato de etilo	12.26	5.16
Metil pirazina	13.63	1.04
3-Hidroxi-2-butanona	14.47	3.49
Ácido acético	22.32	6.03
3-hidroxibutanoato de etilo	24.74	16.21
2-(Metiltio) etanol	25.12	2.75
Ácido propanoico	25.83	0.97
2,3-Butanodiol	27.38	2.61
Fenil oxirano	28.42	1.65
Ácido butanoico	29.33	1.11

Mentol	29.39	0.78
Ácido 3-Metilbutanoico	30.87	4.28
Salicilato de metilo	34.34	0.75
Fenilacetato de etilo	34.97	1.44
Butirato de butilo	37.74	0.33
Alcohol bencílico	38.27	1.75

Tabla 2. Ensayos microbiológicos realizados para evaluar antagonismo por compuestos difusibles y volátiles producidos por COPE52 y ensayos bioquímicos para detectar funciones relevantes en la promoción del crecimiento vegetal.

Endófito bacteriano	IAA (µg/mL)	Actividad de proteasas	Sideróforos	Actividad de ACC desaminasa	Inhibición de *Botrytis cinerea*	
					Compuestos difusibles	Compuestos volátiles
Bacillus toyonensis COPE52	24.08 ± 0.50	28.6 ± 1.6	-	-	11.49 ± 0.84	36.41 ± 2.3

Los agentes de control biológico pueden aislarse de diversas fuentes, por ejemplo, pueden habitar cualquier tejido o superficie vegetal como: flores, frutos, tallos, hojas, raíces, incluso el interior de las semillas de los frutos (Morales-Cedeño *et al*., 2021). También pueden habitar distintos tipos de suelo, desde campos de agricultura, suelos de minas o de ambientes extremos, como suelos salinos, con altas o bajas temperaturas, etc. La mayoría de las cepas que han sido evaluadas como agentes de control biológico contra hongos causales de deterioro postcosecha en frutas y vegetales, han sido precisamente aisladas de frutas y vegetales. En el proyecto de maestría realizado por Morales-Cedeño, 2019, se aisló a la cepa SER3 de la superficie de frutas de fresa. Se decidió caracterizarla definiendo algunas características morfológicas y amplificando el gen ribosomal 16S para establecer su taxonomía y, además, evaluarla como agente de control biológico (figura 4). Los resultados de la morfología mostraron que se trata de una bacteria Gram negativa que forma colonias de un color claro. Sin embargo, los resultados de la comparación del gen ribosomal 16S utilizando la herramienta BLAST, mostraron que tenía un porcentaje de identidad menor al 98.7%, por lo que no se pudo identificar su taxonomía (tabla 3).

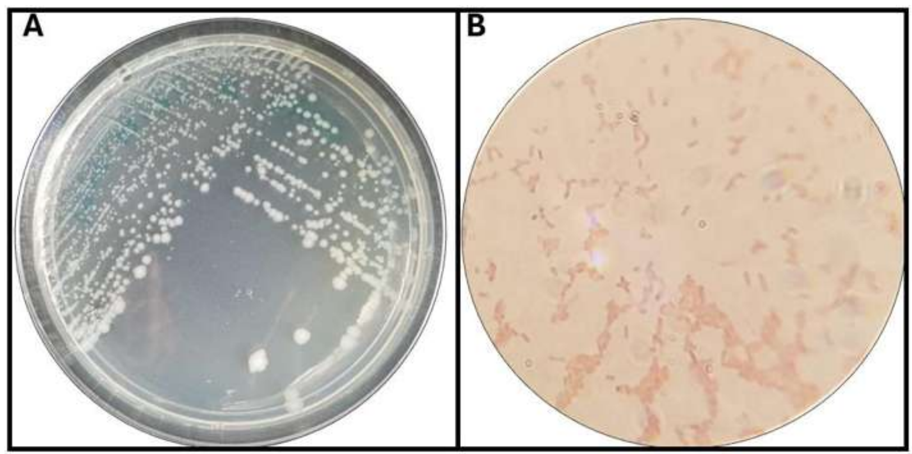


Figura 4. Morfología macroscópica (panel A) y morfología microscópica (panel B) de la cepa SER3 aislada de la superficie de fresas. La morfología microscópica fue observada con microscopio óptico con objetivo de 100x.

Tabla 3: Identificación molecular con PCR del gen ADNr 16S y la herramienta de búsqueda BLAST.

Aislado	Género y especie	Porcentaje de identidad
SER3	*Rahnella aquatilis*	94%

Durante un tiempo considerable el estándar de oro para establecer la taxonomía procariota ha sido la hibridación del ADN. Debido al avance de las nuevas tecnologías han surgido herramientas que han permitido crear un método bioinformático que pueda remplazarla, este método se basa en crear valores análogos a los de la hibridación y se le dio el nombre de índice general de relación del genoma (OGRI), por definición este índice representa una medida que indica que tan similares son dos secuencias genómicas, puede utilizarse para comprobar si una cepa pertenece a una especie conocida calculando la relación entre el genoma de la cepa de interés y el genoma de la cepa tipo de una especie. La identidad promedio de nucleótidos (ANI) y la calculadora de distancia genoma-genoma (GGDC) son dos herramientas que se utilizan para calcular el OGRI. En la figura 5 se muestra el diagrama experimental propuesto por Chun *et al*., 2018 para calcular este índice.

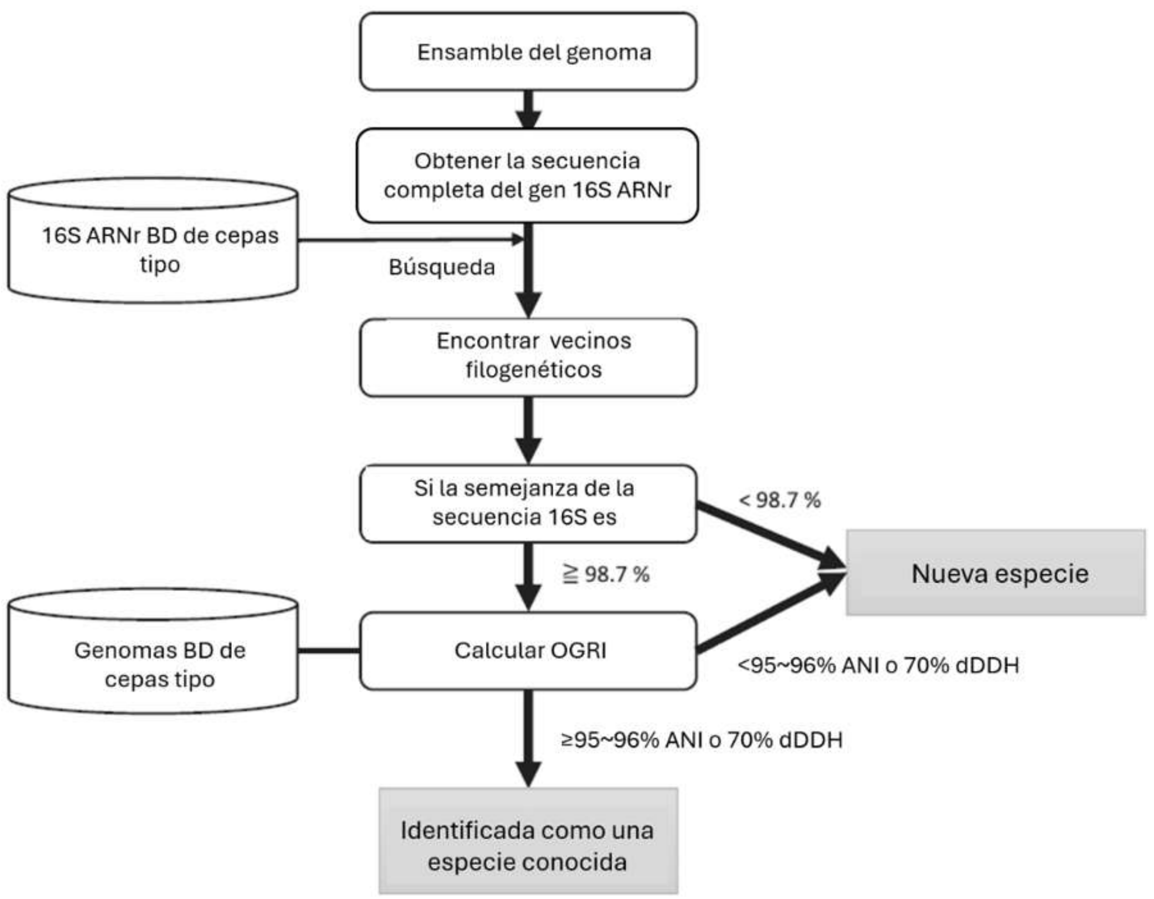


Figura 5. Flujo de trabajo de clasificación procariota a nivel especie basada en el genoma. Para reconocer nuevos géneros, se deben utilizar árboles filo genómicos.

Le Fléche-Matéos *et al*., 2017 utilizaron el índice general de relación del genoma (OGRI) para poder identificar taxonómicamente dos cepas que fueron aisladas de suelos pantanosos en Baden, Alemania. Mediante este método pudieron determinar que se trataban de dos nuevas especies: *Rouxiella badensis* y *Rouxiella silvae*. Para poder caracterizar a dichas cepas, secuenciaron sus genomas completos, utilizaron los algoritmos ANI y GGDC para calcular el OGRI y determinaron algunas características fenotípicas. En la figura 6 se muestra el árbol filogenético que construyeron con secuencias del gen ribosomal 16S. Podemos observar que ambas cepas son cercanas a *Rouxiella chamberiensis*. Los resultados del OGRI mostraron que el porcentaje de similitud utilizando el algoritmo ANI fue menor del 95% y con el algoritmo GGDC el porcentaje fue menor del 70% estos resultados sugirieron que se trataba de nuevas especies. En la tabla 4 se enlistan algunas características bioquímicas y morfológicas que determinaron al caracterizar a *Rouxiella badensis*.

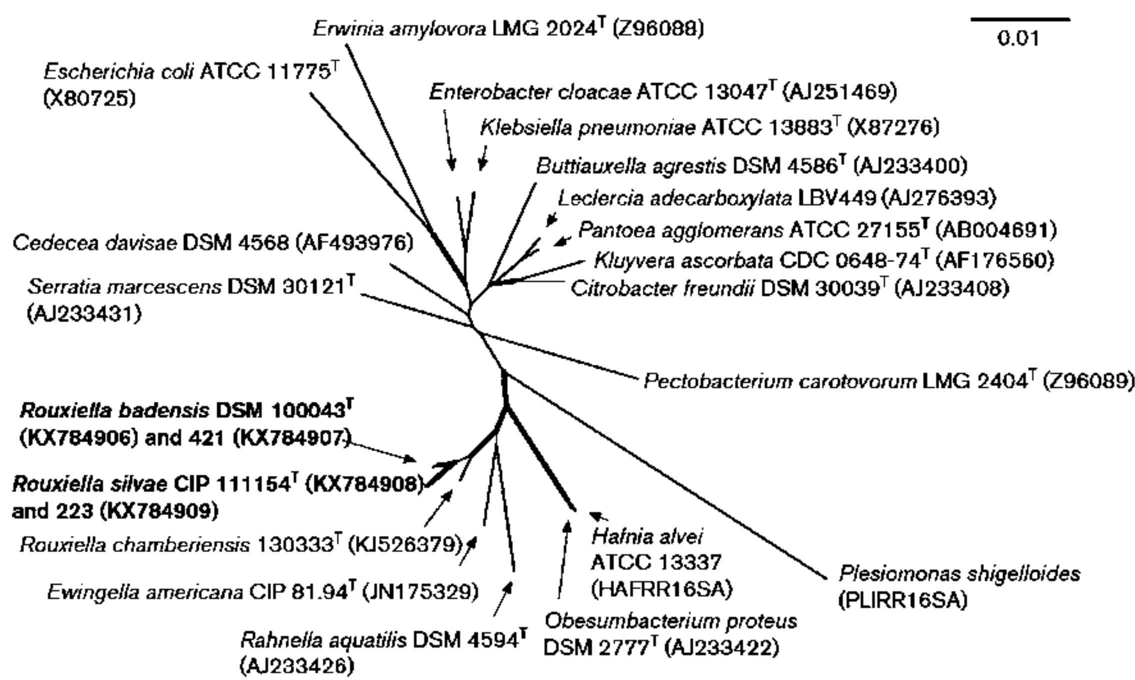


Figura 6. Árbol filogenético construido con secuencias del gen ribosomal 16S. Valores de Bootstrap basados en 1000 replicados.

Tabla 4. Características fenotípicas de *Rouxiella badensis*.

Forma y tinción Gram	Bacilos GRAM (-)
Color de las colonias	Sin pigmento
Crecimiento a 37°C	+
Reducción de nitratos	+
Producción de ácidos a partir de:	+
D-Fucosa	+
Glicerol	+
D-Maltosa	+
D-Melezitosa	+
D-Rafinosa	+
L-Ramnosa	+
D-Sucrosa	+
D-Turanosa	+

Justificación

En un trabajo anterior se aisló a la cepa bacteriana SER3 de la filósfera de frutas de fresa, la cual destacó por su capacidad de antagonizar diversas especies de hongos patógenos postcosecha, incluyendo los géneros *Botrytis*, *Penicillium*, *Fusarium y Alternaria*. Por lo tanto, la cepa SER3 podría tener una función ecológica como antagonista de patógenos fúngicos y ser un promisorio agente de control biológico de enfermedades postcosecha. Sin embargo, se desconocen los elementos genéticos y los potenciales metabolitos involucrados en dicho antagonismo. Por lo que un análisis taxonómico y genómico-funcional de SER3, así como los metabolitos que produce en interacción con hongos, permitirá explorar su principal modo de acción, conduciendo al desarrollo de estrategias que protejan a las frutillas del ataque por patógenos postcosecha.

Hipótesis

La cepa bacteriana SER3 posee rasgos genómico-funcionales asociados al antagonismo de patógenos fúngicos postcosecha.

Objetivos

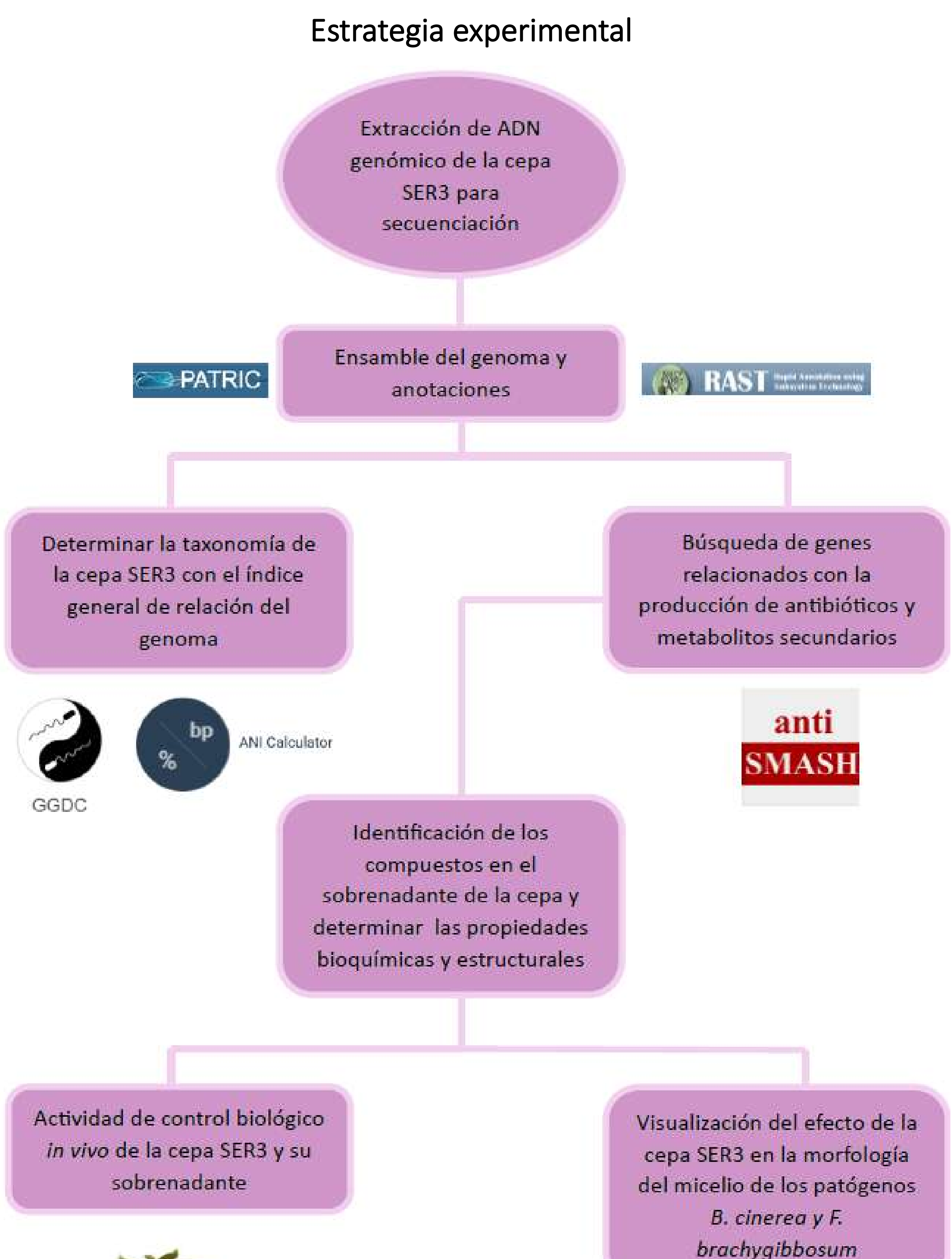
Objetivo general

Definir las características taxonómicas y genómico-funcionales de la cepa SER3 asociadas al antagonismo de patógenos fúngicos postcosecha.

Objetivos particulares

1. Establecer la afiliación taxonómica y filogenómica de la cepa bacteriana SER3.
2. Identificar y validar grupos de genes asociados a la producción de metabolitos antifúngicos encontrados en el genoma de la cepa SER3.
3. Determinar las propiedades bioquímicas y estructurales del o los metabolitos bioactivos previamente identificados en el genoma de la cepa SER3.
4. Cuantificar *in vitro* el control de patógenos fúngicos postcosecha por el o los metabolitos bioactivos producidos por la cepa SER3.

Estrategia experimental



Resultados:

Functional and Genomic Analysis of *Rouxiella Badensis* SER3 as a Novel Biocontrol Agent of Fungal Pathogens

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Functional and Genomic Analysis of *Rouxiella badensis* SER3 as a Novel Biocontrol Agent of Fungal Pathogens

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In recent decades, various bacterial species have been characterized as biocontrol agents for plant crop diseases; however, only a few genera have been predominantly reported in the literature. Therefore, the identification of new antagonists against phytopathogens is essential for boosting sustainable food production systems. In this study, we evaluated the role of strain SER3 from the recently discovered *Rouxiella badensis* as a biocontrol agent. SER3 was isolated from the phyllosphere of decaying strawberry fruit (*Fragaria* × *ananassa*) and showed different grades of antagonism against 20 fungal pathogens of berries, based on confrontation assays, due to the action of its diffusible and volatile compounds. These fungal pathogens were isolated from decayed strawberry, blackberry, and blueberry fruit and were characterized through internal transcribed spacer (ITS) sequencing and homology searches, exhibiting similarity with well-known postharvest pathogens such as *Botrytis*, *Fusarium*, *Geotrichum*, *Mucor*, *Penicillium*, *Alternaria*, and *Botryosphaeria*. Koch's postulates were confirmed for most pathogens by reinfecting berry fruit. SER3 showed good capacity to inhibit the growth of *Botrytis cinerea* and *Fusarium brachygibbosum* in strawberry fruit, affecting mycelial development. To gain better understanding of the genetic and metabolic capacities of the SER3 strain, its draft genome was determined and was found to comprise a single chromosome of 5.08 Mb, 52.8% G + C content, and 4,545 protein-coding genes. Phylogenetic analysis indicated that the SER3 strain is affiliated with the *R. badensis* species, with an average nucleotide identity >96% and a genome-to-genome distance >70%. A comparison of the genomic properties of *R. badensis* SER3 and other close bacterial relatives showed several genes with potential functions in biocontrol activities, such as those encoding siderophores, non-ribosomal peptide synthetases, and polyketide synthases. This is the first study to demonstrate a novel role of the recently discovered *R. badensis* species (and any other species of the genus *Rouxiella*) as a biocontrol agent against postharvest fungal pathogens.

Keywords: genomic analysis, sustainable agriculture, fungal antagonism, postharvest disease, volatile organic compound

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INTRODUCTION

The demand for food is increasing worldwide, resulting in the requirement to produce it under eco-friendly systems to ensure food security (Allen and Prosperi, 2016). However, constant attack by fungal and oomycete phytopathogens reduces the yield and quality of crops, causing huge losses at different stages of the agricultural cycle (Dean et al., 2012; Kamoun et al., 2015). For example, *Botrytis cinerea* has been reported to infect more than 200 plant species and cause losses of more than €1 billion/annum globally (Romanazzi and Feliziani, 2014). Similarly, several species of the genus *Fusarium*, together with *Botrytis*, are among the top 10 pathogens worldwide that can cause serious yield losses in agriculture (Magan et al., 2010; Dean et al., 2012). Likewise, invasion by non-native species of phytopathogens owing to transportation and storage of vegetables and fruit is another factor that affects products postharvest (Fried et al., 2017).

Thus, an efficient alternative against crop infestation, which includes the use of antagonistic biological agents, has been developed to eliminate or reduce the use of pesticides in agriculture (Compant et al., 2005; Backer et al., 2018). One of the advantages of biological agents such as *Trichoderma* or bacteria is that they are safe and environment friendly (Elad, 2000; Wang et al., 2020). This group of beneficial microorganisms associated with plants has emerged as a viable, economical, and efficient alternative to control various pre- and postharvest diseases (Morales-Cedeño et al., 2021). Even antagonism in plant growth-promoting bacteria (PGPB) toward phytopathogens is considered an indirect mechanism to stimulate plant growth (Glick, 2012). Their mechanisms of antifungal action against fungal pathogens include the production of diffusible compounds [e.g., hydrolytic enzymes, siderophores, lipopeptides, phenazines, 1-aminocyclopropane-1-carboxylate (ACC) deaminase] or antibiotics and volatiles compounds (e.g., dimethyl disulfide, hydrogen cyanide, and others) (Hernández-León et al., 2015; Khan et al., 2018; Rojas-Solís et al., 2018). Multiple species of PGPB have been isolated and characterized based on their antagonism toward phytopathogens, including *Pseudomonas* spp. and *Bacillus* spp., among few other genera that are predominantly reported (Höfte and Altier, 2010; Santoyo et al., 2012; Islam et al., 2017). Thus, the search for new antagonistic bacterial species is essential to increase the possibility of developing new biofungicides for commercial application (Córdova-Albores et al., 2020).

