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Impacto de la rizobacteria *Pseudomonas fluorescens* UM270 en el crecimiento, producción y modulación del microbioma endofítico de *Zea mays* en un sistema milpa

TESIS

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Contenido

Resumen

La milpa, un policultivo agroecológico mesoamericano, ha sido reemplazada en gran parte por monocultivos de *Zea mays* híbrido, los cuales enfrentan limitaciones productivas debido a factores bióticos y abióticos. Una estrategia prometedora para mitigar estos problemas es el uso de bacterias promotoras del crecimiento vegetal (BPCV). En este estudio, se evaluó el impacto de la inoculación con *Pseudomonas fluorescens* UM270 en el crecimiento y producción de *Z. mays* dentro de un sistema milpa, asociado con *Phaseolus vulgaris* y *Cucurbita pepo*, además de su efecto en el microbioma endofítico radicular. Se llevaron a cabo experimentos donde *Z. mays* fue inoculado con la cepa UM270 en distintas configuraciones del sistema milpa, analizando parámetros fitométricos, producción de *Z. mays* y composición química del grano. Además, se realizó un análisis del microbioma endofítico mediante secuenciación de 16S rRNA e ITS en la plataforma Illumina. Los resultados mostraron que UM270 mejoró el crecimiento del *Z. mays*, aumentando la concentración de clorofila, altura, biomasa y desarrollo radicular. La combinación de UM270 con fertilizante fosfato diamónico (DAP) incrementó el peso de plantas y del grano en más del 40% en monocultivo y más del 50% en la milpa durante los ciclos agrícolas 2021 y 2023. También se observaron aumentos en los niveles de nitrógeno y fósforo en el grano, así como en potasio y calcio cuando se combinó con DAP. Además, la inoculación modificó el microbioma endofítico radicular. En monocultivo promovió la abundancia de *Burkholderia* y *Pseudomonas*, mientras que en la triada mesoamericana favoreció géneros como *Variovorax* y rizobios fijadores de nitrógeno. Estos hallazgos resaltan el potencial de *P. fluorescens* UM270 como una herramienta biotecnológica para mejorar el rendimiento de *Z. mays* en la milpa, optimizar su microbioma y beneficiar a cultivos asociados, promoviendo la sostenibilidad agrícola.

Palabras clave: biofertilizante, promoción vegetal, sustentable, policultivos, abundancia relativa, bioinformática.

Abstract

The milpa, a Mesoamerican agroecological polyculture, has largely been replaced by monocultures of hybrid *Zea mays*, which face productivity limitations due to biotic and abiotic factors. A promising strategy to mitigate these issues is the use of plant growth-promoting bacteria (PGPB). This study evaluated the impact of inoculation with *Pseudomonas fluorescens* UM270 on the growth and production of *Z. mays* within a milpa system, associated with *Phaseolus vulgaris* and *Cucurbita pepo*, as well as its effect on the root endophytic microbiome. Experiments were conducted in which *Z. mays* was inoculated with the UM270 strain in different milpa configurations, analyzing phytometric parameters, *Z. mays* production, and grain chemical composition. Additionally, an endophytic microbiome analysis was performed using 16S rRNA and ITS sequencing on the Illumina platform. The results showed that UM270 improved *Z. mays* growth by increasing chlorophyll concentration, height, biomass, and root development. The combination of UM270 with diammonium phosphate (DAP) fertilizer increased plant and grain weight by over 40% in monoculture and over 50% in the milpa during the 2021 and 2023 agricultural cycles. Nitrogen and phosphorus levels in *Zea mays* grains also increased, as did potassium and calcium concentrations when combined with DAP. Furthermore, inoculation altered the root endophytic microbiome. In monoculture, it promoted the abundance of *Burkholderia* and *Pseudomonas*, while in the Mesoamerican triad, it favored genera such as *Variovorax* and nitrogen-fixing rhizobia. These findings highlight the potential of *P. fluorescens* UM270 as a biotechnological tool to enhance *Z. mays* yield in the milpa, optimize its microbiome, and benefit associated crops, promoting agricultural sustainability.

I. Introducción

El incremento constante de la población y las malas prácticas agrícolas empleadas en los últimos años, aunado al incremento en el cambio climático, han puesto en riesgo la seguridad alimentaria mundial (Huerta Sobalvarro *et al*. 2018). Hoy en día el sector agrícola se enfrenta a uno de los retos más importantes que es resguardar la seguridad alimentaria al menor costo económico y ecológico posibles, sin embargo, las técnicas actuales utilizadas en el campo como el uso de semillas híbridas en grandes extensiones de monocultivo bajo el uso de maquinaria pesada acompañadas de aplicaciones excesivas de agroquímicos han resultado poco efectivas, porque aun cuando en un inicio el incremento en la producción era notable, al pasar los años se pudieron determinar mediante diversas investigaciones los daños ocasionados a la población humana y a los recursos naturales, dentro de los que se encuentran efectos negativos a la salud de los seres vivos, pérdida de semillas criollas, resistencia a plagas y enfermedades, contaminación de suelo y agua, disminución de la fertilidad del suelo, entre otros (Loucks 2021; Zhang *et al*. 2024).

Por tal motivo, es necesario rediseñar los sistemas de cultivo basando su establecimiento en prácticas sustentables. El sistema tradicional llamado milpa es un ejemplo de ello al ser uno de los modelos más antiguos basado en la sustentabilidad, el cual se caracteriza por integrar policultivos vegetales que se pueden adaptar con facilidad a diferentes tipos de regiones. Sin embargo, el deterioro en el que se encuentran los suelos requiere del uso de ciertas técnicas nuevas que mejoren sus características. Una alternativa es el uso de microorganismo promotores de crecimiento vegetal (MPCV), los cuales han probado su eficacia al mejorar las condiciones de suelos contaminados y además de incrementar la producción de los cultivos (Etesami y Maheshwari 2018; Martínez-Pérez *et al*. 2020).

Dentro de este grupo de microorganismos, se encuentran las rizobacterias promotoras de crecimiento vegetal (RPCV), que se caracterizan por ser bacterias de vida libre, colonizan las raíces de las plantas y promueven su crecimiento mediante diferentes mecanismos de acción como la solubilización de fosfatos,

producción de hormonas, fijación de nitrógeno, además de brindar protección ante patógenos, incrementar la tolerancia a estrés hídrico o salino y participar en la remediación de suelos contaminados con metales pesados. Gracias a su aplicación se ha incrementado la producción de cereales, hortalizas y frutales. Dentro de las bacterias con mayor impacto en el sector agrícola se encuentran *Azospirillum*, *Azotobacter*, *Rhizobium, Bradyrhizobium* y *Bacillus*, éste último gracias a su capacidad de formación de esporas, lo que implica la formación de estructuras capaces de soportar diversas condiciones del ambiente y contribuye significativamente en las formulaciones comerciales (Backer *et al*. 2018; Aloo *et al*. 2022; Gohil *et al*. 2022).

Otro de los géneros perteneciente a las RPCV de gran interés agrícola es *Pseudomonas*, y dentro de este género encuentra la cepa *P. fluorescens* UM270, la cual ha sido previamente caracterizada y evaluada bajo condiciones *in vitro*, comprobando su eficiencia en el control de patógenos y promoción de crecimiento vegetal , así como su influencia en el microbioma rizósferico en diferentes tipos de suelo (Hernández-León *et al*. 2015; Hernández-Salmerón *et al*. 2016; Rojas-Solis *et al*. 2020; Santoyo *et al*. 2024). Sin embargo, se desconoce el efecto de *P. fluorescens* UM270 en el sistema milpa y en el microbioma endófito de las plantas en las cuales ha sido inoculada. Con el conjunto de datos recopilados en el presente trabajo se podrá determinar si esta cepa puede ser candidata potencial para ser un bioinoculante y podría dar la pauta a futuras investigaciones enfocadas en las interacciones ecológicas que existen en el sistema milpa.

II. Antecedentes

2.1 Modelo milpa

La milpa es un sistema de origen prehispánico de importancia social, cultural, económica y ecológica, además de que es la base de la alimentación y la cultura mexicana. Éste se caracteriza por ser un sistema sustentable de temporal donde no se utiliza sistema de riego (la producción depende exclusivamente de la lluvia para su desarrollo), y la labranza es mínima o nula. El cultivo núcleo de este sistema es *Z. mays*, y dentro de los cultivos asociados se encuentra el *P. vulgaris* y *C. pepo* que en conjunto son denominados “triada mesoaméricana”, dado que su integración surgió en Mesoamérica (Montes de Oca y Licea 2008; Romero-Natale *et al*. 2024).

Sin embargo, las especies vegetales asociadas a *Z. mays* varían dependiendo de la región donde son establecidos, los pobladores seleccionan los cultivos que integraran las milpas con base a las diferentes condiciones climáticas y tipos de suelo. Dentro de los cultivos que han formado parte de las milpas se encuentran chile *(Capsicum annuum),* haba (*Vicia faba*), quelites (*Amaranthus spp*)., lenteja (*Lens culinaris*), jamaica (*Hibiscus sabdariffa*), soja (*Glycine max*), chícharo (*Pisum sativum*), entre otros (Méndez-Flores *et al*. 2023). En las últimas décadas se han integrado los huertos de frutales al sistema milpa al asociarse con diferentes especies como leguminosas y cereales, modelo conocido como sistema milpa intercalada con árboles frutales (MIAF) (Cadena Iñiguez *et al*. 2018; Regalado-López *et al*. 2020).

El impacto de los sistemas milpa engloba diferentes sectores como el social y cultural, dentro del establecimiento de las milpas los pobladores realizan diferentes rituales con la finalidad de asegurar la abundancia de su cosecha, lo que ha convertido a las milpas en un espacio de encuentro social donde las familias se reúnen para trabajar, compartir y celebrar. En el ámbito económico impacta gracias a la variedad de productos que de estos sistemas se obtiene, y dentro del área ecológica las milpas presentan grandes beneficios por el conjunto de interacciones ecológicas que dentro del mismo sistema se establecen y por lo tanto participan en el equilibrio del ecosistema (Figura 1) (Vásquez González *et al*. 2018; Leyva-Trinidad *et al*. 2020).

La interacción planta-planta puede ser simbiótica o coopoerativa, un ejemplo de ello es el establecimiento de la triada mesoamericana, donde *Z. mays* sirve como soporte para el cultivo de *P. vulgaris*, que por su parte incrementa la obtención de nitrógeno a *Z. mays* y *C. pepo*, ésta última gracias a su hábito de crecimiento rastrero evita la presencia de malezas y reduce la perdida de humedad del suelo (Terán Contreras y Rasmussen 2009; Sánchez Morales y Romero Arenas 2017).

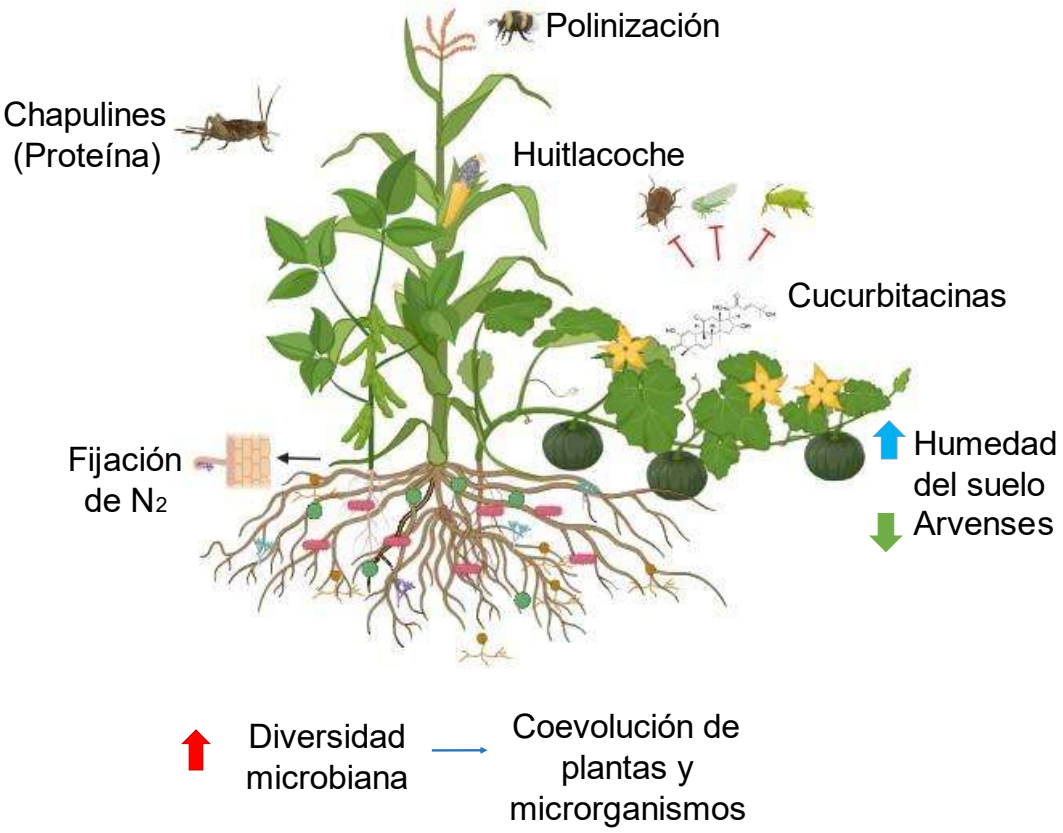


Figura 1. Interacciones ecológicas en el modelo milpa.

Las interacciones planta-insecto son de vital importancia ecológica, así por ejemplo, los insectos polinizadores son directamente beneficiados al tener gran variedad de cultivos a su alcance creando una interacción benéfica durante el proceso de alimentación y polinización (Abbas *et al*. 2022). Otras ventajas alimenticias que aporta esta interacción es la obtención de fuentes proteicas provenientes de insectos como los chapulines (*Sphenarium purpurascens*) que

forman parte de la dieta mexicana (Marín-Morales *et al*. 2022). Por su parte la *C. pepo* emite compuestos alelopáticos llamados cucurbitacinas que alejan insectos plaga y funge como biopesticida (Montesano *et al*. 2018; Bruno *et al*. 2023).

Dentro de la interacción planta-microorganismo se encuentra la asociación *Z. mays* con el hongo comestible huitlacoche *(Ustilago maydis)* que en la industria culinaria es altamente demandado por su peculiar sabor (Macuil Tlachino *et al*. 2021; Yu *et al.* 2023). En el ámbito ecológico la interacción entre las plantas y los microorganismos ha sido clave en su coevolución favoreciendo directamente el incremento en la diversidad microbiana. En los sistemas milpa al estar presentes diferentes especies de plantas existe mayor atracción de microorganimos mediante la exudación radicular, sin embargo, la diversidad y abundancia de microorganismos presentes en la rizósfera o endosfera dependerá de la edad y genotipo de la planta, de las condiciones climáticas, tipos de suelo y prácticas agrícolas (Canarini *et al*. 2019; Chamkhi *et al*. 2021).

Sin embargo, hoy en día los suelos destinados al establecimiento de las milpas han sido reemplazados por grandes extensiones de monocultivos que usan altas dosis de agroquímicos de los que se conoce su efecto tóxico para los seres vivos. Otra de las causas es el abandono de las superficies destinadas a las milpas por la falta de la fertilidad del suelo y las variaciones climáticas que afectan a los cultivos, generando repercusiones desde el ámbito social hasta el económico (Camacho 2017; Santiago Vera *et al*. 2021).

La recuperación de áreas de cultivo requiere integrar diferentes prácticas de cultivo basadas en la sustentabilidad, una de las técnicas implementadas desde hace algunas décadas es el uso de MPCV, que han logrado mitigar los efectos ocasionados en suelos salinos y contaminados con metales pesados como cadmio, cobre, etc., además de mejorar la salud de las plantas mediante diferentes mecanismos y por ende incrementar la producción agrícola (Hernández-Montiel *et al*. 2017; Gopi *et al*. 2020; Ibarra-Villarreal *et al*. 2021).

Dentro de los MPCV se encuentran los hongos micorrízicos, bacterias rizósfericas y bacterias endófitas. Estos microorganismos se han usado para la protección de plantas ante la presencia de patógenos y como promotores de

crecimiento vegetal, destacando géneros como *Trichoderma, Azospirillum, Penicillium, Bacillus, Burkholderia, Pseudomonas*, etc., (Agbodjato *et al*. 2021; de Almeida *et al*. 2021; Galeano *et al*. 2023).

2.2 Bacterias promotoras de crecimiento vegetal

La relación entre las plantas y las bacterias ha existido desde el inicio de la evolución de las plantas, sin embargo, los efectos entre dicha relación pueden ser positivos, negativos o neutros. En los últimos años ha otorgado gran importancia a dilucidar la relación positiva entre las plantas y las bacterias, a estas se les conoce como bacterias promotoras de crecimiento vegetal (BPCV) (Larsen *et al*. 2015; Ajijah *et al*. 2023).

Las plantas exudan diferentes sustancias que sirven como quimioatrayentes para las bacterias dentro de las que se encuentran azúcares, aminoácidos, ácidos aromáticos, ácidos alifáticos, ácidos grasos, esteroles, fenoles, metabolitos secundarios, proteínas y enzimas (Molina-Romero *et al.* 2015; Balyan y Pandey 2024). La composición del exudado cambia a medida que la planta se desarrolla o responde a estímulos exógenos, así como al genotipo de la planta, y en conjunto determinan el microbioma de la zona rizósferica (Vranova *et al*. 2013; Lyu y Smith 2022).

Una vez que se lleva a cabo el proceso de atracción por quimioatrayentes, las bacterias se adhieren a las plantas y algunas de ellas penetran a la planta a través de los estomas, heridas, áreas de emergencia de raíces laterales, nódulos, fisuras y mediante la producción de enzimas hidrolíticas capaces de degradar la pared celular de los vegetales, y logran colonizar el interior de la planta (Santoyo *et al*. 2016).

Las bacterias que promueven el crecimiento vegetal lo hacen mediante dos tipos de mecanismos, los directos que están enfocados en la nutrición de la planta y producción de moléculas señal y los indirectos que se enfocan en el biocontrol de patógenos (Molina-Romero *et al*. 2015). Los mecanismos directos incluyen factores relacionados con la promoción del crecimiento vegetal como la producción de hormonas (auxinas, citocininas, etileno, ácido abscísico y giberelinas),

biosolubilización de fosfatos, reducción de hierro y adquisición de nutrientes esenciales (Olanrewaju *et al*. 2017; Ali *et al*. 2024). Los mecanismos indirectos incluyen los mecanismos de biocontrol (antibióticos, sideróforos, enzimas líticas, producción de metabolitos), emisión de compuestos orgánicos volátiles, resistencia sistémica inducida, entre otros (Massawe et al. 2018; Álvarez-García *et al.* 2020).

2.3. *P. fluorescens*

Las bacterias del género *Pseudomonas* habitan en una amplia variedad de ambientes, lo cual es el reflejo de su diversa capacidad metabólica, lo que les ha permitido adaptarse a condiciones variables del ambiente. Así mismo, dicho género se considera ambivalente (puede generar efectos positivos o negativos), debido a que algunas especies establecen relaciones benéficas con las plantas y otras patogénicas con plantas, animales y humanos (Sánchez Cariillo y Guerra Ramírez 2022).

En el sector agrícola se ha comprobado la eficacia de algunos miembros de este género en la promoción de cultivos mantenidos bajo condiciones adversas; por ejemplo se ha comprobado que *P. koreensis* MG209738 inhibe al patógeno *Cephalosporium maydis* que afecta a *Z. mays* en campo, también se ha evaluado su efecto en el biocontrol de *Rhizoctonia solani* otro de los patógenos que afecta negativamente a los cultivos (Rana *et al*. 2019; Ghazy y El-Nahrawy 2021)**.** Además se ha comprobado su efecto positivo en cultivos de *Z. mays* bajo estrés por sequía en campo, ya que se demostró mitiga éste estrés y aumenta la concentración de clorofila en las plantas (Mubeen *et al*. 2021).

2.4 Aislamiento y caracterización genómica de la cepa *P. fluorescens* UM270

El aislamiento de BPCV provenientes de ambientes extremos o suelos rizósfericos, ha contribuido de manera significativa a la formulación de bioinoculantes que han tenido impacto positivo en el incremento de la producción agrícola mediante mecanismos directos e indirectos (Sharma *et al.* 2021; Waheed *et al*. 2024).

Además, con el inicio de la era genómica se ha podido examinar un genoma completo de cualquier cepa BPCV con ello conocer los metabolitos de importancia ecológica, así como destacar los genes que codifican enzimas beneficiosas, involucradas, por ejemplo, en la absorción nutricional de las plantas y en la modulación de hormonas, así como definir que grupos de genes biosintéticos codifican antimicrobianos. Con los análisis genómicos se puede determinar si las BPCV tienen potencial para convertirse en un bioinoculante (Flores *et al.* 2020; Petrillo *et al*. 2021).

De tal manera la cepa UM270 de *P. fluorescens* fue aislada de suelo rizósferico de plantas de *Medicago truncatula,* en un campo agrícola ubicado en la ciudad de Morelia, Michoacán. La cepa UM270 es una bacteria Gram negativa, no esporulante, móvil, con forma de bastón, considerada mesófila, que crece mejor en un pH de 6-8.5, siendo el óptimo de 7-8 y a una temperatura de 28°C (Hernández-León *et al*. 2015; Hernández-Salmerón *et al*. 2016).

Dentro del estudio de la minería de datos, se realizó el agrupamiento jerárquico de la cepa UM270 basado en FastANI (herramienta de software para el cálculo de la identidad promedio de nucleótidos), se incluyeron un total de 155 secuencias genómicas descargadas de la base de datos del NCBI RefSeq (Secuencias de Referencia del Centro Nacional de Información Biotecnológica) (Li *et al*. 2021). Este conjunto de datos incluyó 133 representantes de cada una de las especies de *Pseudomonas* y 22 genomas de *P. fluorescens*, del conjunto de éstos genomas se analizó su distancia genómica con la cepa *P. fluorescens* UM270, mediante comparaciones por pares con los genomas utilizando FastANI v1.32 (Jain *et al*. 2017), que implementa los cálculos de ANI.

Una vez realizadas todas las comparaciones por pares, se generó un árbol de agrupamiento jerárquico (Figura 2) basado en las distancias obtenidas con FastANI (Hernández-Salmerón y Moreno-Hagelsieb 2022), empleando las funciones hclust y el método divisivo (diana), implementados en el programa R Core Team (2021). El árbol representativo fue graficado en iTOL (árbol de la vida interactivo) (Letunic and Bork 2021).

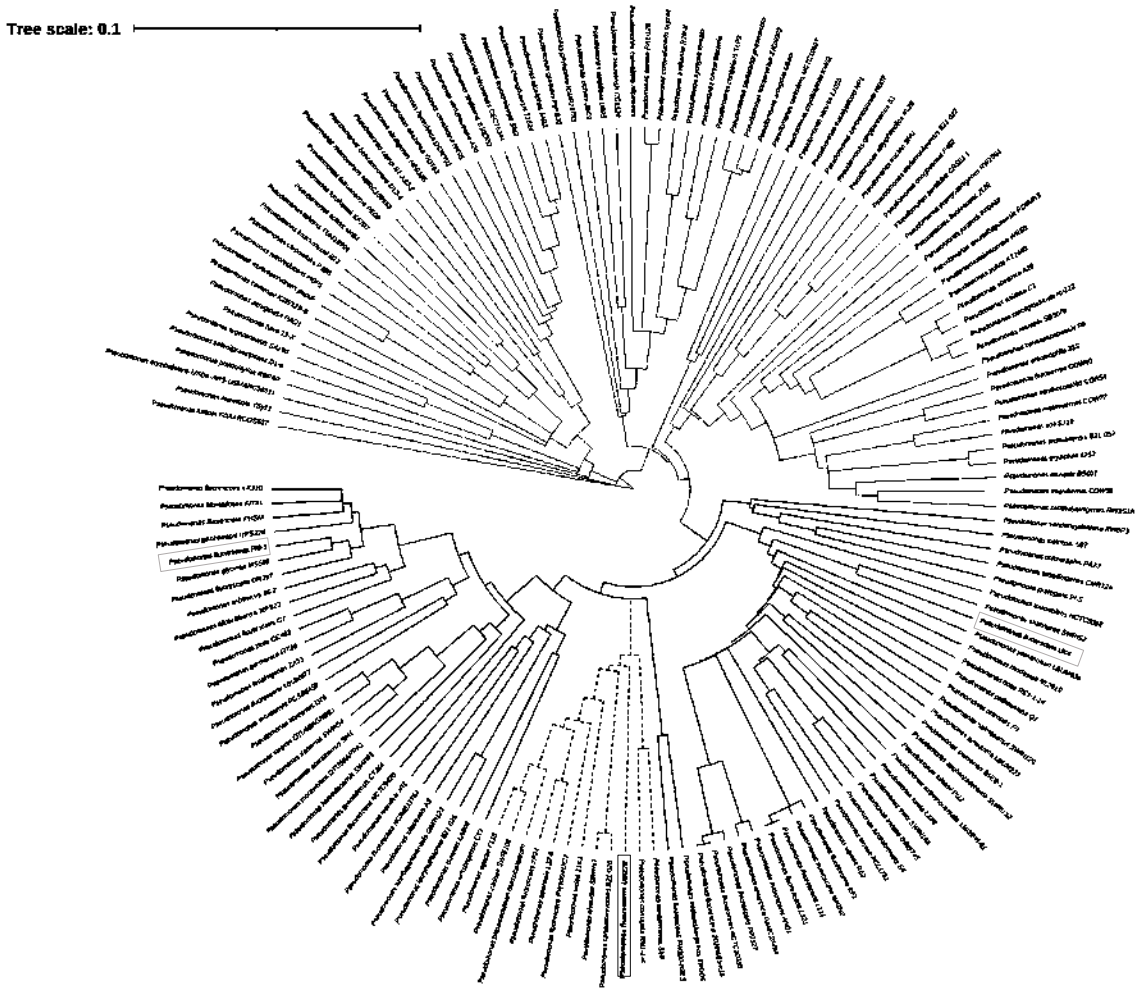


Figura 2. Agrupamiento jerárquico basado en FastANI que muestra las distancias genómicas entre representantes de todas las especies de *Pseudomonas*. Las cepas de tipo *P. fluorescens* están resaltadas en gris. La cepa UM270 está resaltada en negritas (Santoyo *et al*., 2025).

Su genoma contiene 5509 genes, de los que 5396 codifican para proteínas (97% de su genoma es codificante). Dentro de los genes encontrados hay involucrados en el metabolismo de carbohidratos, proteínas, crecimiento y división celular, así como un pequeño grupo seleccionados con las actividades de colonización y supervivencia en el ambiente del suelo. También se han comparado los genes de *P. fluorescens* UM270 con 7 genomas completos secuenciados para especies de *Pseudomonas*, en este caso se encontraron 599 únicos en dicha cepa,

dentro de los que se encuentran involucrados con la síntesis de ácido indol acético y ácido fenilacético, señalización, colonización y competencia de la rizósfera (Hernández-Salmerón *et al*. 2016, 2017).

2.4.1 Actividad antifúngica de *P. fluorescens* UM270 en ensayos *in vitro* de biocontrol

Una de las ventajas de la aplicación de las BPCV en la agricultura, es que disminuyen la severidad e incidencia de los microorganismos patógenos que afectan a los cultivos dentro de los que se encuentran *Cephalosporium maydis* y *Rhizoctonia solani* que ocasionan pérdidas en la producción de cultivos de *Z. mays* (Rana *et al*. 2019; Ghazy y El-Nahrawy 2021).

La cepa UM270 produce diferentes compuestos que participan en la inhibición de microorganismos patógenos dentro de la producción de compuestos volátiles que emite esta cepa se encuentran metanotiol, dimetilsulfuro, tiazol, tialocetato de metillo y trisulfuro de dimetilo que contienen metabolitos azufrados y de los que se conoce su efecto antagonista de patógenos, dentro de los mecanismos difusibles se encuentra la producción de sideróforos, 2-4-diacetilfloroglucinol y cianuro de hidrogeno (Figura 3) (Hernández-León *et al*. 2015).

Hernández León et al., 2015., evaluaron los efectos antifúngicos de la cepa UM270, mediante la producción de compuestos difusibles y volátiles en un sistema dual. La inoculación de la cepa UM270 inhibió en un 86% y 53% al patógeno *B. cinérea*, además de evaluar el efecto de su inoculación en plantas de *Medicago truncatula in vitro* expuestas ante el patógeno *B. cinerea*, se determinó que con la inoculación de la cepa disminuyeron los síntomas y la necrosis de la raíz de las plantas de *M. truncatula*.

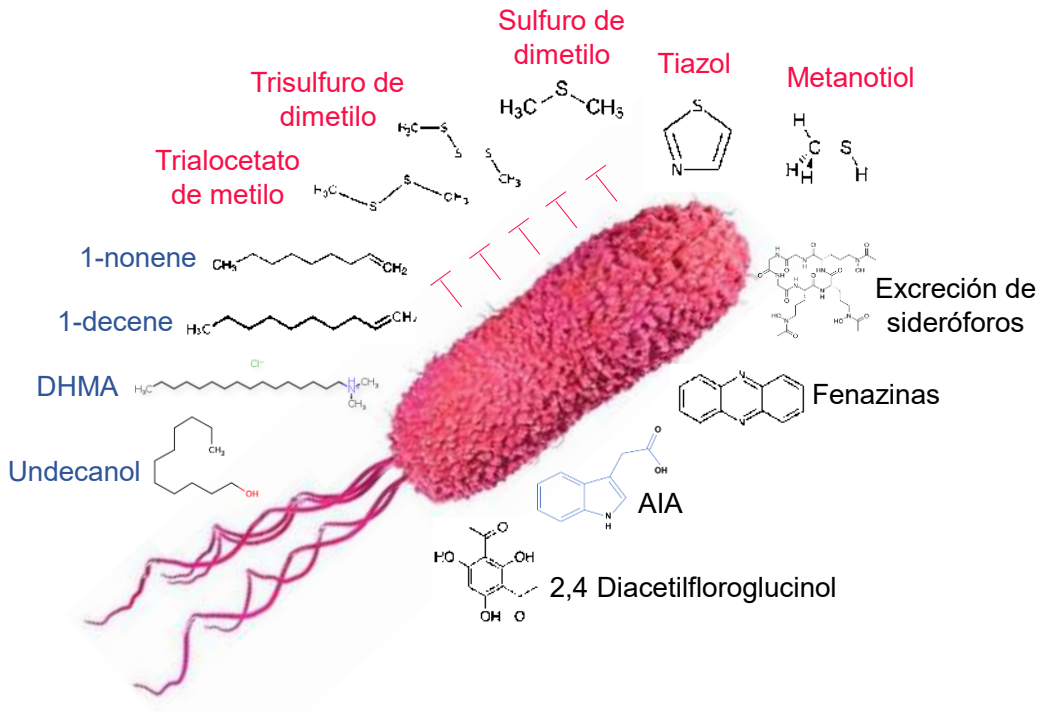


Figura 3. Compuestos identificados y reportados en la cepa *P. fluorescens* UM270 (Hernández-León *et al*. 2015).

En otro experimento enfocado en el biocontrol se ha evaluado el efecto antagónico de la cepa UM270 hacia los hongos fitopatógenos *B. cinerea, Fusarium oxysporum, F. solani* y *R. solani*., y los resultados muestran que la cepa UM270 inhibió el crecimiento del micelio de *B. cinerea* (45%), *F. solani* (25%) y *R. solani* (24%) en diferentes porcentajes, mientras que en *F. oxysporum* (1%) no hubo inhibición significativa. También se determinó la expresión de los genes *phlD* y *hcnC* en la cepa UM270 en presencia de los fitopatógenos durante bioensayos de antagonismo *in vitro*. Se determinó que los patógenos modulan la expresión del gen *phlD* diferencialmente, ya que mientras *B. cinerea* induce su expresión, los demás patógenos la reprimen. En el caso de la expresión del gen *hcnC*, *B. cinerea* y *F. oxysporum* no afectaron su expresión, mientras que *F. solani* y *R. solani* la inhibieron. Estos resultados sugieren que los fitopatógenos pueden modular la expresión de genes importantes para la síntesis de compuestos antimicrobianos en *P. fluoresces* UM270 (Hernández-Salmerón *et al*. 2018).

Asimismo, la cepa UM270 se ha evaluado en el control de hongos en poscosecha, cuyos resultados mostraron un efecto por encima del 35% sobre los hongos *Botrytis* sp., *B. cinérea*, *Geotrichum candidum*, *Cladosporum* sp., *G. phurueaensis, F. brachygibbosum*, *Penicillium crustoum*, *P. expansum, Alternaria alternata* y *Alternaria* sp. De los que se seleccionaron los hongos *F. brachygibbosum*, *B. cinerea* y *A. alternata* para evaluar su efecto sobre frutos de fresa (*Fragaria L*) y uva (*Vitis vinífera*) previamente inoculados con la cepa UM270, en frutos de *Fragaria* L., se logró inhibir en un 60, 55 y 65% la incidencia de cada uno de los hongos respectivamente y en el caso de *V. vinífera* disminuyó la incidencia en el hongo *F. brachygibosum* (Morales-Cedeño *et al*. 2023).

Otro de los análisis realizados con la cepa UM270 fue la determinación de la inducción de la expresión génica de *T. atroviride* codificando proteínas efectoras durante la interacción con la cepa UM270, inoculados en *Arabidopsis* y en presencia del fitopatógeno *F. brachygibbosum,* se comprobó que el consorció de *T. atroviride* con UM270 mejoró el crecimiento de *Arabidopsis,* además de incrementar la expresión génica relativa de los efectos de *Trichoderma* en interacción micoparasitaria a los 5 y 7 días después de ser inoculado *Trichoderma*, UM270 y *Fusarium* (Guzmán-Guzmán *et al*. 2024).

2.4.2 Efecto de *P. fluorescens* en la promoción de crecimiento vegetal en ensayos *in vitro*

Uno de los pasos para la selección de las BPCV es el establecimiento de cultivos *in vitro* en interacción con los inóculos y con ellos se puede realizar la selección de aquella BPCV con mejores efectos de promoción. Por ello, se evaluaron plantas de *M. truncatula* en condiciones *in vitro,* inoculadas con la cepa UM270. En el experimento se determinó que mediante los mecanismos directos e indirectos, la cepa UM270 promovió el crecimiento de la planta y de la raíz, e incrementó la concentración de clorofila (Hernández-León *et al*. 2015).

2.4.3 Interacción sinérgica de *P. fluorescens* UM270 con otras BPCV en condiciones controladas

Evaluar el efecto sinérgico entre cepas promotoras de crecimiento vegetal es de vital importancia para determinar la eficacia de los biofertilizantes que son formulados en base a consorcios bacterianos. Se ha comprobado que mediante la aplicación de consorcios se puede incrementar el efecto entre cepas y por ende, mejorar la producción de cultivos (Kaushal *et al*. 2023).

En un experimento *in vitro* se inoculó la cepa *P. fluorescens* UM270 en consorcio con *B. thuringiensis* UM96 para evaluar su antagonismo, sin embargo, se observó un efecto sinérgico. Posteriormente se evaluaron los efectos de la inoculación de ambas cepas individualmente y en consorcio en tomate de cáscara (*Physalis ixocarpa Brox*. ex Horm), por su parte la inoculación con la cepa UM270 en semillas de *P. ixocarpa Brox*. ex Horm, incrementó la longitud de hipocótilo y raíz, así como el peso fresco total de las plántulas, por su parte con la inoculación individual de la cepa UM96 únicamente presento efectos de promoción en la longitud de raíz, cabe mencionar que la cepa UM96 no se ha reportado con efectos de promoción vegetal, solo actividad que limita el crecimiento de patógenos y por ende puede tener efecto indirecto. En cuanto al consocio de la cepa UM270 y UM96, no hubo un efecto de promoción en las plántulas de *P. ixocarpa Brox*. ex Horm (Rojas-Solis *et al*. 2016).

2.4.4 Respuesta de *P. fluorescnes* UM270 bajo condiciones de estrés salino

La salinidad causa un desequilibrio iónico que obstaculiza la absorción de agua, afectando la fotosíntesis y otros procesos metabólicos, lo que en última instancia da como resultado una proporción inferior de las semillas germinadas y un retraso en el crecimiento de las plantas, afectando directamente la producción de los cultivos y a los microbiomas asociados a los cultivos (Nishu *et al.* 2022; Zahra *et al*. 2024).

Las cardiolipinas (CL) son fosfolípidos de membrana esenciales para la adaptación bacteriana a diversas condiciones ambientales incluido el estrés salino. En un experimento con mutantes de deleción de los genes *clsA* y *clsB* en la cepa

UM270, se observó que ambas mutaciones redujeron significativamente la síntesis de CL (58% y 53%, respectivamente). Aunque la disminución de CL afectó ligeramente el crecimiento celular en condiciones salinas, no fue crítica para la supervivencia. También se evaluó la promoción del crecimiento en plantas de *S. lycopersicum*, las cepas mutantes mostraron reducciones en la producción de ácido indol-3-acético (AIA), pero mantuvieron la excreción de sideróforos y aumentaron la formación de biopelículas, incluso bajo estrés salino. Estos resultados subrayan el papel de las CL en la adaptación y la función promotora de UM270, aunque no son indispensables en condiciones extremas (Rojas-Solis *et al*. 2023b).

2.4.5 Adaptación de *P. fluorescens* UM270 a condiciones de estrés inducidas por metales pesados

En la actualidad, existe un deterioro significativo en las tierras agrícolas, dentro de las causas se encuentra la contaminación por metales pesados, lo que limita la cantidad de suelo destinado para la agricultura. Uno de los elementos que se encuentra mayoritariamente como contaminante es el arsénico que ha alcanzado valores nocivos en diferentes áreas. Una de las alternativas que hoy en día se emplean para contrarrestar los efectos tóxicos de algunos metales pesados es el establecimiento de plantas y microorganismos asociados que sirvan para remediar y mejorar las características de los suelos (Guarino *et al*. 2020; Poria *et al.* 2022). En pastos (*Cynodon dactylon* y *Eleusine indica*), se ha comprobado que el uso de bacterias endófitas *Jeotgalicoccus huakuii* y *B. amyloliquefaciens* mejoran la producción de biomasa del pasto y mejoran la bioacumulación de mercurio, por ende surgen como una alternativa eficaz en temas de fitorremediación (Ustiatik *et al*. 2022).

Se evaluó el crecimiento de la cepa UM270 en medio Luria Bertani suplementado con sales de arsénico y mercurio, bajo la presencia de ambos metales se mantuvo la producción de AIA, solubilización de fosfatos, secreción de sideróforos y la formación de biopelículas. Con el uso de las sales se modificaron la mezcla de los compuestos volátiles como 2-butanona, 2-3-butanediol, dimetilsulfuro, etc., que han sido reportados como promotores del crecimiento

vegetal. También se inocularon plantas de *Z. mays* con las cepas UM270 y *B. Paralicheniformis* ZAP17 y adicionadas con sales de arsénico y mercurio, en consorcio las cepas UM270 y ZAP17 la biomasa vegetal y longitud del brote de *Z. mays* , con ello se determina su importancia en la promoción de crecimiento vegetal en suelos con metales pesados (Rojas-Solis *et al*. 2023a).

2.4.6 Efecto de *P. fluorescens* UM270 sobre el crecimiento y el microbioma rizosférico de *Z. mays*, así como su impacto en *Vaccinum* en invernadero

El microbioma juega un papel primordial en la agricultura sostenible al realizar múltiples actividades que promueven el crecimiento de las plantas, incluyendo la fijación, mineralización, solubilización y movilización de nutrientes, producción de sideróforos, etc. (Suman *et al.* 2022b).

La inoculación la cepa UM270 en plantas de *Z. mays* sembradas bajo condiciones controladas, en diferentes tipos de suelo (arcilloso, franco arenoso y franco), provenientes de Huiramba y Uruapan Michoacán y Yuriria Guanajuato., confirmo el efecto en la promoción en *Z. mays* al incrementar el peso de raíces y brotes, la concentración de clorofila y biomasa total de las plantas, además de modular el microbioma rizósferico al incrementar las poblaciones de proteobacteria y acidobacteria y disminuir actinobacteria y bacteroidetes (Santoyo *et al.* 2024).

Asimismo, bajo condiciones de invernadero se ha evaluado el efecto de la inoculación de la cepa UM270 en plantas de arándano (*Vaccinium* sp., var Bilox), bajo diferentes tratamientos con la adición de dos fertilizantes de lenta liberación el nitrofosfato y el basacote (16 unidades de nitrógeno, 8 unidades fósforo, 12 unidades de potasio, y 8 unidades de magnesio), se determinó que al inocular la cepa UM270 en adición con nitrofosfato aumentó el peso seco de la planta, mientras que con basacote disminuyó el peso seco de la raíz. Con estos resultados se determinó que el efecto de la cepa al estar en contacto con diferentes fertilizantes diferencial y que esto repercute positiva o negativamente en los mecanismos de promoción del crecimiento de la cepa UM270 (Cortes-Solis *et al*. 2023).

2.4.7 Inoculación de *P. fluorescens* UM270 en condiciones de campo

La eficiencia de los bioinoculantes depende de diferentes factores dentro de los que se encuentra el ambiental, que define las interacciones biológico-ambientales, el tipo de especies vegetales que incluye la etapa de desarrollo de la planta, otro de los factores determinantes son las características físico-químicas del suelo ya que el tipo de suelo, pH y fertilidad son cruciales en la supervivencia de diferentes especies de microorganismos. Además de la diversidad microbiana presente en el suelo previó a la inoculación , así como los métodos de aplicación en campo que van desde aspersiones foliares, paletizados de semilla, aplicaciones líquidas al suelo, o por medio de enmiendas, entre otros (Mącik *et al*. 2020; Suman *et al*. 2022a).

En un experimento en campo se llevó a cabo la inoculación de la cepa UM270 en plantas de *P. ixocarpa* Brot. ex Horm en el municipio de Uruapan, Michoacán. Con la inoculación aumentó la altura, el diámetro del tallo y el peso fresco de las plantas y, por ende, incrementó la producción en un 65.54%, con lo que se concluyó que el efecto de la cepa bajo condiciones de campo fue positivo en este cultivo (Villaseñor-Tulais *et al*. 2023).

2.4.8 Inoculación de *P. fluorescens* UM270 en el modelo milpa en campo

La inoculación de las BPCV se realiza mayoritariamente en cultivos intensivos, principalmente en híbridos, sin embargo, es necesaria su aplicación para la mejora de la producción de razas criollas para conservar la biodiversidad y obtener productos agrícolas a base de sistemas sustentables. En este caso la cepa UM270 se inoculó durante las temporadas 2021 y 2023 en el cultivo de *Z. mays* bajo diferentes sistemas milpa (*Z. mays*, *Z.mays* + *P. vulagris*, *Z. mays* + *C. pepo*, *Z,mays* + *P. vulgaris* + *C. pepo*), incrementó la concentración de clorofila, altura de las plantas, longitud de las raíces y el peso seco de las plantas de maíz, además de incrementar más del 40% la producción de grano (Rojas-Sánchez *et al*. 2024b). Además alteró el microbioma endófito de las raíces al estimular la presencia de los géneros *Burkholderia* y *Pseudomonas* (*Z. Mays* + UM270), mientras que, en la triada mesoamericana estimuló una mayor diversidad endófita y la presencia de géneros

como *Burkholderia*, *Variovorax* y los géneros de rizobios fijadores de N como *Rhizobium*, *Mesorhizobium* y *Bradyrhizobium* (Rojas-Sánchez *et al*. 2024a).

El conjunto de datos obtenidos durante los ensayos previos de la cepa P. *fluorescens* UM270 brindaron, resultados positivos en su uso como promotora del crecimiento vegetal, tanto en pruebas *in vitro*, maceta y campo, además de presentar rasgos potenciales para su uso como biofungicida, mediante el antagonismo de patógenos rizósfericos y de poscosecha., gracias a los mecanismos que posee. No obstante, aún falta dilucidar cuál es la formulación más eficiente para su posible comercialización (Figura 4).

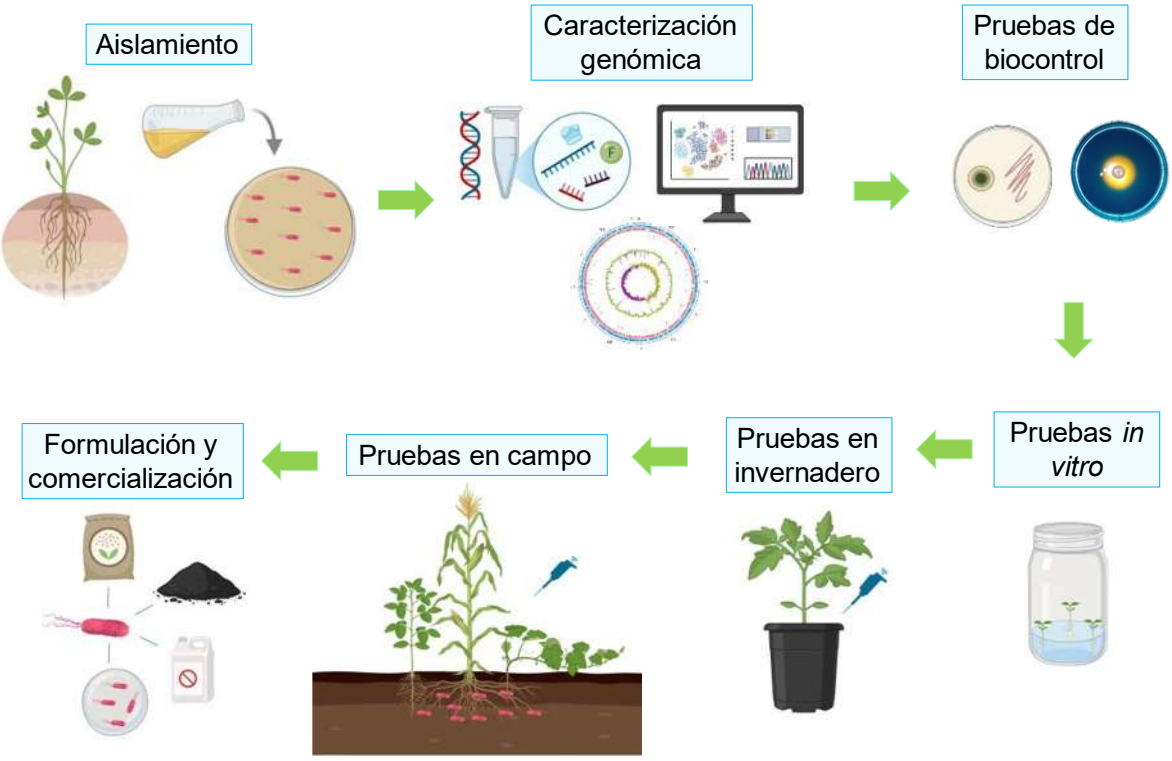


Figura 4. Proceso de selección de *Pseudomonas fluorescens* UM270 como BPCV

III. Justificación

Zea mays L. es uno de los cereales más importantes a nivel mundial; sin embargo, su cultivo se ve afectado por factores ambientales y bióticos, lo que ha llevado al uso excesivo de agroquímicos con efectos tóxicos en los seres vivos y el ecosistema. Una alternativa sustentable para mitigar estos efectos y aumentar la producción de *Z. mays* es el uso de agentes biológicos, como la cepa UM270 de *Pseudomonas fluorescens*. Esta cepa ha demostrado ser una excelente bacteria promotora del crecimiento vegetal (BPCV) en condiciones *in vitro* e invernadero, con actividad antagonista contra hongos patógenos, incluidos aquellos que afectan a *Z. mays*. Sin embargo, aún se desconoce si la cepa mantiene sus efectos benéficos en condiciones de campo, especialmente en el modelo milpa, y su impacto en la diversidad del microbioma endofítico de *Z. mays*. Explorar estos efectos en campo podría permitir el desarrollo de un biofertilizante sostenible, ofreciendo una alternativa ecológica para la producción de *Z. mays* dentro de un sistema de cultivo amigable con el ecosistema, como la milpa.