In this study, we propose a novel ecological role for *Rouxiella badensis* strain SER3 as an antagonist of postharvest pathogens of berries. *R. badensis*, together with *Rouxiella silvae*, was recently proposed as a new bacterial species by Le Fléche-Matéos et al. (2017). Some genera phylogenetically close to *Rouxiella*, such as *Serratia* and *Rahnella*, have previously been described as antagonists and PGPB. For example, Koo and Cho (2009) isolated and characterized a strain of *Serratia* sp. SY5, which had the ability to stimulate the growth of maize seedlings under stressful conditions. In addition, Sun et al. (2020) observed that *Rahnella aquatilis* strain MEM40, isolated from the rhizosphere of a rice plant, showed plant growth promoter effects and antagonism against phytopathogens such as *Magnaporthe oryzae* and *F. graminearum*. The only report on the genus *Rouxiella* described its role as an inhibitor of human pathogenic bacterial growth, but this strain has not been fully characterized, and its taxonomic assignation remains at the genus level (Nam et al., 2020). The isolation of a possible *R. badensis* strain 70 (among other 43 isolated endophytic strains) as an antagonist of pathogenic bacteria and fungi has also been reported. However, the characterization of strain 70 was based only on a partial 16S rDNA sequence (1,023 bp); thus, elucidation of its taxonomic affiliation requires further analysis (Wang et al., 2019). Herein, we present the isolation and characterization of a novel ecological role of *R. badensis* as a biocontrol agent against 20 fungal phytopathogens of berries (*Fragaria* × *ananassa*, *Vaccinium* spp. var. Biloxi, *Rubus* subgenus *Eubatus*), also isolated and characterized in this study. Furthermore, the *R. badensis* SER3 genome was sequenced to support its taxonomic affiliation and mined for detecting biosynthetic gene clusters that could be involved in its biocontrol capabilities.

MATERIALS AND METHODS

Isolation and Characterization of Postharvest Fungal Pathogens

Endophytic fungal pathogens were isolated from berries, including strawberries (n = 33), blackberries (n = 36), and blueberries (n = 49), which were collected from commercial markets. Berry fruits were surface sterilized according to a previous study (Contreras et al., 2016). Briefly, berries were immersed in 70% ethanol for 30 s, then washed with sodium hypochlorite (NaOCl) solution (2.5% available Cl^-) for 5 min, and then rinsed with ethanol (70% v/v) for 30 s. Finally, the fruits were washed five times with sterile distilled water. Aliquots of sterile distilled water used in the final rinse were cultured on plates containing nutrient agar (NA) medium (Merck). The plates were examined for bacterial growth after incubation at 28°C for 4 days. The sterilized fruits were used in decaying experiments to isolate potentially endophytic fungal pathogens. Briefly, groups of 5–10 fruits (strawberries, blackberries, and blueberries) were placed in disinfected containers, closed, and kept in the dark at room temperature. Fruit weight and firmness were measured on days 1, 5, and 10 (until the growth of fungal mycelium was detected) with an analytical balance (Benchmark Scientific, Inc., Sayreville, NJ, United States) and a penetrometer (Model GY-1, Hangzhou Scientific Instruments), respectively. Koch's postulates of fungal endophytes were confirmed for most of the pathogens (except *Trichoderma*) as follows: berry fruits were sterilized as described above, placed inside sterile glass bottles, and inoculated with the spores obtained from each fungal culture (~1×10^5 spores/ml). Mycelial growth of fungi on the fruit was visualized after 5–10 days. Further characterization was performed to confirm fungal identity.

Genomic DNA was extracted from fungal isolates as per the protocol by Mahuku (2004), followed by polymerase chain reaction analysis to amplify the intergenic spacer (ITS) regions with the following primers: ITS4 (5′-TCCTCCGCTTATTGATATGC-3′)

and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) (Hernández-León et al., 2015). The amplified ITS regions of each of the 20 fungal isolates were sequenced at Macrogen, Seoul, South Korea. Sequences and most probable taxonomic affiliation were deposited in GenBank, and the accession numbers are shown in **Table 1**.

Isolation of SER3 and Confrontation Bioassays

Strain SER3 was isolated from the phyllosphere of strawberry fruit and selected for antagonism against the fungal pathogen *F. brachygibbosum* in a prescreening assay in dual culture (**Supplementary Figure 1**). The strain was grown at 30°C for 24 h on NA medium and maintained at 4°C.

Fungal antagonism by strain SER3 was evaluated as previously reported for Petri dish-based bioassays (Hernández-León et al., 2015). Briefly, SER3 was streaked onto potato dextrose agar (PDA) plates in a cross shape, and then, four mycelial plugs (6-mm diameter) from each of the 20 fungal isolates were deposited in the center of each quadrant. The plates were incubated in the dark at 30°C, and the mycelial growth diameter was measured on day 6.

The antifungal effects of the volatile compounds produced by strain SER3 were also evaluated in Petri plates. SER3 [100 μl, at $\sim 1 \times 10^6$ colony forming units (CFU)] was inoculated on one side of the divided Petri plates, and in the other sections, mycelial plugs of each studied fungus (6-mm diameter) were inoculated. The inoculated plates were incubated, and mycelial growth was measured as described above. Both experiments were independently performed in triplicate, and the inhibition percentage was calculated using the following formula:% growth inhibition = $[(Ac - Ab)/Ac] \times 100$, where Ac is the control mycelial area, and Ab is the mycelial area under treatment.

TABLE 1 | Fungal strains isolated from strawberries, blackberries and blueberries, with the closest identity based on the ITS sequence homology searches.

Strain	Closest Genbank species identity	Identity (%)	Access number	Source of isolation
62BCV	*Botrytis cinerea*	99.8	MN365049.1	Strawberries
62C	*Botrytis* sp.	99.4	MN365050.1	
4BF	*Fusarium brachygibbosum*	99.2	MN365015.1	
HBF	*Fusarium brachygibbosum*	98.3	MN365017.1	
FRB	*Geotrichum candidum*	98.3	MN394447.1	
1BF	*Mucor circinelloides*	99.1	MK880497.1	
22	*Mucor fragilis*	99	MN365051.1	
FRA	*Mucor fragilis*	99	MN364941.1	
1F	*Penicillium crustosum*	96.8	MN080331.1	
230	*Penicillium expansum*	99.6	MN393696.1	
5F	*Penicillium expansum*	99.6	MN080332.1	
4AF	*Trichoderma* sp.	98.8	MN365013.1	
2Z	*Alternaria alternata*	99	MN397936.1	Blackberries
7Z	*Geotrichum phurueaensis*	98	MN397937.1	
1A	*Alternaria alternata*	99.6	MK881030.1	Blueberries
3A	*Alternaria* sp.	99.4	MN393668.1	
4A	*Alternaria alternata*	96.2	MN410562.1	
6A	*Alternaria alternata*	97.3	MN365025.1	
5A	*Botryosphaeria rhodina*	99.4	MN364705.1	
1BOA	*Cladosporium* sp.	98.8	MN364646.1	

Fungal Growth Inhibition Bioassay on Strawberry Fruit and Microscopy Visualization

The strawberries were washed with running water and subsequently placed in a container with 70% ethanol for 1 min. The ethanol was decanted, and then, the berries were washed with 2.5% sodium hypochlorite for 1 min. This process was repeated three times, and finally, the strawberries were rinsed thrice with sterile deionized water.

Following the above procedure, strawberries were allowed to dry in a laminar flow hood, and an incision of approximately 3 mm length, width, and depth was made on each fruit with the tip of a sterile scalpel. Four treatments were performed, using the following: (i) sterile distilled water as the negative control; (ii) a mycelium plug, 7 mm in diameter, of the phytopathogen *B. cinerea* 62BCV or *F. brachygibbosum* 4BF as a positive control; and (iii) a bacterial suspension of SER3 (100 μl, $\sim 1 \times 10^6$ CFU) and a mycelium plug, 7 mm in diameter, of each phytopathogen; and (iv) the supernatants (100 μl) of strain SER3 obtained from nutrient broth after an overnight culture and the mycelium of the two studied phytopathogens grown for 24 h at 29°C. After treatment, the strawberries were placed in closed sterile plastic containers and maintained at room temperature for 3 days.

For microscopy visualization, strain SER3 was simultaneously striated with phytopathogenic fungi (*B. cinerea* 62BCV or *F. brachygibbosum* 4BF) in separate Petri dishes containing PDA. The bacteria were streaked on the cross-shaped dishes, and a 7-mm portion of the mycelium was deposited in the center of each quadrant, as previously mentioned. Subsequently, a mycelium sample was stained with lactophenol blue and safranin and visualized under a Velab VE-BC3 Plus optical microscope.

SER3 Genome Sequencing and Analysis

A single colony of strain SER3 was picked from a streaked NA plate (BD Bioxon), which was maintained at 30°C overnight. SER3 genomic DNA was extracted following standard protocols (Mahuku, 2004) and further purified using a Wizard® Genomic DNA Purification Kit (Promega, Fitchburg, WI, United States). The quality and quantity of the extracted DNA were evaluated with agarose gel electrophoresis and using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, United States), respectively. Genomic DNA from SER3 was sequenced commercially (MR DNA, Shallowater, TX, United States) by using the Illumina HiSeq technologies platform (2 × 300 bp). FastQC analysis, version 0.11.5, of the raw reads was employed to perform quality control (Andrews, 2010). Trimmomatic, version 0.32, was used to remove bases of low quality and adapter sequences (Bolger et al., 2014). Genome assembly was performed with contigs obtained through the PATRIC[1] genome

[1] https://www.patricbrc.org/

service and SPAdes assembler version 3.10.0 (Bankevich et al., 2012). The draft genome of SER3 was reordered according to the reference genome of *Rahnella aquatilis* KM12 (NCBI project accession number: ASM395610v2). PLACNETw was used to explore the presence of plasmids in the SER3 genome (Vielva et al., 2017).

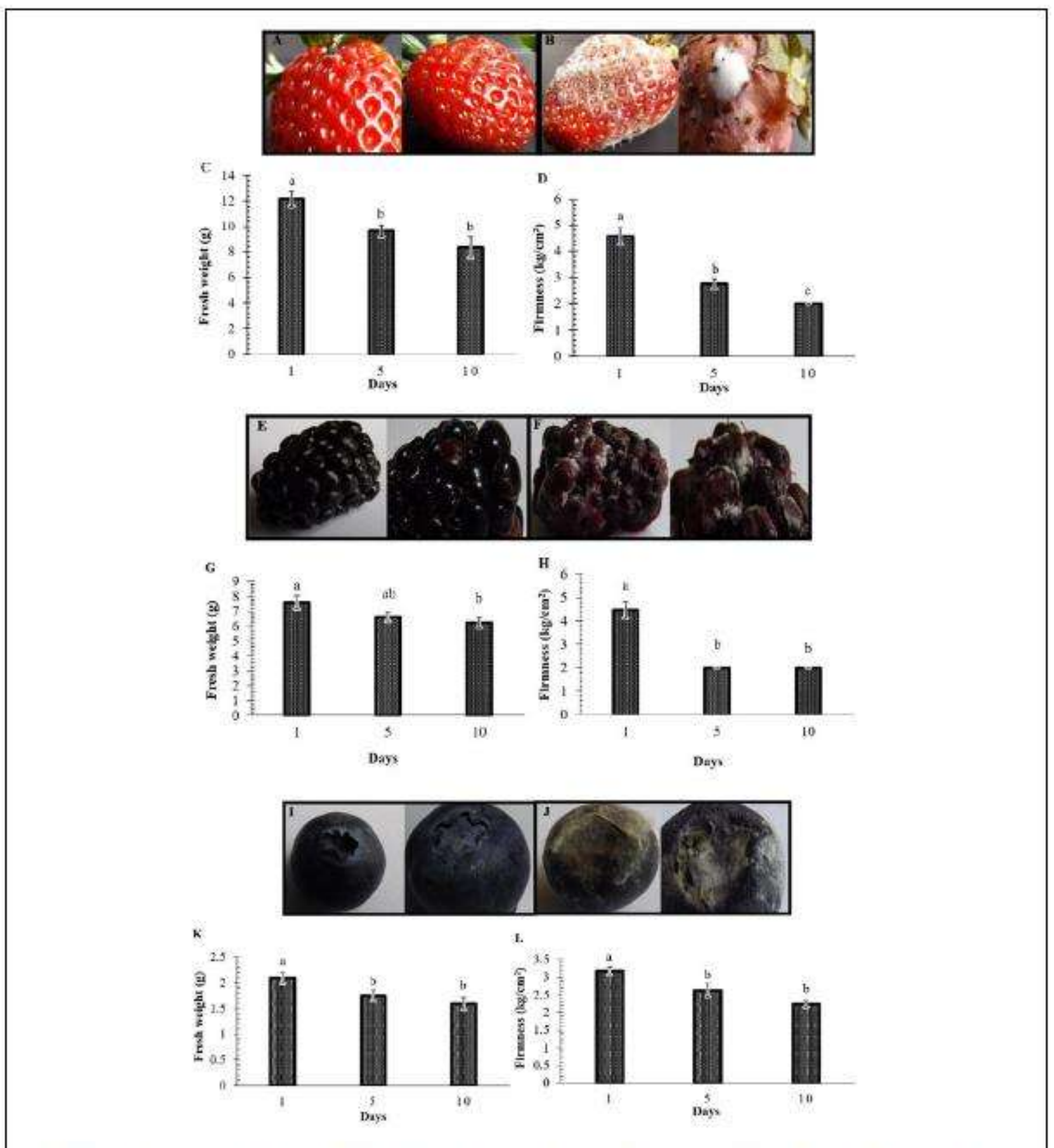

FIGURE 1 | Postharvest decay in berries represented by loss of weight and firmness. Representative fruit are shown in Panels **(A,B,E,F,I,J)**. **(A,E,I)** Fruit at the beginning of the study (control). **(B,F,J)** Fruit in decay on days 5 and 10. Panels **(C,G,K)** show the decrease in the weight of strawberries, blackberries, and blueberries, respectively. Panels **(D,H,L)** show the decline in firmness. Bars represent means ± standard deviation ($n = 12$). The letters indicate that the means differ significantly according to Duncan's multiple range test ($p < 0.05$).

Taxonomic Affiliation of Strain SER3

The 16S rRNA gene sequence was obtained from the genome and used in basic local alignment search tool (BLAST) homology searches to assign the possible taxonomic affiliation of strain SER3. After that, a genome-level approach was adopted, employing average nucleotide identity (ANI) > 95–96% (Yoon et al., 2017) and a genome-to-genome distance calculator (GGDC) > 70% (Meier-Kolthoff et al., 2013). This genome-level approach was based on strains having cutoff values for species delimitation established for the 16S rRNA gene (>98.7%) (Chun et al., 2018).

Phylogenomic Analysis of SER3

Phylogenomic relationships of *R. badensis* SER3 and the bacterial strains with high similarity according to ANI and GGDC values were analyzed using the REALPHY pipeline (Bertels et al., 2014). The neighbor-joining method was used for tree construction, and the nucleotide distance was measured using the Jukes–Cantor model. Furthermore, bootstrap analysis with 1,000 replications was performed.

Genome Annotation and Mining for Plant Growth-Promoting and Biocontrol Traits

The assembled genome was annotated using the Rapid Annotation of the Subsystem Technology (RAST) server[2]. Genome mining was performed by biosynthetic gene cluster (BGC) prediction using antiSMASH 4.0 (Blin et al., 2017) for *R. badensis* SER3 and other close bacterial genomes and manually

[2]http://rast.theseed.org/FIG/rast.cgi

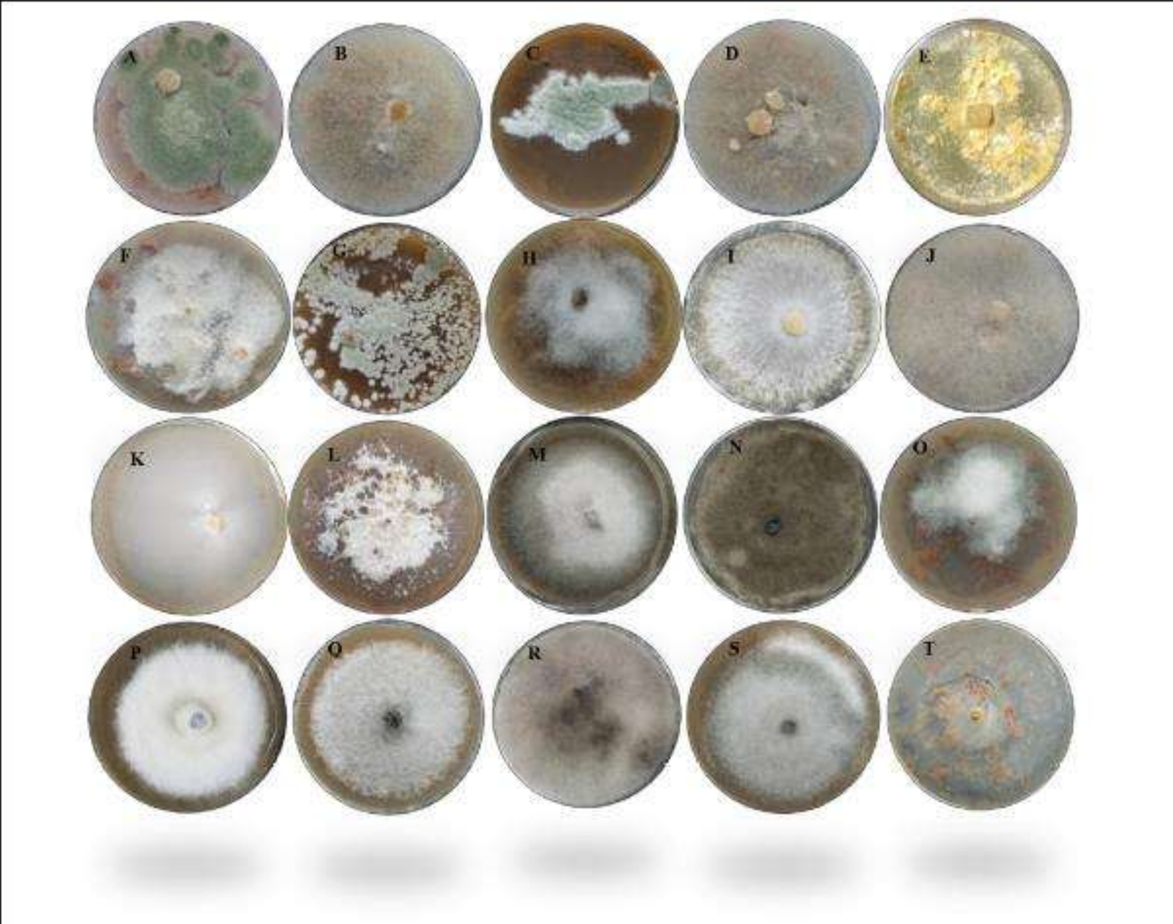

FIGURE 2 | Morphological appearance of the 20 fungal strains isolated from fruit berries. **(A)** *Penicillium crustosum* 1F. **(B)** *Mucor circinelloides* 1BF. **(C)** *Penicillium expansum* 230. **(D)** *Mucor fragilis* 22. **(E)** *Trichoderma* sp. 4AF. **(F)** *Fusarium brachygibbosum* 4BF. **(G)** *Penicillium expansum* 5F. **(H)** *Botrytis* sp. 62C. **(I)** *Botrytis cinerea* 62BCV. **(J)** *Mucor fragilis* FRA. **(K)** *Geotrichum candidum* FRB. **(L)** *Fusarium brachygibbosum* HBF. **(M)** *Alternaria alternata* 1A. **(N)** *Cladosporium* sp. 1BOA. **(O)** *Alternaria alternata* 2Z. **(P)** *Alternaria* sp. 3A. **(Q)** *Alternaria alternata* 4A. **(R)** *Botryosphaeria rhodina* 5A. **(S)** *Alternaria alternata* 6A. **(T)** *Geotrichum phurueaensis* 7Z. The fungal growth was observed at different times, ranging from 5 to 8 days.

inspected from the annotations generated by the RAST server[3] (Aziz et al., 2008), specifically the RASTtk pipeline.

RESULTS

Isolation and Characterization of Postharvest Phytopathogens

In this study, the decay of berries over time showed a reduction in fresh weight and fruit firmness between days 5 and 10, consistent with the appearance of decaying symptoms caused by fungal pathogens (**Figure 1**). Following the decay, 20 berry fungi were isolated. **Figure 2** shows the morphological appearance of the isolated fungal strains. Sequencing of the ITS from the isolated fungi showed high homology with *B. cinerea*, *Botrytis* sp., *F. brachygibbosum*, *Geotrichum candidum*, *Geotrichum phurueaesis*, *Mucor circinelloides*, *Mucor fragilis*, *Penicillium crustosum*, *Penicillium expansum*, *Trichoderma* sp., *Alternaria alternata*, *Alternaria* sp., *Botryosphaeria rhodina*, and *Cladosporium* sp. (**Table 1**). To determine the infection rates of fungi, including those not reported as the main postharvest phytopathogens of berries, Koch's postulates were confirmed, thus corroborating their role in postharvest fungal infections (**Supplementary Figure 2**).