IV. Hipótesis

La inoculación de *Zea mays* con la rizobacteria *Pseudomonas fluorescens* UM270 modula la composición del microbioma endofítico radicular, promueve el crecimiento del cultivo y aumenta su producción en un sistema milpa.

V. Objetivo general

Evaluar la inoculación de *P. fluorescens* UM270 sobre el microbioma endofítico radicular de *Z. mays* y su efecto en el crecimiento y la producción de grano bajo un modelo milpa.

5.1 Objetivos específicos

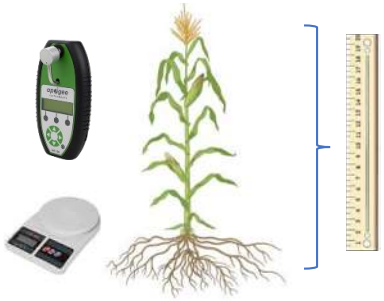
1. Analizar el efecto de *P. fluorescens* UM270 sobre el crecimiento y producción de *Z. mays* bajo un sistema milpa durante los ciclos 2021 y 2023
2. Determinar el efecto de la inoculación de *P. fluorescens* UM270 sobre el microbioma radicular endofítico de *Z. mays.*

VI. Estrategia Experimental

Pseudomonas fluorescens UM270

Inoculación

Análisis de parámetros fitométricos y de producción

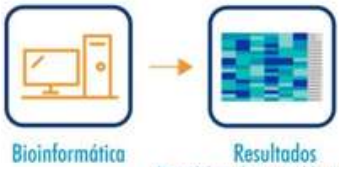


Rendimiento

Composición química:
N, P, K, Mg, Ca, Fe, Mn y Zn

Análisis del microbioma endófito

MR DNA



Secuenciación illuminas 16S e ITS

VII. Resultados

7.1 Evaluación en campo de una *Pseudomonas* promotora del crecimiento vegetal sobre los componentes fitométricos, nutricionales y de rendimiento de *Z. mays* en un agrosistema de milpa.

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FULL-LENGTH RESEARCH ARTICLE

Field Assessment of a Plant Growth-Promoting *Pseudomonas* on Phytometric, Nutrient, and Yield Components of Maize in a Milpa Agrosystem

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Abstract The traditional milpa system, a polyculture originating in Mesoamerica, centers around maize (*Zea mays* L.), associated with pumpkin (*Cucurbita* sp.) and beans (*Phaseolus vulgaris* L.). The application of plant growth-promoting rhizobacteria (PGPR) under a milpa agrosystem has been little explored. In this study, a maize crop in a milpa system was fertilized with the PGPR *Pseudomonas fluorescens* UM270 during the 2021 and 2023 seasons, and various phytoparameters (plant height, root length, chlorophyll concentration, root dry weight and total plant dry weight), total production, and grain nutrition were evaluated. The results showed that UM270 improved chlorophyll concentration and increased plant height, root length, and dry weight in maize plants. Co-fertilization with UM270 and diammonium phosphate (DAP) significantly improved plant and corn cob weight compared to controls with single fertilizations in both the 2021 and 2023 seasons. Notably, corn production increased by more than 40% in the corn monoculture inoculated with UM270 compared to the uninoculated plants. The UM270 + DAP cofertilization in the monoculture was also increased by more than 50% in both cycles. When analyzing the nutritional content of the corn cob, nitrogen and phosphorus increased with the inoculation with UM270, while other elements, such as potassium and calcium, were higher in treatments co-inoculated with UM270 + DAP. Based on our research, this study is the first to report the milpa as a suitable model for bioinoculation with PGPR, demonstrating its potential to increase maize yield and benefit other associated crops.

Keywords Bioinoculation · PGPR · Rhizobacteria · *Phaseolus vulgaris*

Introduction

In the middle of the twentieth century, there was an increase in agricultural production and this exceeded the current increase in population. This transcendental change in agriculture was called "green revolution", and represented a boost in the world's most developed and later less developed nations [39, 48]. The main objective was to exponentially increase production through the use of hybrid seeds in large monocultures, planted with heavy machinery. To supply the nutrients required by the plants, different synthetic sources were applied as fertilizers [3, 35].

Maize has been one of the most impactful crops since the beginning of the Green Revolution, due to its nutritional value and high demand in the food, balanced feed, and pharmaceutical industries. In recent years, its use in bioethanol production has further cemented its status as one of the most important cereals worldwide [12]. However, its establishment as a monoculture has increased the presence and resistance of pests and diseases, and the soils are deteriorating and leading to increased soil toxicity due to the large amounts of agrochemicals that are supplied during the development of the crop [5, 26, 40, 50].

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Today it is necessary to implement different crop systems that include the use of technologies that are friendly to the environment and counteract the effects caused since the beginning of the green revolution [10, 46]. One of the systems that has regained importance in recent years is the traditional "milpa" system, one of its characteristics is that they apply minimum or zero tillage, do not need irrigation systems, and are based on the establishment of maize cultivation associated with other crops such as beans and pumpkin. [2, 11], where maize serves as a support for the entangling of beans through the production of nodules, increases nitrogen fixation that benefits maize and pumpkin, and the latter provides soil protection by reducing the growth of weeds, which retains moisture, and through the production of allelopathic compounds (cucurbits) released by the leaching of the rain, they keep insects away. It has been one of the most used systems over the years in Mexico, and its importance encompasses cultural, economic, social, biological, and environmental aspects [29, 44]. Additionally, the traditional milpa model system is key to conserving soil biological diversity. Research indicates that the plants in this system have co-evolved with microbial biodiversity, enhancing soil fertility and ecosystem health [13, 49].

The main objective of the milpa system is self-consumption. Due to changes in culture and environmental conditions, productivity has declined. To ensure food security, research into new production methods to increase milpa productivity is essential. One option is the use of microorganisms that promote plant growth, which in recent years has proven effective as biofertilizers, biopesticides, and biofungicides [6, 32].

The interaction between microorganisms and plants depends on the species and age of the plant, soil characteristics, and climate. Plant growth-promoting rhizobacteria (PGPR) are among the plant growth-promoting microorganisms [33, 47, 51]. Mechanisms by which PGPRs act as bioinoculants include phosphate solubilization, nitrogen fixation, phytohormone production, and iron reduction. PGPR protect crops by producing antibiotics, siderophores, lytic enzymes, and volatile organic compounds, and by triggering systemic resistance in plants [27, 38, 52]. However, the survival and proliferation of these non-native microorganisms in the soil are necessary for them to exert their mechanisms on plants [4].

Some of the most studied PGPR genera in the maize rhizosphere include *Burkholderia, Bacillus, Azotobacter, Streptomyces*, *Paenibacillus*, *Sphingobium*, and *Pseudomonas*. Pseudomonads stand out for their effectiveness as plant growth promoters in maize plants, fungicides against diseases such as *Rhizoctonia solani*, biostimulants that mitigate water stress, and bioremediators of copper toxicity in maize crops [9, 12, 41, 45]. *Pseudomonas fluorescens* strain UM270 has various PGP mechanisms, such as the production of siderophores, antibiotics, volatiles, ACC deaminase activity, biofilm formation, and phosphate solubilization. It has been proven that it is an excellent promoter of plant growth in vitro in plants, including *Solanum lycopersicum*, *Physalis ixocarpa*, *Medicago truncatula*, and antagonists of fungal pathogens such as *Botrytis cinerea* and *Fusarium oxysporum* [20–23]. However, its beneficial effects in the field are unknown and under a milpa model. Therefore, the objective of this work was to evaluate the effect of *P. fluorescens* UM270 inoculation on maize growth, plant nutrition, and production in a milpa agrosystem during two growth cycles (2021 and 2023).

Materials and Methods

Experimental Site

The experiment was conducted in Santa Clara del Cobre, located within the municipality of Salvador Escalante, Michoacán, México (Fig. 1), at 19° 24′ 23″ North and 101° 38′ 24″ West, with an elevation of 2239 m. The climate prevalent in this area is classified as humid subtropical (Köppen climate classification, Cwa). Maize production in this region follows a seasonal pattern, with cultivation of native varieties, including white, black, yellow, and pink maize. Soil analyses were conducted prior to the experiments to determine their physicochemical properties (such pH, textural class, organic matter, elements like P, K, N, Mg, among others), with samples sent to INIFAP-Celaya (Mexico) for processing.

Biological Material

Zea mays L., *Phaseolus vulgaris* L., and *Cucurbita* sp. seeds utilized in this experiment were sourced locally from the municipality of Salvador Escalante, Michoacán, México where the study took place and were sourced from local producers. The bioinoculant used was the UM270 strain, which has been previously isolated and characterized [20].

Chemical Fertilizer

Diammonium phosphate (DAP) 18–46-0 was applied. It was purchased from a local company. It is a granular inorganic fertilizer and an excellent source of phosphorus (P) and nitrogen (N), which is highly soluble and dissolves in the soil solution, developing an alkaline pH around the granule.

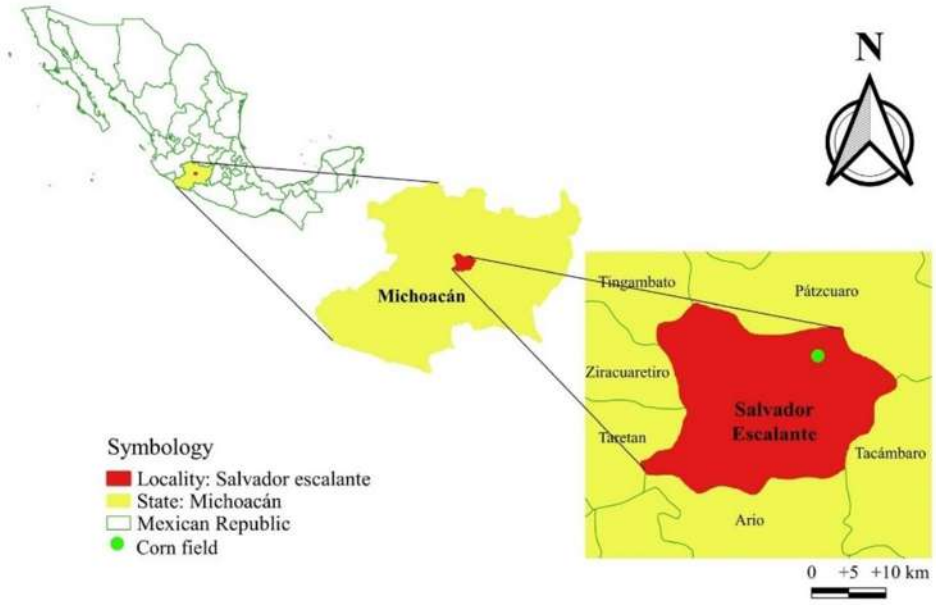

Fig. 1 Geographic location map of the experimental site for maize cultivation (Green dot) under the milpa model in Santa Clara del Corn cobre, Michoacán, Mexico

Inoculum Preparation and Seed Treatments

Inoculum preparation was carried out as follows. Briefly, *P. fluorescens* strain UM270 was activated by inoculating a bacterial loop into a flask containing 500 mL of Nutrient Broth (BD BIOXON). The flask was then placed on a shaker set to 120 rpm and incubated at 28 °C for 24 h until it reached an optical density at 560–600 nm of 1. Subsequently, the supernatant was separated from the bacterial pellet, and the pellet was resuspended in a solution containing 0.1 mM magnesium sulfate. Finally, colony-forming units (CFUs) per mL were determined.

Seed preparation consisted of a superficial disinfection process involving washing with 70% ethanol, 5% sodium hypochlorite, and sterile distilled water. The seeds used for the treatments in the presence of the bacterial strain were inoculated at a concentration of approximately $1 \times 10^3 - 1 \times 10^4$ CFU per seed.

Establishment of the Experiment in the Field

Maize planting was carried out in May during 2021 and 2023, with the entire cultivation stage ending in December of each year. Native maize seeds known as 'white maize' were used (Fig. 2). This variety is selected in the area for its characteristics of nixtamalization and tortilla flavor. After two weeks, guide beans and pumpkin were planted. One month after the maize planting, a second inoculation within the same treatment with the UM270 strain at a concentration of 1×10^8 UFC was carried out on the crops with the inoculated seeds, and after another month, a third inoculation was carried out at the same concentration.

The dose of DAP fertilizer applied to the selected crops was 200 kg/ha in the respective treatments. The maize crop was fertilized at the time of sowing; the second fertilization was carried out 1 month later by applying the same doses. Weed management was performed manually through weekly selective weeding, and vegetative development of the plants was monitored every 15 days. The maize harvest was carried out in December, and the bean and pumpkin were harvested when they reached physiological maturity and left to dry in the open air under shade.

The maize phenological scale in which the crop was evaluated included the following stages: emergence stage (VE), stages of development from the first to the nth leaf (V1 to V(n)), panicle stage (VT), reproductive stages that carry out the process from aqueous grain to hard grain (R1 to R5), and finally, the stage of physiological maturity (R6). The evaluated phytometric parameters were chlorophyll concentration, plant height, root length, plant dry weight, root dry weight, and maize ear weight.

Grain Yield and Chemical Composition Analysis

To determine grain yield, the number of maize ears per hectare was calculated by counting the number of ears in an

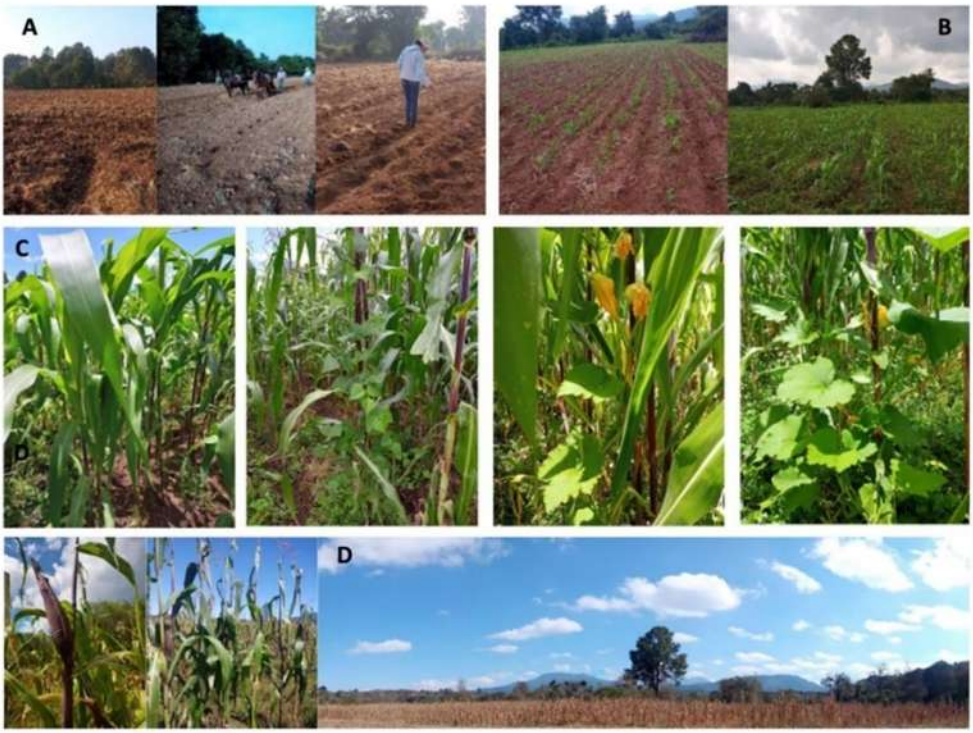

Fig. 2 Composite pictures of the sowing of maize in a milpa system. Panel A represents the process of preparing the land that consists of making the fallow, to later carry out the plowing and planting of corn with beans and pumpkin. The letter B represents the vegetative growth stage of plants. Panel C represents, from left to right, the planting of corn, corn co-cultivated with beans, corn with pumpkin, and corn with beans and pumpkin. Complete cycle of maize cultivation. Panel D represents the reproductive stage of the maize cycle (Representative photographs taken during the 2021/2023 seasons)

area of 10 m^2, and the number of grains per ear was determined by counting the number of rows in each ear and the number of grains per row. The final number of kernels per ear was calculated by multiplying the number of rows by the number of kernels in each row. Finally, the number of grains per hectare and the weight of a thousand grains were measured.

The chemical composition of the maize corn cob was analyzed after the harvest of the crop in December, and the samples obtained were sent to INIFAP-Celaya, Mexico for processing. The parameters evaluated were concentration of nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulfur (S), iron (Fe), zinc (Zn), manganese (Mn), copper (Cu), and sodium (Na). The beans were harvested in September, as soon as they reached physiological maturity and were left to dry under shade in open air until they reached 14% humidity, after which they were weighed. Pumpkins were harvested between July and August as necessary. This crop was harvested twice a week in the form of flowers and green fruits until plant senescence. Afterward, the fruit was weighed, and the number of flowers was counted.

Experimental Design

The experiment was implemented over an area of 5600 m^2. The experimental design was completely randomized with 10 treatments, where the three crops were planted at various densities. According to recommendations from the producers in the region, eight Maize plants m^2 were planted. The composition of the polycultures was calculated as follows: planting a Maize plant is equivalent to 0.75 bean plants and 0.25 pumpkin plants.

The treatments evaluated were:

(1) *Zea mays* L. (M)
(2) *Zea mays* L. + *Phaseolus vulgaris* L. (M + P)
(3) *Zea mays* L. + *Cucurbita* sp. (M + C)
(4) *Zea mays* L., *Phaseolus vulgaris* L., *Cucurbita sp.* (TM)
(5) *Zea mays* L. + diammonium phosphate (M + DAP)

(6) *Zea mays* L. + UM270 (M + UM270)
(7) *Zea mays* L. + UM270 + *Phaseolus vulgaris* L. (M + P + UM270)
(8) *Zea mays* L. + UM270 + *Cucurbita* sp. (M + C + UM270)
(9) *Zea mays* L. + UM270 + *Phaseolus vulgaris* L. + *Cucurbita* sp. (TM + UM270)
(10) *Zea mays* L. + UM270 + diammonium phosphate (M + DAP + UM270)

Statistical Analysis

The data obtained were analyzed by analysis of variance, and the variables showing significant differences were further analyzed using Tukey's test ($p < 0.05$) with STATISTICA 12 software. Additionally, correlation analysis and a heat map were performed using the METABOANALYST 6.0 platform. The data were subjected to *t*-test ANOVA with autoscale samples and a Pearson distance measure with a complete clustering method.

Results

Physicochemical Traits of the Soil

The physicochemical characteristics of the soil are presented in Table 1, where it can be observed that the pH levels are 5.42 and 6.2, organic matter was measured at 7.18 and 7.9 respectively, and phosphorus levels were low. Additionally, calcium, manganese, copper, magnesium, and zinc levels were found to be in a moderately low range. K and Fe levels were within the medium range. However, for the establishment of corn, soils with a pH range of 5.5–7.8 were required. Beyond these values, the crop may exhibit symptoms of excess micronutrient toxicity. Corn's adaptability to various soil types contributed to successful cultivation in the conditions described in this study (cycles 2021 and 2023).

Table 1 Physicochemical characteristics of the experimental soil and minimal changes during the two cycles evaluated

Physical soil propieties	Season 2021	Season 2023
Textural class	Clay	Clay
Base saturation percent	62.2%	53.2
Field capacity	48.9%	46.9
pH	5.42	6.2
Organic matter	7.18%	7.9
Fertility		
N-Inorg	42.08 ppm	41
Phosphorous (P)	1.96 ppm	1.83
Potassium (K)	258 ppm	248
Calcium (Ca)	709.16 ppm	689.13
Magnesium (Mg)	119.54 ppm	125.34
Sodium (Na)	12.87 ppm	11.87
Iron (Fe	9.54 ppm	9.1
Zinc (Zn)	0.72 ppm	0.82
Manganese (Mn)	2.24 ppm	2.48
Copper (Cu)	0.46 ppm	0.42
Boron (B)	N. D	N. D

Maize Growth Promotion by Biofertilization with Strain UM270

During both corn crop cycles, different parameters were evaluated, such as plant height, root length, chlorophyll concentration (SDAP units), root dry weight, total plant dry weight and corn cob weight (Suppl. Table 1 and Figs. 3 and 4). In general, all treatments under the different milpa systems, with and without inoculum, showed an increase in chlorophyll concentration compared to the corn monoculture control treatment (without inoculum). The monoculture treatments fertilized with DAP, the Mesoamerican triad model (TM), and the corn-squash coculture increased the chlorophyll concentration by more than 50% during both cycles (Fig. 3A-A2), where significant differences were found between treatments ($p < 0.05$).

The height of the plants for both cycles increased in the treatments inoculated with the UM270 strain, highlighting the treatments of monoculture fertilized with DAP, TM, and corn-squash co-culture, which increased by more than 26% and by up to 56% during both cycles (Fig. 3B-B2), and significant differences were found between the treatments ($p < 0.05$). Similarly, root length increased in treatments inoculated with strain UM270, with an increase of more than 27% in each cycle (Fig. 3C-C2).

The dry weight of the plant with respect to the TM increased by more than 100% in the treatments inoculated with the UM270 strain, including the corn-squash co-culture, TM, and corn monoculture fertilized with DAP. for both cycles (Fig. 4D-D2). However, regardless of the type of milpa model with or without inoculum or DAP fertilization, there were significant differences ($p < 0.05$), and the dry weight of the plant increased with respect to the corn control treatment for both cycles.

There were no significant differences in the root dry weight after inoculation with strain UM270 (Fig. 4E-E2). Curiously, the dry weight of the corn cob presented significantly different ($p < 0.05$), highlighting the treatments inoculated with UM270, which included corn monoculture, corn-bean co-culture, and fertilized corn monoculture were found. with DAP, which increased their weight by more than 40% during the 2021 cycle. In the 2023 cycle,

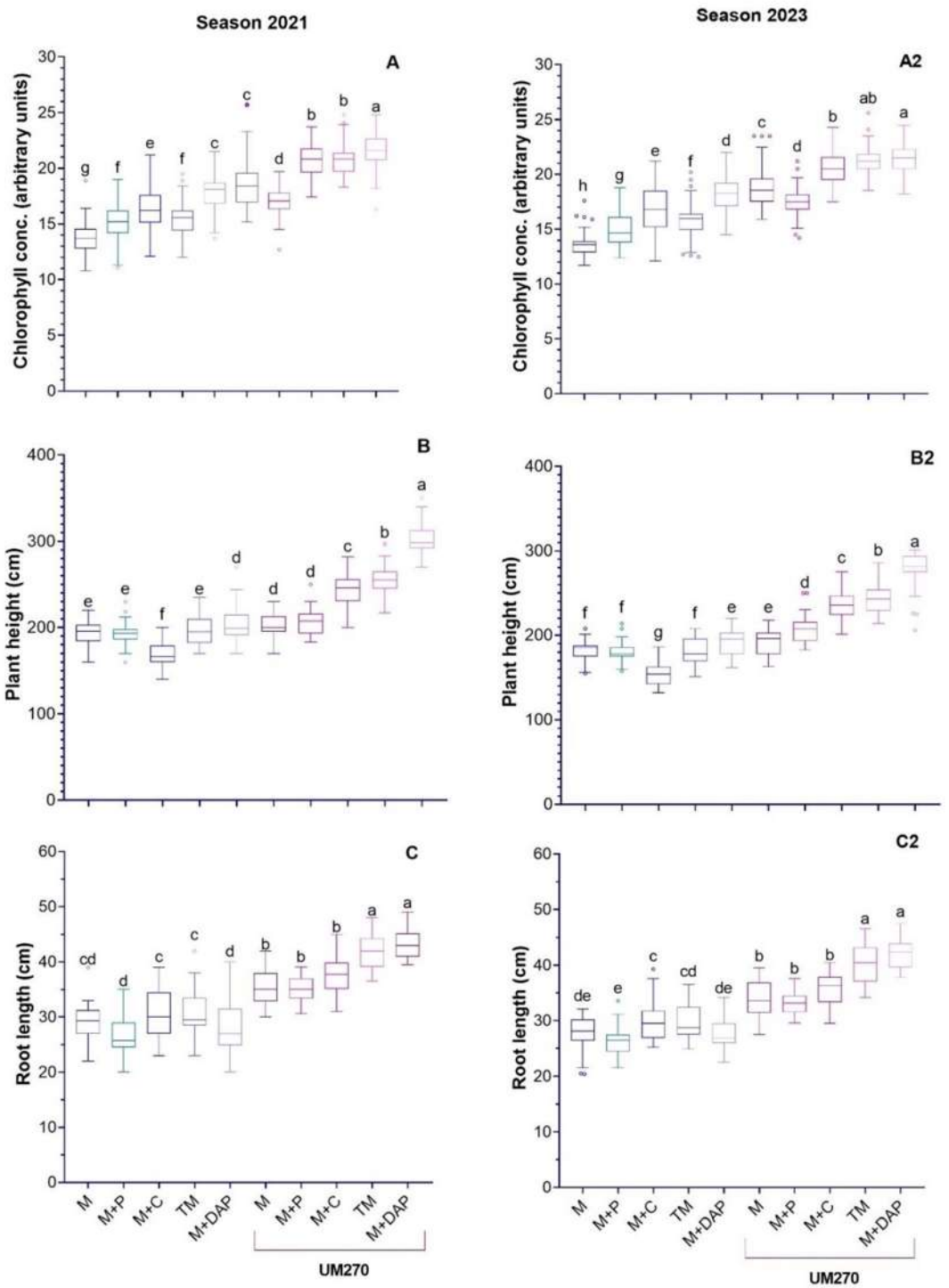

Fig. 3 Effect of biofertilization of maize plants by *fluoresces* UM270 under the milpa model. Different letters indicate significant difference calculated by a Tukey test ($p < 0.05$) $n = 100$

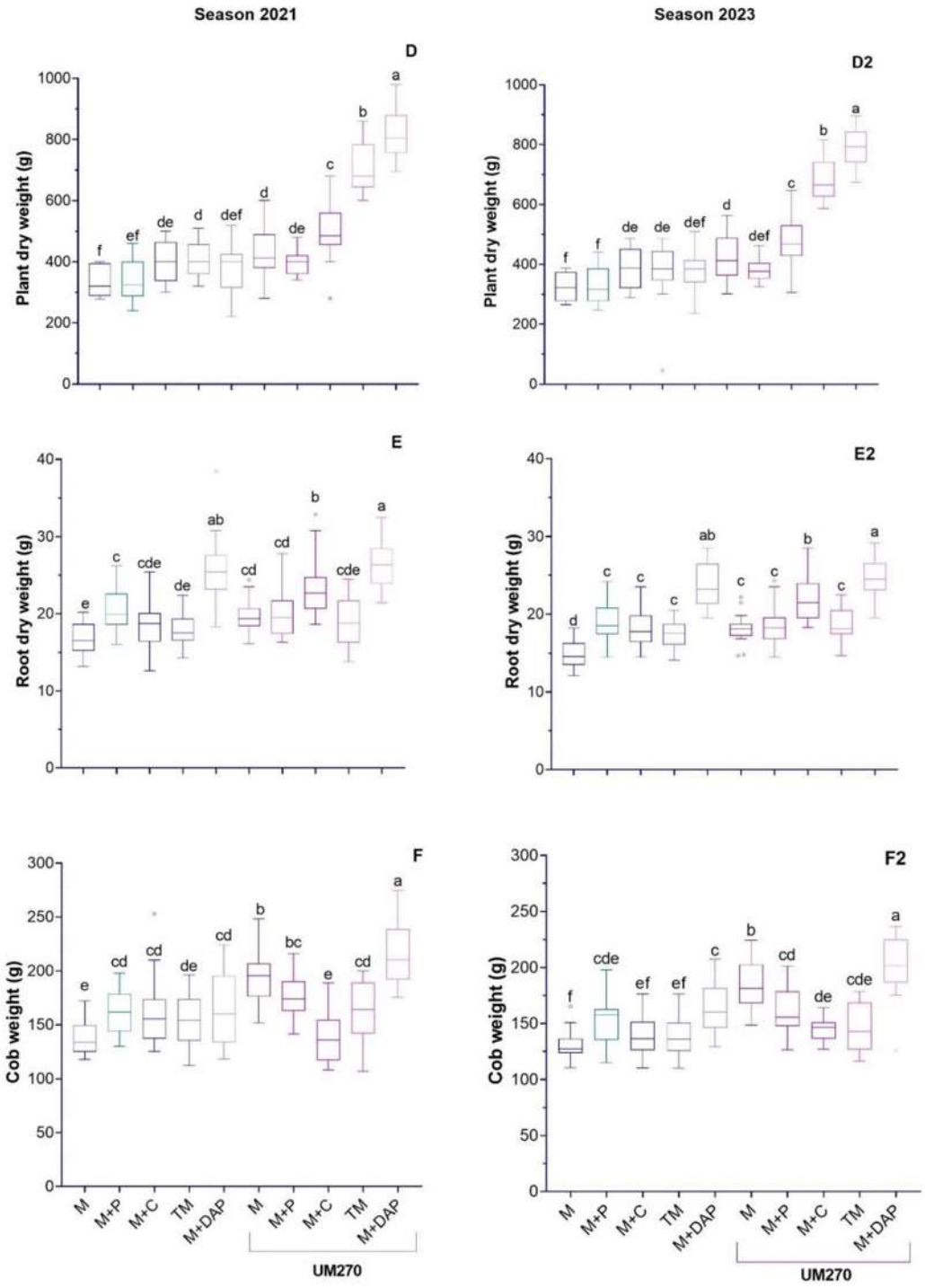

Fig. 4 Effect of biofertilization of maize plants by *P. fluoresces* UM270 under the milpa model. Different letters indicate significant difference calculated by a Tukey test ($p < 0.05$) $n = 100$

treatments inoculated with UM270 included corn monocultures with and without DAP; on the other hand, the corn monoculture fertilized with DAP without inoculum increased by 56%, 42%, and 25%, respectively (Fig. 4F-F2).

Based on the correlation analysis between treatments, it can be determined that during the 2021 cycle, the corn-bean co-culture and the corn monoculture, both inoculated with UM270, were positively correlated. In the same way the corn monoculture fertilized with DAP and the TM, both inoculated with UM270, were positively correlated (Fig. 5).

During the 2023 cycle, the TM, corn monoculture, and fertilized DAP were positively correlated, and both treatments were inoculated with UM270 (Fig. 5).

The results of the heat map that considers the six phytometric parameters show that the treatment of the corn monoculture inoculated with UM270 and fertilized with DAP presented the highest values of all the parameters in both cycles. The treatments where UM270 biofertilization was applied increased the values of the parameters depending on the type of model evaluated (Fig. 6). Based on the results of the principal component analysis, it can be determined that the first axis of the PCA explains 30.5 and 30% of the variation and the second 16.6 and 16.2% for the 2021 and 2023 cycles, respectively. During the first cycle, they grouped the height of the plant, root growth, and chlorophyll concentration during the second cycle, only the dry weight of the plant was not grouped with the other phytometric parameters (Fig. 7).

Maize Yield

The increase in maize yield was evaluated at the end of the harvest in both cycles. During the first cycle, the treatments that showed an increase in maize yield were those inoculated with UM270, maize in co-culture with bean plants, and maize fertilized with DAP by 41.96%, 28.28%, and 58.13%, respectively, in comparison with the maize plants controls (uninoculated) (Table 2). During the second cycle, the corn monocultures inoculated with the UM270 strain and the co-fertilized one (UM270-DAP) were the ones that presented the greatest increase in production by 42.03% and 56.59%, respectively (Table 2). This result indicates that biofertilization with *P. fluorescens* UM270 has great potential to increase maize crop yield.

Phaseolus vulgaris L. and *Cucurbita* sp. Yield

Bean yield was determined under the milpa model; the interaction, given in the corn-bean co-culture inoculated with the UM270 strain increased by 12.5% and 13.32% in the 2021 and 2023 cycles, respectively, compared to the corn-bean co-culture without inoculum (Table 3). Biofertilization with the UM270 strain increased pumpkin yield, indicating the treatment of the TM with an increase of 30.27% and 20.90% in the 2021 and 2023 cycles, respectively, compared to the corn-squash co-culture without inoculum (Table 4).

Nutritional Composition of the Maize Corn Cob

The nutritional composition of maize grains is presented as the mean of the two seasons (Table 5). One of the elements analyzed was the concentration of total N, which presented significant differences ($p > 0.05$) between treatments. Notably, maize co-cultured with pumpkin and inoculated with the UM270 strain showed an 18.8% increase in nitrogen concentration compared to the maize control treatment and the traditional TM system. The same behavior was observed, but with an increase of 14.87%.

P is another of the treatments that was evaluated, and in this case the treatments of the TM, that of maize fertilized with DAP, and that of maize in co-culture with beans and inoculated with the bacterial strain, stood out for presenting the highest concentration, increasing by 52.94%, 43.38%, and 45.58% compared to the maize control treatment. In this case, we determined that even without inoculation with the bacterial strain, the TM system could increase P concentration.

K increased in maize treatments inoculated with strain UM270 and in maize inoculated and fertilized with DAP, showing an increase of 20.47% and 16.5%, respectively, compared to the control treatment of maize alone. Ca presented its highest concentration in the maize treatment inoculated with the strain and added with DAP fertilizer, increasing by 56% compared to the maize control treatment. Mg, Zn, Mg, Cu, and Na did not show significant differences between the treatments. S had the highest concentration in the TM treatment, increasing by 41.09%. Fe presented its highest concentration in the maize treatment co-cultivated with beans, where it increased by 509.48% compared with the maize control treatment.

The correlation analysis between the different milpa models in the nutritional content of the corn cob, the treatments without TM inoculum, corn, corn-bean co-culture, and corn monoculture fertilized with DAP were positively correlated, whereas the treatments inoculated with the TM, strain UM270, and corn-bean co-culture were positively correlated (Fig. 8A). In the heat map based on the elements in the corn cob, it was observed that Fe had the highest values in the control treatment of the corn monoculture, even though there was no increase in the values given by the inoculation of the UM270 strain, which can be determined depending on the type of system, and

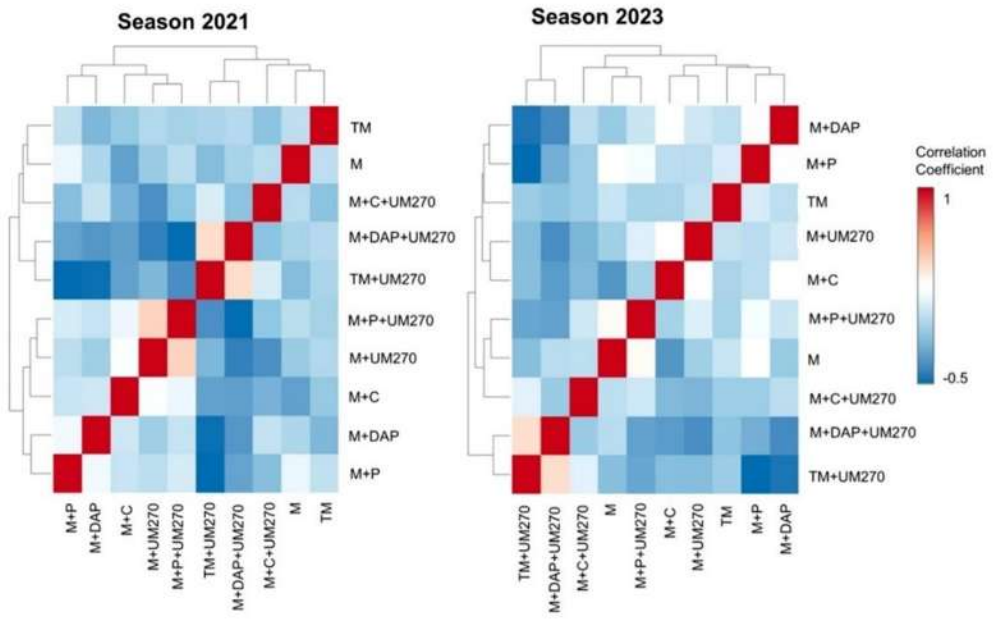

Fig. 5 Pearson correlation matrix between treatments of different cornfield models biofertilized with *P. fluorescens* UM270, based on the phytometric parameters of plant growth promotion. Correlations are shown in blue (negative) and red (positive)

the different parameters evaluated increased in different ways (Fig. 8B).

Discussion

Corn is one of the most important cereals worldwide, and its nutritional value makes it key to food security including its cultivation via the Milpa model, which is key to generating a variety of agricultural products in a small space and stimulating soil biodiversity [18, 19, 44]. No agrochemicals were applied in the milpa; therefore, its production was highly eco-friendly. However, the threat of pathogens and their cultivation in nutrient-poor soils can reduce their efficient production. Therefore, it is necessary to use and apply biological fertilizers and fungicides to naturally restore agroecosystems [16, 17, 42].

The results of this study demonstrate that the application of a biofertilizer based on the PGPR *P. fluorescens* UM270 under the Milpa model (TM or Mesoamerican Triad), among other treatments, including corn-pumpkin co-inoculation, managed to increase all analyzed parameters (e.g., concentration of chlorophyll, biomass, root length, corn plant height, and corn cob weight) compared to the control treatment of corn monoculture without bacterial inoculum. Similar beneficial effects have been observed in corn crops under a monoculture system with the application of biocapsules formulated with chitosan and PS2 and PS10 (*Bacillus* spp.) [7]. The effect of *P. fluorescens* UM270 on maize plants also increased the chlorophyll concentration, regardless of the type of Milpa system treatment established in the field.

Plant height growth increased only in the treatments in which the UM270 strain was inoculated. Similarly, root growth increased only in the treatments inoculated with the UM270 strain, which indicated that the plant-microorganism interaction promoted the growth of maize plants roots, regardless of which Milpa model was established. The dry weight of the plant increased only in the treatments inoculated with the UM270 strain in corn-squash, TM and corn monoculture fertilized with DAP, with the results of this parameter it can be determined that depending on the established Milpa system, the weight increases of plant height by the end of the cycle. Likewise, previous studies have shown that in maize crops under a monoculture system by inoculating consortia of plant growth-promoting bacterial strains such as *Bacillus* and *Pseudomonas*, they increase the growth of maize plants compared to the treatments where only an inoculant was applied [37]. Strains like *Pseudomonas geniculata*, *Pseudomonas psychrotolerans*, *Bacillus circulans*, *Pseudomonas putida*, and *Pseudomonas pseudoalcaligenes*, increase the growth of corn plants through various mechanisms under abiotic conditions both in vitro and in the field; however, it is worth mentioning that the majority of crops where bioinoculants are applied are under the establishment of

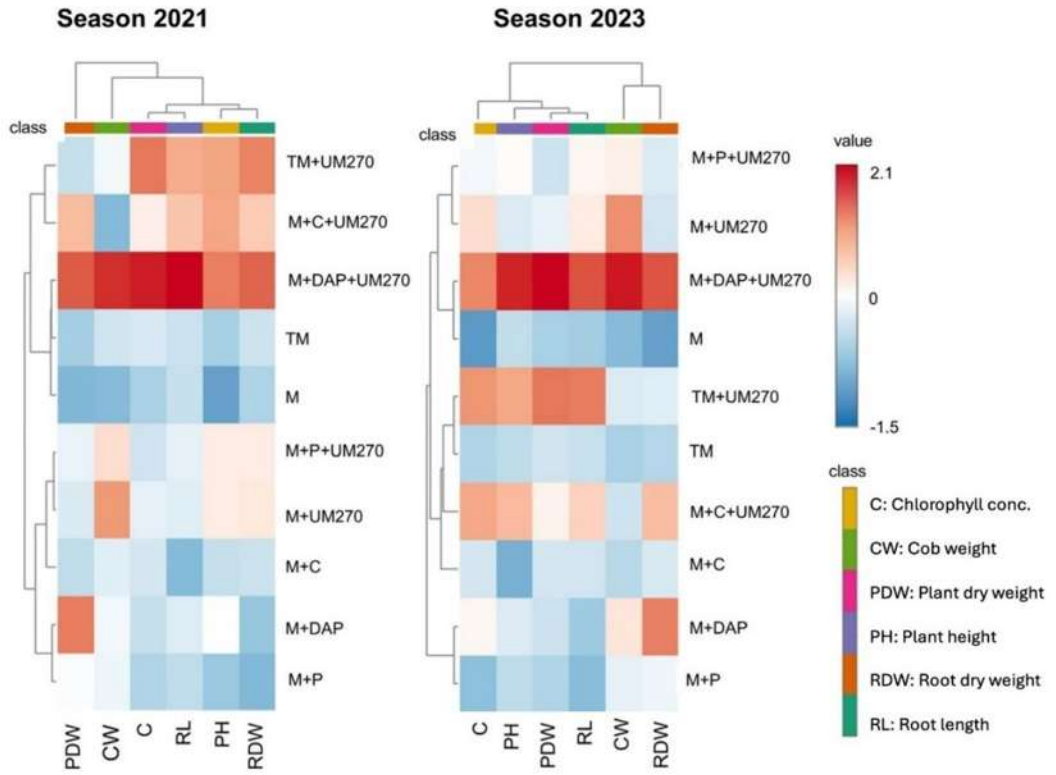

Fig. 6 Heat map diagram that represents the effect of biofertilization of *P. fluorescens* UM270 on corn under different milpa models and its phytometric parameters (chlorophyll concentration, plant height and root growth, plant dry weight and root and corn cob weight

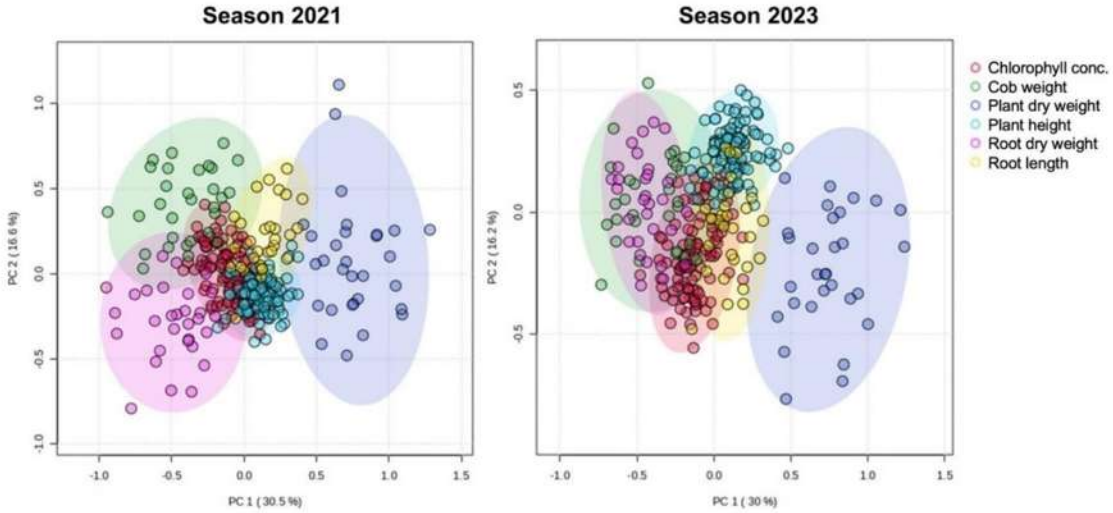

Fig. 7 Principal component analysis for the phytometric parameters of plant growth promotion among treatments of different milpa models biofertilized with *P. fluorescens* UM270

Table 2 Corn yield under a milpa system inoculated with the PGPR *Pseudomonas fluorescens* strain UM270

Treatments	Corn yield (Kg/ha) Season 2021	Increased percentage in corn yield	Corn yield (Kg/ha) Season 2023	Increased percentage in corn yield
Zea mays L. (M)	2817.11e		2598.07f	
Zea mays L. + *Phaseolus vulgaris* L. (M + P)	3329.88 cd	18.20	3022.13cde	16.35
Zea mays L. + *Cucurbita* spp. (M + C)	3270.50 cd	16.09	2818.02ef	8.46
Zea mays L. + *Phaseolus vulgaris* L. + *Cucurbita* spp. (M + P + C)	3171de	12.56	2751.86ef	5.91
Zea mays L. + DAP (M + DAP)	3104.52 cd	10.20	**3263.93c**	**25.62**
Zea mays L. + UM270 (M + UM270)	**3999.27b**	**41.96**	**3690.20b**	**42.03**
Zea mays L. + *Phaseolus vulgaris* L. + UM270 (M + P + UM270)	**3614.01bc**	**28.28**	3210.40 cd	23.56
Zea mays L. + UM270 + *Cucurbita* spp. (M + C + UM270)	2795.92e	− 0.75	2909.45de	11.98
Zea mays L. + *Phaseolus vulgaris* L. + *Cucurbita* spp. + UM270 (M + C + UM270)	2939.83 cd	4.35	2977.42cde	14.60
Zea mays L. + UM270 + DAP (M + DAP + UM270)	**4454.92[a]**	**58.13**	**4068.54a**	**56.59**

To have a comparative evaluation of the beneficial effect of the bioinoculant based on UM270, several treatments were fertilized with diammonium phosphate (DAP). Different letters indicate significant difference calculated by a Tukey test ($p < 0.05$) $n = 3$. See text for further details

Table 3 Common bean yield under a milpa system inoculated with the PGPR *Pseudomonas fluorescens* strain UM270

Treatments	Bean yield (Kg/ha) Season 2021	Bean yield (Kg/ha) Season 2023
Zea mays L. + *Phaseolus vulgaris* L. (M + P)	1000 b	985 b
Zea mays L. + *Phaseolus vulgaris* L. + *Cucurbita* spp. (TM)	1065.50 ab	1002.30 b
Zea mays L. + *Phaseolus vulgaris* L + UM270 (M + P + UM270)	**1125 a**	**1116.3 a**
Zea mays L. + *Phaseolus vulgaris* L. + *Cucurbita* spp. + UM270 (TM + UM270)	937.5 b	875.4 c

Different letters indicate significant difference calculated by a Tukey test ($p < 0.05$) $n = 3$. See text for further details

Table 4 Pumpkin yield under a milpa system inoculated with the PGPR *Pseudomonas fluorescens* strain UM270

Treatments	Pumpkin yield (Kg/ha) Season 2021	Pumpkin yield (Kg/ha) Season 2023
Zea mays L. + *Cucurbita* spp. (M + C)	15,300 b	14,325 bc
Zea mays L. + *Phaseolus vulgaris* L. + *Cucurbita* spp. (TM)	13,855 bc	13,266 c
Zea mays L. + *Cucurbita* spp. + UM270 (M + C + UM270)	17,850 b	16,585 b
Zea mays L. + *Phaseolus vulgaris* L. + *Cucurbita* spp. + UM270 (TM + UM270)	**19,932 a**	**17,320 a**

Different letters indicate significant difference calculated by a Tukey test ($p < 0.05$) $n = 3$. See text for further details

hybrid seeds in monoculture systems and through irrigation systems, which contrasts with the system established in this work, where native seeds were used in a traditional and agro-sustainable system [8, 14, 28, 43, 45, 54].