[3]http://rast.nmpdr.org

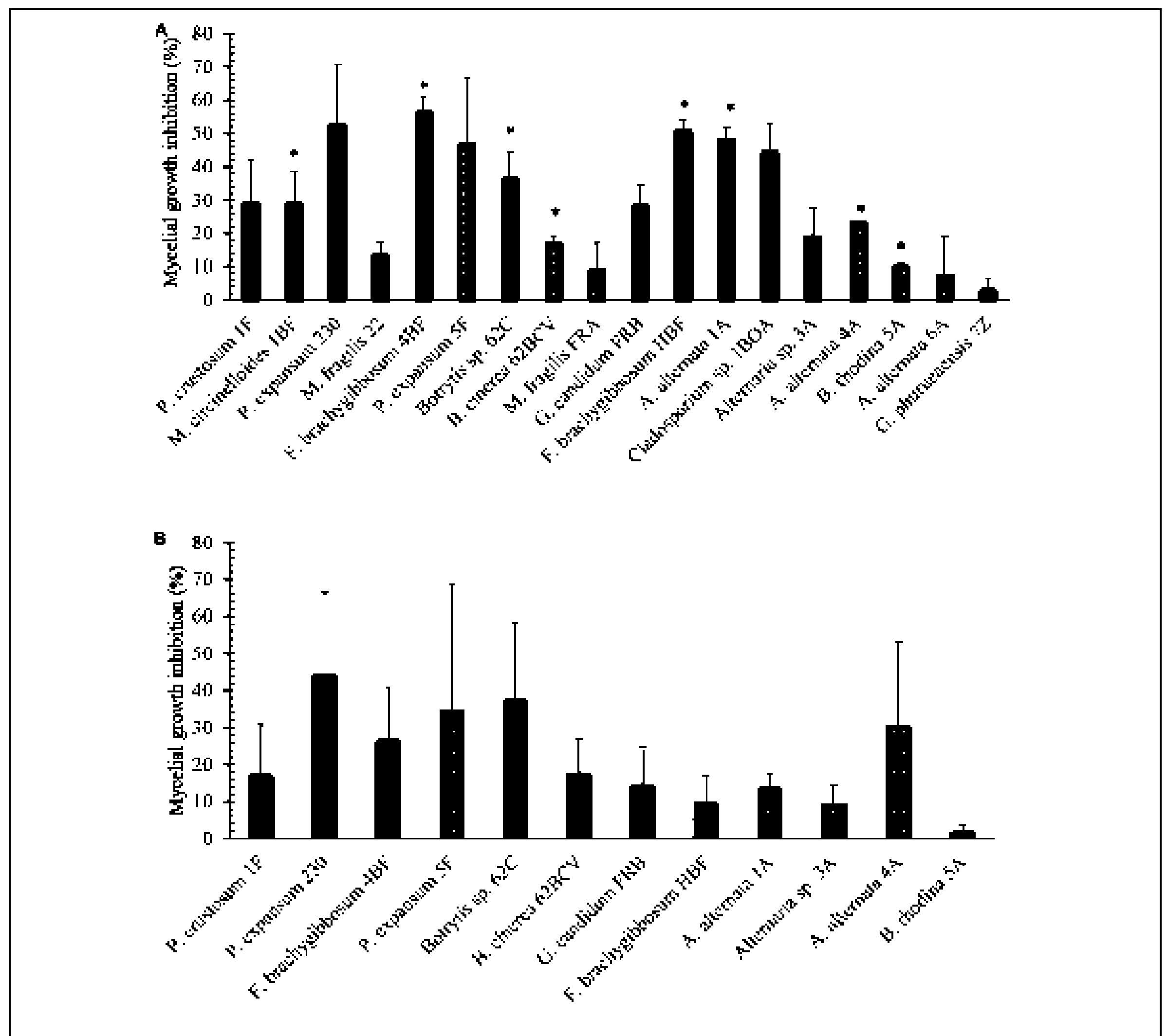

FIGURE 3 | Percentages of inhibition of fungi isolated from berries postharvest caused by **(A)** diffusible and **(B)** volatile compounds of the SER3 strain. The experiments were carried out independently three times. Bars represent mean ± SE. Asterisks indicate significant difference compared to the respective controls, using Student's *t*-test ($p < 0.05$).

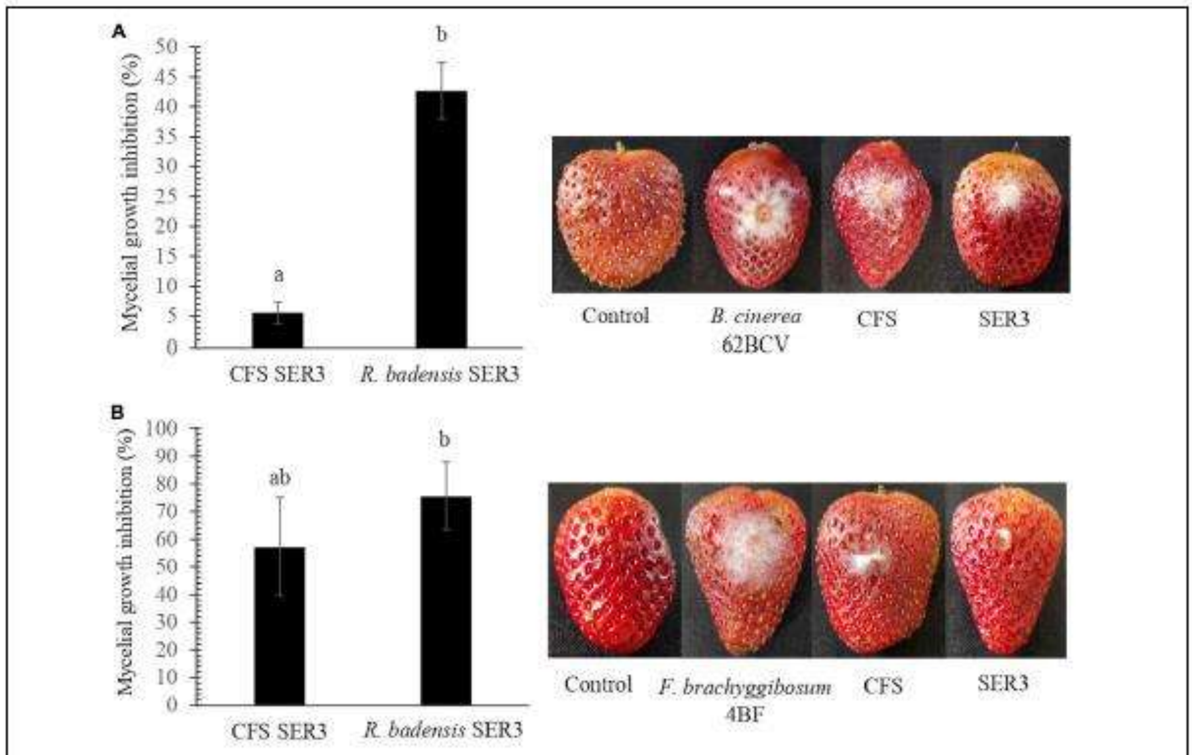

FIGURE 4 | Biocontrol effects of SER3 and its supernatant on strawberries. **(A)** Mycelial growth inhibition of *Botrytis cinerea* by the cell-free supernatant (CFS) and SER3. **(B)** Mycelial growth inhibition of *Fusarium brachyggibosum* by the CFS and SER3. Treatments consisted of sterile distilled water (control) and inoculation with pathogens, CFS from SER3, and cell suspensions of SER3. Experiments were independently performed three times, and bars represent mean ± SE ($n = 9$). Letters indicate significant difference, based on Duncan's multiple range test ($p < 0.05$).

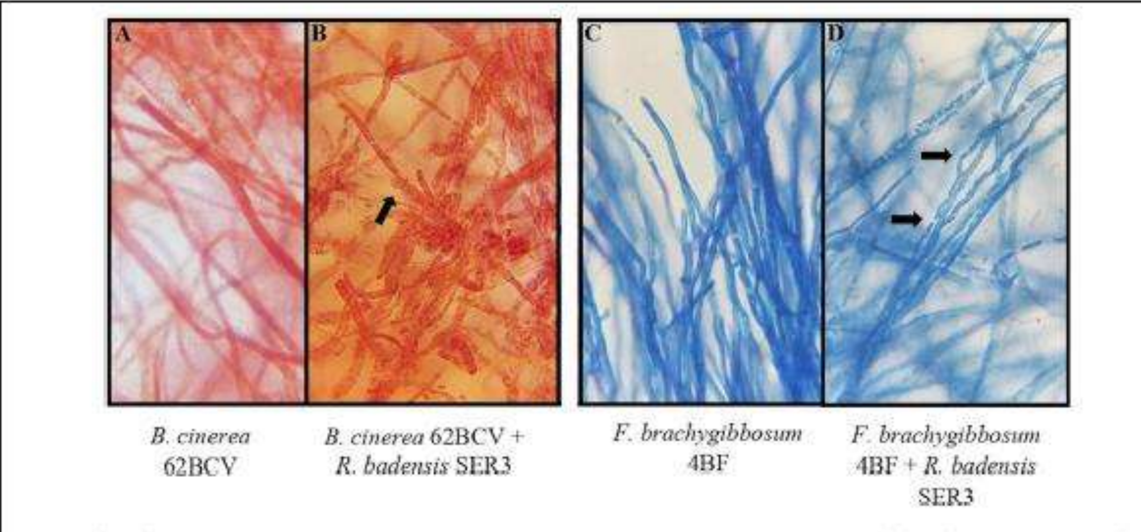

FIGURE 5 | Effect of SER3 on mycelial morphology of **(A,B)** *B. cinerea* and **(C,D)** *F. brachyggibosum*. Panels **(A,C)** represent the controls, Panels **(B,D)** show the interaction between the bacterial strain and each of the pathogens. Arrows indicate distortion of hyphae (× 100 magnification).

Confrontation Assays

Once the growth and infection capacity of fungal phytopathogens was confirmed in strawberry and blueberry fruit, confrontation tests were performed using strain SER3. SER3 remarkably inhibited mycelial growth through the action of diffusible compounds against eight phytopathogens, such as *Alternaria alternata*, *Botryosphaeria rhodina*, *Mucor circinelloides*, *Botrytis* spp., *and Fusarium* spp. (**Figure 3A**). Although an inhibitory trend was observed in the growth of some phytopathogens by the action of volatile compounds from SER3, results showed that the inhibition was not significant (**Figure 3B**).

In vivo Phytopathogen Inhibition Assay Using Strain SER3

To evaluate the potential antagonism of strain SER3 against phytopathogens on fruit, two important postharvest phytopathogens (*B. cinerea* 62BCV and *Fusarium brachygibosum* 4BF) were selected. **Figure 4** shows that SER3 produced significant mycelium growth inhibition of *B. cinerea* 62BCV through direct interaction (42.66%), while the cell-free supernatant (CFS) inhibited only 5.55% of phytopathogen growth. With *F. brachygibosum* 4BF, mycelial growth was inhibited by 75.68%, while CFS restricted mycelial growth by 57.37%. Microscopic analysis of the mycelia of each fungal phytopathogen showed deformations and protrusions in their hyphae on application of the bacterial strain or the cell-free supernatant, whereas typical hyphae were observed in the control in the absence of strain SER3 or its CFS (**Figure 5**).

Genome Features of Strain SER3

To gain better understanding of the potential traits of strain SER3 involved in postharvest phytopathogen biocontrol, its genome was sequenced. The SER3 genome consisted of 47 contigs, and the quality of the assembly was evaluated with Quast[4], with approximately 5.08 Mb, a GC content of 52.8%, and 4,545 open reading frames, among other genes that code for ribosomal genes (**Table 2** and **Figure 6**). Similar numbers are also found in other *Rouxiella* genomes. The genome sequences were deposited in GenBank under the following accession numbers: NZ_CP049603.1; BioProject, PRJNA224116; and BioSample, SAMN14066751.

[4]http://quast.sourceforge.net/quast

TABLE 2 | Genome characteristics of strain SER3.

Size (Mb)	5.08
GC%	52.8%
Protein	4,545
rRNAs	4
tRNAs	61
Other RNA	6
Gene	4,684
Pseudogene	69
Scaffolds	1
Contigs	47
N50	255,898
L50	8

Taxonomic Affiliation of SER3

Based on the sequences of the 16S rRNA gene, SER3 showed 100% identity with the type strains of *R. badensis* DSM 100043^T (**Supplementary Figure 3**). A phylogenomic approach confirmed the close relationship with the *R. badensis* DSM 100043 type strain (**Figure 7**). Moreover, a comparison at the genomic level of strain SER3 through ANI > 95–96% and the GGDC > 70% also showed that it is strongly affiliated with *R. badensis* (**Table 3**).

Search for Biocontrol Gene Clusters in SER3 and Related Genomes

The antiSMASH program was used to determine the potential compounds involved in the postharvest biocontrol of phytopathogens by *R. badensis* SER3 and other closely related species, including two *R. badensis* strains (DSM 100043 and WG36), *Rhanella* spp., and *Serratia* spp. In the SER3 genome, biosynthetic gene clusters involved in siderophores (100%) and polyenes (77%) were observed. In addition, compounds such as thiopeptides, non-ribosomal peptide synthetases (NRPS), and polyketide synthases (PKS) were identified (**Table 4**). Similar biosynthetic clusters and percentages were observed in the other two *R. badensis* genomes analyzed, corroborating their close phylogenomic similarity. A 100% similarity was observed for siderophore biosynthetic clusters in *Rhanella* spp. and *Ewingella americana* CCUG 14506, and in *Obesumbacterium proteus* DSM 2777 and *Hafnia* spp. with a good similarity score (75%) in their respective genomes.

DISCUSSION

Berries (strawberries, blackberries, and blueberries) have a very short shelf life after harvest. Therefore, they must be immediately distributed for use, preferably under cold chain, which considerably hinders their international commercialization. High postharvest fruit losses due to phytopathogenic fungal diseases are related to high humidity levels, increased nutrients, low pH values, and low intrinsic resistance to postharvest decomposition and fungal diseases (Dukare et al., 2019). During the decomposition process, the fruit loses weight and decreases in firmness and quality, resulting in economic losses. In many instances, synthetic chemical compounds are used as coatings to avoid pathogen infections and extend their shelf life; however, toxic residues can be hazardous to human health, in addition to restricting the global commercialization of berries. Therefore, it is important to describe the phytopathogens that affect postharvest berries as well as to develop sustainable alternatives for their biological control (Abeer et al., 2013).

Here, 20 fungal strains were isolated from berries and characterized by ITS sequencing and homology searches. They showed similarity to *Botrytis* or *Fusarium*, among others. Previous reports have shown that several of these genera are phytopathogens that cause pre- and postharvest diseases in various crops, including strawberries (Dukare et al., 2019).

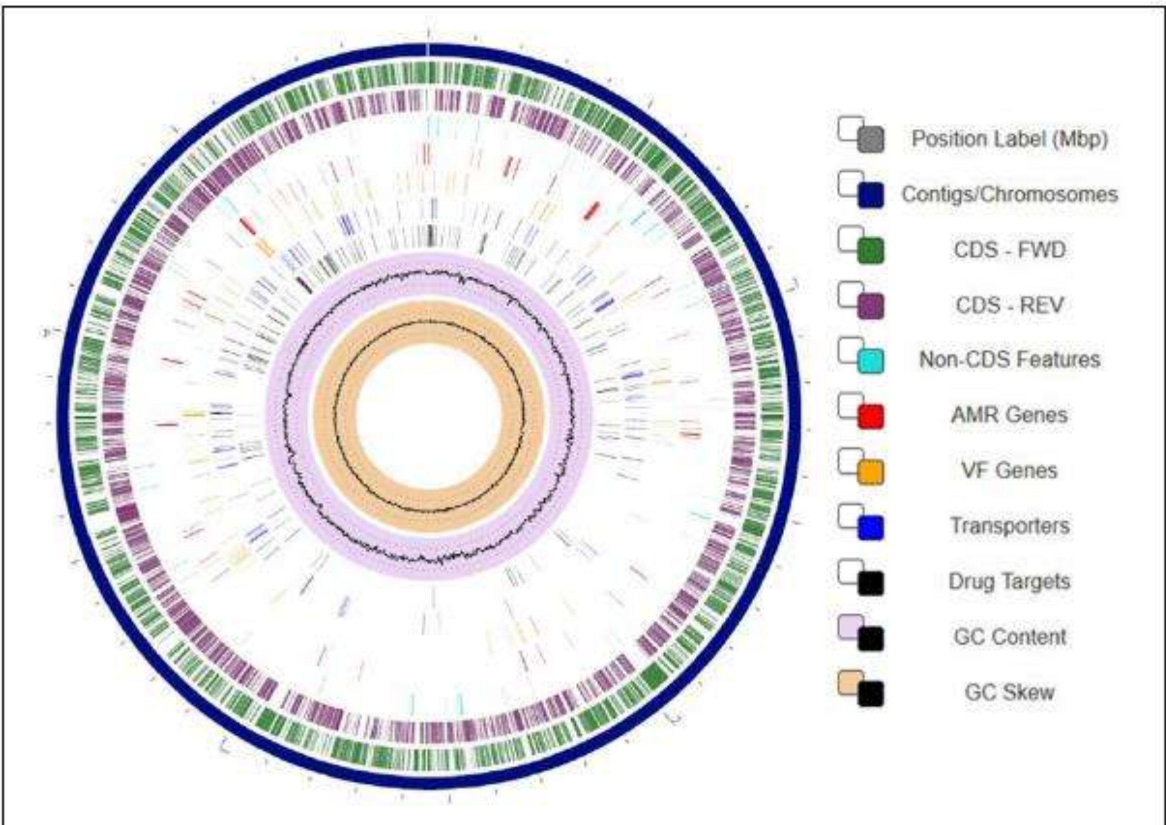

FIGURE 6 | Circular visualization of the *Rouxiella badensis* strain SER3 genome. Numbers represent megabases (Mbp). From outside to the center: 47 contigs were assembled to form a scaffold representing the chromosome (navy blue track). Genes on forward strand (green), genes on reverse strand (purple), non-CDS features (turquoise), resistance genes (red), virulence factor genes (orange), transporters (blue), drug targets (black), GC content (lilac and black), and GC skew (khaki and black). To display the circular viewer, we used genome annotation service from PATRIC (RAST tool kit).

In particular, *B. cinerea* can easily infect berries such as strawberry, blueberry, blackberry, raspberry, cranberry, and bilberry fruit, causing drastic losses after harvest (Leroux et al., 2002; Romanazzi and Feliziani, 2014; Petrasch et al., 2019). Another type of ascomycete fungus that causes damage to various crops is *Fusarium*, which is best known for affecting the roots and some aerial parts of plants, such as stems, and causes vascular browning, leaf epinasty, stunting, progressive wilting, defoliation, floral damage, and subsequent plant death (Dean et al., 2012). Herein, two strains with highly similar identity (98.3 and 99.2%) to *F. brachygibbosum* were isolated from strawberry fruit (**Table 1**). To our knowledge, *F. brachygibbosum* has not been reported as a fruit phytopathogen; therefore, this would be the first report as a postharvest pathogen in fruit such as strawberries. Further studies on the morphology of *F. brachygibbosum* and analysis of other molecular markers are being conducted by our research group to corroborate this hypothesis.

Other fungal genera found in berries were *Alternaria*, *Cladosporium*, *Geotrichum*, *Mucor*, and *Penicillium*, which have already been reported as causative agents of postharvest disease in these fruit (Koike et al., 2003; Tournas and Katsoudas, 2005; Gordon et al., 2016; López et al., 2016; Pastrana et al., 2017; Petrasch et al., 2019). It should be noted that fungi belonging to beneficial species such as *Trichoderma* have also been found in berries (Santoyo et al., 2021). In the present work, the strain *Trichoderma* sp. AF4 did not produce any apparent damage when reinoculated in strawberries and showed similar results as the uninoculated controls. Moreover, preliminary studies performed in our laboratory suggest that AF4 restricts the growth of some postharvest berry pathogens.

In agreement with the aforementioned studies, the fruit microbiome has been reported to contain not only pathogenic species but also microorganisms that can naturally help fight postharvest diseases, thus reducing losses through increased shelf life and fruit quality (Droby and Wisniewski, 2018). Consequently, we isolated the SER3 strain from the surface of a strawberry fruit, and it showed antifungal activity against *Fusarium* (**Supplementary Figure 1**). Furthermore, during activity evaluation, SER3 exhibited significant antagonism against the postharvest pathogens isolated herein. Moreover, the volatile

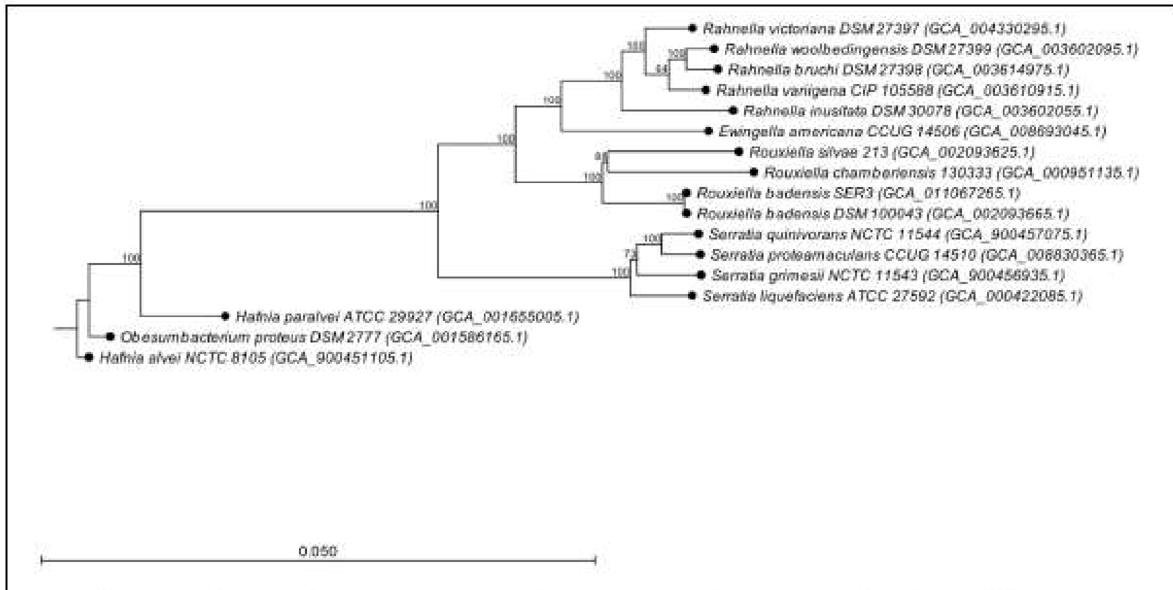

FIGURE 7 | Phylogenomic tree based on the whole genome sequence data of *Rouxiella badensis* strain SER3, including the relationship with other bacterial species. The phylogenetic tree was constructed using the neighbor-joining algorithm. Bootstrap analysis of 1,000 replications was performed and is expressed as a percentage. *Hafnia alvei* NCTC 8,105 was used as an outgroup.

compounds of SER3 also exhibited inhibition of mycelial growth, although to a lesser extent, with significant inhibition being observed only against two species, viz., *P. expansum* and *F. brachygibbosum*. These results suggested that SER3 antagonizes the phytopathogens through the action of diffusible (mainly) and volatile compounds, which is consistent with other studies showing similar mechanisms of action in other bacterial species (Hernández-León et al., 2015; Wallace et al., 2017). The inhibition of mycelial growth of postharvest phytopathogens was corroborated by *in vivo* tests on strawberry fruit using *B. cinerea* and *F. brachygibbosum*. Following the coinoculation of the SER3 strain and *B. cinerea* or *F. brachygibbosum*, the hyphae presented deformations and protrusions on the surface. This type of damage in the fungal pathogen hyphae has been observed in other studies and is associated with a reduction in fungal pathogenicity (Wallace et al., 2017; Emanuel et al., 2020).