The correlations between treatments during both cycles allowed to determine that the treatments with the UM270 bioinoculum correlate with each other, as is the case of the corn monoculture with the corn-bean co-culture. On the other hand, the monoculture fertilized with DAP was

Table 5 Chemical composition of maize cob analyzed under a milpa system

Treatment	Element										
	N (%)	P (%)	K (%)	Ca (%)	Mg (%)	S (%)	Fe (ppm)	Zn (ppm)	Mn (ppm)	Cu (ppm)	Na (ppm)
M	1.6^{abx}	0.136^{b}	0.503^{b}	0.05^{b}	0.143^{a}	0.073^{ab}	49.14^{d}	29.02^{c}	4.46^{a}	3.89^{a}	0.01^{a}
	SE = 0.072	SE = 0.016	SE = 0.028	SE = 0.005	SE = 0.014	SE = 0.008	SE = 4.03	SE = 1.17	SE = 0.64	SE = 0.27	SE = 0
M + P	1.486^{bc}	0.16^{ab}	0.523^{ab}	0.048^{b}	0.14^{a}	0.076^{ab}	**299.5^{a}**	31.40^{c}	4.66^{a}	5.14^{a}	0.01^{a}
	SE = 0.08	SE = 0.015	SE = 0.029	SE = 0.007	SE = 0.005	SE = 0.023	SE = 15.80	SE = 2.81	SE = 0.56	SE = 0.22	SE = 0
M + C	1.683^{abc}	0.155^{ab}	0.55^{ab}	0.05ab	0.15^{a}	0.091^{ab}	47.83^{d}	30.01^{a}	5.24^{a}	4.62^{a}	0.01^{a}
	SE = 0.057	SE = 0.012	SE = 0.02	SE = 0.006	SE = 0.01	SE = 0.014	SE = 4.8	SE = 2.46	SE = 0.83	SE = 0.24	SE = 0
TM	1.613^{abc}	**0.208^{a}**	0.523^{ab}	0.053^{b}	0.153^{a}	**0.103^{a}**	92.28^{bc}	29.68^{c}	3.63^{a}	4.34a	0.01^{a}
	SE = 0.071	SE = 0.021	SE = 0.031	SE = 0.003	SE = 0.003	SE = 0.008	SE = 10.95	SE = 1.25	SE = 0.25	SE = 0.29	SE = 0
M + DAP	1.578^{bc}	**0.195^{a}**	0.52^{b}	0.053^{b}	0.143^{a}	0.051^{b}	57.46^{cd}	29.21^{c}	3.74^{a}	4.03^{a}	0.01^{a}
	SE = 0.093	SE = 0.023	SE = 0.015	SE = 0.003	SE = 0.008	SE = 0.013	SE = 5.26	SE = 2.8	SE = 0.38	SE = 0.114	SE = 0
M + UM270	1.423^{c}	0.163^{ab}	**0.606^{x}**	0.063^{ab}	0.146^{a}	0.076^{ab}	77.62^{bc}	30.55^{c}	4.88^{a}	4.9^{a}	0.01^{a}
	SE = 0.06	SE = 0.014	SE = 0.017	SE = 0.008	SE = 0.003	SE = 0.006	SE = 8.5	SE = 2.84	SE = 0.39	SE = 0.53	SE = 0
M + P +UM270	1.7^{ab}	**0.198^{a}**	0.568^{ab}	0.056^{ab}	0.163^{a}	0.083^{ab}	109.17^{b}	29.73^{c}	4.8^{a}	4.63^{a}	0.01^{a}
	SE = 0.129	SE = 0.016	SE = 0.022	SE = 0.003	SE = 0.016	SE = 0.02	SE = 24.54	SE = 2.97	SE = 1.0	SE = 0.82	SE = 0
M + C + UM270	**1.853^{a}**	0.16^{ab}	0.54^{ab}	0.046^{b}	0.153^{a}	0.083^{ab}	57.26^{cd}	27.90^{c}	4.73^{a}	4.17^{a}	0.01^{a}
	SE = 0.066	SE = 0.015	SE = 0.015	SE = 0.003	SE = 0.014	SE = 0.006	SE = 4.83	SE = 1.13	SE = 0.42	SE = 0.39	SE = 0
TM + UM270	1.7^{ab}	0.185^{ab}	0.543^{ab}	0.046^{b}	0.13^{a}	0.075^{ab}	72.72^{cd}	28.24^{c}	5.09^{a}	4.00^{a}	0.01^{a}
	SE = 0.06	SE = 0.017	SE = 0.026	SE = 0.003	SE = 0.005	SE = 0.018	SE = 2.70	SE = 1.85	SE = 0.70	SE = 0.28	SE = 0
M + DAP + UM270	1.616^{abc}	0.171^{ab}	**0.586^{a}**	**0.078^{a}**	0.15^{a}	0.066^{ab}	54.45^{d}	30.98^{c}	5.49^{a}	4.89^{a}	0.01^{a}
	SE = 0.059	SE = 0.016	SE = 0.035	SE = 0.017	SE = 0.01	SE = 0.012	SE = 3.91	SE = 1.68	SE = 0.64	SE = 0.34	SE = 0

Letters in bold mean significant differences regarding control treatments. Different letters indicate significant difference calculated by a Tukey test ($p < 0.05$) $n = 3$. See text for further details

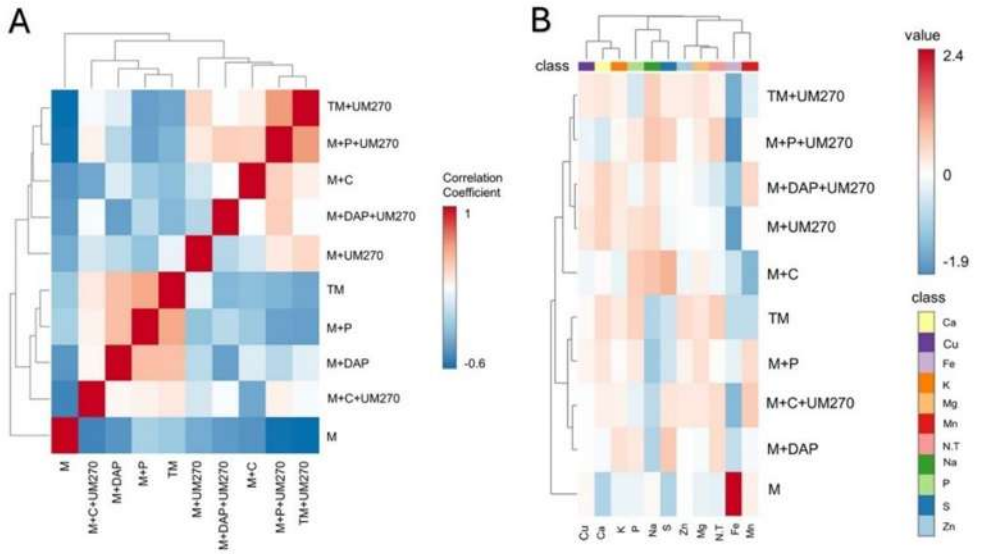

Fig. 8 Pearson correlation matrix between treatments of different cornfield models biofertilized with *P. fluorescens* UM270, based on the concentration of the elements of the corn cob (Panel A). Correlations are shown in blue (negative) and red (positive). Heat map diagram representing the effect of *P. fluorescens* UM270 biofertilization on the nutrient content of corn corn cob under different milpa models (Panel B)

correlated with the TM. The heat map shows that the highest parameters during both cycles are presented in the same way in the treatments with the inoculum. This indicated that the strain's effect on corn growth depends on the interaction between plants, and plants with microorganisms.

One of the most important parameters for field crops is the dry weight of the cob, which directly influences grain yield. In the TM, corn production increased by 12.56 and 5.91% compared with the control treatment during the 2021 and 2023 cycles, respectively. By adding the UM270 inoculum to the corn monoculture, the production increased by 41.96 and 42.03 for 2021 and 2023 cycles, respectively. respectively. Similarly, in the co-cultivation of corn and beans with the UM270 inoculum, it was determined that there was an increase in production of 28.28 and 23.56 during both cycles.

Previous reports have shown that inoculation with PGPR such as *Sinorhizobium* sp. A15, *Bacillus* sp. A28 and *Sphingomonas* spp. A55, isolated from the maize rhizosphere in the same area and inoculated separately, increased maize growth and grain yield between 22 and 29% [8]. The increase in corn yield is determined due to abiotic and biotic factors, but it has been proven that through the application of PGPR, corn yield increases even under stress conditions in the field [15, 34, 41]. In addition, promoting corn yield through the establishment of multispecies cropping increases productivity per unit area and, in turn, reduces the number of weeds and pathogens present [36].

Regarding bean production, it can be observed that the corn-bean interaction and the UM270 bioinoculant increased bean production by 13.32%, compared to the corn-bean co-culturepl. For its part, the pumpkin yield increased in the biofertilized treatments by 30.29% (2021 cycle) and 20.90% (2023 cycle). In previous field studies, the effect of plant promotion in wheat crops was determined due to the effect of bioinoculation of the *B. subtilis* strain [25]. Furthermore, under controlled conditions, the benefits of plant–plant interactions have been observed in intercrops such as corn-bean, where nodulation in broad bean was stimulated. In *Cajanus cajan-Zea mays* co-culture, an increase in the production of corn proteins and in the corn-bean co-culture, the induction of genes for nodulation in bean plants was determined, and in the case of maize genes for the degradation of mucilage and feluric acid, among other compounds [1, 30, 53].

The chemical composition of the grain provides a basic parameter for determining its nutritional quality [31]. Calcium, phosphorus, potassium, and nitrogen are among the elements with the greatest nutritional importance. Here, an increase was observed in certain elements, such as N and P, whereas K and Ca improved in treatments with UM270 + DAP. In a recently published study, Pereira et al. (2020) observed an increase in N and P in maize plants when inoculating with two PGPR, *Cupriavidus*

necator 1C2 and *P. fluorescens* S3X. Although the nutritional grade of the corn grain was not analyzed, only that of the plants under conditions of water deficit was analyzed. In our study, it is possible that the UM270 strain, which is a phosphate solubilizer, increases its nutrient use efficiency, shoot biomass, and concentration in the grain cob. The nutritional value of seeds enhanced by bacterial inoculation has also been tested in other crops, such as faba beans [55] and wheat [24], among others.

Conclusions

Incorporating biofertilizers, such as the *P. fluorescens* strain UM270, into Milpa models not only enhances plant growth promoting parameters and improves grain nutrition by providing specific elements (e.g. K and P), but also boosts overall crop yield. This approach offers significant economic and agroecological benefits to local farmers by providing an alternative to reducing dependency on synthetic fertilizers and leading to more sustainable agricultural practices.

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Author's Contribution BRS: investigation, methodology and formal analysis. MCOM and GS: Conceptualization, resources, supervision, writing, review, and editing. All the authors have read and agreed to the published version of the manuscript.

Declarations

Conflict of interest The authors declare no conflicts of interest.

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7.2 Diversidad del microbioma endosferico y rizósférico de la raíz de *Z. mays* modulado por la inoculación con *P. fluorescens* UM270 en un sistema milpa.

plants

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Article

Diversity of the Maize Root Endosphere and Rhizosphere Microbiomes Modulated by the Inoculation with *Pseudomonas fluorescens* UM270 in a Milpa System

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Abstract: Milpa is an agroecological production system based on the polyculture of plant species, with corn featuring as a central component. Traditionally, the milpa system does not require the application of chemicals, and so pest attacks and poor growth in poor soils can have adverse effects on its production. Therefore, the application of bioinoculants could be a strategy for improving crop growth and health; however, the effect of external inoculant agents on the endemic microbiota associated with corn has not been extensively studied. Here, the objective of this work was to fertilize a maize crop under a milpa agrosystem with the PGPR *Pseudomonas fluorescens* UM270, evaluating its impact on the diversity of the rhizosphere (rhizobiome) and root endophytic (root endobiome) microbiomes of maize plants. The endobiome of maize roots was evaluated by 16S rRNA and internal transcribed spacer region (ITS) sequencing, and the rhizobiome was assessed by metagenomic sequencing upon inoculation with the strain UM270. The results showed that UM270 inoculation of the rhizosphere of *P. fluorescens* UM270 did not increase alpha diversity in either the monoculture or milpa, but it did alter the endophytic microbiome of maize plant roots by stimulating the presence of bacterial operational taxonomic units (OTUs) of the genera *Burkholderia* and *Pseudomonas* (in a monoculture), whereas, in the milpa system, the PGPR stimulated greater endophytic diversity and the presence of genera such as *Burkholderia*, *Variovorax*, and N-fixing rhizobia genera, including *Rhizobium*, *Mesorhizobium*, and *Bradyrhizobium*. No clear association was found between fungal diversity and the presence of strain UM270, but beneficial fungi, such as *Rizophagus irregularis* and *Exophiala pisciphila*, were detected in the Milpa system. In addition, network analysis revealed unique interactions with species such as *Stenotrophomonas* sp., *Burkholderia xenovorans*, and *Sphingobium yanoikuyae*, which could potentially play beneficial roles in the plant. Finally, the UM270 strain does not seem to have a strong impact on the microbial diversity of the rhizosphere, but it does have a strong impact on some functions, such as trehalose synthesis, ammonium assimilation, and polyamine metabolism. The inoculation of UM270 biofertilizer in maize plants modifies the rhizo- and endophytic microbiomes with a high potential for stimulating plant growth and health in agroecological crop models.

Keywords: bioinoculants; PGPR; milpa system; plant bacteriome; endophytes

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1. Introduction

Milpa is a traditional open-field polyculture system that is still preserved as the main production system in various regions of Mexico and Latin America. It consists of the rotation of several plant species, with corn (*Zea mays* L.) featuring as the central crop, and it may include other plant crops such as Mexican husk tomatoes (*Physalis* spp.), common beans (*Phaseolus vulgaris* L.), pumpkins (*Cucurbita* spp.), and others [1]. The milpa system

usually does not require the input of agrochemicals; therefore, its production depends on its own ecological resources (e.g., recycling of organic matter and biological control mechanisms). It can be affected by potential pathogens in addition to being grown in soils that can be nutritionally poor and very irregular in their orography, such as those that exist in the southeastern region of Mexico. The milpa, like other crop rotation systems [2], is a system that favors synergy between different species, as well as their short- and long-term rotation, stimulating better overall yields and generating resilience to external disturbances such as attack by pathogens and stressful abiotic conditions [3].

Likewise, by having several vegetable crops, milpa can generate greater species richness [2–4], and thisinvolves microorganisms in the soil and rhizosphere zones. Recently, Ariza-Mejía et al. [5] evaluated the rhizosphere diversity of two Physalis species (ixocarpa and philadelphica), maize grown in milpa, and bulk soil, finding a wide diversity of bacterial genera associated with *Physalis*, such as *Nocardioides*, *Streptomyces*, *Pseudonocardia*, and *Solirubrobacter*. On the other hand, the microbiome associated with corn plants has been widely analyzed under different environmental conditions (e.g., pH or soil type) [6,7], genotypes/varieties [8], and interaction zones, such as the rhizosphere [9,10], endosphere [11], and phyllosphere [12], among others. From these studies, it has been determined that the structure of the microbial communities of maize in the rhizosphere is highly dependent on the genotype of the plant, and its variation can also be modified by other factors, such as organic and inorganic fertilization. This was confirmed by Peiffer et al. [9], who evaluated the bacterial diversity of the rhizosphere of 27 inbred varieties of modern maize, which exhibit wide genetic diversity when grown under field conditions. Based on this work, it was noted that bacterial groups such as Proteobacteria, Bacteroidetes, Actinobacteria, and Acidobacteria were among the most abundant and poorly heritable.

Plant growth-promoting rhizobacteria (PGPR) play an important role in agricultural systems such as biofertilizers, biostimulants, and bioprotectants [13]. In the case of maize, this crop has been used as a study model because of its importance worldwide as one of the most cultivated grains in the world. Therefore, there are multiple studies where PGPRs have been inoculated into corn crops, observing increases in their growth and production, even under stressful conditions such as drought [14]. Likewise, studies have shown that certain PGPRs can also protect corn from attack by pathogens, trough mechanisms like antibiosis (e.g., production of diffusible and volatile organic compounds), competition for spaces, nutrient deprivation, and 1-amino cyclopropane-1-carboxylic acid desaminase activity, in addition to stimulating immune defense mechanisms.

Another interesting topic to analyze is the impact of PGPR inoculation on the assembly and diversity of microbial communities associated with corn. For example, Ferrarezi et al. [15] recently evaluated the inoculation of a bacterial corsortium made up of *Bacillus thuringiensis* RZ2MS9 and *Burkholderia ambifaria* RZ2MS16, observing that it did not significantly alter the microbiome associated with corn. Similarly, the authors compared the inoculation of the consortium with the *Azospirillum brasilense* Ab-V5 strain, which is widely commercialized and applied to corn crops to increase production [16]. The authors concluded that there are multiple inconsistencies when expanding studies from greenhouse and field conditions; therefore, it is recommended to expand similar studies under different environmental conditions.

Despite multiple studies on the corn microbiome, the impact of PGPR inoculation on the composition and structure of the microbiota associated with different interaction zones, such as the rhizosphere and endosphere, is still not well understood. Therefore, in this study, the impact of the inoculation of the beneficial bacterium *P. fluorescens* strain UM270 on the root endophytic microbiome, as well as on the rhizobiome of corn plants in an open and polyculture system (such as the cornfield) was characterized.

2. Results

2.1. Endobiome Analysis of Maize Roots

When inoculated into plant cultures, PGPR can modify the endophytic microbiome and stimulate the growth and fitness of the host. Thus, we evaluated whether the diversity and structure of the endobiome were modulated by the bioinoculation of maize plants in a monoculture system (maize roots + UM270) and in polyculture (maize roots + UM270 + Milpa system), using uninoculated maize roots as a control (Figure 1). The analysis was performed in triplicate using the composite samples.

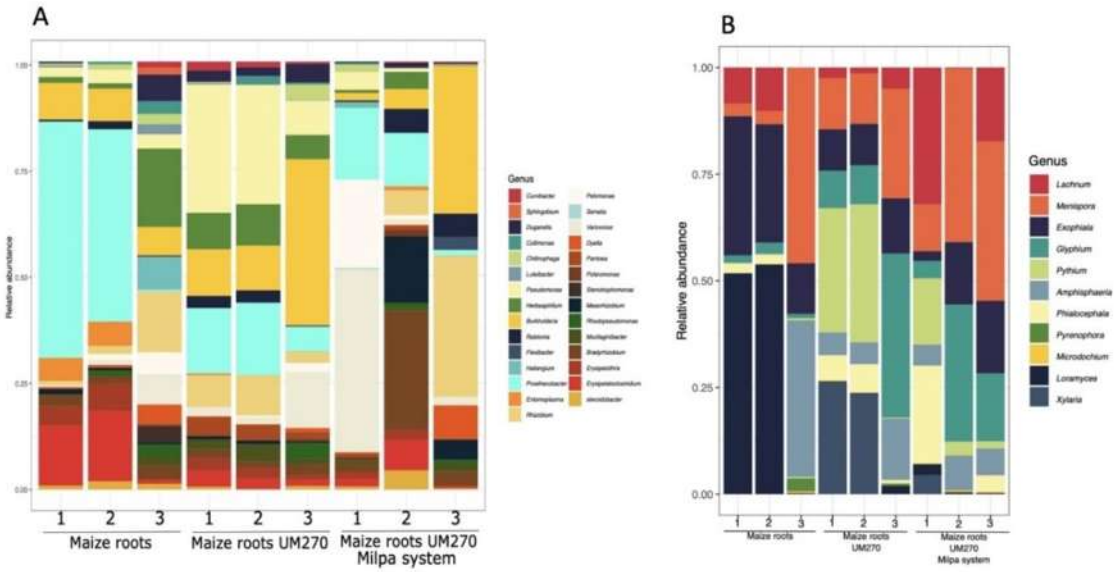

Figure 1. Relative abundances of bacterial (**A**) and fungal (**B**) taxa among the endophytic communities from maize plant roots cultivated in a milpa system.

The results suggested that *P. fluorescens* UM270 inoculation changed the endophytic microbiome of maize roots (Figure 1; maize roots + UM270) compared to uninoculated plants (Figure 1; maize roots treatment). Interestingly, maize roots inoculated with strain UM270 showed unexpected and very different endobiome diversities. Uninoculated maize roots showed a high abundance of OTUs belonging to the genera *Prosthecobacter* and *Curvibacter*, whose presence decreased in inoculated treatments. On the other hand, the bacterial OTUs of the genera *Burkholderia* and *Pseudomonas* were stimulated in a monoculture (Figure 1A; maize roots + UM270), whereas, in the milpa system (Figure 1A; maize roots + UM270 + Milpa system), the abundance of plant-associated genera, such as *Burkholderia, Variovorax*, and N-fixing rhizobia genera, such as *Rhizobium, Mesorhizobium*, and *Bradyrhizobium*, increased.

Figure 1B shows the fungal diversity found in the maize roots from each treatment, including those biofertilized with UM270, either in mono- or polyculture (Milpa system). As noted, no significant association was correlated with the presence of the UM270 strain; however, it was interesting to detect a high abundance of mycorrhizal fungi, such as Rizophagus irregularis or the plant growth-promoting fungus *Exophiala pisciphila*.

The increase in the number of these OTUs was better observed in Figure 2 (panels A and B) for bacteria and fungi, respectively. Some OTUs, shown in gray color, unexpectedly increased in the milpa system (Treatment 3, maize roots + UM270 + Milpa system), which belong to *Burkholderia* and *Variovorax* genera. Other N-fixing bacteria were also increased in plants inoculated with the UM270 strain, but not at the same level detected in the

genera *Burkholderia* and *Variovorax*. It was also noted that some OTUs, such as *Candidatus* Phytoplasma (a phytoplasma taxon associated with aster yellows disease), were also increased in one of the composed samples. However, no disease symptoms were detected in the maize plants.

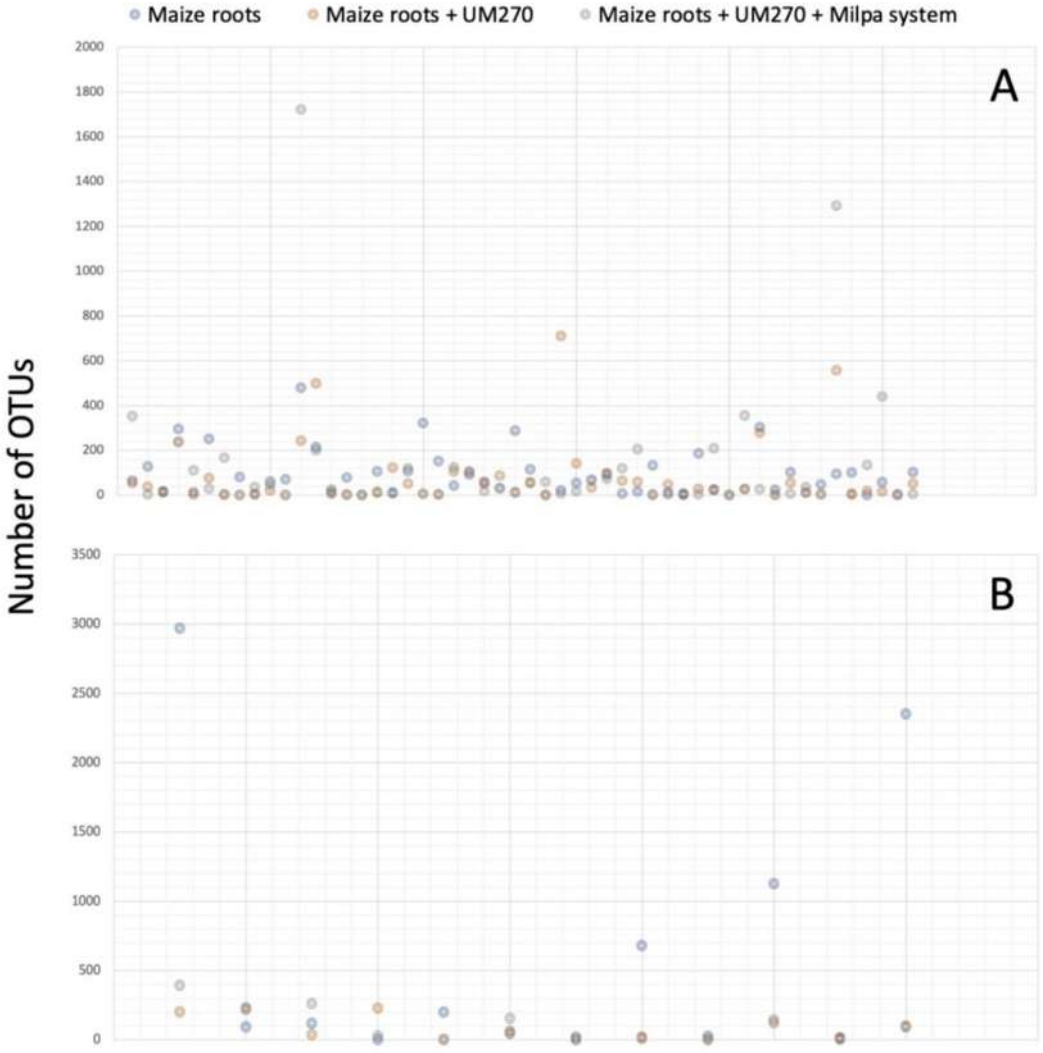

Figure 2. Number of bacterial (**A**) and fungal (**B**) OTUs detected in each of the three treatments.