TABLE 3 | OGRIs values obtained from the genome comparison of strain SER3 and closely related species.

Species/Strain	16S ≥98.7%	ANI ≥96%	GGDC ≥70%
Rouxiella badensis DSM 100043^T	100	99.69	98.20
Rahnella variigena CIP 105588^T	99.51	76.41	21.10
Obesumbacterium proteus DSM 2777^T	99.31	73.14	21.30
Hafnia paralvei ATCC 29927^T	99.31	72.56	20.40
Rouxiella chamberiensis 130333^T	99.31	80.56	23.80
Rahnella bruchi DSM 27398^T	99.31	76.23	20.90
Rahnella woolbedingensis DSM 27399^T	99.31	76.28	21.00
Rahnella inusitata DSM 30078^T	99.21	76.47	20.80
Rouxiella silvae 213^T	99.21	80.88	24.00
Serratia liquefaciens ATCC 27592^T	99.12	75.04	20.60
Serratia grimesii NBRC 13537^T	99.12	73.94	20.40
Ewingella americana ATCC 33852^T	99.12	76.81	21.30
Serratia proteamaculans CCUG 14510^T	99.12	74.66	20.20
Serratia quinivorans NCTC 11544^T	99.03	74.81	20.30
Rahnella victoriana DSM 27397^T	98.98	76.56	21.10
Hafnia alvei ATCC 13337^T	98.72	72.74	21.10

Given the relevant biocontrol properties of strain SER3, its genome was sequenced, and its taxonomic affiliation was assigned based on ANI and GGDC. Based on these parameters, SER3 was established to belong to the *R. badensis* species. *R. badensis* is a relatively new species described in 2017; it is a Gram-negative bacillus that forms whitish colonies, can grow optimally at 37°C, reduces nitrates, and produces acid from different sugars (Le Flèche-Matéos et al., 2017). To investigate the possible antifungal mechanism of *R. badensis* SER3, its genome was analyzed using the antiSMASH server (Blin et al., 2017), which led to the prediction of various antibiotic compounds and antifungal compounds such as siderophores, NRPS, and PKS. These three compounds are extracellular and are produced by a wide range of biocontrol bacterial species, such as *Bacillus* and *Pseudomonas*, and close relatives of *R. badensis*, such as *Rahnella aquatilis*, which have been characterized as antifungals (Calvo et al., 2007; Chen et al., 2007; Santoyo et al., 2012; Carmona-Hernandez et al., 2019). Likewise, NRPS and PKS are not exclusive to bacterial strains but can also be synthesized by phytopathogenic and beneficial fungi, such as *Trichoderma* (Mukherjee et al., 2012). Other compounds reported to have

TABLE 4 | AntiSMASH analysis and prediction of biosynthetic compounds in *R. badensis* SER3 and related bacterial genomes.

Bacterial species/strain	NRPS	Siderophore	Thiopeptide	Arylpolyene	T1PKS	T3PKS	transAT-PKS	Hserlactone	Redox-cofactor	transAT-PKS-like	thioamitides	Nrps-like	Terpene	Betalactone	RRE-containing	Ladderane	RIPP-like	Pyrrolnitrin
Rouxiella badensis SER3	38%	100%	14%	77%	+	16%	40%	+	13%	–	–	–	–	–	–	–	–	–
Rouxiella badensis DSM 100043	38%	100%	14%	72%	+	16%	40%	–	13%	–	–	–	–	–	–	–	–	–
Rouxiella badensis WG36	23%	100%	+	77%	+	12%	+	–	13%	+	–	–	–	–	–	–	–	–
Rouxiella silvae 213	–	–	14%	77%	–	–	–	–	+	–	+	20%	–	–	–	–	–	–
Rouxiella silvae Leaf50	38%	–	14%	77%	–	–	–	+	+	–	+	–	–	–	–	–	–	–
Rouxiella chamberiensis 130333	38%	–	14%	–	–	–	40%	+	+	–	–	–	100%	–	–	–	–	–
Rahnella bruchi DSM 27398	+	100%	14%	77%	–	–	–	+	–	–	–	–	–	+	13%	+	–	–
Rahnella woolbedingensis DSM 27399	–	100%	14%	77%	–	–	–	–	–	–	–	–	–	+	+	–	–	–
Rahnella inusitata DSM 30078	38%	100%	14%	66%	–	–	–	–	–	–	–	–	–	+	–	–	–	–
Rahnella variigena CIP 105588	–	100%	14%	72%	–	–	–	+	–	–	–	–	–	+	13%	–	–	–
Rahnella Victoriana DSM 27397	–	100%	14%	77%	–	–	–	2%	–	–	–	–	–	–	13%	–	–	–
Ewingella americana CCUG 14506	–	100%	14%	77%	–	–	–	–	–	–	–	–	–	+	–	–	+	–
Serratia liquefaciens ATCC 27592	57%	+	14%	83%	–	–	–	–	–	–	–	+	–	+	–	–	–	–
Serratia proteamaculans CCUG 14510	57%	+	–	73%	–	–	–	+	–	–	–	–	75%	+	–	–	–	–
Serratia quinivorans NCTC 11544	57%	+	+	77%	–	–	–	+	–	–	–	80%	–	+	–	–	–	–
Serratia grimesii NCTC 11543	57%	+	14%	77%	+	–	–	–	–	–	–	–	–	+	–	–	+	100%
Obesumbacterium proteus DSM 2777	–	75%	14%	–	–	–	–	+	–	–	–	–	–	+	–	–	–	–
Hafnia paralvei ATCC 29927	–	75%	14%	–	–	–	–	6%	–	–	–	–	–	+	–	–	–	–
Hafnia alvei NCTC 8105	–	75%	14%	+	–	–	–	+	–	–	–	–	–	+	–	–	–	–

been found in the *R. badensis* SER3 genome were a cluster of desferrioxamine-type siderophores (100% similarity), which are iron-chelating compounds (Boiteau et al., 2019) and can restrict the growth of pathogens (Kloepper et al., 1980; de los Santos-Villalobos et al., 2012). They have been reported in a wide range of biocontrol and plant growth promoter species (Crowley, 2006; Wang et al., 2020). Interestingly, the same compounds, such as siderophores, NRPS, and arylpolyene compounds, with similar percentages of identity were detected using antiSMASH in two other *R. badesis* genomes. Similarly, close relatives of *R. badensis*, such as *Rahnella*, also presented good similarity percentages with clusters for the synthesis of siderophores, thiopeptides, and arylpolyenes in their respective genomes. Other bacterial species, including those belonging to genera such as *Ewingella* (Roy Chowdhury et al., 2007), *Obesumbacterium* (Amin et al., 2014), and *Hafnia*, contain highly similar clusters for the synthesis of potential compounds, such as siderophores (100%). These results support the proven role of SER3 in the biocontrol of fungal pathogens and similar roles reported by Calvo et al. (2007) and Chen et al. (2007) in *Rahnella* and *Serratia* genera. To our knowledge, the potential role in the biocontrol of plant fungal pathogens has not been described for the rest of the bacterial species analyzed here with antiSMASH (**Table 4**).

CONCLUSION

In this study, a functional analysis of the biocontrol activities of the novel strain SER3 against postharvest pathogenic fungi of berries was performed, which showed a high genomic and phylogenetic identity with *R. badensis*. Thus, we propose a new ecological role for this species and other species of the genus *Rouxiella*. Notably, SER3 genome provides some indications of the antifungal modes of action; however, other mechanisms of biocontrol by *R. badensis* SER3 cannot be excluded, since other antifungal activities, such as the activity of lytic enzymes, have not been explored. In addition, antiSMASH analysis for other species analyzed in this study provides some clues of possible antagonistic action toward plant pathogens, although this hypothesis requires further investigation. Lastly, isolation of SER3 presents a new option in the biocontrol of postharvest pathogens of berries and provides new opportunities to investigate its role as a promoter of plant growth through direct mechanisms.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, NZ_CP049603.1.

AUTHOR CONTRIBUTIONS

LRM-C conducted the experiments, analyzed the data, and prepared the figures and tables. SS-V and GS conceived and designed the experiments and analyzed the data. GS wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.709855/full#supplementary-material

Supplementary Figure 1 | Effect of diffusible compounds of SER3 following direct co-inoculation with the Fusarium pathogen. The bacterial strain was streaked onto plates in a cross shape, and mycelial plugs 4 mm in diameter were deposited at the center of the quadrants formed. Experiments were independently performed a minimum of three times. The plates were incubated, and mycelial growth was measured on day 3. The percentage of growth inhibition was measured as follows:% growth inhibition = [(Ac – Ab)/Ac] × 100, where Ac is the control mycelial area, and Ab is the mycelial area with treatment.

Supplementary Figure 2 | Koch's postulates. Berries were infected (*n* = 18) with spore solutions (1×10^5 spores/mL). Panel **(A)** shows blueberries inoculated with *Cladosporium* sp. 1BOA spores, while panel **(B)** shows strawberries inoculated with *Penicillium expansum* 230 spores, and panel **(C)** shows strawberries inoculated with *Mucor circinelloides* 1BF spores.

Supplementary Figure 3 | Phylogenetic tree based on the 16S ribosomal gene sequence of *Rouxiella badensis* strain SER3, including the relationship with other bacterial species (nucleotide sequence can be accessed in GenBank: CP049603). A phylogenetic tree was constructed using the maximum-likelihood algorithm. Bootstrap analysis of 1000 replications was performed and expressed as a percentage, and the most common enterobacteria were used as an outgroup.

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Resultados adicionales

Inhibición de *Fusarium brachygibbosum* y producción de sideróforos en medio cromo-azurol (CAS)

Detectamos producción de sideróforos en ensayos de confrontación de la cepa SER3 con el patógeno *Fusarium brachygibbosum* en medio cromo-azurol (CAS). Tanto la bacteria como el patógeno producen sideróforos (figura 7). Al inocular la bacteria y el hongo al mismo tiempo observamos que no hay diferencias significativas en el crecimiento del hongo ni en el tamaño del halo de sideróforos que éste produce. Sin embargo, al inocular la bacteria 24 horas antes que el hongo, el crecimiento de éste se reduce un 18% y el halo de sideróforos producido por este patógeno también disminuye su tamaño, mientras que el halo de la bacteria es ligeramente mayor comparado que cuando se inoculan al mismo tiempo. Cuando la cepa SER3 se inocula 48 horas antes que Fusarium su crecimiento se muestra inhibido en un 28%. El halo de sideróforos producido por este hongo es significativamente menor comparado con el control y con los tratamientos donde se inocularon al bacteria y hongo al mismo tiempo y la bacteria 24h antes que el hongo. También observamos que, si la bacteria se inocula 72h antes que el patógeno el porcentaje de inhibición es del 31%, el halo de sideróforos por parte del patógeno se mantiene aproximadamente del mismo tamaño comparado con el tratamiento donde la bacteria se inocula 48h antes que el patógeno. El halo de sideróforos producido por la bacteria es de mayor tamaño comparado con los tratamientos donde se inocularon bacteria y hongo al mismo tiempo y la bacteria 24 y 48 horas antes que el patógeno.

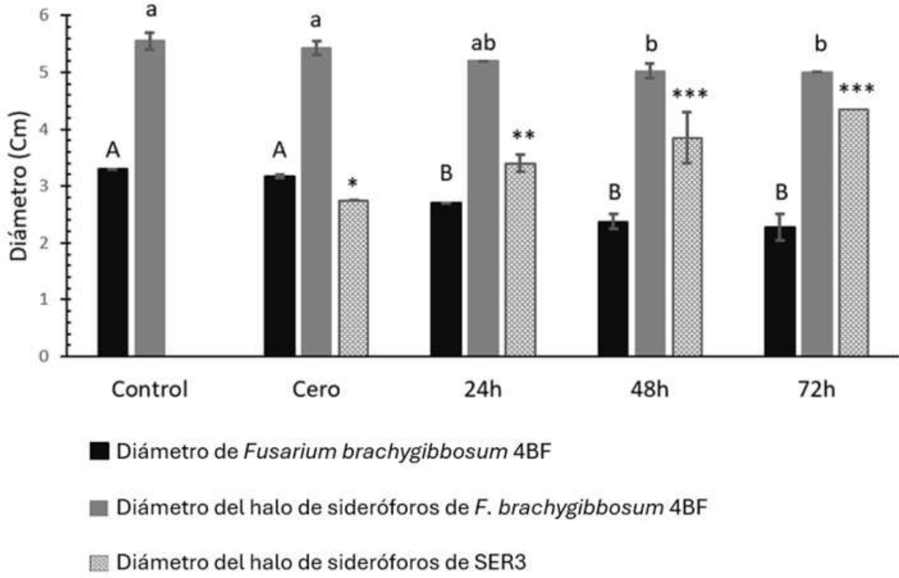

Figura 7: Ensayos de confrontación entre *Rouxiella badensis* SER3 y *Fusarium brachygibbosum* en medio CAS. Las horas representan los tiempos de inoculación de la bacteria antes de la inoculación del patógeno. Las barras representan la media de los tratamientos ± error estándar (n=2). Las letras y los asteriscos indican que los promedios difieren significativamente de acuerdo con la prueba de rango múltiple de Duncan ($p<0.05$).

Para comprobar que la deficiencia de hierro provocada por los sideróforos son la causa de la inhibición del crecimiento de *Fusarium brachygibbosum*, se realizaron los mismos experimentos, pero se le agregó una solución de $FeCl_3$ para ver si el crecimiento del hongo se restablecía al proporcionar hierro. Si comparamos estos resultados con los del experimento anterior en términos de porcentaje, el crecimiento del hongo se restablece al 100% cuando se administra la solución de $FeCl_3$. Los resultados se muestran en la figura 8.

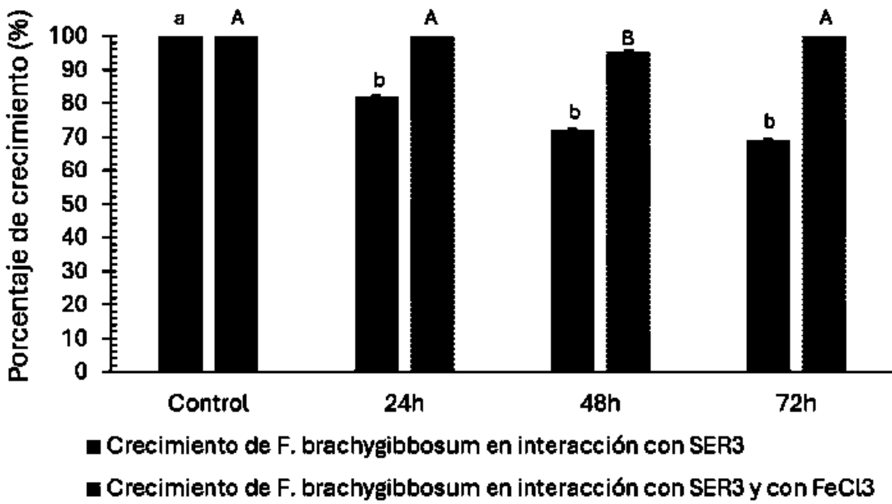

Figura 8: Ensayos de confrontación entre *Rouxiella badensis* SER3 y *Fusarium brachygibbosum* en medio CAS. Las horas representan los tiempos de inoculación de la bacteria antes de la inoculación del patógeno. Después de 72h de interacción entre la bacteria y el hongo en cada tratamiento, se adicionó una solución de $FeCl_3$. Las barras representan la media de los tratamientos ± error estándar (n=2). Las letras indican que los promedios difieren significativamente de acuerdo con la prueba de rango múltiple de Duncan ($p<0.05$).

Tipos de sideróforos detectados con espectrofotometría UV-vis en el sobrenadante de la cepa SER3

El espectro obtenido con el sobrenadante de la cepa SER3 en caldo nutritivo muestra un pico entre los 400 y 450nm lo que indica la presencia de sideróforos tipo hidroxamato de acuerdo con (Neilandsl, 1981). La imagen del espectro obtenido se muestra en la figura 9.

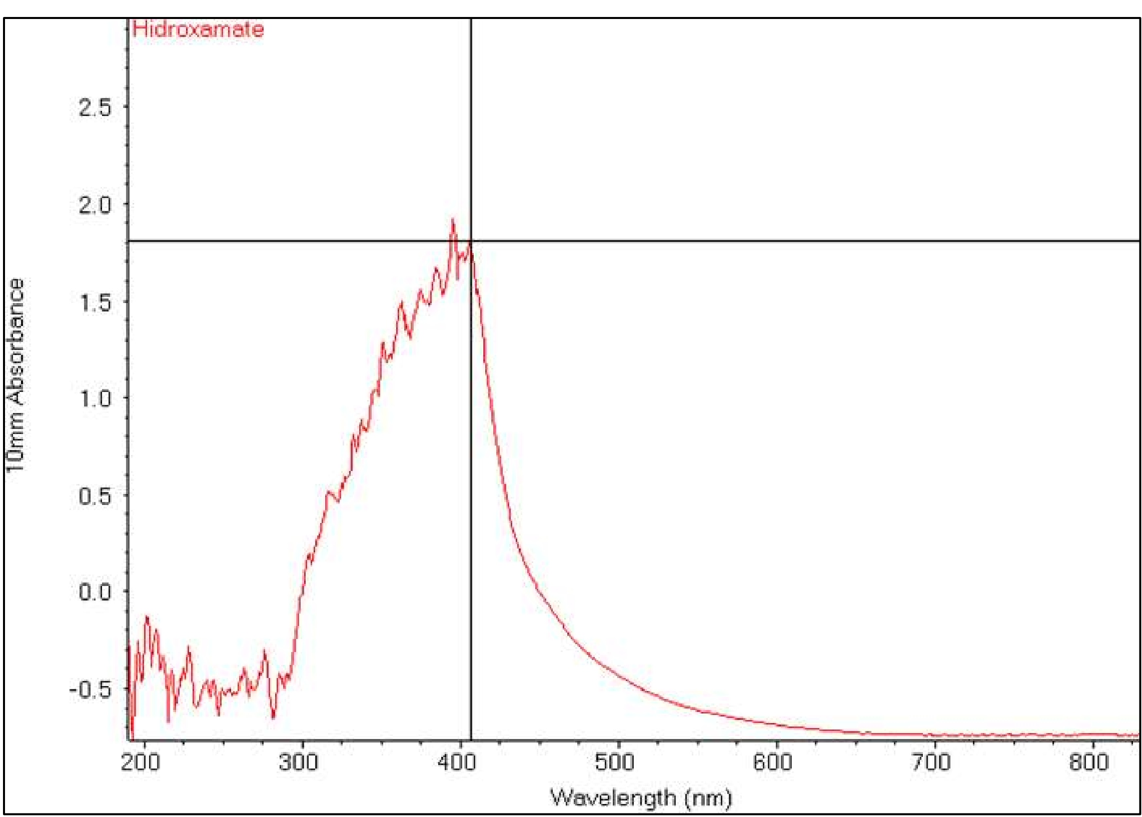

Figura 9: Espectro obtenido con espectrofotometría UV-vis realizado en el sobrenadante de la cepa SER3 en caldo nutritivo. La presencia de un pico entre los 400 y 450nm indica la presencia de sideróforos tipo hidroxamato.

Mientras que la presencia de un pico a una longitud de 495nm indicaría la presencia de sideróforos tipo catecolato. Este no fue encontrado en el sobrenadante de la cepa en caldo nutritivo. La imagen del espectro obtenido se muestra en la figura 10.

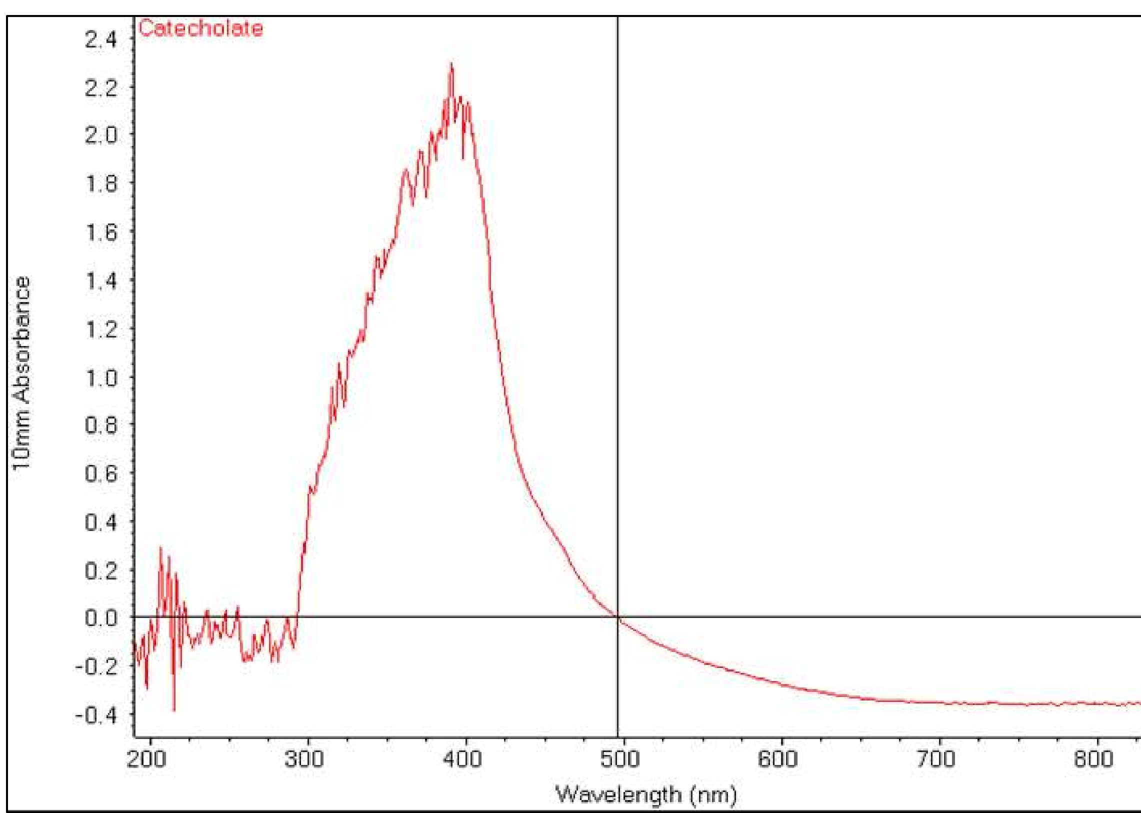

Figura 10: Espectro obtenido con espectrofotometría UV-vis realizado en el sobrenadante de la cepa SER3 en caldo nutritivo. La presencia de un pico en los 495nm indicaría la presencia de sideróforos tipo catecolato.

Dado que la producción de sideróforos se estimula en mayor proporción por la deficiencia de nutrimentos (particularmente Fe^{+}), se realizaron los mismos experimentos de espectrofotometría UV-vis utilizando el medio de cultivo M9.

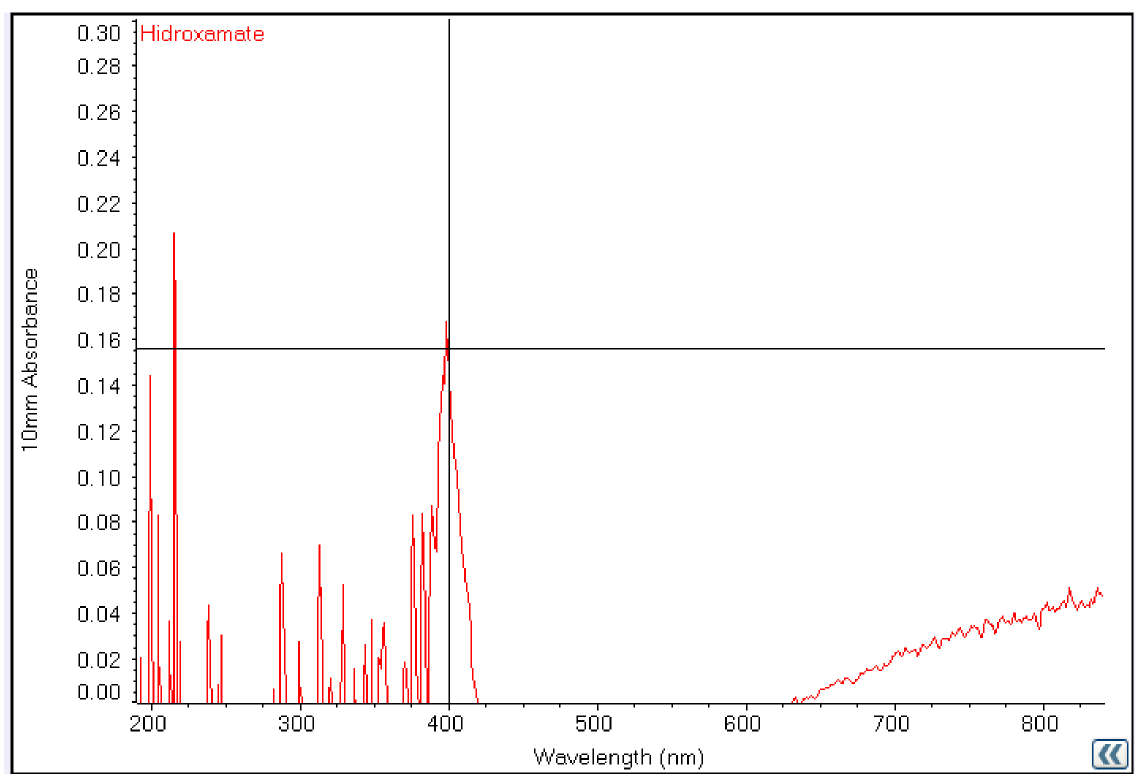

Figura 11: Espectro obtenido con espectrofotometría UV-vis realizado en el sobrenadante de la cepa SER3 en caldo M9. La presencia de un pico entre los 400 y 450nm indica la presencia de sideróforos tipo hidroxamato.

El espectro muestra la presencia de un pico entre los 400nm y 450nm detectando la presencia de sideróforos tipo hidroxamato, (figura 11). Mientras que no se observó pico a los 495nm indicando que no hay presencia de sideróforos tipo catecolato (figura 12).

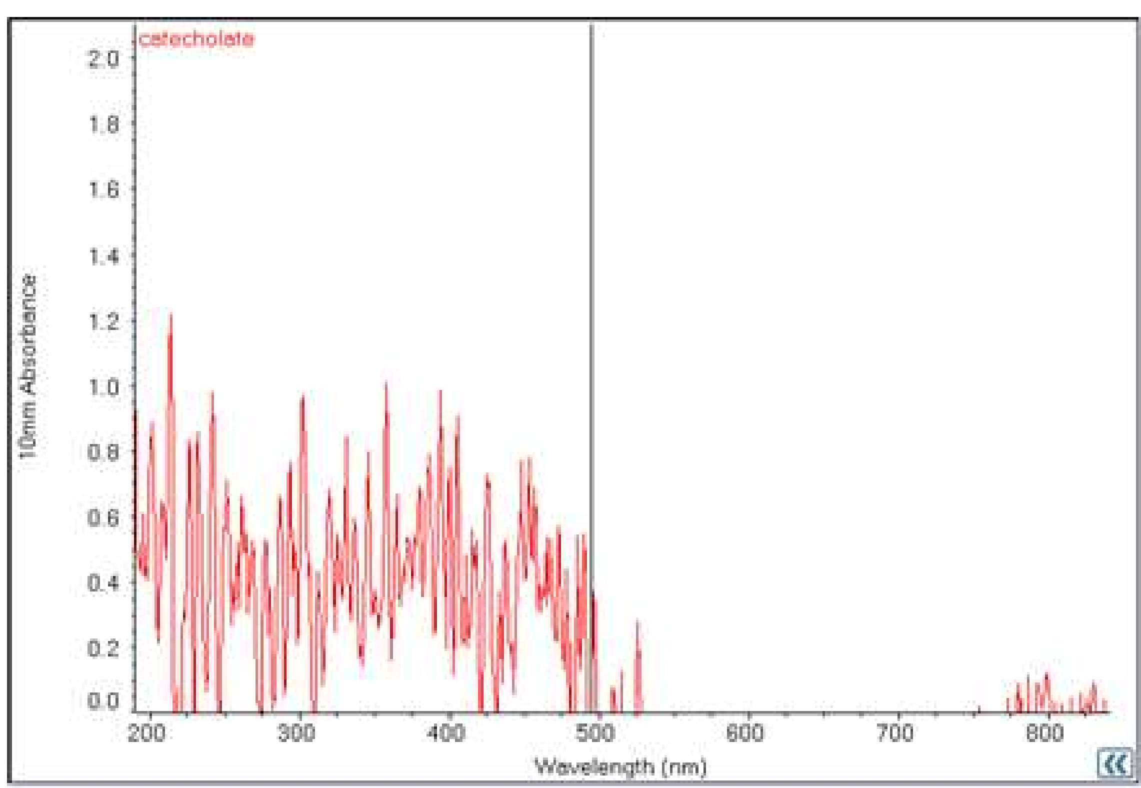

Figura 12: Espectro obtenido con espectrofotometría UV-vis realizado en el sobrenadante de la cepa SER3 en caldo M9. La presencia de un pico en los 495nm indicaría la presencia de sideróforos tipo catecolato.

Grupo de genes involucrados en la producción de Desferrioxamina E (nocardamina) y su posición en el genoma de *Rouxiella badensis* SER3

El grupo está compuesto por 4 genes, en la figura 13 se observa su posición en el genoma de *R. badensis* SER3. El gen putativo *dfoJ* codifica para la enzima lisina/ornitina descarboxilasa dependiente de piridoxal-fosfato. Descarboxila lisina produciendo cadaverina. El gen putativo *dfoA* codifica para la enzima amina monooxigenasa. Conduce la oxigenación en uno de los grupos amino terminales de la cadaverina para producir 1-amino-5-(N-hidroxi)-aminopentano. El gen putativo *dfoC* realiza la reacción de acoplamiento de succinil-CoA con el 1-amino-5-(N-hidroxi)-aminopentano para formar ácido N-5-aminopentil-N-(hidroxil)-succinámico, que es trimerizado y ciclado en una reacción dependiente de ATP por la proteína dfoC para formar desferrioxamina E. El gen putativo *dfoS* codifica para una proteína de transporte de membrana MFS (superfamilia de facilitadores principales) responsable de exportar de la célula la desferrioxamina E.

Figura 13: Grupo de genes involucrado en la síntesis y excreción del sideróforo desferrioxamina E, también conocido como nocardamina E y su posición en el genoma de la cepa SER3.

Efecto de la cepa SER3 en la morfología de las hifas de *Botrytis cinerea* y *Fusarium brachygibbosum* observado con microscopio electrónico de barrido

Utilizando el microscopio electrónico de barrido (MEB) observamos coincidencias con las imágenes que se tomaron con el microscopio óptico, es decir, las hifas en contacto con la cepa SER3 muestran deformidad, sin embargo, con el MEB también observamos otros detalles que se pueden apreciar en las figuras 14 y 15. La figura 14 muestra las hifas de *F. brachygibbosum* en contacto con la bacteria muestran rupturas y protuberancias, además las hifas de *B. cinerea* (figura 15) tienen una forma aplanada. Esto nos indica que los compuestos de la cepa SER3 que difunden al medio provocan una deformidad en las hifas de los patógenos y por lo tanto se inhibe su crecimiento.

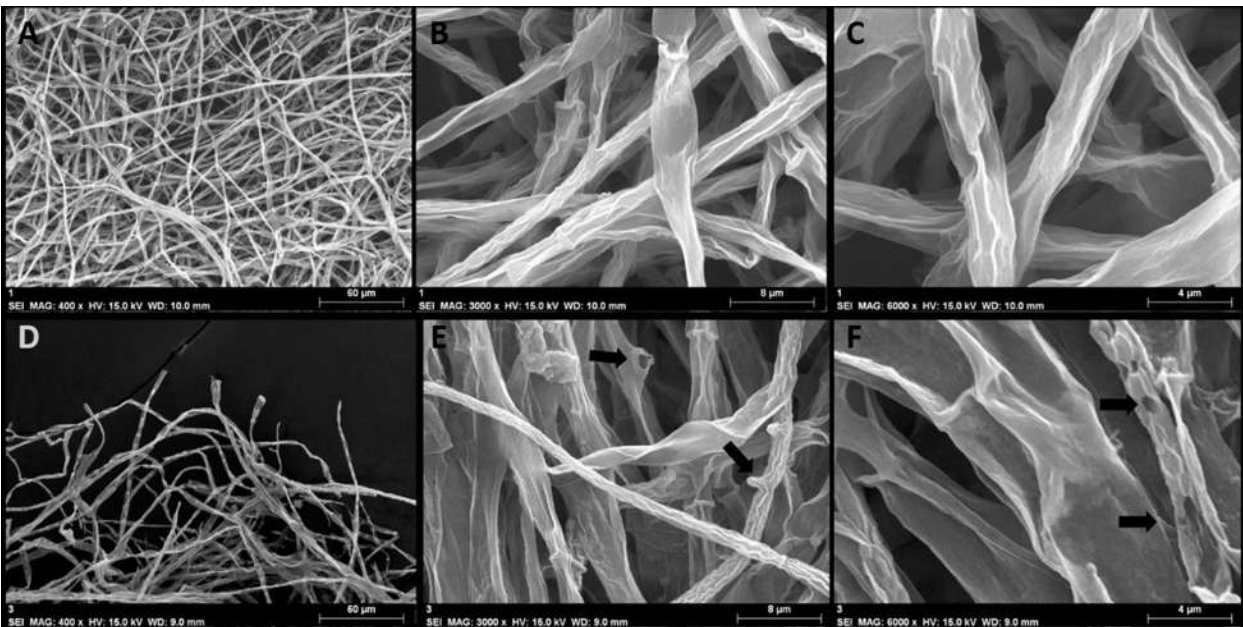

Figura 14: Hifas de *Fusarium brachygibbosum* 4AF. Panel (A), (B) y (C) corresponden a las hifas en condiciones control vistas a 400x, 3000x y 6000x, respectivamente. Panel (D), (E) y (F) corresponden a las hifas que estuvieron en co-cultivo con la cepa SER3 vistas a 400x, 3000x y 6000x, respectivamente.

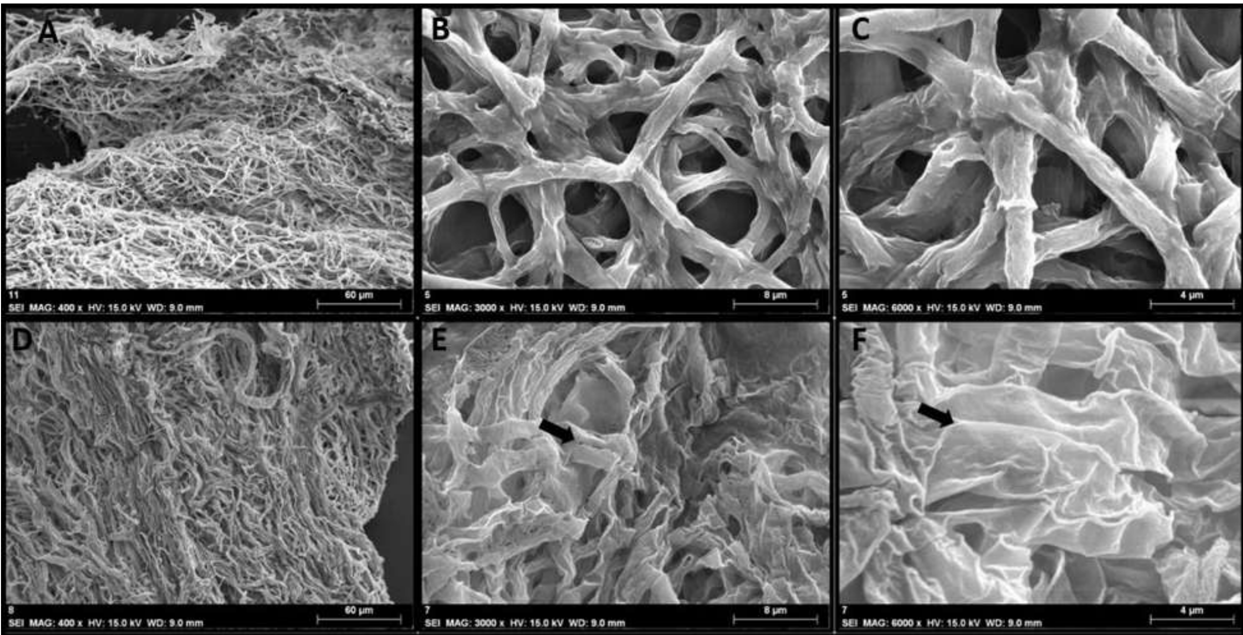

Figura 15: Hifas de *Botrytis cinerea* 62BCV. Panel (A), (B) y (C) corresponden a las hifas en condiciones control vistas a 400x, 3000x y 6000x, respectivamente. Panel (D), (E) y (F) corresponden a las hifas que estuvieron en co-cultivo con la cepa SER3 vistas a 400x, 3000x y 6000x, respectivamente.

Discusión general

Los agentes de control biológico o antagonistas microbianos pueden ser aislados de diversas fuentes, sin embargo, las fuentes más comunes son la superficie o interior de las frutas o las plantas. Cuando una cepa es propuesta como agente de control biológico importante caracterizarla de la manera más completa posible, es decir, hacer uso de las diversas metodologías como ensayos microbiológicos, bioquímicos y moleculares para conocer su taxonomía, conocer los potenciales genes involucrados en los mecanismos de acción que ejerce, comprobar su capacidad antagonista en co-cultivos y en experimentos *in vivo*, realizar pruebas bioquímicas para identificar los metabolitos que produce, verificar que estos no sean tóxicos para las personas o animales. Incluso hacer pruebas para proponer alguno de estos metabolitos como nuevo compuesto antimicrobiano que pueda ser utilizado de forma aislada, en vez de utilizar las células bacterianas.

La cepa SER3 con la que se trabajó en el presente proyecto, fue aislada de la superficie de frutas de fresas, después de realizar co-cultivos para evaluar su capacidad de control biológico se decidió caracterizarla y determinar los mecanismos de acción que utiliza contra los hongos patógenos. Definir las características taxonómicas y genómico-funcionales de la cepa SER3 asociadas al antagonismo de patógenos fúngicos postcosecha fue el objetivo general de este proyecto. Para lograrlo fue necesario utilizar metodologías microbiológicas, bioquímicas y moleculares como es sugerido por (Massart *et al*., 2015).

Dentro de las metodologías moleculares está la secuenciación de alto rendimiento. Esta herramienta nos permite avanzar rápidamente en diagnóstico, taxonomía, epidemiología, genómica comparada, virulencia, descubrimiento de genes o variantes de interés y asociación de microorganismos con deterioro de alimentos e infecciones transmitidas por alimentos (Quijada *et al*., 2020). En este trabajo mediante la secuenciación de alto rendimiento y el uso de herramientas bioinformáticas pudimos ensamblar el genoma completo de la cepa SER3, obtuvimos 47 contigs que fueron ensamblados en un solo scalfold. Una vez que obtuvimos el genoma, realizamos las anotaciones, es decir, localizamos y encontramos funciones de los genes presentes. Algunas de las características del genoma es que tiene un tamaño de 5.08Mb, tiene un contenido de GC del 52.8%

Los algoritmos Identidad Promedio de Nucleótidos por sus siglas en inglés (ANI) y la calculadora distancia Genoma-Genoma (GGDC), son herramientas que nos permiten calcular el índice General de la Relación del Genoma (OGRI), este índice nos permite reemplazar la técnica de hibridación del ADN que por mucho tiempo fue el estándar de oro para establecer la taxonomía procariota. El OGRI, es reconocido ahora como la herramienta para definir especies procariotas.

De acuerdo con Chun *et al*., 2018, una vez que el genoma completo es ensamblado, se localiza la secuencia completa que corresponde al gen ribosomal 16S, se hace una comparación de ésta con secuencias de cepas tipo, de esta manera encontramos especies filogenéticamente cercanas a

nuestra cepa de interés, si el porcentaje de identidad es menor al 98.7% podemos definirla como una nueva especie, pero si es mayor a este valor, se debe calcular el Índice General de Relación del Genoma (OGRI). Para calcular este índice, es necesario descargar secuencias de genomas completos de cepas tipo filogenéticamente cercanas a nuestra cepa de interés de acuerdo con los resultados de la comparación del gen ribosomal 16S. La comparación de genomas completos debe hacerse con los algoritmos ANI y GGDC, si los valores de éstos son menor de 95% y 70% respectivamente, se define como nueva especie, pero si son mayores a este valor entonces se define como una especie conocida, a la especie con mayor porcentaje de identidad.

Mediante el uso de esta técnica pudimos identificar a la cepa SER3 como *Rouxiella badensis* SER3 de manera certera, ya que el porcentaje de identidad comparando el gen ribosomal *16S* de la cepa SER3 con el de *Rouxiella badensis* DSM 10043 fue de 100% y utilizando los algoritmos ANI y GGDC para comparar los genomas completos de estas dos cepas los porcentajes de identidad fueron 99.6% y 98.2%, respectivamente.

Otra herramienta bioinformática que aporta valiosa información a partir del genoma ensamblado es el programa anti-SMASH, éste identifica grupos de genes y su posición para la producción de antibióticos y metabolitos secundarios, por lo tanto, nos permite conocer los potenciales metabolitos que una bacteria puede producir. Sin embargo, es importante mencionar que se debe comprobar la presencia de los metabolitos de interés mediante alguna otra técnica, por ejemplo, pruebas bioquímicas, cromatografía, o incluso la expresión de los genes utilizando RT-qPCR o microarrays. De esta manera podemos constatar que los genes detectados con anti-SMASH se están expresando y en qué condiciones pueden hacerlo, por ejemplo, si la presencia o ausencia de un patógeno influye en la expresión de un gen o en la producción de un metabolito determinado.

Los resultados de anti-SMASH mostraron que en el genoma de la cepa SER3 hay grupos de genes para la producción de diversos metabolitos de los cuales resaltan los sideróforos con un porcentaje de semejanza del 100% y los aril polienos con un porcentaje de similitud del 77%. Estos porcentajes indican que la mayor parte de genes que constituye al grupo están presentes y que por lo tanto el metabolito que expresan puede ser sintetizado, es decir, porcentajes mayores al 70% indicarían grupos de genes con actividad biológica funcional. Los aril polienos son policétidos bacterianos que contienen una parte arilo terminal conectado a un ácido polienocarboxílico que en algunos casos está esterificado con un sistema de dialquilresorcinol (DAR). Se han descrito como pigmentos, compuestos antioxidantes similares a los carotenoides. Se han descrito 4 tipos: xantomonadina, flexirrubina, arcuflavina y aril polieno tipo flexirrubina (Grammbitter *et al*., 2019). Su estructura tiene cierta similitud con el antifúngico anfotericina B por los polienos, hasta el momento no hay estudios que demuestren que los aril polienos tienen actividad de control biológico. Estos compuestos no se venden de manera comercial y su extracción requiere de técnicas sofisticadas y costosas, por lo que este proyecto se enfocó hacia el otro metabolito con actividad biológica funcional detectada con antiSMASH, los sideróforos. Los resultados de anti-SMASH mostraron dos tipos de sideróforos: uno tipo catecolato, que

corresponde a la turnerbactina, este mostró un porcentaje menor al 70% aun así se decidió verificar su presencia en el sobrenadante del cultivo líquido libre de células de la cepa SER3, sin embargo, éste no fue encontrado en ninguno de los cultivos líquidos realizados, ni en medio nutritivo ni en medio pobre en nutrimentos. El otro sideróforo es un tipo hidroxamato que corresponde a la desferrioxamina E también conocida como nocardamina E, este tipo de sideróforo sí fue detectado con espectrofotometría uv-vis de nanodrop en los dos medios de cultivos utilizados, tanto en el medio pobre en nutrimentos como en el nutritivo. El espectro obtenido nos brinda la información sobre el tipo de sideróforo que se encuentra presente en la muestra. En el trabajo realizado por De los Santos-Villalobos *et al*., 2012 se identifican los tipos de sideróforos producidos por *Burkholderia cepacia* XXVI utilizando espectrofotometría uv-vis, donde describen que al agregar una solución de cloruro de hierro al 2% al sobrenadante libre de células y detectar un pico en el espectro a una longitud de onda de 495nm indica la presencia de sideróforos tipo catecolato, mientras que la presencia de un pico a una longitud de onda entre los 400nm y 450nm indica la presencia de sideróforos tipo hidroxamato. Con estos resultados y los proporcionados por antiSMASH, se sugiere que el sideróforo turnerbactina (tipo catecolato) no se produce y que el sideróforo tipo hidroxamato detectado con espectofotometría uv-vis corresponde a la desferrioxamina E.