Figure 3A,B also show significant differences at the genus level of the OTUs found in the diversity of the *endo*bacteriom*e* that was modulated by the interaction with the PGPR UM270. For example, in the top five genera modulated, *Prosthecobacter*, *Burkholderia*, *Pseudomonas*, *Rhizobium*, and *Variovorax* were found, whereas a decrease in the relative abundance of *Prosthecobacter* was observed in the maize roots + UM270 treatment of 2.6-fold, while in the *Maize* roots + UM270 + Milpa system, there was a decrease of 5.6-fold. In contrast, in the other genera, there was a increase in *Burkholderia* OTUs of 2.6-fold in the maize roots + UM270 treatment and a 1.5-fold change in the milpa system. The increase in *Pseudomonas* in the Maize roots + UM270 treatment of 7.5-fold increase is also surprising,

while there was no change in the milpa. *Rhizobium* and *Variovorax* also showed an increase of approximately 2- and 5-fold, respectively, in the milpa treatment. Such differences in the top five genera demonstrated a significant difference relative to the control plants (uninoculated) according to the χ^2 test ($p < 0.05$).

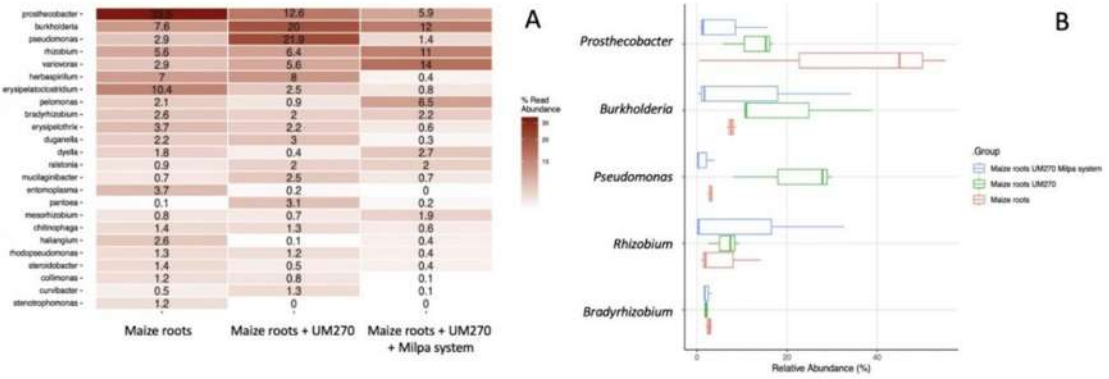

Figure 3. Heatmap of relative bacterial abundances of endophytic bacteriome detected in maize roots (Panel (**A**)), as well as the top five bacterial out treatments (Panel (**B**)).

In the case of the OTUs belonging to fungi, such evident results were not found when there was a correlation with the inoculation of the rhizobacterium *P. fluorescens* strain UM270. In contrast, the abundance of some possible species decreased with inoculation of the UM270 strain (such as in the case of the bacterial OTU *Prosthecobacter*). However, this hypothesis requires additional studies to detect certain antagonistic effects among the endophytic OTUs.

2.2. Index Diversity Analysis

In this study, three of the main ecological indices were analyzed, including Shannon and Simpson indices, as shown in Figure 4, for bacterial and fungal endobiomes. The results showed that the inoculation of the UM270 bacterium in the monoculture and polyculture treatments of maize resulted in quite different alpha diversity, and also that the inoculation altered such measures. Although the alpha diversity in bacteria did not show an evident increase, this was not the case with the alpha diversity for fungi, where an increase in the evaluated indices was noted with respect to the control experiment, where there was no interaction with PGPR UM270.

Figure 5 shows the shared bacterial and fungal OTUs among the three treatment groups. The results showed that 41 bacterial and nine fungal OTUs were shared among the treatments; however, only two bacterial OTUs were found to be unique in maize roots without inoculation, and only one in roots inoculated with UM270 grown in a milpa system. No unique OTUs were found in the fungal endobiomes among treatments.

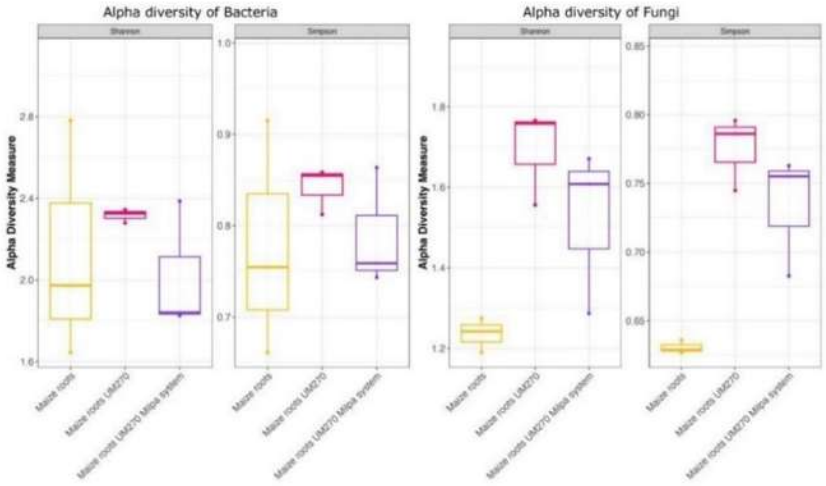

Figure 4. Alpha diversity indexes (measured by Shannon and Simpson diversity indexes) of bacterial and fungal endophytic communities from maize plant roots under three different experiments. Maize roots uninoculated (controls), maize roots inoculated with *P. fluorescens* UM270, and maize roots inoculated with *P. fluorescens* UM270 under a milpa system growth.

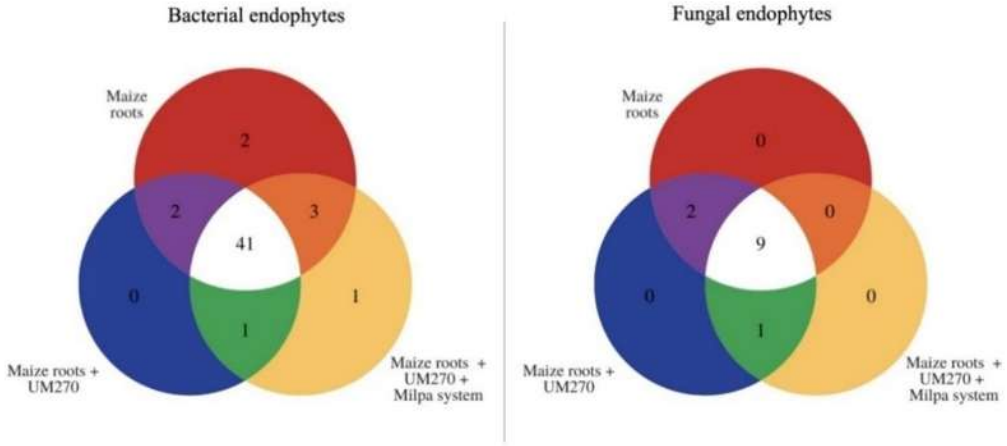

Figure 5. Shared OTUs among treatments. Regarding the unique OTUs found in the treatments where the UM270 strain was inoculated in a Milpa system, only one was found; On the other hand, there were no unique OTUs in corn roots inoculated with UM270. The maize root endobiome showed only two OTUs that were unique, while, for the diversity of fungal endophytes, no unique OTUs were found in each of the three treatments.

2.3. Endobiome Network Analysis

Network analysis was performed to evaluate possible species interactions among the three endobiomes (Figure 6). By identifying unique, common, and co-occurring species, we can better understand the potential ecological relationships among different species and their influence on the overall health and function of endobiomes. This information can be used to develop targeted interventions to promote a healthy endobiome and prevent imbalances in microbial communities [17].

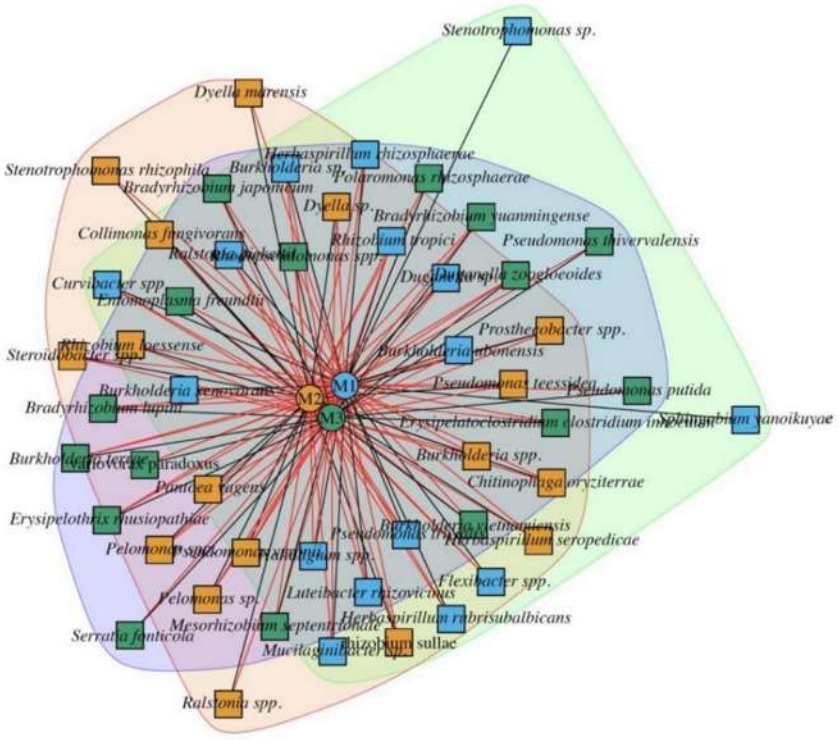

Figure 6. Network analysis of endophytic bacterial communities (endobiomes) from maize plant roots cultivated in a milpa system, inoculated or not with *P. fluorescens* UM270. The boxes represent individual endobiomes: M1 (maize roots), M2 (maize + root UM270), and M3 (maize + UM270 + milpa system). In the network, black lines indicate species that are unique and not present in the endobiomes. In contrast, the red lines indicate interactions or co-occurrences of species in the endobiomes.

Interestingly, despite the different maize treatments, multiple species were present in the endobiomes. This suggests that there are fundamental relationships between certain microbial species and maize plants that are unaffected by specific treatments.

2.4. Metagenomics of the Rhizosphere

Figure 7 shows the taxonomic profile of the rhizosphere metagenomes, including the maize roots (A1), maize roots + UM270 (A2), and maize roots + UM270 + Milpa systems (A3). In general, according to other analyses, no significant differences were found between the three treatments. The Proteobacteria group was the most abundant, followed by Firmicutes and Actibobacteria. However, when performing a heatmap analysis of functional activities detected in the microbial metagenomes based on SEED classifications, some differences were observed in the rhizospheres affected by the UM270 inoculation. For example, functions related to trehalose biosynthesis, ammonium assimilation, and polyamine metabolism are overrepresented in uninoculated maize roots. Figure 8 shows a heatmap of the different levels of analysis of metagenome functional annotations.

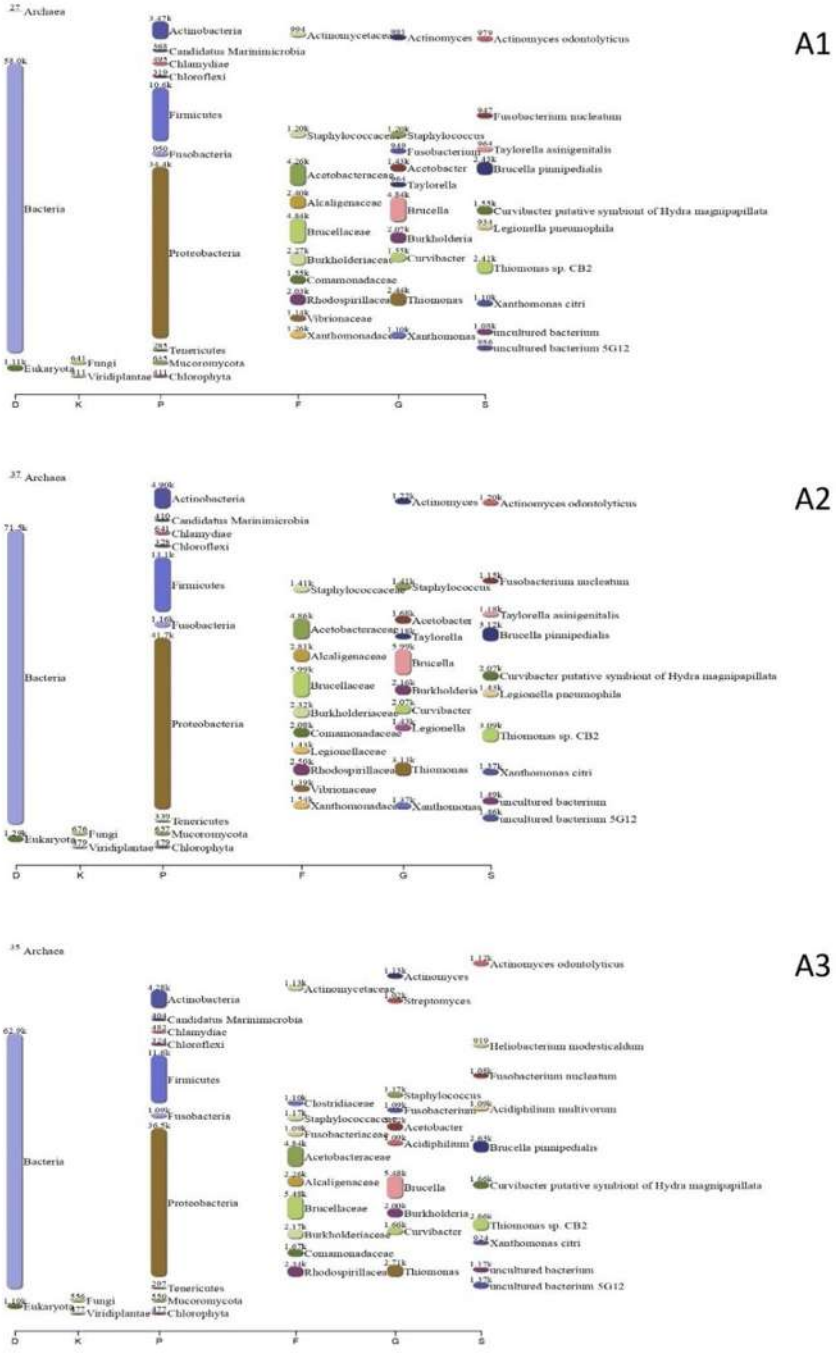

Figure 7. Taxonomic profiles of the rhizosphere metagenomes of maize (A1); maize inoculated with UM270 (A2), and maize inoculated with UM270 in a Milpa system (A3). The *x*-axis reports the taxonomic levels: D: domain; P: phylum; C: class; O: order; F: family; G: genus; S: species. Numbers correspond to the assigned contigs.

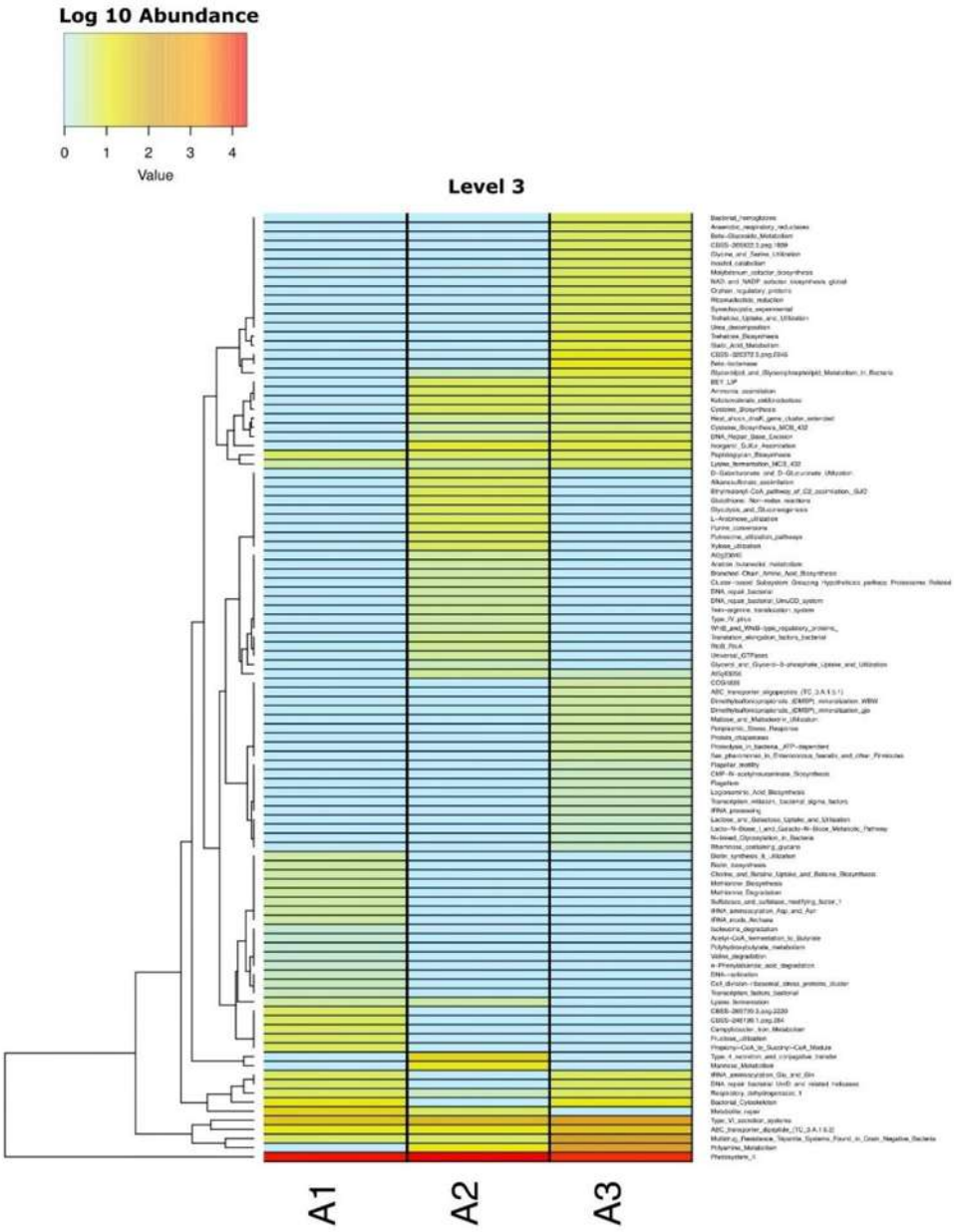

Figure 8. Heatmap of functional activities detected in the rhizosphere metagenomes of maize (A1); maize inoculated with UM270 (A2), and maize inoculated with UM270 in a Milpa system (A3).

3. Discussion

The results of this study show that the application of a biofertilizer based on the rhizobacterium *P. fluorescens* UM270 under the milpa model modulates the microbial diversity of root endophytes and rhizosphere microbiomes, and there was also a negative interaction between the inoculation of PGPR UM270 and the genus *Prosthecobacter*. *Prosthecobacter* has

been associated with medicinal plants as an endophytic organism; however, the role of this bacterium, particularly the strains associated with plants, has been little explored [18]. In contrast, inoculation with beneficial microbial agents associated with plants can engage with other synergistic microbes [19]. Although the mechanism is not very clear, a recent study showed that pre-inoculation of pepper seedlings with the *Bacillus velezensis* strain NJAU-Z9 induced changes in the structure of the rhizosphere microbiome in a field experiment, stimulating communities of genera such as *Bradyrhizobium*, *Chitinophaga*, *Streptomyces*, *Lysobacter*, *Pseudomonas*, and *Rhizomicrobium* [20]. Recently, the endophytic bacteriome of *Medicago truncatula* was modified by the interaction of the biocompound N,N-dimethylhexadecylamine (DMHDA) produced by PGPRs such as *Arthrobacter* sp. UMCV2, and *Pseudomonas fluorescens* UM270. The results showed that bacterial groups such as β-proteobacteria and α-proteobacteria were more abundant in the root and shoot endophytic compartments, respectively [21]. Here, we observed that some genera, such as *Burkholderia* and *Variovorax*, and N-fixing rhizobia genera, such as *Rhizobium*, *Mesorhizobium* and *Bradyrhizobium*, were more abundant in maize cultures inoculated with rhizobacteria UM270. Therefore, it is also possible that these nitrogen-fixing bacteria were stimulated by nodulation factors released by bean plants, and, in turn, improved the acquisition of nitrogen, one of the elements that increased in the ear. It should be noted that it has been reported that intercropping between crops of faba beans (*Vicia faba* L.) and maize can result in overyielding and enhanced nodulation by faba beans [22]. Similarly, co-inoculation with *Rhizobium pisi* and *Pseudomonas monteilii* has been an effective biofertilization strategy for common bean production in Cuban soils [23]. Other studies have also shown synergism between rhizobia and PGPRs to increase the growth and production of maize and beans under different environmental conditions [24–27]. An increase in the abundance of nitrogen-fixing genera in the maize endophytic microbiome affected by inoculation with PGPR UM270 was not clearly detected in the rhizospheric microbiome, perhaps because of the capacity (and preference) of these rhizobia to colonize legume rhizospheres (such as *Phaseolus vulgaris*). Unfortunately, this is a limitation of our study; however, further studies are required to analyze other rhizospheres.

The beneficial mycorrhizal fungus *Rizophagus irregularis* was potentially detected in the maize roots in the three treatments analyzed, including the milpa system. Some previous studies shows that *R. irregularis* can promote the growth of bean plants under greenhouse conditions, as well as under field conditions, having positive effects on maize, soybeans and wheat [28,29]. In 2022 [30], Chen and coauthors reported that *R. irregularis* is capable of modulating soil bacteriomes, in addition to modulating corn growth under salt stress conditions. In another study [31], a strain of *R. irregularis* was co-inoculated with a *Bacillus megaterium* strain, showing that the dual consortium improved maize tolerance to combined drought and elevated temperatures stresses by enhancing photosynthesis, root hydraulics, and regulating hormonal responses. Similarly, the endophytic fungus *Exophiala pisciphila*, particularly the H93 strain, has been an excellent promoter of plant growth in maize. One action of *E. pisciphila* is to improve plant nutrition by solubilizing phosphates [32]. Other species found as endophytes of maize were *Menispora tortuosa*, *Glyphium elatum* or *Phialocephala subalpina*, to mention a few, but they have been more associated with woody plants [33–35]; however, it would be interesting to explore its symbiotic functions with plants of agricultural interest.

Some of the bacterial species identified in this study were well known plant growth-promoting bacterial endophytes. It is present only in certain endobiomes, particularly in untreated maize with the PGPR UM270. These species include *Stenotrophomonas* sp., *Sphingobium yanoikuyae*, and *Burkholderia* spp. For example, *B. unamae* can use phenol and benzene as sole carbon sources; additionally, strains of *B. kururiensis* can metabolize trichloroethylene, 2,4,6-trichlorophenol and decompose phenol, benzene and toluene. Another strain of *B. tropica* degrades benzene, toluene, and xylene. Furthermore, the *B. xenovorans* strain LB400T is one of the most potent aerobic microorganisms and can degrade polychlorinated biphenyl (PCB). Some of these strains have been associated with crop

plants, such as corn (in the case of *B. unamae* [36]). The unique occurrence of these bacteria suggests that they may play a role in corn plant growth, development, and health, particularly in the absence of external treatments. Interestingly, the same bacterial species were also detected in the rhizosphere metagenome. However, unlike the diversity found in the endospheric zone, no significant differences were observed in the rhizosphere. Therefore, diversity was very similar, with few differences.

One of the common genera associated with maize plants is *Stenotrophomonas* sp., which has a wide range of metabolic capabilities and can survive under a variety of environmental conditions [37]. Some species of *Stenotrophomonas* have been found to be plant growth-promoting bacteria that can increase the growth and yield of crops such as maize. For example, some strains of *Stenotrophomonas* have been found to produce indole acetic acid, a plant hormone that stimulates the growth and development of maize roots.

Burkholderia xenovorans is another bacterial species found in the endobiomes of maize. This species degrades various environmental pollutants, including pesticides and herbicides. This suggests that *Burkholderia xenovorans* may play a role in detoxifying soil and protecting maize plants from the harmful effects of these chemicals [38].

Sphingobium yanoikuyae is a bacterial species that degrades polycyclic aromatic hydrocarbons (PAHs) and environmental pollutants that are toxic to plants [39]. This suggests that *Sphingobium yanoikuyae* may protect maize plants from the harmful effects of PAHs in soil. In the M2 condition (*maize + root UM270*), bacteria such as *Dyella marensis*, *Stenotrophomonas rhizophila*, and *Ralstonia* spp. co-occurred with M1 (*maize roots*), but not with M3 *(maize + UM270 + milpa system)*. The co-occurrence of *Dyella marensis*, *Stenotrophomonas rhizophila*, and *Ralstonia* spp. with M1, but not with M3, suggests that the addition of M3 may alter the microbial community structure in the maize endobiome and instead favor the development of other microbial species. *Dyella marensis* is a bacterial species that occurs in soil and is known for its ability to degrade a wide range of environmental pollutants. *Stenotrophomonas rhizophila* is another bacterial species known to promote plant growth, and it has been found in the endobiomes of several plant species, including corn. Some strains of *Stenotrophomonas rhizophila* produce plant hormones and enzymes that can stimulate root growth and plant development [37]. Plant growth-promoting properties have also been observed in some *Ralstonia* species, such as the production of plant hormones and enzymes that stimulate root growth and nutrient uptake. In the M3 system, *Pseudomonas putida*, *Pseudomonas thivervalensis*, and *Serratia fonticola* co-occurred only in M1 and not in M2. *Pseudomonas putida* is present in the endobiomes of several plant species, including maize, and may play a role in promoting plant growth and health [40]. *Pseudomonas thivervalensis* is a less well-studied bacterial species; however, some strains have been found to produce compounds that can inhibit the growth of plant pathogens [41]. *Serratia fonticola* is a bacterial species found in various environments, including soil and water [42].

Beta diversity detected in the endophytic microbiome of maize roots in monoculture and biofertilised with *P. fluorescens* UM270 showed the lowest biodiversity variation with respect to the other treatments. Although it can be argued that polyculture (or cropping practices) and fertilization with biological agents can stimulate greater endophytic diversity [43], the uninoculated maize monoculture also showed high variation. In general terms, it is important to highlight that, among the three treatments carried out in this work, the one associated with milpa is the most variable in terms of diversity and abundance, as all the triplicates vary from each other. In contrast, the most homogeneous triplicates were those of the inoculated "monoculture". Similarly, it is important to point out that field experiments can generate wider variations than those performed under controlled conditions. However, the objective of this work was to get closer to the "reality" of field work, where abiotic conditions may not be so controlled, examining the inoculation of a bacterial agent, such as the UM270 strain, in such conditions in order to determine its performance.

As mentioned above, the taxonomic affiliations of the three rhizospheres analyzed in this study showed no significant differences. However, at the functional level, increases in trehalose biosynthesis, ammonium assimilation, and polyamine metabolism were observed. Trehalose (a-D-glucopyranosyl-1, 1-a-D-glucopyranoside) is a non-reducing disaccharide present in a wide variety of known organisms, some of which are known as anhydrobionts, including plants, fungi, and bacteria. Some plants can revive in the presence of water within a few hours of being completely dehydrated for months or years [44]. Trehalose-producing bacteria, such as rhizobia, can increase the biomass of maize and bean plants under drought conditions [45,46]. Similarly, ammonia assimilation is also related to nitrogen-fixing bacteria, such as *Rhizobium*, and its function seems to be relevant in this milpa system, where legume plants are co-cultivated with maize [47]. Enzymes such as Glutamine Synthetase (GS) and Glutamate Synthase (GOGAT) are important for the assimilation of ammonium; therefore, their search in rhizospheric soil in the cornfield would be relevant for determining their function in these environments. Polyamines play an important role in plant-bacteria communication, as well as in beneficial processes such as PGPR. In a recent review, Dunn and Becerra-Rivera [48] mentioned that polyamines are compounds that act as physiological effects and signal molecules in plant-bacteria interactions, so these functions can be found in rhizospheric environments modulated by the presence of *P. fluorescens* UM270 and could be an area that requires additional attention and research. Thus, the presence of PGPR plays an important role in its presence in rhizospheric environments, stimulating the synthesis of polyamines in other potentially beneficial microbes.

4. Materials and Methods

4.1. Experimental Site

The experiment was conducted in the town of Santa Clara del Cobre in the municipality of Salvador Escalante, Michoacán, Mexico. It is located at 19° 24′ 23″ North, 101° 38′ 24″ West, at an altitude of 2239 m. The prevailing climate is humid subtropical (Köppen climate classification: Cwa).

Prior to the experiment, soil analysis was performed to determine its physicochemical characteristics. This analysis determined that the type of soil is clay and that it is composed of a percentage of 40% sand, 41.96% clay, and 18% silt.

4.2. Biological Material

Seeds of *Zea mays* L., *Phaseolus vulgaris* L., and *Cucurbita* spp. used in this experiment were obtained from the same municipality where the experiment was established and provided by local producers. The UM270 strain was used as a bioinoculant, and it was previously isolated and characterized [49].

4.3. Inoculum Preparation

Bacterial activation of *Pseudomonas fluorescens* strain UM270 was carried out by removing a hoe from the bacteria and placing it in a flask with 500 mL of Nutrient Broth (BD BIOXON, Franklin Lakes, NJ, USA), keeping it under constant agitation at 120 rpm at 28 °C for 24 h until an optical density (560–600 nm) of 1 was reached. The separation of the supernatant and the bacterial pellet was carried out to subsequently suspend it in solution with 0.1 mM magnesium sulfate ($MgSO_4$), while a count of colony forming units (CFU) per milliliter was carried out during serial dilutions on Nutrient Agar media (BD BIOXON).

4.4. Seed Treatments

Seed preparation consisted of a superficial disinfection process involving washing with 70% ethanol, 5% sodium hypochlorite, and sterile distilled water [50]. The seeds used for the treatments in the presence of the bacterial strain were inoculated at a concentration of approximately 1×10^3 CFU per seed. Control seeds were inoculated with $MgSO_4$ solution only. The standard deviation of each inoculum was never greater than 10%. The average CFU per seed was extended in triplicate experiments (three seeds/replication)

in which a seed with bacterial inoculum was placed by immersion in a nutritious liquid culture (5 mL). After vortexing, dilutions were made in nutrient agar at 28 °C for 48 h to quantify CFU/seed. Nine seeds were analyzed during serial dilution.

4.5. Establishment of the Experiment under Field Conditions

The land preparation was carried out using the minimum essential procedures, which consist of clearing the land, followed by fallowing, then a pass with a harrow and furrowing, aiming to not overturn the surface layer of the soil, using animal traction. After this traditional task, maize planting was carried out on 11 May 2021, and the entire stage of cultivation ended in December of the same year. Native maize seeds known as "white maize" were used. This variety was selected for its nixtamalization and tortilla flavor characteristics. After two weeks, guide beans and pumpkins were planted. One month after planting the maize, a second inoculation with the *P. fluorescens* UM270 strain at a concentration of 1×10^8 UFC was performed on the crops with the inoculated seeds, and, after another month, a third inoculation was performed at the same concentration. The *P. fluorescens* UM270 inoculations were applied in liquid form between 10 and 20 cm from the stem of each maize plant.

4.6. Experimental Design

The experimental design was completely randomized, featuring three treatments in which three crops were planted at different planting densities. According to the recommendations of the producers in the region, eight maize plants per m^2 were planted, with 100 plants in each treatment. The composition of the polycultures was calculated as follows: planting a maize plant is equivalent to 0.75 bean plants and 0.25 pumpkin plants. The treatments evaluated were: (1) *Zea mays* L. (maize roots); (2) *Zea mays* L. + UM270 (maize roots + UM270); and (3) *Zea mays* L. + UM270 + *Phaseolus vulgaris* L. + *Cucurbita* spp. (maize roots + UM270 + Milpa system).

4.7. Endophytic DNA Extraction and Illumina Sequencing

Three samples composed of ten healthy maize plant roots (1 g of lateral root tissue from each plant) were pooled to isolate genomic DNA and sequence the endophytic microbiome, including bacteria and fungi. Briefly, soil particles were removed and root tissues were washed and superficially sterilized by immersion in 70% ethanol for 30 s, then in a 2.5% solution of commercial bleach for 5 min, followed by at least five times washing with sterile distilled water. To further confirm the sterilization process, an aliquot from the last rinse of sterile distilled water was cultured on plates with a nutrient agar medium and incubated at 28 °C for 72 h. No growth of bacterial or fungal colonies was observed in the plates after incubation. Then, plant root tissues were macerated using mortars in liquid nitrogen under sterile conditions, following the DNA extraction protocol published by Mahuku (2004) [51], and further purified using a DNA purification kit (PROMEGA). The quantity and quality of the DNA were confirmed by electrophoresis on agarose gels stained with GelRed and visualized under UV light using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Rockford, IL, USA). Nine samples (three from each treatment) with good quantity and purity were sequenced using the Illumina MiSeq platform at the Mr. DNA company (Houston, TX, USA). DNA libraries were constructed by amplifying the V3-V4 hypervariable region (Primers: 515F GTGYCAGCMGCCGCGGTAA; 806R GGACTACNVGGGTWTCTAAT) of the 16S rRNA gene and ITS regions (Pimers: ITS1F CTTGGTCATTTAGAGGAAGTAA; ITS2R GCTGCGTTCTTCATCGATGC) using Mr. DNA. Subsequently, these amplicons were tagged and attached to PNA PCR Clamps to reduce plastid/mitochondrial DNA amplification [52].

4.8. Data Processing

The taxonomic levels of phyla and genera were examined and are indicated for the 16S rRNA gene and ITS sequences obtained with paired-end reads. The sequences were

aligned and processed using a Parallel-META 3.5 workflow [53]. Operational taxonomic unit (OTU) clustering was performed using the SILVA database integrated into Parallel-META 3.5 using a 97% homology criterion. There must be at least two sequences: the minimum zero abundance criterion is 10%, and the average abundance threshold is 0.1%. The maximum and minimum abundances were set to 0.1% and 0%, respectively [53].

4.9. Analysis Alpha and Beta Diversities

Statistical analyses of sequence richness and diversity were performed using the Simpson and Shannon estimators, respectively, implemented in the Phyloseq package (v1.42.0) [54]. In addition, taxonomic composition was visualized using boxplots and heatmaps using ampvis2 (v2.5.5) [55]. Beta-diversity was determined using Vegan (v2.6-4) [56].

4.10. Endobiome Network Analysis

Endobiome network analysis involves the construction and analysis of networks that represent relationships between different species within the endobiome. For this analysis, we used the igraph library, a network analysis library for R. The library provides a wide range of tools and functions for network construction, analysis, and visualization.

4.11. Metagenomic DNA Isolation and Analysis of Soil Rhizosphere

Metagenomic DNA was isolated as previously described [57]. Briefly, Metagenomic DNA was extracted from the rhizospheric soil samples ($n = 5$) using the Mo Bio PowerSoil® DNA Isolation Kit and further purified with the Mo Bio PowerClean DNA Cleanup Kit. The DNA was then quantified, and its quality was assessed using a NanoDropTM 2000 c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The samples were sent to the Genomic Services Center of the MR DNA (Shallowater, TX, USA). Metagenomic analyses were conducted in a similar manner as previously published, following the same quality controls, assembly, and taxonomic and functional annotations [57].

4.12. Sequence Accession Numbers

The raw sequences are available at NCBI under BioProject accession number PRJNA901513 and Sequence Read Archive (SRA) accession numbers SRR22351342, SRR22351344, SRR22351348, SRR22351343, SRR22351346, SRR22351345, SRR22351347, and SRR22351341.

4.13. Statistical Analysis

The data obtained were analyzed by analysis of variance, and the variables that presented significant differences were analyzed by Tukey's test ($p < 0.05$) using the statistical package SAS (Statistical Analysis System) version 9.2.

5. Conclusions

Finally, the endobiome network allowed for the identification of different bacterial species present in the three maize treatment types, indicating the presence of fundamental relationships between certain microbial species and maize plants that were not affected by the specific treatments. In addition, some unique bacterial species have been identified in specific endobiomes (e.g., *Stenotrophomonas* spp. or *Burkholderia* spp.), some of which are also present in the rhizosphere, indicating their possible roles in the growth, development, and health of maize plants, especially in the absence of external treatments.

The addition of biofertilizers to maize plants grown under mild conditions, such as the *P. fluorescens* UM270 strain, modulates the rhizosphere and root endophytic microbiome. One of the potential mechanisms employed by the UM270 strain to stimulate plant growth may be the recruitment of other beneficial microorganisms through signaling molecules (e.g., polyamines). However, this hypothesis requires further investigation through the isolation and characterization of the synergistic activities of the inoculated strain UM270 and the associated microorganisms of maize plants.

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VIII. Discusión general

La milpa es una unidad de producción agrícola que sirve como ejemplo de policultivos milenarios y sustentables, que engloban una serie de interacciones ecológicas de las que, además de obtener una gran variedad de productos agrícolas, conserva la diversidad de especies. Sin embargo, la falta de fertilidad de los suelos y el efecto de factores bióticos y abióticos que afectan a los cultivos presentes en las milpas como *Z. mays* , se requiere el uso de MPCV que mejoren la producción y mitiguen los efectos negativos por estrés hídrico, salino, insectos plaga y patógenos en las milpas (Torres-Calderón *et al.* 2018; Regalado López *et al*. 2020; Sánchez Cariillo y Guerra Ramírez 2022).

Los resultados de este estudio comprueban el efecto de la cepa *P. fluorescens* UM270 en el crecimiento de *Z. mays* bajo diferentes tipos de sistemas milpa en campo como los co-cultivos *Z.mays* + *P. vulgaris*, *Z.mays* + *C. pepo*, *Z.mays* + *C. pepo* + *P. vulgaris* (triada mesoamericana). Se comprobó que durante ambos ciclos la inoculación de UM270 incrementó los parámetros de concentración de clorofila, altura, crecimiento de raíz, peso seco de las plantas y raíces comparado con los tratamientos sin inóculo. Efectos similares se han observado en monocultivos de *Z. mays* inoculados con biocápsulas de *Bacillus* sp., en campo. De igual forma, en un estudio previó se evaluó la inoculación de un consorcio bacteriano con *Bacillus* y *Pseudomonas*, el cual incrementó el crecimiento de *Z. mays* en comparación con las plantas no inoculadas (Olanrewaju y Babalola 2019; Chaudhary *et al*. 2021).

Con base en los parámetros fitómetricos se puedo determinar que el tratamiento de *Z. mays* y + UM270 + DAP se correlaciona positivamente con el tratamiento de la triada mesoamericana más el inóculo, este comportamiento se observó durante ambos ciclos (2021 y 2023). Por otra parte, el monocultivo de *Z. mays* + UM270 se correlacionó positivamente con el co-cultivo *Z. mays* + *P. vulgaris* + UM270, esta correlación se presentó únicamente durante ciclo de cultivo 2023. Lo que indica que los efectos en el crecimiento de *Z. mays* depende de la interacción planta-planta y planta-microorganismos. Efectos similares se han reportado en un modelo milpa en donde la interacción entre *V. faba* y *Z. mays* promueve la

producción de *Z. mays* en suelos deficientes de fósforo (Yan *et al*. 2014). Respecto a la correlación planta-microorganismo existen diferentes estudios que han comprobado el efecto béneficio de BPCV tal es el caso de *B. subtilis* que al ser inoculado en trigo (*Triticum*), promovió el incremento en la clorofila, tamaño de la espiga e incluso proteína del grano, comprobando su capacidad para colonizar la rizósfera de este cultivo y, por ende, mejorar su producción (Ibarra-villarreal *et al*. 2023).

Uno de los parámetros evaluados fue la producción de grano, en el tratamiento *Z. mays* + UM270 incrementó un 41.96 y 42.03% para los ciclos 2021 y 2023, respectivamente. De igual forma, en el cocultivo de *Z. mays* + *P. vulgaris* + UM270, hubo un incremento en la producción de grano de 28.28 y 23.56% durante ambos ciclos. Por su parte la co-fertilización de UM270 + DAP, incremento la producción de grano en un 58.13 y 56.59% durante el ciclo 2021 y 2023 respectivamente. En diferentes evaluaciones se ha determinado la eficacia de los sistemas milpa al obtener mayor producción en menor espacio, además con intercultivos como chícharo (*Cajanus cajan*) + *Z. mays*, aumentó la producción de mioinositol, prolina y se observó una mayor formación de biopelículas en plantas de *C. cajan*. Por su parte Z. *mays* se beneficia de ésta interacción al aumentar el contenido de la galactosa, D-glucopiranósido y arginina (Ebel *et al.* 2017; Aguilar Jiménez *et al*. 2019; Vora *et al.* 2021).

La producción de *P. vulgaris* y *C. Pepo* también se vieron beneficiados con la inoculación de la cepa UM270, en el co-cultivo *Z. mays* + *P. vulgaris* se observó un incremento en la producción durante ambos ciclos de un 12.5 y 13.32%, respectivamente, con respecto al tratamiento sin inóculo. Por su parte, la inoculación en el tratamiento triada mesoamericana incrementó la producción de la calabaza en un 30.29% para el ciclo 2021 y 20.90% para el ciclo 2023. Resultados similares se han reportado al establecer cultivos intercalado de *Z. mays* + *P. vulgaris* e inoculados con *Rhizobium*., aumenta la nodulación y biomasa de frijol (Cardoso *et al*. 2007). En el análisis de la composición química (N, P, K, Mg, Ca, Fe, Mn y Zn) del grano, se determinó que no existe una correlación entre la cepa UM270 y la concentración química del grano.

Por otro lado, la inoculación de la cepa UM270 bajo el modelo de milpa moduló la diversidad microbiana de los endófitos de la raíz y los microbiomas de la rizosfera, además de que también hubo una interacción negativa entre la inoculación de la cepa UM270 y el género *Prosthecobacter*, que ha sido asociado como endófito de plantas medicinales, sin embargo, el papel de esta bacteria, particularmente las cepas asociadas a plantas, ha sido poco explorado (Liu *et al.* 2018). Sin embargo, existen asociaciones simbióticas que mejoran la estructura del microbioma de la rizósfera tal es el caso de la cepa de *B. velezensis* NJAU-Z9 que modula comunidades bacterianas como *Bradyrhizobium, Chitinophaga, Streptomyces, Lysobacter, Pseudomonas* y *Rhizomicrobium* (Zhang *et al*. 2019).

En este experimento se observó que géneros, como *Burkholderia*, *Variovorax*, y géneros de rizobios fijadores de N, como *Rhizobium*, *Mesorhizobium* y *Bradyrhizobium*, fueron más abundantes en cultivos de maíz inoculados con UM270. Por lo tanto, también es posible que estas bacterias fijadoras de nitrógeno fueran estimuladas por factores de nodulación liberados por las plantas de frijol y, a su vez, mejoraran la adquisición de nitrógeno, no obstante, para demostrar esta hipótesis es necesario realizar experimentos. En el cultivo intercalado como *V. faba* y *Z. mays*, se ha comprobado que mejora la productividad nodulación y fijación de nitrógeno en *V. faba* mediante interacciones radiculares interespecíficas entre las especies (Li *et al*. 2016). Diferentes estudios se han reportado con efectos positivos sobre la coinoculación de *R. pisi* y *P. monteilii*, en la producción de cultivos de *P. vulgaris* (Sánchez *et al*. 2014).

En este estudio se encontró la presencia de hongos promotores de crecimiento vegetal como *R. irregularis* en los tres sistema milpa, del que se conoce incrementa la producción de cultivos como *Z. mays*, *Glycine max* y *Triricum*, además de tener la capacidad de modular bacteriomas de suelos salinos relacionados con el cultivo de *Z. mays* (Hidalgo Rodríguez *et al.* 2019; Renaut *et al*. 2020). Otro de los hongos detectados en estos sistemas es *Exophiala pisciphila*, y en particular la cepa H93, que ha sido un excelente promotor del crecimiento de plantas en *Z. mays* (Xu *et al*. 2020). Otras especies de hongos encontradas como endófitas de *Z. mays* fueron *Menispora tortuosa*, *Glyphium elatum* o *Phialocephala subalpina*, por

mencionar algunas, pero han sido más asociadas a plantas leñosas (Lorenzo and Messuti 2005; Réblová *et al*. 2006; Schlegel *et al.* 2016). Sin embargo, sería interesante explorar sus funciones simbióticas con plantas de interés agrícola.

Algunas de las especies bacterianas identificadas en este estudio son endófitos bacterianos promotores del crecimiento vegetal bien conocidos. En el tratamiento M1 (monocultivo de *Z. mays* sin inóculo) incluyen *Stenotrophomonas* sp., *Sphingobium yanoikuyae* y *Burkholderia* spp, que se conoce tienen efecto en la promoción de crecimiento de cultivos de *Z. mays*, fungen como fitorremediadoras ante contaminantes como pesticidas, además de ser degradadoras de hidrocarburos aromáticos policíclicos (Chen et al. 2021; Leite *et al.* 2022; Chauviat *et al.* 2023).

En el tratamiento M2 (*Z. mays* + UM270), bacterias como *Dyella marensis*, *S. rhizophila* y *Ralstonia spp.* co-ocurrieron con M1 pero no con M3 (triada mesoamericana + UM270). En el caso de M3, especies como *P. putida*, *P. thivervalensis* y *Serratia fonticola* coexistieron solo con M1, destacando la capacidad de estas bacterias para promover el crecimiento vegetal y por su efecto biocontrol ante patógenos. Lo anterior sugiere que el conjunto de interacciones dadas entre los microorganismos y las plantas pueden ser útiles para el reclutamiento de otros MPCV (Costa-Gutierrez *et al.* 2022; Chauviat *et al.* 2023).

A nivel funcional, se observó un aumento en la biosíntesis de trehalosa, asimilación de amonio y metabolismo de poliaminas, procesos clave para la adaptación de las plantas a condiciones de estrés, como la sequía. Las bacterias productoras de trehalosa, como los rizobios, pueden aumentar la biomasa de plantas de *Z. mays* y *P. vulgaris* ante condiciones de sequía (Rodríguez-Salazar *et al*. 2009). De igual manera, la asimilación de amoniaco también está relacionada con bacterias fijadoras de nitrógeno, como *Rhizobium*, y su función parece ser relevante en este sistema de milpa en donde las plantas leguminosas se co-cultivan con *Z. mays* (Schulte *et al*. 2021).

IX. Conclusión

Pseudomonas fluorescens UM270 promovió el crecimiento y la producción de *Zea mays* en sistemas milpa, favoreciendo géneros bacterianos benéficos como *Burkholderia*, *Rhizobium* y *Bradyrhizobium*. Sus efectos pueden incrementar la productividad agrícola y reducir la dependencia de fertilizantes sintéticos, consolidándola como una alternativa sostenible.

X. Perspectivas

- Desarrollar un bioinoculante encapsulado con *Pseudomonas fluorescens* UM270 como agente principal para mejorar su estabilidad y eficacia en campo.
- A partir de los efectos positivos observados en sistemas milpa, explorar el uso de consorcios microbianos que incluyan *P. fluorescens* UM270 junto con otros microorganismos nativos del sistema, con potencial para la promoción del crecimiento vegetal, biocontrol y bioestimulación.
- Analizar el transcriptoma y metaboloma en las interacciones planta-UM270-patógeno para identificar genes y metabolitos clave en la resistencia al estrés biótico.