La presencia de sideróforos también fue detectada mediante otro ensayo bioquímico utilizando el medio cromo azurol (CAS), en donde un cambio en la coloración del medio de azul a naranja indica la presencia de sideróforos. Utilizando este medio también se realizaron ensayos de confrontación con el patógeno *Fusarium brachygibbosum* 4BF y la cepa SER3 a distintos tiempos; inoculando bacteria y hongo al mismo tiempo, la bacteria 24h antes que el hongo y así mismo 48h y 72h antes que el hongo. Los resultados mostraron que no había diferencia significativa en el crecimiento del hongo cuando se inoculan ambos microorganismos al mismo tiempo, sin embargo cuando la bacteria se inocula 24h antes que el patógeno el crecimiento de éste se ve inhibido en un 18%, al comparar la producción de sideróforos del patógeno y la producción de sideróforos de la bacteria con el tratamiento donde se inocularon al mismo tiempo, observamos que los sideróforos del hongo disminuyen discretamente y los de la bacteria aumentan. Cuando la bacteria se inocula 48h antes que el hongo el porcentaje de inhibición es del 28%, y si comparamos la producción de sideróforos del hongo y de la bacteria de este tratamiento con el del tratamiento de 24h y con el de inoculados al mismo tiempo observamos disminución significativa en la producción de sideróforos del hongo y aumento de la producción de sideróforos por parte de la bacteria. En el tratamiento de 72h observamos un efecto similar donde la inhibición del hongo es del 31%. Estos resultados muestran que el tiempo de inoculación de la bacteria afecta la producción de sideróforos del hongo y su crecimiento, y que al tener mayor tiempo de inoculación que el hongo la bacteria tiene una mayor producción de sideróforos, ya que tiene el tiempo suficiente para adaptarse y consumir el poco hierro presente en el medio, así que cuando el hongo es inoculado, hay una menor cantidad de hierro que puede utilizar y se restringe su crecimiento. Además, si se agrega una solución que contenga hierro, el crecimiento del hongo se restablece, lo que comprueba que la deficiencia de hierro provocada por los

sideróforos es la responsable de inhibir el crecimiento, por lo que se sugiere que la competencia por espacio y nutrientes es uno de los mecanismos de acción que utiliza la cepa contra este patógeno.

También se realizó un análisis de grupos de genes para la producción de metabolitos secundarios de las bacterias filogenéticamente cercanas a la cepa SER3 con el programa anti-SMASH y se hizo una comparación con los resultados que se obtuvieron previamente con la cepa SER3. Con esta comparación pudimos observar que los grupos de genes del género *Rahnella* son muy similares a los del género *Rouxiella* en el tipo de metabolitos que pueden llegar a producir y en los porcentajes de semejanza de los grupos de genes. El género *Rahnella* ha sido descrito como agente de control biológico, y debido a los resultados obtenidos con este análisis, sugerimos que el género *Rouxiella* también tiene actividad de control biológico.

Los experimentos *in vivo* que se realizaron con fresas mostraron que, al estar presente la cepa hay porcentaje de inhibición tanto de *Botrytis cinerea* como de *Fusarium brachygibbosum*, sin embargo, la bacteria parece tener un mayor efecto inhibitorio ante *Fusarium*. Por otra parte, los experimentos realizados con el sobrenadante de la cepa (filtrados libres de células), también mostraron efectos de inhibición, estos también fueron más notables en el patógeno *Fusarium* que en *Botrytis*, esto puede ser debido a que, quizás, *Fusarium* sea más susceptible a la falta de hierro ocasionada por los sideróforos de SER3. Al comparar ambos resultados (los de sobrenadante y los de células bacterianas), podemos observar que se logran mayores porcentajes de inhibición al utilizar la cepa (células bacterianas) que el sobrenadante. Este resultado es frecuentemente observado en los experimentos de control biológico en frutas y vegetales en etapa postcosecha. Algunos autores lo atribuyen a que al estar presente la célula bacteriana se puede estar realizando más de un mecanismo de acción, y por lo tanto es difícil atribuir el efecto a un mecanismo en concreto. En el caso de nuestro experimento, este efecto puede deberse a que cuando la célula está presente, la producción de sideróforos es constante, mientras que al utilizar el sobrenadante sólo hay una determinada concentración.

Al visualizar con el microscopio óptico las hifas de los patógenos que estuvieron en co-cultivo con la cepa SER3, observamos deformidad de las hifas comparadas con las hifas de los patógenos que no estuvieron en contacto con la cepa. Cuando visualizamos las hifas con el microscopio electrónico de barrido (MEB) corroboramos los resultados, observamos deformidad de las hifas, sin embargo, estas imágenes nos brindan aún más detalles, como las protuberancias formadas y las rupturas que se visualizan en las figuras 14 y 15. Debido a estas alteraciones se restringe el crecimiento de los patógenos.

En este trabajo pudimos caracterizar a la cepa SER3 como *Rouxiella badensis*, realizamos análisis *in silico* para conocer su maquinaria genética relacionada al control biológico y mediante algunas técnicas bioquímicas pudimos constatar la producción de uno de sus metabolitos, los sideróforos y sugerir que se trata en particular de la desferrioxamina E también conocida como nocardamina E. Sin embargo, queda como perspectiva, realizar una cromatografía líquida acoplada a espectrometría de masas (LC-MS), que nos podrá confirmar la presencia de la desferrioxamina E

en el sobrenadante del cultivo. Además de constatar los resultados obtenidos con anti-SMASH y quizás demostrar la presencia de algún otro metabolito que pueda tener efecto de control biológico y que pudiera estar presente en el filtrado libre de células, algo similar a lo sucedido en el trabajo de Tian *et al*., 2020, donde encontraron genes relacionados a la síntesis de péptidos antimicrobianos que no se detectaron en la LC-MS. En algunos trabajos se ha mencionado que la expresión de ciertos genes se relaciona con la presencia de ciertas condiciones, por ejemplo, la presencia o ausencia de los patógenos, la temperatura, la presencia o ausencia de ciertos nutrimentos, la respuesta de defensa del hospedero, etc. Recordando que para caracterizar los mecanismos de acción de un agente de control biológico de deben utilizar las técnicas microbiológicas, bioquímicas y moleculares, de esta manera se puede tener un panorama amplio y no pasar por alto algún mecanismo o verificar en qué condiciones son efectivos los mecanismos de acción identificados.

Conclusión

La cepa bacteriana SER3 identificada como *Rouxiella badensis*, posee rasgos genómico-funcionales asociados al antagonismo de patógenos fúngicos postcosecha.

Perspectivas

- Analizar el filtrado libre de células de *Rouxiella badensis* SER3 con cromatografía líquida acoplada a espectrometría de masas (LC-MS) para comprobar que el sideróforo tipo hidroxamato detectado con espectrofotometría uv-vis se trata efectivamente de la desferrioxamina E (nocardamina E) como lo sugieren los resultados predichos por antiSMASH.
- Extraer los aril polienos y evaluarlos para comprobar si poseen actividad antifúngica.
- Hacer un análisis con LC-MS del filtrado libre de células de un cultivo dual *de Rouxiella badensis* SER3 y *Fusarium brachygibbosum* para comparar el cambio en la producción de metabolitos secundarios.

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Anexo I

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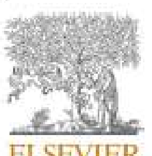

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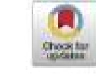

Plant growth-promoting bacterial endophytes as biocontrol agents of pre- and post-harvest diseases: Fundamentals, methods of application and future perspectives

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ABSTRACT

Sustainable agriculture requires the recruitment of bacterial agents to control diverse plant diseases such as bacterial endophytes. Bacterial endophytes colonize and inhabit internal plant tissues without causing any apparent damage. Within the plant, these bacteria exert multiple beneficiary effects, including direct stimulation of plant growth by the action of phytohormones or the production of metabolites. However, bacterial endophytes also protect their plant host through biocontrol pathogens or by inducing plant innate immune system. The present work makes a systematic and in-depth review on the current state of endophytic bacterial diversity, their plant colonization strategies, and their potential roles as protective agents against plant diseases during pre- and post-harvest stages of crop productivity. In addition, an exploration of their beneficial effects on sustainable agriculture by reducing/eliminating the use of toxic agrochemicals was conducted. Finally, we propose diverse effective strategies for the application of endophytic bacteria as biological agents during both pre- and post-harvest stages, with the aim of protecting crop plants and their agricultural products.

1. Introduction

Agricultural diseases lead to considerable losses in the production of fruits and vegetables, during their cultivation, handling, transportation, and storage. To control these diseases, agricultural crops are commonly treated with synthetic fungicides, which despite their effectiveness in controlling diseases caused by phytopathogens, are not sustainable alternatives due to their high economic and environmental costs (Sharma et al., 2009). Several studies show that they leave harmful residues in the soil, water, and the atmosphere, in addition to inducing resistance in phytopathogenic strains (Villarreal-Delgado et al., 2018). Thus, the development of efficient and environmental friendly technologies focused on the elimination or reduction of the application of synthetic fungicides in agriculture is highly desirable (Santoyo et al., 2012). Additionally, the export of organic fruits and vegetables is a widely accepted practice in world markets, and hence new technologies and alternatives to the use of chemicals must be generated. One of the promising alternatives to achieve this goal is the use of microbial biocontrol agents (Vinale et al., 2007; Santoyo et al., 2016).

Biocontrol agents, or bacterial antagonists, are microorganisms that decrease the harmful effects of pathogens. These microorganisms belong to the group of plant growth promoting bacteria (PGPB), which consists of bacteria that exert beneficial effects on plants (Lugtenberg and Kamilova, 2009). PGPB establish beneficiary relationships (which are sometimes very specific) with various plant species, stimulating their growth through direct and indirect mechanisms (Ullah et al., 2019). For example, they facilitate the acquisition of nutrients by plants, such as, biological nitrogen fixation and mobilization of immobilized nutrients as phosphorus and iron through the production of organic acids and siderophores, respectively (Glick, 2012). In addition, they aid in the

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modulation of hormone concentrations, such as auxins (indolacetic acid or AIA) and cytokinins, in plants. Another important mechanism to promote plant growth by PGPB is to reduce plant ethylene levels, which is a hormone produced by plants under certain stress conditions such as floods, water, or saline-induced stress, infection caused by pathogens, among others (Gamalero and Glick, 2015). 1-aminocyclopropane-1-carboxylate (ACC) produced by PGPB is the immediate precursor of the hormone ethylene in plants and is hydrolysed by the enzyme ACC deaminase (Orozco-Mosqueda et al., 2020). The bacterial enzyme ACC deaminase functions by degrading ACC molecule into α-ketobutyrate and ammonia, and hence an increase in the production of ethylene is avoided under conditions of stress and attack by pathogens, which in turn promotes the growth and facilitating the survival of plants (Santoyo et al., 2020). Also, plant growth can be increased through indirect action of PGPB as the growth or activity of phytopathogens is prevented or inhibited through various strategies, such as, competition for space and nutrients, antibiosis, lytic enzyme production, toxin inhibition, and induction of plant defense mechanisms (Solanki et al., 2019; Weyens et al., 2009). The group of bacteria that stimulate plant growth and can colonize and survive without causing harm to the plant, are known as plant growth-promoting bacterial endophytes (PGPBE) (Santoyo et al., 2016).

The way to suppress the activity of plant pathogens by PGPBE (and PGPB) can be carried out during two periods, either before (pre-harvest) or after (post-harvest) harvesting the fruit or vegetable product. Usually, the pre-harvest period could include prophylactic actions to eliminate potential pathogens that reside in the agricultural soil, as well as during planting and plant development until obtaining the fruit, seed or plant product by inoculating biocontrol agents (or other bio-compounds) (Hernández-León et al., 2015; Solanki et al., 2019).

Once the fruits or vegetables have been collected, they have to be transported and stored, which continues to make them susceptible to pathogen attack. Economic losses in both periods can vary but can signify more than 50 % of the total harvest (Sharma et al., 2009).

Several of the pathogens found during pre-harvest periods may continue to affect product quality during post-harvest. For example, *Botrytis cinerea*, which causes Gray mold disease, affects more than 200 species and plant products. Other important pathogens are *Colletotrichum musae*, the cause of Anthracnose or Blossom end rot diseases in banana; *Penicillium expansum* (Blue mold in apple) or *Alternata alternata* (Alternaria rot in Cherry), to name a few. A long list of pre- and post-harvest pathogens can be found in other excellent works (Sharma et al., 2009; Shafi et al., 2017).

Hence, the isolation, characterization, and application of biological control agents in the field and in post-harvest crops should be a priority for sustainable food production for all the countries in the world, especially those developing countries, since this will allow sustainable agriculture generation in the long term. In fact, "Noah's arks" should be created in every country or region to conserve, preserve, and study microorganisms from various environments with multiple effects on different plant species and in various environmental conditions, including soil conditions with poor nutrients, water scarcity, or metal contamination (de los Santos-Villalobos et al., 2018a, b).

2. Plant associated bacteria

Plant-bacteria associations have been studied for many decades, which suggest that bacteria positively impact plant growth and health while plants can "select" their microbiome or core microbiome to obtain beneficial bacterial colonizers, including those living within plant tissues, called endophytes (Santoyo et al., 2016; De Souza et al., 2016). Some authors have proposed a functional definition of endophytic behavior, considering any bacterium as an endophyte if it can be isolated from the disinfected surface of a plant tissue or extracted from the interior of the plant, without causing any apparent visible damage to the plant (Hardoim et al., 2015). In ecological terms, it is not clear whether residing within plant tissues is an advantage for endophytic bacteria, as opposed to living freely (saprophytically) in the area surrounding the plant roots, such as rhizospheric bacteria or living on the surfaces of the aerial parts or the plant (phyllosphere bacteria) (Liu et al., 2017a, b; Massoni et al., 2020). The rhizosphere is the soil zone containing a complex environment where plant roots interact physical and chemically with the soil. It is defined as the area of the soil whose properties are influenced by the presence and activity of the roots (Richardson et al., 2009). For example, soil nutrients such as phosphorus, are transferred to the root surface through the rhizosphere, or in the case of roots associated with mycorrhizal fungi, through the mycorrhizosphere, prior to acquisition (Richardson et al., 2009). Plants modify the physicochemical properties and biological composition of the rhizosphere through several mechanisms, which include acidification through proton extrusion and the release of root exudates. Along with changes to soil pH, root exudates influence the availability of nutrients for the plant (Richardson et al., 2009; Hardoim et al., 2015; Liu et al., 2017a, b).

Bacterial endophytes could have advantages over bacteria inhabiting the rhizosphere, since living within the tissues of a plant represents an opportunity to always be in contact with plant cells (and their metabolites), and therefore they can readily exert a direct beneficial effect (Santoyo et al., 2016). However, bacteria that reside in the rhizosphere have the potential to enter and colonize the plant roots; thus, the root ecosystem has been widely known as one of the main sources for endophytic bacterial colonization (Hardoim et al., 2015).

Different mechanisms are used by endophytic bacteria to enter plant tissues, particularly the roots. The most common mode of entry is through lateral or primary fissures and various wounds in the tissues that occur because of plant growth. The wounds occurring at the roots cause leakage of plant metabolites and become sites, which attract these bacteria (Santoyo et al., 2016).

Another plant zone of great importance and that has not been explored as it should be, is the phyllosphere. According to recent work by Massoni et al. (2020), it is suggested that the resident microbiota on the surfaces of plant organs such as leaves or flowers, may be more conserved than previously thought. Therefore, its adaptive role to the phyllosphere could be more plant-specific with close interactions of ecological or agronomical importance (Crombie et al., 2018; Herpell et al., 2020).

3. Colonization of the plant endosphere

The plant roots are exposed to soil bacteria during their development and growth, allowing bacteria to enter the plant and clearing the route for colonization of the seeds (spermosphere) (Truyens et al., 2014; Schiltz et al., 2015). For this regard, several bacterial traits are important for plant colonization and endophytic capacity. Chemotaxis-induced motility leading to root colonization is probably one of the most important mechanisms determining the endophytic potential of soil bacteria (Bacilio-Jiménez et al., 2003). The next step in the colonization route towards the seeds requires bacteria to enter the root and become endophytes either through passive penetration at the root tip, by the emergence of lateral roots or by pathogen entry sites, or by active penetration using cell wall degrading enzymes, such as cellulase and pectinase (Hurek et al., 1994; Ebeltagy et al., 2000; James et al., 2002). In addition, transportation of proteins for uptake of nutrients synthesized by plants, secretion and distribution systems involved in switching from a free lifestyle to an endophytic lifestyle, transcriptional regulators for metabolic adaptation, quorum sensing, and other detoxification mechanisms used in the protection against oxidative stress induced before or after host infection have been shown to be determinants of competent endophytes (Sessitsch et al., 2012; Ali et al., 2014).

Bacterial colonization in the plant interior is attractive since the host nutrients can be used efficiently and without competition from high bacterial numbers colonizing outside the roots (Liu et al., 2017a, b). In

addition, endophytic bacteria are more efficient in being protected from abiotic stress than rhizospheric bacteria (Hardoim et al., 2015). Once certain endophytes reach inside the plant, they are able to disperse systemically and ultimately reach flowers, fruits, and seeds. Some endophytes use the root xylem of their hosts to reach their meristems, where they are assisted by the movement of their flagella and by the plant transpiration current (James et al., 2002; Compant et al., 2005), while others use the nutrient-rich intracellular spaces, but this requires the secretion of cell wall degrading enzymes (Truyens et al., 2014). The bacteria that become seed endophytes are not exclusively soil derived, since several alternative entry (and hosting) sites might be present including the caulosphere (stem), phyllosphere (leafs surface), anosphere (flowers), spermosphere (seeds), and carposphere (fruits) (Fig. 1) (James et al., 2002; Berg et al., 2005; Compant et al., 2010, 2011). In a recent review on endophytes that inhabit the carposphere, interesting views on the microbiome associated with fruits are presented, either as epiphytes (on the surface) or as endophytes, which can be used as postharvest biocontrol agents (Droby and Wisniewski, 2018). Fig. 1 shows the complete picture of the different colonizable zones or ecosystems present in plants by bacterial endophytes.

Some of the key traits that distinguish endophytic PGPB from rhizospheric bacteria can be predicted through a bioinformatic approach. In a previous study, Ali et al. (2014) compared the genomic DNA sequences of a rhizospheric bacterium and an endophytic bacterium, both of the genus *Burkholderia* spp. The authors identified the genes encoded by the rhizospheric strain and these were subtracted from the

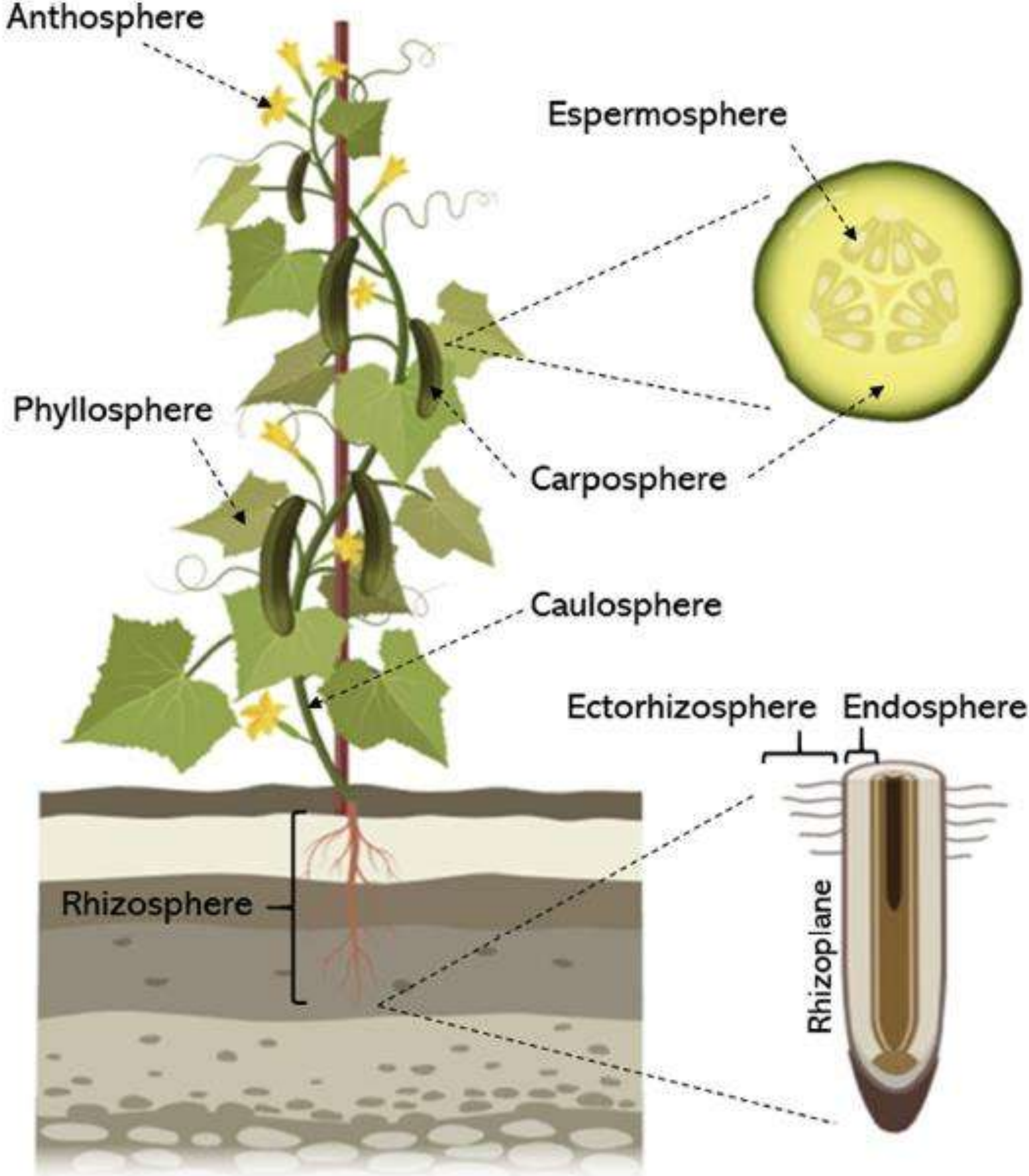

Fig. 1. Colonizable microecosystems by endophytic bacteria, including caulosphere (stem), phyllosphere (surface of leaves), anosphere (flowers), spermosphere (seeds), and carposphere (fruit). The root endosphere, which is considered part of the rhizosphere, is one of the internal zones of the plant where highly dense populations of bacteria can be found, compared to other tissues. Bacterial endophytes inhabiting these internal regions in host plants might exert beneficiary effects on plant growth, as well as, direct protection from potential infecting pathogens, or by inducing systemin resistance.

endophytic strain. Then, the remaining putative endophytic genes were compared with the complete genomes of eight different endophytic PGPB. According to the study, genes that were identified to be common to all these strains were considered to be potentially involved in endophytic behavior. Most of the 40 genes identified by this study, which were common to all the strains encode for protein functions as suggested by previous biochemical/functional studies, which are involved in endophytic behavior including genes encoding transporter proteins, secretion systems, plant polymer degradation, transcriptional regulators, detoxification, and redox potential maintenance, among other hypothetical genes.

Since microorganisms play an important role in preserving and preparing the environment for germination, seeds can benefit from the microorganisms associated with them. (Chee-Sanford et al., 2006). Thus, when the seeds begin to germinate, they absorb water and secrete exudates for attracting other rhizospheric bacteria. Based on this, the seedlings can benefit from the activity of PGPB (Nelson, 2004). Bacteria already present within the seed also play important roles in the evolution of the microbial community present in the seedling, since they can produce phytohormones, such as indolacetic acid, cytokinins, or gibberellins, which efficiently stimulate their development (Johnston-Monje and Raizada, 2011). Hence, seed-borne endophytes are of particular importance since they are passed between successive plant generations *via* vertical transmission. Thus, the seed-borne endophytes ensure their presence and colonization in the progeny. This vertical transmission of bacterial endophytes (and also other microbes such as fungi or oomycetes), defined as the direct transfer of the parents, should be selected against pathogenic microorganisms and favor mutualism for the survival and reproduction of the host (Truyens et al., 2014; Shahzad et al., 2018).

4. Diversity of bioprotective bacterial endophytes

Determining the diversity of bacterial endophytes is as complicated as analyzing each of the nearly 300,000 plant species that exist on the earth (Santoyo et al., 2016). However, studies on endophytic diversity are biased towards plant species with an agronomic importance which are widely cultivated and consumed; for example, sugarcane (*Saccharum officinarum*), corn (*Zea mays*), rice (*Orysa sativa*), wheat (*Triticum* spp.), potato (*Solanum tuberosum*), or soy (*Glycine max*) to name a few. In a recent study, Santoyo et al. (2016) analyzed the diversity of bacterial endophytes that promote plant growth, and suggested that the most common species reported in multiple studies are *Arthrobacter, Bacillus, Burkholderia, Enterobacter, Methylobacterium, Microbacterium, Micrococcus, Paenibacillus, Pantoea, Phyllobacterium, Pseudomonas, Rhanella, Rhodanobacter, Sphingomonas,* and *Stenotrophomonas*. Hence, it is advisable to isolate bacterial endophytes in plants that have ecological rather than agricultural importance, particularly in places where environmental conditions are extreme and the possibility that a beneficial microbiome for the plant has been selected for millions of years to survive in such conditions. There are several studies, which looked at identifying endophytes in different hosts and reported on their beneficiary interactions with plants of agronomic importance (Hardoim et al., 2008; Sturz et al., 2000). Hence, these studies highlight the importance of specific bacterial-host benefits, and also state that those bioprotective bacterial endophytes could exert antagonistic activity towards multiple pathogens that affect other crops during pre- and post-harvest periods.

5. Biocontrol mechanisms

Plant growth-promoting bacterial endophytes have the ability to restrict the growth of plant pathogens. This is because they synthesize a series of antibiotic compounds and enzymes with antagonistic activity towards phytopathogens, as well as igniting the innate plant defense system, known as induced systemic resistance (ISR) (Pérez-Montaño et al., 2014). Other biocontrol mechanisms may be the ability to colonize plant tissues, however this is difficult to study in quantitative terms (even in inhabitants of the rhizosphere). However, there is indirect evidence that competition and colonization of spaces by beneficial bacteria can limit the severity and incidence of plant diseases (Glick, 2012). The biocontrol mechanisms detailed below can be shared between endophytic bacteria with other bacteria that inhabit the bulk soil, the rhizosphere or phyllosphere, so the possibility of exploring exclusive mechanisms of endophytic agents in a comparative way has not been evaluated so far.

5.1. Antibiosis

The ability to synthesize various antimicrobial compounds has been extensively studied to inhibit, restrict, or eliminate the growth of phytopathogenic organisms (Liu et al., 2017a, b). *Pseudomonas* and *Bacillus* are the main bacterial genera studied for their ability to produce antibiotics, such as 2,4-diacetylfloroglucinol acid, phenazine-1-carboxylic acid, phenazine-1-carboxamide, pyroluteorine, pyrrolnitrine, oomycin A, viscosinamide, butyroaminectone, kyanoaminectone, zymicrolactone, zymicrolactone A, aerugina, rhamnolipids, cepacyamide A, ecomycins, pseudomonic acid, azomycin, and cepafungins (Santoyo et al., 2019). A large number of strains of the genus *Pseudomonas* are recognized for producing a wide variety of antibiotics that contribute to the suppression of agricultural diseases, for example *P. flourescens* produces pyoluteorin and 2,4-diacetylfloroglucinol to suppress root rot of the tobacco, caused by *Thielaviopsis basicola*; on the other hand, 2,4-diacetylfloroglucinol also contributes to the suppression of take-all disease in wheat caused by *Gaeumannomyces graminis*. Finally, pioluteorin and pyrrolnitrin have been shown to effectively suppress watercress disease caused by *Pythium ultimum* and *Rhisoctonia solani*, respectively (Milner et al., 2019).

5.2. Lytic enzymes

these enzymes are involved in the cellular wall degradation of phytopathogenic microorganisms, being one of the most reported biological control mechanisms mainly against fungal pathogens (Villarreal-Delgado et al., 2018). The fungal cellular wall (including plant pathogens) consists of glycoproteins, polysaccharides, and other components that vary depending on the fungal species (Bowman and Free, 2006). The polysaccharide fraction comprises up to 80 % of the cell wall (Latgé, 2007). These polysaccharides have a determining structural role in the stiffness of the cell wall, through an extensive network of glycosidic bonds; therefore, the interference in these bonds by lytic enzymes can deteriorate the cell wall of phytopathogenic fungi, causing their lysis and cell death (Jadhav et al., 2017). Among the most studied lytic enzymes produced by biological control agents are chitinases, cellulases, proteases, and β-1,3-glucanases, which modify, perforate, and/ or degrade the structure of the cell wall (Mota et al., 2017). Mishra and Arora (2012) reported the role of an extracellular chitinase -isolated from *P. aeruginosa*- in the biological control of *Xanthomonas campestris*, which causes black rot disease. Furthermore, strains of *Pseudomonas* isolated from the chickpea rhizosphere have been reported to produce chitinases and cellulases with antagonistic activity against *Rhisoctonia solani* and *Pythium aphanidermatum* (Sindhu and Dadarwal, 2001). Similarly, *Bacillus subtilis* has been reported for its biological control capacity against *R. solani*, the causative agent of black scabies in potatoes, through the production of chitinases (Saber et al., 2015).

5.3. Induction of systemic response (ISR) in plants

this mechanism can be induced by chemical signals (elicitors) produced by beneficial microorganisms (Pérez-Montaño et al., 2014). ISR signaling is dependent on jasmonic acid and ethylene (Kannojia et al., 2019). So far, not all the molecular mechanisms that regulate plant-beneficial microbe interactions have been described; however, the main

routes by which these agents regulate ISR in plants have been identified: i) phytohormones, ii) pathogen-associated molecular patterns (PAMPs)/ microbe- associated molecular patterns (MAMPs), and iii) several elicitors (volatile organic compounds, siderophores, phytases, miRNAs, among others) (Abdul Malik et al., 2020; Rodriguez et al., 2019). ISR has been evidenced in tobacco plants, where PR2 (encodes a β-1,3 glucanase) and PR3 (encodes a chitinase) were activated in response to volatile compounds produced by *Bacillus*, conferring resistance to *Rhisoctonia solani* and *Phytophthora nicotianae* (Kim et al., 2015). In addition to PR genes, *Bacillus* activates other protection mechanisms in plants, which include structural changes in the cell wall by the accumulation of lignin (Singh et al., 2016), or the production of secondary metabolites such as flavonoids, phytoalexins, auxins and/ or glucosinolates (Pretali et al., 2016). Thus, ISR has been reported in a variety of crops (beans, carnations, cucumbers, radishes, tobacco, and tomatoes), significantly reducing the pathogenicity of several plant pathogens, including fungi, bacteria, and viruses (Kannojia et al., 2019).

5.4. Production of δ-endotoxins

The δ-endotoxins, produced by *Bacillus thuringiensis* (Bt), are parasporal bodies proteins made up of polypeptide units of different molecular weights, from 27 to 140 kDa (Villarreal-Delgado et al., 2018). Bt toxins are produced during the sporulation phase, the Cry (crystal) protein is known for its specific toxic effects on a target organism (most belong to the insect order); likewise, Cyt (cytolytic) proteins have been reported with toxic effects on a wide variety of insects, mainly diptera; however, its cytotoxicity against mammalian cells has also been verified (Anaya et al., 2020). The mechanism of action of Cry proteins starts once they are proteolytically processed through proteases that are found in the midgut of the host, separating a section of amino acids in the N-terminal region and at the C-terminal end (depending on the nature of the Cry protein), and thus releasing active and toxic fragments that interact with the receptor proteins present in intestinal cells of the insect, signaling the formation of a pre-pore oligomeric structure and consequently the lytic pore, which generates an osmotic imbalance, and then destroying the intestinal epithelium and consequently causing cell death (Xu et al., 2014). Currently, at least 13 strains of Bt are used in agriculture, some of them are facultative bacterial endophytes (Regnault-Roger, 2012).

5.5. Siderophores production

These metabolites are produced by microbes in response to a limitation of iron in the environment; thus, some biological control agents produce these low molecular weight (400–1500 Da) receptor protein structures with high affinity for iron. Siderophores are secondary metabolites that act as sequestrants of iron, due to their high dissociation constant by this element (between 10^{22} and 10^{55}). Siderophore-producing biological control agents can use iron by two mechanisms: i) directly through the Fe^{3+}-siderophore complex through the cell membrane, or ii) reduced extracellularly to Fe^{2+} complexes (Hider and Kong, 2010). This allows these agents to regulate the concentration of iron in their habitats through the sequestration of that element (Fe^{3+} -siderophore), causing iron not to be available for phyto-pathogenic microorganisms, and restricting its growth (Kannojia et al., 2019). Currently, several bacterial strains have been reported for their ability to control plant diseases through the siderophores production, limiting the growth and colonization of iron-dependent phytopathogenic microorganisms (Fgaier and Eberl, 2011). Yu et al. (2011) reported that *B. subtilis* CAS15 antagonized the growth (19–94 %) of 15 fungal phytopathogens belonging to the genus *Fusarium*, *Colletotrichum*, *Pythium*, *Magnaporthe* and *Phytophthora*, through the production of catecholate-type siderophores (Bacillibactin). On the other hand, de los Santos-Villalobos et al. (2012) reported the siderophore-producing capacity of *Burkholderia anthina* XXVI, which are involved in the inhibition of the causal agent of anthracnose in mango, *Colletotrichum gloeosporioides*, at a minimum inhibitory concentration of 0.64 μg ml^{-1}.

5.6. Production of lipopeptides

these molecules consist of a cyclic peptide linked to a β-hydroxy or β-amino fatty acid chain, classified into 3 different families (iturins, phengicines, and surfactins), based on their amino acid sequence and fatty acid length (Falardeau et al., 2013; Valenzuela-Ruiz et al., 2020). Lipopeptides are synthesized by multi-enzyme complexes called non-ribosomal peptide synthetase (NRPS), which are independent of messenger RNA (Chowdhury et al., 2015; Valenzuela-Ruiz et al., 2019), which are low-Molecular weight compounds with amphiphilic characteristics that protect to plants during several phenological stages by directly suppressing the growth of pathogens or inducing systemic resistance (Hashem et al., 2019). Recently, Coutte et al. (2017) reported 263 different lipopeptides synthesized by 11 microbial genera, among which the *Bacillus* genus was the most abundant producer with 98 different lipopeptides, those were involved in the biological control of a wide range of phytopathogens (bacteria, fungi and oomycetes), causing diseases in agricultural importance crops (Ongena and Jacques, 2008). For example, multiple isoforms of phengicines and iturins have been reported in cell-free extracts of liquid cultures of *B. subtilis* GA1, with the ability to inhibit *Botrytis cinerea* in apple fruits (Toure et al., 2004).

6. Biocontrol of plant pathogens during pre-harvest period

Various microorganisms, that form the microbiome of both the plant and the fruit, and those that belong to environments not related to crops have been extensively studied to test pre-harvest biocontrol activity. In this section, we mention recent studies concerning pre-harvest biocontrol, which include *in vitro* assays, as well as, *in planta* experiments (greenhouse or field trials) but without reaching the harvest stage. Conventionally, microbial antagonists are isolated from the environment where the pathogen is present and are generally considered more effective for disease control than isolates from unrelated environments. Hence, Veldman et al. (2018) evaluated the effects of 77 bacterial isolates against *Fusarium mangiferae*, which is the causative agent of mango malformation, a serious disease that affects crops during pre-harvest. Some of these isolates were obtained from mango orchards and others from mine effluents. Surprisingly, it was shown that isolates obtained from mine effluents were more effective against *Fusarium mangiferae* than those obtained from mango orchards. The authors suggest that this was because mine isolates exhibited a better combination of antagonistic mechanisms than rhizospheric strains, such as, competition for nutrients and spaces, production of volatile compounds, phenolic compounds, and siderophores. This result generates a hypothesis that hostile environments such as mines, select highly competitive and antagonistic organisms. More research is needed to prove this hypothesis.

However, evidence suggests that microorganisms associated with plants and fruits have a high ability to restrict the growth of pathogens, being in some cases up to 100 %. This was seen in a study conducted by Sharma et al., 2018, where the authors obtained 383 isolates from the rhizosphere of mustard plants (*Brassica juncea* L.), of which only 6 isolates showed significant antagonistic activities. However, the HMR25 isolate showed a complete control of the blight disease caused by *Alternaria brassicae*, in addition to promoting plant growth, possibly because this isolate produces a volatile compound cyanhydric acid (HCN) and siderophores. Both these compounds have been widely reported as plant pathogen antagonists (Hernández-León et al., 2015; Nellkner et al., 2019).

Endophytes that inhabit certain plant species might function as biocontrol agents for plants that have certain evolutionary kinship. For example, Verma et al., 2018 obtained bacterial isolates from *Leersia orysoides* seeds, a wild relative of rice (*Orysa sativa*), and demonstrated that the bacteria obtained from *L. orysoides* are compatible with rice and

most of these were endophytic bacteria. These bacterial endophytes induced gravitropic response of the root, increased root and stem growth, stimulated the formation of root hairs, and also protected the seedlings from infection caused by soil pathogens such as *Fusarium oxysporum*.

In a recent work, Chowdhury and Bae (2018) screened 256 bacterial endophytes isolated from mountain-cultivated ginseng plants (*Panax ginseng* Meyer), where 12 of them were selected by showing good antagonism against 6 ginseng pathogens (*Alternaria panax*, *Botrytis cinerea*, *Cylindrocarpon destructans*, *Phytophthora cactorum*, *Pythium* sp., and *Rhisoctonia solani*) using a dual culture assay. The endophyte *Burkholderia stabilis* EB159 (PG159) exerted the greatest inhibitory activity against all ginseng pathogens. In addition, this strain was able to suppress leaf spot disease caused by *B. cinerea*. Interestingly, defense-related ginseng genes were significantly upregulated in plant leaves treated with the strain PG159, thereby showing a potential mechanism to induce protection against the studied phytopathogen.

Transgenic soybean (*Glycine max* L. Merrill) is one of the most cultivated crops around the world, particularly in countries like Brazil where the plant production is around 85 million ton per year (Babujia et al., 2015). Some transgenic crops are not exempted from pathogenic infection. For example, de Almedia-Lopes et al. (2018) searched for bacterial antagonist against the pyhtopathogens *Sclerotinia sclerotiorum*, *Phomopsis sojae*, and *Rhisoctonia solani* in conventional and transgenic glyphosate-resistant soybean crops. The authors suggested that bacterial endophytes isolated from conventional and transgenic soybeans were significantly different in population diversity and in their antagonistic capacity. Endophytes belonging to *Bacillus* and *Burkholderia* genera were the most effective isolates in controlling bacterial and fungal pathogens *in vitro*. Although the antimicrobial activities of certain supernatants were tested in this work, it would be interesting to check whether the endophytes have biocontrol activities during post-harvest cultivation. Similarly, it would be relevant to conduct a more extensive study on the endophytic diversity by massive sequencing methods.

Certain bacterial species, along with fungi or oomycetes, can cause serious crop losses. For example, *Agrobacterium tumefaciens* is the causal agent of crown gall disease in several crop fruits worldwide, including peach. To control this pathogen, Li et al. (2019) recently screened 305 bacterial endophytes for antagonistic activities and reported that 54 endophytes showed significant inhibition against *A. tumefaciens*. Some pathogen antagonists were most frequently detected in peach resistant plants as compared to susceptible cultivars. These results are significant as it is suggested that the proportions of "defensive" bacteria could be more abundant in pathogen-resistant plants, such as *A. tumefaciens*. These previous results confirm, in a different environment, that the proportions of antagonistic bacteria (pseudomonads) are responsible for inhibiting the growth of pathogens in disease suppressing soils (Mendes et al., 2011).

Another most common prolific bacterial pathogen is *Pseudomonas syringae*, which usually inhabits in the superficial tissues of plants (the phyllosphere) and subsequently penetrate the tissues through wounds or natural openings such as stomata. In addition, *P. syringae* contains multiple virulence factors, such as, small-molecule toxins, exopolysaccharides, or cell wall-degrading enzymes (Santoyo et al., 2016; Xin et al., 2018). Therefore, it is very important to find bacterial endophytes against this pathogen. For this regard, Wicaksono et al. (2018) screened for endophytes with antagonistic activity against *P. syringae* pv. actinidiae in the medicinal plant Mānuka (*Leptospermum scoparium*) from New Zealand, which produces essential oils with antimicrobial properties. In particular, authors aimed to determine whether endophytes from L. *scoparium* could be transferred to a different plant, *A. deliciosa* (kiwifruit) and whether their biocontrol activities are maintained. Interestingly, three endophytes were transmissible to kiwifruit plants by wound inoculation where they inhibited colonization by *P. syringae* pv. *actinidiae*, thereby, reducing the Canker disease severity. Recently, Etminani and Harighi (2018) published an investigation reporting the isolation of two endophytic strains from wild Pistachio trees (*Pistacia atlantica* L.), Pb78 and Sp15, with inhibitory effects against two pathogens, *Pseudomonas syringae* pv. *syringae* and *Pseudomonas tolaasii*. Other strains also exhibited direct mechanisms to induce root formation on carrot slices. Endophytic strains were characterized and taxonomically associated to *Pantoea*, *Bacillus*, *Pseudomonas*, *Serratia*, and *Stenotrophomonas* genera.

Recently, a novel *Bacillus* species was isolated as an endophytic bacterium associated with wheat, *Bacillus cabrialesii* TE3T, which is preserved in a national microbial culture collection, Colección de Microorganismos Edáficos y Endófitos Nativos (COLMENA, www.itson.mx/colmena) (de los Santos-Villalobos et al., 2018a, b). This strain was identified as a plant growth-stimulating bacterium, since wheat plants inoculated with strain TE3T increased their chlorophyll content, 57.2 SPAD Unit *vs.* 43.1 SPAD Unit (non-inoculated control). In addition, this strain displayed the ability to solubilize phosphates (43.2 % $\pm$ 1.7 %), produce indoles (1.4 % $\pm$ 0.1 %), and grow under thermal, saline and water stress conditions. TE3T strain was also identified as a biological control agent against an emerging wheat phytopathogen (*Bipolaris sorokiniana*) in the Yaqui Valley, causing spot blotch, and reducing the infection [scale 1 (minimum) to 9 (maximum)] to 3–5, and the number of lesions/cm2 to 3.06 $\pm$ 0.6 in contrast to plants inoculated only with *B. sorokiniana*, which showed a disease severity of 8–9 and 6.46 $\pm$ 1.46 lesions/cm^2 (Villa-Rodriguez et al., 2019).

Thus, to summarize, previous works have reported the relevance of bacterial endophytes in displaying an *in vitro* or *in planta* antagonism, all during pre-harvest periods. However, biotic or abiotic conditions might vary in long-term experiments (Santoyo et al., 2017), where biological control of pathogens might include waiting for harvesting the product, as well as, transportation and subsequent commercialization. Therefore, in the following paragraphs we will analyze studies that include this important stage of biological control, the post-harvest period.

7. Biocontrol of post-harvest pathogens by PGPBE

Pathogenic infections result in considerable deterioration of fruits and vegetables during their handling, distribution, and post-harvest storage periods and reduce their shelf lives. The deterioration caused by fungi is primarily responsible for significant losses during the storage of fruits and vegetables. Fruit infections by fungal pathogens, both in the field and after harvest, lead to postharvest decay. In addition to the deterioration of quality and economic losses, fruits infected with fungal pathogens might represent an imminent risk to human health, as several genera of fungi, such as, *Aspergillus*, *Alternaria*, *Fusarium*, and *Penicillium*, produce mycotoxins (Dukare et al., 2018).

Due to the abovementioned reasons and also since the chemical methods are not usually favorable, the use of plant growth-promoting bacterial endophytes (PGPBE) with antagonistic activities is a good strategy to control post-harvest decay. Aiello et al. (2019) recently evaluated the biocontrol properties of *Pseudomonas synxantha* DLS65, an isolated endophyte isolated from kiwi fruit tissues, against *Monilinia fructicola* and *Monilinia fructigena*, which are causal agents of post-harvest brown rot of fruit with bone. Antagonism assays were performed *in vitro* and *in vivo* and the results showed that *P. synxantha* DLS65 inhibits 100 % of mycelial growth of *Monilinia fructicola* and *Monilinia fructigena* in petri dishes with potato dextrose agar (PDA). In addition, growth of these two microbes in peaches growth was partially reduced (49.8 % and 24.9 %, respectively) using agar extracts. The production of volatile organic compounds by DLS65 strain reduced mycelial growth of studied phytopathogenic fungi, both *in vitro* and *in vivo*. For this regard, *in vivo* tests showed that *P. synxantha* is effective even at low storage temperatures (10 and 0 °C). According to the authors, competition for space and nutrients, production of diffusible metabolites and volatile organic compounds play an important role in the antagonism of *P. synxantha* against *Monilinia fructicola* and *Monilinia fructigena*.

In another recent study, Stocco et al. (2019) detected and quantified

the presence of the pathogen *Alternaria* during different stages of vine cycle, as well as, in Red Globe variety table grapes. This pathogen was present from the flowering to the post-harvest storage periods in Mendoza province in Argentina. They compared alternative treatment strategies for the use of SO2 using chitosan and *Metschnikowia pulcherrima* RCM2 yeast, and noticed that the pathogen *Alternaria alternata* was found at all stages of the phenological cycle of the Red Globe variety table grapes. They also noticed that the highest incidence occurred during the flowering period, followed by post-harvest periods at 90, 60, and 30 days of cold storage (0–0.5 °C). They compared alternative treatments to the use of SO2, such as, chitosan and *Metschnikowia pulcherrima* RCM2 yeast. The strain RCM2 causes a reduction in the incidence of the disease during the market period, but when comparing these results with those obtained using the traditional chemical method (SO2), the chemical method was more efficient in reducing the incidence of the disease. However, modification of the amount of inoculum and the time of inoculation are factors that should be considered for future trials. Chitosan was effective as a biocontrol agent in *in vivo* post-harvest tests against *A. alternata* and the results obtained were similar to those obtained with the SO2 method. Hence, the authors suggest that chitosan might replace traditional chemical methods to reduce the incidence of *A. alternata* in post-harvest grapes.

Certain bacterial agents have the ability to colonize on fruits and survive at low temperatures; hence, they could be found in fruits that are naturally stored for long periods. For example, *Aureobasidium pullulans* ApB was found on the surface of apples stored for 6 months. The strain ApB was selected among other isolates due to its antagonistic activity against *Botrytis cinerea*. With regard to the pathogen inhibitory mechanism, the authors propose that siderophore production would be involved, although more studies are required to test this hypothesis. However, the strain ApB failed to grow above 35 °C, which is an important feature to be highlighted in the biosecurity agent application (Vero et al., 2009). In a previous work, *Aureobasidium pullulans* ApB strain was not isolated from the internal tissues of plants or fruits; however, there are other studies that show that the species *Aureobasidium pullulans* is a common endophyte of sweet cherries. In fact, multiple isolates belonging to *A. pullulans* showed post-harvest biocontrol activities against *Botrytis cinerea* and *Monilinia laxa*, protecting fruits such as sweet cherries and table grapes, showing a decrease in their decomposition that goes from 10 % to 100 % (Schena et al., 2003).

Plant growth-promoting bacilli species are commonly associated to plants, either inhabiting the rhizospheric or endophytic environments (Santoyo et al., 2012). Thus, several studies have isolated and evaluated antifungal agents of bacteria from these genera to control post-harvest diseases. For example, Chen et al. (2019) isolated diverse bacilli strains, including *B. amyloliquefaciens* RS-25, *B. licheniformis* MG-4, *B. subtilis* Z-14, and *B. subtilis* Pnf-4, as well as their culture filtrates and extracts, which showed significant inhibitory effects against the gray mold caused by *B. cinerea* on post-harvest tomatoes, strawberries, and grapefruits. Multiple antagonistic traits were evaluated as potential mechanism of action, such as, lipopeptide production along with cellulase and protease activities.

In a recent work, another bacilli strain, LYLB4, was isolated from pear fruits and tested for its antifungal activity against post-harvest pathogens present in pears, such as, *Alternaria brassicae*, *Botrytis cinerea*, *Fusarium graminear*, *F. oxysporum* f. sp. *cubense*, *Pyricularia orysae*, *Rhisoctonia solani* Kuhn, and *Verticillium dahliae* Kleb. During *in vivo* assays (on wounded pears), LYLB4 strain significantly reduced the disease severity caused by *Botryosphaeria dothidea* and *Rhisopus stolonifer*. Interestingly, MALDI-TOF mass spectrometry analysis of surface extracts isolated from whole cells of strain LYLB4 revealed differential production of iturin family lipopeptides including C_{16} Bacillomycin D and C_{17} Bacillomycin D. Such lipopeptide production might be the cause of antagonism against the fungal pathogens tested (Wu et al., 2019).

Bananas are the most produced and consumed fruit worldwide, but they require the most care after harvest. In addition, banana is highly susceptible to diseases like anthracnose, which is caused by *Colletotrichum musae*, the main post-harvest pathogen of bananas. Therefore, it is relevant to develop biocontrol strategies using bacterial antagonists against *C. musae*. Another antagonistic *Bacillus* strain was isolated from sisal plants grown in Brazil (among other rhizospheric and endophytic strains) and tested for its activity to protect banana (*Musa* spp.) fruits (Damasceno et al., 2019). The bacillus strain was characterized as *B. velesensis* BLE7. The results showed that BLE7 showed similar activities to thiabendazole. Since *B. velesensis* is not pathogenic to humans, authors suggest its use as a bacterial agent, which can be directly sprayed on to the fruit or by applying the fruit immersion technique (Damasceno et al., 2019). Table 1 shows recent studies describing the role of bacterial endophytes during pre- and post-harvest protection.

8. Application methods of bacterial endophytes

Once a potential and effective antagonist is identified or selected, it is necessary to look for a method by which it is effectively applied to control or suppress the desired phytopathogen. In general, microbial antagonists are applied in two different stages: pre-harvest application and post-harvest application (Singh & Sharma 2009). In many cases, pathogens infest fruits and vegetables in the field, and these latent infections become the biggest factor of decay during transportation and storage (Singh & Sharma 2009). The purpose of the application of biological control agents in pre-harvest stages is to prevent colonization on the surface of the fruit, so wounds made during the harvest step can be colonized by the antagonist before the pathogen (Ippolito and Nigro, 2000). However, after harvesting, since fruits and vegetables are perishable products, they suffer an accelerated process of aging and degradation, which can be accelerated by colonization of pathogens, characterized by a worsening of physical state, including dehydration, weight loss, wrinkling, color change or rotting, among other characteristics, in addition to the loss of organoleptic and nutritional properties due to the metabolic functions of the fruit/vegetable itself. Fig. 2 shows the methods to apply bacterial endophytes during pre- and post-harvest, as well as the objectives of each of the stages.

In a recent review, a number of studies were mentioned where antagonistic microbial agents were applied during pre-harvest stages, and also had a protective effect during post-harvest stages. For example, Silva and De Costa (2014) evaluated the pre-harvest application of *Burkholderia spinosa* strains to control various phytopathogens in banana fruits (*Musa* spp.). Likewise, other works also demonstrated great potential in the pre-harvest application of antagonists such as *Pantoea* (and other agents) to control post-harvest diseases caused by pathogens such as *Penicillium digitatum* and *Penicillium italicum*, which affect citrus fruits (Carmona-Hernandez et al., 2019). However, a subsequent application of these bacteria might be required later to improve protection during transportation and handling of the fruit. Therefore, immediately after harvesting, application of the bacterial agents is useful to control diseases during transportation and storage. In this method, various microbial strains are applied as aerosols or by immersion of fruits and vegetables in solutions prepared with the antagonist (Barkai-Golan, 2001; Irtwange, 2006). Hence, the antagonist is not exposed to the field conditions as is the case during pre-harvest application and it can be maintained under controlled conditions.

As mentioned earlier, an important factor for the effectiveness of controlling post-harvest diseases is the endophytic agent involved and it must continue to produce its antagonistic effect at low temperatures during storage (Stocco et al., 2019). Temperatures can vary, from 0 °C to 10 °C. Therefore, extending the shelf life of fruits and vegetables during the post-harvest periods is very important to increase the marketing period and obtain better prices in the market. The positive consumer response is restricted to products that meet certain quality requirements, such as, good visual appearance, good taste, adequate physical qualities, and resistance to cold storage and transportation (Mditshwa et al.,

Table 1
Examples of antagonism and biocontrol action against pre- and post-harvest pathogens by plant growth-promoting bacterial endophytes (PGPBE).

Period of protection	Biocontrol microorganisms	Target pathogen/disease	Potential mechanisms	Crop/Fruits application	Method of application	Reference
Pre-harvest protection	*Pseudomonas fluorescens* P142	Bacterial wilt by *Ralstonia solanacearum* (biovar 2, race 3)	Induction of plant systemic resistance	Tomato	Tomato seeds immersed in culture suspension	Elsayed et al. (2020)
	Pseudomonas sp. R4R21AP, T1R12P, T1R21, T4MS32AP, T4MS33	Bacterial canker by *Pseudomonas syringae* pv. *actinidiae* (Psa)	Antibiosis	Kiwifruit	Sprayed	Wicaksono et al. (2018)
	Pseudomonas protegens Pb3, Pb24, Pb71, Pb78, *Stenotrophomonas maltophilia* Sm25, Sm97, *Bacillus anthracis* Ba66, *B. pumilus* Bp108 *Serratia plymuthica* Sp15, *Pantoea Brenneri* Pb1	*Pseudomonas syringae* pv. Syringae, *Pseudomonas tolaasii*	Protease activity, HCN and siderophore production	Wild pistachio tree	*In vitro* essays	Etminani and Harighi (2018)
	Rhizobium, Pseudomonas, Bacillus, and Pantoea	Crown gall disease by *Agrobacterium tumefaciens*	Antibiosis	Peach	*In vitro* essays	Li et al. (2019)
	Bacillus subtilis GLB191 and *B. pumilus* GLB197	Downy mildew disease by *Plasmopara viticola*	Antibiosis, competition for space and nutrients	Grapevine	Sprayed	Zhang et al. (2017)
	Burkholderia stabilis PG159	*A. panax, B. cinerea, C. destructans, P. cactorum, Pythium sp., R. solani*	Antibiosis, induce different defense related genes	Ginseng	Dipping roots	Chowdhury and Bae (2018)
	Bacillus sp., *Burkholderia* sp.	*Sclerotinia sclerotiorum, Phomopsis sojae, Rhizoctonia solani.*	Antibiosis	Soybean	*In vitro* essays	de Almeida Lopes et al. (2018)
	Bacillus amyloliquefaciens LE109, *B. subtilis* LE24 and *B. tequilensis* PO80	Citrus canker by *Xanthomonas citri*	Antibiosis	Lime	Sprayed	Daungfu et al. (2019)
	Bacillus subtilis 7PJ-16	Mulberry fruit sclerotiniose pathogens	Antibiosis	Mulberry fruit	Sprayed	Xu et al. (2019)
	Biocontrol Bacterial endophyte	**Target pathogen/disease**	**Potential mechanisms**	**Crop/Fruits application**	**Method of application**	**Reference**
Post-harvest protection	*Lactobacillus plantarum* CM-3	Grey mold by *Botrytis cinerea*	Competition for nutrients and space	Strawberry	Cell suspensions inoculated on strawberry fruits	Chen et al. (2020)
	Bacillus Subtilis 10−4 *Bacillus Subtilis* 26D	Late blight by *Phytophthora infestans,* Fusarium wilt and dry rot by *Fusarium oxysporum*	Competition for nutrients and space	Potato	Immersing	Lastochkina et al. (2020)
	Pseudomonas synxantha DLS65	Postharvest brown rot by *Monilinia fructicola & Monilinia fructigena*	Competition for nutrients and space, production of diffusible toxic metabolites and VOCs	Stone fruit	Cell suspensions inoculated on peach fruits	Aiello et al. (2019)
	Bacillus amyloliquefaciens RS-25, *Bacillus licheniformis* MG-4, and *Bacillus subtilis* Pnf-4	*Botrytis cinerea*	Antibiosis, cellulase and protease activities, biofilm production	Tomato, strawberry, and grapefruit	Cell suspensions inoculated on fruits	Chen et al. (2019)
	Bacillus velesensis, Citrobacter freundii, Enterobacter asburiae_01, E. asburiae_02, E. cloacae, E. kobei, E. xiangfangensis, Pantoea dispersa, Stenotrophomonas maltophilia	Anthracnose by *Colletotrichum musae*	VOCs production and lytic enzymes activity	Banana	Sprayed	Damasceno et al. (2019)
	Bacillus sp. LYLB4	Pear ring rot disease by *Botryosphaeria dothidea.* Rhizopus soft rot by *Rhizopus stolonifer*	Antibiosis	Pear	Cell suspensions inoculated on pear fruits	Wu et al. (2019)
	Pantoea dispersa	Black rot by *Ceratocytis fimbriata*	Antibiosis	Sweet potato	Cell suspensions inoculated on tuberous roots	Jiang et al. (2019)
	Bacillus velesensis strains BUZ-14, I3 and I5	*Botrytis cinerea, Monilinia fructicola, M. laxa, Penicillium italicum, P. digitatum* and *P. expansum*	VOCs production	Grape, apricot, mandarin, apple	Cell suspensions inoculated on fruits	Calvo et al. (2020)
	Bacillus amyloliquefaciens NCPSJ7	Gray mold by *Botrytis cinerea*	Antibiosis and polyphenol oxidase, peroxidase, chitinase, and β-1,3-glucanase activities	Grapes	Cell suspensions inoculated on grapes	Zhou et al. (2020)

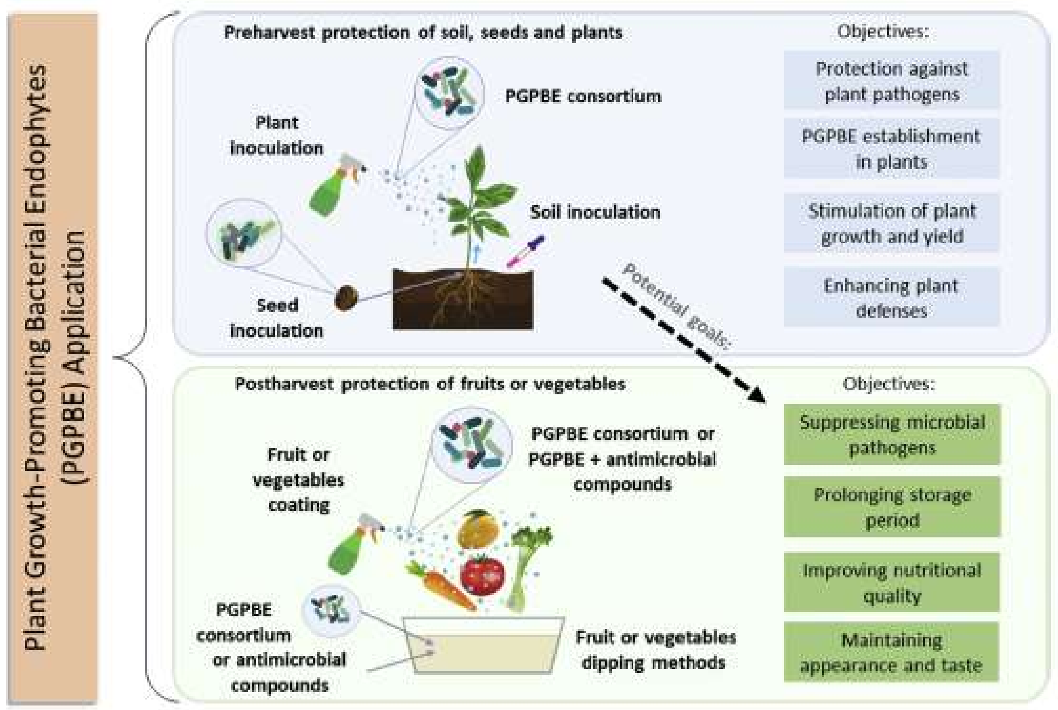

Fig. 2. Methods of application of bacterial endophytes during pre- and post-harvest stages. The objectives of each period are shown in the boxes on the right. During the pre-harvest inoculation stage, potential post-harvest protection could be generated for products such as fruits and vegetables, leading to protection against microbial pathogens, improved quality and nutritional aspects.

2017). For this regard, selecting endophytic isolates from endemic plants that inhabit areas near the poles, where low temperatures are present for much of the year, can be a viable option to find antagonistic agents with these intrinsic characteristics. Bacterial endophytes, as well as bacteria of another origin, such as rhizospheres, can withstand temperatures of 0 °C or less, without significantly decreasing their viability (Duan et al., 2013).

In some other cases, compounds produced by potential human pathogens, such as *Pseudomonas aeruginosa*, can be used in the biocontrol of plant pathogens (Sandani et al., 2019). However, additional considerations should be taken with this type of antagonist, since its use is riskier than other bacteria, which are harmless to humans.

Unfortunately, only a small number of the microbial antagonists reported in the literature to control postharvest diseases of fruits and vegetables (under laboratory conditions) are marketed. There could be several reasons for this, but two of the main barriers are as follows: the relative ineffectiveness of the antagonists compared to chemical control procedures, and the lack of economic incentives (Wilson and Wisniewski, 1989). However, once an effective antagonist is identified, a search for the methodology for its formulation, storage, and application is conducted. For example, the *Bacillus subtilis* B-3 strain was the first patented organism as a post-harvest biological control agent for stone fruits in the United States of America (Pusey and Wilson, 1984). Pusey et al. (1988) conducted a pilot test applying *Bacillus subtilis* under simulated commercial conditions for the control of brown rot in peaches, in which the bioagent was effectively incorporated into the wax normally used in packaging lines. *Botrytis* rot was effectively controlled by this procedure. Other commercial products have been developed, commercialized, and shown to be highly effective with respect to their bio-control activities. For example, "BioSave " has been developed from a saprophytic strain of *Pseudomonas syringae* by "EcoScience Corp., Orlando, USA, which is highly useful for controlling blue and gray mold in apples and pears (*Pyrus communis* L.) (Janisiewicz and Jeffers, 1997; Janisiewicz and Korsten, 2002).

In Mexico, the first biofungicide was developed by the Biotechnology Institutes of UNAM and CIATEJ (Galindo et al., 2013). This biofungicide is based on the bacteria *Bacillus subtilis* and can be applied in various crops, such as mango, avocado, papaya, lime, lemon, mandarin, orange, grapefruit, eggplant, chili, tomato, peel tomato, pumpkin, squash, squash, chayote, melon, cucumber, watermelon, strawberry, blueberry, blackberry and raspberry, during both pre- and post-harvest stages. It is applied as a powder containing *Bacillus* spores. The production of spores by some biofungicidal agents is an advantage over other non-sporulant species, since they can remain dormant until they find the right conditions to reproduce in the field.

9. Perspectives of the role of bacterial endophytes on sustainable agricultural production

Pathogens causing pre- and post-harvest diseases are attacked by agricultural producers with commercial chemicals to reduce their negative impacts. However, there is a worldwide trend towards the disuse of chemicals that can harm the health of the consumer, as well as, the farmer who applies these products to the crops (Seufert et al., 2012). Therefore, it is necessary to increase the options for pre- and post-harvest protection of crops with non-toxic products, which are friendly to the environment and also to animal and human health, such as the use of bacterial endophytes. Previously, we enlisted a series of advantages for the use of agrochemicals. Even combinations between antagonistic bacterial endophytes and other products could be a feasible option (Romanazzi et al., 2006, 2007).

Both endophytic and rhizospheric bacteria have attracted attention based on their activities to promote plant growth and biocontrol strategies. However, endophytic bacteria have certain advantages. By

entering the plant tissues, they protect themselves from the external environment, which represents less competition with the microbiota found in the rhizosphere. In addition, endophytes are efficiently transmitted to subsequent generations of the plant through the seeds (vertical transmission) providing the progeny with beneficial endosymbionts. In this regard, innovative strategies have been proposed to generate seeds with beneficial bacterial endophytes, which can act as bioprotectors of future diseases during plant growth in the field and during transmission in seeds and fruits (Mitter et al., 2017). This microbiome engineering strategy has high expectations to leave agrochemicals behind (Orozco-Mosqueda et al., 2018).

10. Conclusions

The green revolution resulted in two main advances; improvements in chemical inputs to plants, including pesticides, herbicides, and chemical fertilizers; and also improved crop plants through targeted breeding and genetic manipulation. However, such green revolution strategies have high environmental costs, mainly by the overuse of chemicals in agricultural systems (Backer et al., 2018). Therefore, a fresh green revolution or a new bio-revolution is needed to solve for previous environmental problems. Thus, endophytic antagonistic bacteria emerge as a potential strategy since bacterial endophytes have proven to be efficient agents for controlling crop diseases during pre- and post-harvest periods. Even when physical and chemical treatment strategies continue to be carried out, in certain cases in a combination with the application of compounds produced by bio-protective endophytes, with the intention of increasing their protective effectiveness against pathogens in fruits and vegetables, it is expected for the reduction, or ideally, the disuse of toxic chemicals. Finally, comprehensive mechanisms and a collaborative strategy between academia and industry are required to successfully achieve these goals in order to generate a long-term sustainable agriculture production.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2020.126612.

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Anexo II

Article

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).

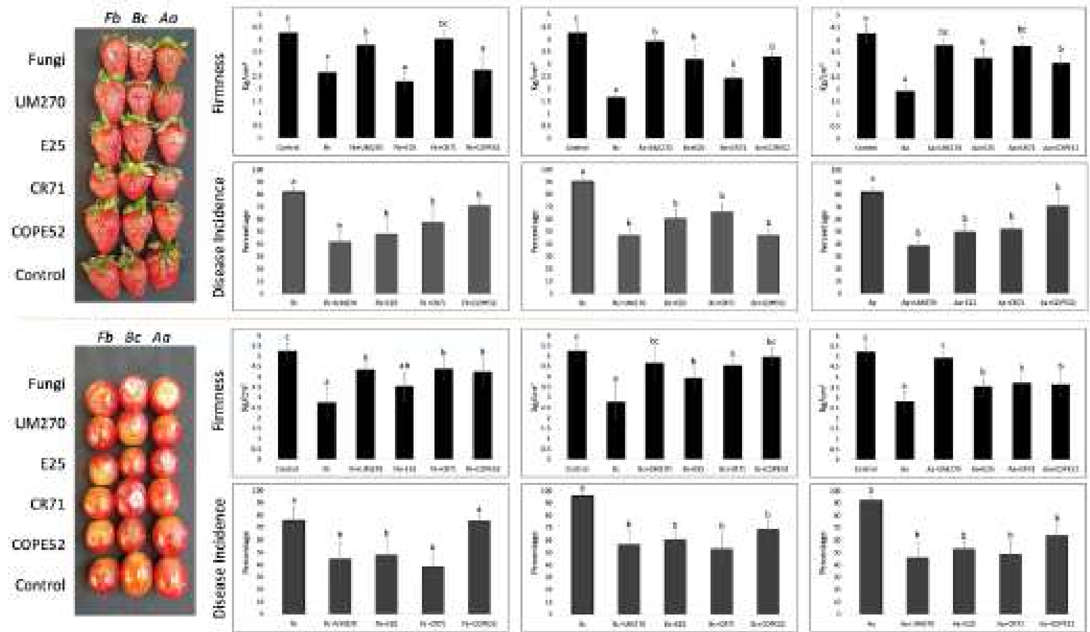

Figure 1. Biocontrol assay for strawberries and grapes. The top panel shows firmness and disease incidence for strawberries. The bottom panel shows firmness and disease incidence for grapes. The first column of the graphics shows results with *Fusarium brachygibbosum*, the second column with *Botrytis 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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