XI. Referencias

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XII. Anexos de publicaciones

agronomy

MDPI

Review

Plant Growth-Promoting Bacteria as Bioinoculants: Attributes and Challenges for Sustainable Crop Improvement

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Abstract: Plant growth-promoting bacteria (PGPB) are excellent biocontrol agents and stimulators of plant growth, nutrition, and production. Therefore, these plant-associated bacteria are considered an excellent alternative to reduce or eliminate the use of toxic agrochemicals. In this work, we review the current state of the beneficial mechanisms (direct and indirect), including the production of antibiotic compounds and enzymes, facilitation of resource acquisition, or production of stimulating phytohormones/metabolites. Some aspects of the formulation technology and bioinoculant efficiency of diverse PGPBs (e.g., rhizobacteria, phyllobacteria and endophytic bacteria) in the field are also discussed. However, the commercialization and application of these biological agents in agriculture occur mainly in developed countries, limiting their success in developing regions. The possible causes of the delay in the application of bioinoculants for sustainable agriculture and the plausible solutions are also discussed in this study. Finally, the use of PGPBs is currently a priority for sustainable production in agriculture.

Keywords: plant growth-promoting endophytes; rhizobacteria; phyllosphere; agrochemicals; sustainable agriculture; biostimulants

1. Introduction

In the mid-20th century, an almost exponential growth in human population occurred in various countries worldwide. This increasing number of individuals caused an unprecedented demand for food and required an agricultural production that had not been seen before. In this regard, the "green revolution" helped meet this demand for food and products through the use of chemical fertilizers and pesticides, which led to a decrease in infections of plant crops caused by pathogens [1]. However, the excessive use of agrochemicals, which was more evident in some underdeveloped regions, has wreaked havoc on the environment and on human and animal health [2]. Even with the excessive use of agrochemicals, agricultural losses due to pathogens have not ceased, with up to 25% of the total world production being lost annually [3]. It has been proposed that food production by 2050 will need to double the present production. To achieve this goal, new alternatives that would result in an increase in agricultural production via eco-friendly, sustainable, and nontoxic strategies have been sought [4,5]. Thus, various strategies, such as the production of genetically modified organisms (mainly plants), the generation of crosses that are naturally resistant to pests, and the use of natural compounds and

plant-beneficial microorganisms have been proposed [6]. The beneficial microorganisms that may be part of bioinoculants, whether these are biofertilizers, biocides, or biostimulants, may be beneficial fungi such as *Trichoderma* spp., arbuscular mycorrhizal fungi, and rhizospheric or endophytic bacteria [7–9]. A long list of commercialized bacterial inoculants, based mainly in plant growth-promoting bacteria (PGPB), has been reviewed by Glick [10]., and includes *Agrobacterium radiobacter*, *Azospirillum brasilense*, *Azospirillum lipoferum*, *Azotobacter chroococcum*, *Bacillus firmus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus mucilaginous*, *Bacillus pumilus*, *Bacillus* spp., *Bacillus subtilis*, *Bacillus subtilis* var. *amyloliquefaciens*, *Burkholderia cepacia*, *Delftia acidovorans*, *Paenibacillus macerans*, *Pantoea agglomerans*, *Pseudomonas aureofaciens*, *Pseudomonas chlororaphis*, *Pseudomonas fluorescens*, *Pseudomonas solanacearum*, *Pseudomonas* spp., *Pseudomonas syringae*, *Serratia entomophila*, *Streptomyces griseoviridis*, *Streptomyces* spp., *Streptomyces lydicus*, and *Rhizobium* spp. However, the list is still growing, and new bacterial species with beneficial properties for sustainable agriculture are being described [11]. This work tries to explain the different mechanisms used by plant-associated bacteria, with special emphasis on bacteria inhabiting the rhizosphere (rhizobacteria), phyllosphere (phyllobacteria) and the plant endosphere (endophytic bacteria), as well as the different challenges to be applied in the field through efficient formulation that ensure the survival and action of their bacterial agents.

2. An Overview of Plant-Associated Bacteria

Bacteria can be associated and interact from below- or above-ground areas of the plant. Likewise, bacteria can penetrate the internal compartments of the plant and live inside. All these plant-associated bacteria might be able to exert beneficial mechanisms, such as direct and indirect (biocontrol) plant growth promotion (PGPBs).

2.1. The Rhizobacteria

The rhizosphere is the area of the soil that surrounds the root and is influenced by the excretion of root compounds. Root exudates contain vitamins and, amino acids, among other nutrients that can be acquired by rhizospheric bacteria, increasing their populations of those that have the greatest advantage to take them [12]. The term "rhizosphere" was coined by Lorenz Hiltner, and since its description, he had described it as a microenvironment where the bacteria that inhabit it ("bacteriorhiza") could interact and significantly influence plant nutrition [13]. Additionally, Hiltner had also suggested the visit of "uninvited guests," that adjust to the specific root exudates. According to Hartmann and colleagues [13], Hiltner also hypothesized that "the resistance of plants towards pathogenesis is dependent on the composition of the rhizosphere microflora". This idea has been confirmed by multiple studies, as the rhizosphere microbiome has a preponderant role in plant protection through the stimulation of the plant's immune system and the direct control of potential phytopathogens [14].

2.2. The Phyllobacteria

Plants can be colonized below-ground by rhizobacteria and above-ground by a variety of microorganisms, including bacteria. This aerial habitat, which includes leaves, stems, flowers or fruits surfaces that can be colonized by microbes is termed the phyllosphere, and the inhabitants are called epiphytes. One of the most common inhabitants of phyllosphere are bacteria, which are known as phyllobacteria (or phyllosphere bacteria). Usually, phyllobacterial species may face a lot of environmental changes (even during the same day) compared to rhizosphere or endophytic bacteria (see below), including wet or dry conditions, as well as tolerating UV radiation [15]. However, some specific points of colonization in the leaves are particularly protecting sites for phyllobacteria from these harsh abiotic factors, such as trichomes, veins, cell wall junction of epidermis and stomata. In addition, phyllobacteria can uptake nutrients for survival from phyllosphere and exert protection to the plant from the pathogens attack. For example, Pseudomonas species are able to

avoid the Botrytis conidia attachment by changing the surface wettability [16]. Besides, phyllobacteria can stimulate the ISR plant system and stimulate plant growth [17].

2.3. The Bacterial Endophytes

Plants form associations with microorganisms in each and every corner of our planet. The microorganisms that interact with a plant are collectively known as the plant microbiome. The group of microorganisms that interact "more" closely with plants and live within their tissues are known as endophytes. Endophytes, or the plant endobiome if we refer to endophytic communities, have been proposed to have a long-lasting relationship as old as 400 million years [18,19]. Therefore, these microorganisms have evolved different types of plant-endophyte interactions, such as neutralism or commensalism, mutualism, and symbiosis. Of course, there are also harmful interactions with pathogenic endophytes, such as parasitism [20]. However, endophytes must have a non-pathogenic relationship with the plant. In other words, endophytes must be isolated from the surface of sterilized tissues and not produce any apparent damage to the host plant [6]. Thus, it is important to differentiate between pathogenic and non-pathogenic endophytes, since those that have beneficial interactions with the plant are the ones to be used in sustainable agriculture.

In general, two types of endophytes have been proposed. First, the long-term relationship endophytes, which are part of the core of endophytes "selected" by the plant. Long-term associations can be inherited vertically through seeds. Second, the short-term association endophytes, which are represented by those endophytes that tend to colonize the host through random mechanisms, such as colonization of cracks or damaged areas in the plant, mainly roots. This may be a way to facilitate their penetration of the plant internal tissues. To learn more about these colonization mechanisms and the types of interactions, several recent contributions are recommended [7]. Some authors refer to these short- and long-term associations as nonsystemic or systemic, respectively [21]. It is noteworthy to mention that these classifications are based on taxonomic characteristics, mode of transmission, lifestyle, host defense response, ecological functions, evolutionary pattern, and diversity.

3. Beneficial Activities of PGPB

The beneficial and promoting mechanisms of plant growth have been widely reported and reviewed in previous studies [7,10,22]. However, for the purpose of this work, it is relevant to review them to mention their importance in the application of PGPBs as bioinoculants in various agricultural systems. Rhizobacteria and bacterial endophytes, among other beneficial microorganisms, can stimulate the growth and health of plants through direct and indirect mechanisms that are described below (Figure 1).

3.1. Direct Mechanisms

The direct mechanisms of plant growth promotion include the facilitation of nutrient acquisition and the synthesis of hormones [10]. One of the main problems that plants face in acquiring nutrients is the poor solubility of the elements in the soil. For example, phosphorus is scarce in many soils worldwide, besides being in insoluble forms, limiting its use by plants. Plants generally obtain soluble phosphorus in two forms, monobasic and dibasic. Phosphorus is present in the soil as inorganic minerals, such as apatite, or as one of the several organic forms, including inositol phosphate, phosphomonoesters, and phosphotriesters [23]. Inorganic phosphorus is applied in the field as a chemical fertilizer, along with other elements such as nitrogen. However, as phosphorus is mostly insoluble, the plant does not use it and it leaches, contaminating the ground water reserves [24]. Therefore, the use of phosphate solubilizing PGPB, including genera such as *Achromobacter*, *Agrobacterium*, *Bacillus*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Gluconacetobacter*, *Mycobacterium*, *Pseudomonas* and *Serratia*, play a fundamental role in solubilizing insoluble forms of phosphorus, mainly through mechanisms such as the production of acid phosphatases, which help to mineralize organic phosphorus in the soil [22,23]. Likewise, the production

of organic acids such as gluconic acid and citric acid by PGPB help in the solubilization of phosphorus, in such a way that, when plants acquire these solubilized or mineralized molecules, their growth and production can be stimulated [24]. Moreover, production of organic and inorganic acids such as citrate, oxalate, acetate, sulfuric acid, carbonic acid and nitric acids by PGPB, also stimulates the solubilization of other elements, such as zinc and potassium, which are essential for soil fertility and crop improvement [22].

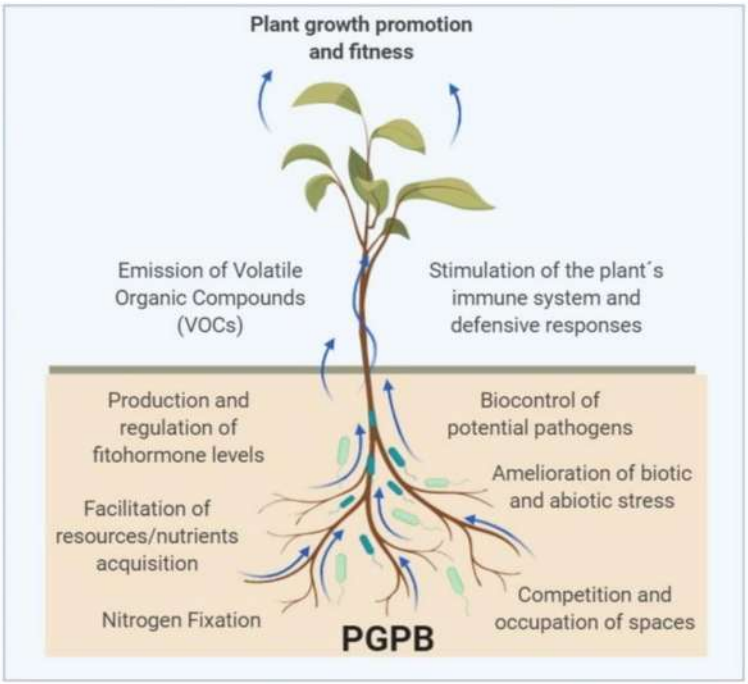

Figure 1. Beneficial mechanisms exerted by plant growth-promoting bacteria (PGPB) to stimulate healthy plant growth and fitness.

An element that is abundant in nature is iron, whose acquisition requires the formation of Fe-siderophore complexes. Siderophores are iron-chelating compounds secreted by bacteria that reduce iron (Fe^{3+} to Fe^{2+}) intra and intercellularly and can be used either by the plant or the endophyte. Siderophores have a relevant function when Fe is scarce in the environment [6]. One of the main endophytic bacterial genera is *Pseudomonas*. Characteristically, *Pseudomonas* species fluorescence is due to their different kinds of siderophores, such as pyochelin, pseudobactin, and pyoverdine. Several studies have shown that microbial siderophores can directly increase plant growth through the improvement in iron acquisition, since this element has several important biological functions for the cell [25]. For example, *P. fluorescens* strain C7 produces siderophores of the pyoverdine type, which forms a pyoverdine-Fe complex. This complex may be taken up by the plant *Arabidopsis thaliana* and increase its growth [26]. Microbial siderophores are synthesized by various taxa and may participate through indirect mechanisms in plant growth [27,28], as reviewed below.

Nitrogen (N) is another essential element for the development and production of fruits and seeds in plants of agricultural interest [29]. Leguminous plants may symbiotically interact with soil bacteria collectively known as rhizobia, which include the genera *Bradyrhizobium*, *Sinorhizobium/Ensifer*, *Mesorhizobium*, *Rhizobium*, *Azorhizobium*, *Neorhizobium*, and *Pararhizobium*. These are free-living bacteria (diazotrophic) that may penetrate

plant tissues through the exchange of chemical signals and form nodules. Nodules are globular or cylindrical structures where rhizobial endophytes reside and are capable of fixing atmospheric nitrogen and converting it into ammonia, an assimilable form of nitrogen for the plant [30]. Some non-nitrogen-fixing bacteria, such as *Pseudomonas*, may stimulate the legume-rhizobia symbiosis in addition to increasing levels of nitrogen fixation, thus improving plant growth and nutrition. For example, the high activity levels of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase detected in *Pseudomonas* are essential to enhance the nodulation process in rhizobia, showing that a beneficial interaction between bacteria may also benefit the plant [31]. Similar activities have been observed in other plant-bacteria models [32].

The production of phytohormones and other diffusible or volatile compounds that modulate plant growth is a relevant factor for potential endophytes that are candidates for being used as biostimulant products in agricultural crops [6]. The main hormones that stimulate plant growth are auxins, such as volatile organic compounds (VOC). Each of them has special functions to stimulate plant growth, in addition to being synthesized by the plant and fulfilling various physiological processes. For example, IAA participates in processes such as seed germination, formation of lateral roots, gravidity, and photosynthesis; it affects photosynthesis and the production of metabolites and other relevant compounds involved in the development of the plant [6,10]. Gibberellins and cytokinins also modulate a wide variety of processes, such as germination of seeds and cell elongation, primarily in the stem. The production of other compounds that stimulate plant growth and development, such as those of the diffusible or volatile type, has stood out in the last decade for their relevant role in plant-bacteria interactions, such as acetoin, 2,3-butanediol, and *N*,*N*-dimethylhexadecylamine [33–35]. Table 1 shows a summary of works describing some of the main direct mechanisms of plant growth promotion in bacteria.

3.2. Indirect Mechanisms

Indirect mechanisms include antagonism of PGPB towards potential phytopathogens. Restricting the growth or eliminating pathogens is an indirect mechanism for PGPB to promote the growth and health of the plant. PGPB contain an entire arsenal of compounds and enzymes that have the ability to restrict or eliminate pathogens. For example, the siderophores produced by bacteria of the genus *Pseudomonas* have the ability to chelate the Fe available from the medium, restricting it to pathogens. This mechanism was one of the first described in plant growth-promoting bacteria and has been reported in various studies [36].

Another mechanism widely used by bacterial endophytes with antifungal activity is the production of enzymes, such as chitinases, cellulases, and β-1,3-glucanases that degrade the fungal cell wall. Chitinase degrades chitin, an insoluble linear polymer of β-1,4-N-acetyl-glucosamine, known to be the major component of the fungal cell walls. Various bacteria that are part of the plant protective endobiome include species of *Bacillus*, such as *B. licheniformis*, *B. cereus*, *B. subtilis*, and *B. thuringiensis*. Martínez-Absalón et al. [37] demonstrated the relevant role of chitinase production in *B. thuringiensis* UM96. These researchers showed that, when using a chitinase inhibitor compound in UM96 strain supernatants, the biocontrol function towards the plant pathogen *Botrytis cinerea* decreased. *B. cinerea* is known to cause gray mold in more than 200 plant species. Other Gram-negative species, such as *Pseudomonas*, also produce various enzymes that directly attack the fungal cell wall [38].

Bacilli is one of the most studied groups of isolated endophytic and rhizospheric bacteria; the members of this group have been characterized as potential biocontrol agents [34]. The antagonistic abilities of Bacilli include the synthesis of various enzymes with antibiotic activity including peptides of ribosomal origin (polyketide synthases), such as subtilin, subtilosin A, TasA, and sublancin. Besides, Bacilli produce peptides of non-ribosomal origin, which are synthesized by non-ribosomal peptide synthetases, such as bacillaene, bacilysin, chlorotetain, difficidin, mycobacillin, and some rhizocticins [39]. The production

of other volatile compounds such as ethylene, methyl salicylate, and methyl jasmonate may induce and control plant defense responses [33]. Plant defense responses stimulated by bacterial endophytes are widely reported in the literature, and their main function is to increase a series of actions that allow the plant to defend from the attack of pathogens [40]. Some recent works that exemplify the benefits of plant growth promoting bacteria and their application in different plant crops are listed in Table 1.

Table 1. Examples of works highlighting direct and indirect mechanisms of biocontrol and plant-growth promotion in plant-associated bacteria.

Bacterial Species and Strain	Mechanism and/or Benefit in the Host Plant	Plant Host Species	Type of Test or Applied Technique	Reference
Bacillus subtilis ES748, ES749 and a bacterial consortium	Synergistic interaction between species	*Arabidopsis thaliana*	Generation of mutants, colonization and maintenance assays in vitro	[41]
Agrobacterium rhizogenes K599	Improvement in the acquisition of nitrogen and change in the secretion of organic compounds	*Phaseolus vulgaris* L.	In vitro colonization assays and microbial community analysis	[42]
Pseudomonas umsongensis, *Arthrobacter defluvii*, *Streptomyces gardneri*, *Microbacterium yannicii*, *Variovorax ginsengisoli*, *Cupriavidus laharis*, *Bosea vestrisii*, *Bosea robiniae*	Production of phytohormones, secretion of siderophores and stimulating effects	*Zea mays* L., *Populus nigra* and *Arabidopsis thaliana*	Detection of siderophores, phytohormones and colonization and permanence tests in vitro and in the field	[1,43]
Sinorhizobium meliloti 2011	Improved nitrogen acquisition, secretion of signaling compounds	*Medicago truncatula*	Detection of nitrogen-fixing nodules and in vitro colonization assays	[44]
Bacillus cereus YL6	Improved phosphorus acquisition and phytohormone biosynthesis	*Glycine max*, *Triticum vulgare*, *Brassica rapa* subsp. *pekinensis*	Phosphorus solubilization test, in vitro and field colonization test of plant species	[45]
Pseudomonas aeruginosa NXHG29	Dual antagonism, quorum sensing, and biofilm formation	*Nicotiana tabacum*	In vitro colonization and antagonism assays	[46]
Pseudomonas stutzeri E25, *Stenotrophomas maltophilia* CR71	Antagonism, secretion of volatile organic compounds and synergism between bacterial species	*Physalis ixocarpa*, *Lycopersicon esculentum* cv Saladette	In vitro antagonism, promotion, colonization and volatile compound detection assays by GC-MS	[47]
Bacillus cereus SA1	Production of phytohormones, secretion of organic acids. Improved the biomass and chlorophyll content.	*Glycine max*	Detection of phytohormones, HPLC compound determination and assays in plants.	[48]
Pseudomonas fluorescens UM270	Genes involved in signaling, antioxidant activities, secretion systems, and biofilm production	not applicable	Genomic comparison *Pseudomonas* strains	[49]
Bacillus megaterium, *Enterobacter* C7	Improvement in the acquisition of Na, Ca, Mg, production of antioxidants, phytohormones and secretion of secondary metabolites	*Solanum lycopersicum*	Detection of phytohormones, evaluation in the change of metabolic profiles by GC-MS and in vitro colonization assay	[50]
Bacillus subtilis SWR01	Genes involved in swarm signaling and motility	*Solanum lycopersicum*	Generation of mutants and in vitro colonization assay	[51]
Bacillus thuringiensis UM96, *Pseudomonas fluorescens* UM16, UM240, UM256, UM270	Synergistic interaction between species and plant growth stimulation of plants	*Physalis ixocarpa*	In vitro colonization assay	[2,52]

Table 1. *Cont.*

Bacterial Species and Strain	Mechanism and/or Benefit in the Host Plant	Plant Host Species	Type of Test or Applied Technique	Reference
Bacillus altitudinis KP-14	Production of phytohormones, secretion of siderophores, Improvement in the acquisition of phosphorus, Antagonism, secretion of volatile organic compounds	*Miscanthus* × *giganteus* (*Mxg*), *Brassica alba*	Detection of phytohormones and siderophores, phosphorus solubilization test, in vitro antagonism assays, volatile compound detection assays and assays in plants	[53]
Bacillus amyloliquefaciens NJN-6	Organic compound secretion and biofilm generation	*Musa paradisiaca*	Chemotaxis Assays, In Vitro Colonization Assay, and HPLC Compound Determination	[54]
Rhizobium etli G12, *Pseudomonas trivialis*, *Pseudomonas jessenii*, *Serratia plymuthica*, *Bacillus subtilis* Sb4-23, Mc5-Re2, Mc2-Re2	Antibiosis, biofilm formation, chemotaxis, phytohormone production, secretion of toxic compounds to nematodes and induced systemic resistance	*Solanum lycopersicum* cv moneymaker	In vitro colonization and antagonism assays	[55]
Paenibacillus polymyxa CF05	Production of phytohormones, secretion of antioxidants and phenolic compounds	*Solanum lycopersicum* cv Zheza 203	Antioxidant detection assays, in vitro and greenhouse antagonism and colonization assays	[56]
Bacillus subtilis HJ5	Antibiosis and biofilm production	*Gossypium herbaceum*	In vitro colonization and antibiosis assays	[57]
Pseudomonas sp. DSMZ 13134	Improvement in the acquisition of phosphorus, secretion of siderophores, antimicrobial compounds and induction of systemic resistance	*Hordeum vulgare*	Phosphorus solubilization test, siderophore detection, antagonism and colonization test in vitro	[58]
Kliebsella pneumoniae NG14	Improved nitrogen acquisition and biofilm production	*Oryza sativa* L.	Detection of genes associated with nitrogen metabolism and in vitro colonization assay	[59]
Azospirillum brasilense SP245, SK048, SK051, SK454	Genes involved in motility	*Triticum vulgare*	Generation of mutants and in vitro colonization assay	[60]

4. Formulation of Bioinoculants and Recommendations on Their Application

There are several techniques to generate bioformulations containing PGPBs. There is solid (e.g., powder) or liquid formulations, where in some cases the application is more efficient and it is usual to maintain a good number of colony-forming units (CFUs), either in the soil or in the phyllosophere. Likewise, if the intention is to attack soil fungi or oomycetes, it is appropriate to apply the bioinoculant with antifungal activity in the soil, near the root. On the other hand, if the potential infection by pathogens is in the aerial part of the plants, the ideal is to do foliar application in liquid or powder [61].

In certain situations, the formulation depends on the objective of the application either in the field or in the greenhouse, whether in large or small areas of land. Likewise, the inoculation time is also relevant, either prophylactically or when there is already a certain infection caused by pathogens. In the case of the latter situation, the protective effect has not been as effective as the direct application of biocidal agrochemicals, which usually kill or directly damage pathogens, whether they are bacteria, fungi, oomycetes or viruses. The ideal in any case is to use bioinoculants before observing disease symptoms in plant crops, since the evidence suggests that their action is better when the antagonist is previously present, managing to protect even the products during post-harvest [62].

Now, if what the producer want is to apply a bioinoculant with a stimulating action on plant growth and development (biofertilizer or biostimulant), the best option is to apply it on seeds or during the first stages of plant growth (seedlings), in order to exert a greater promoting effect [63]. Likewise, some bioinoculants can have dual action, that is, certain species of bacteria such as *Pseudomonas* or *Bacillus* can exert direct action by stimulating plant growth and at the same time, antagonizing pathogens and/or stimulating plant defenses [34].

Although it has been proposed that the production of bioinoculants can be cheap and that the final product can also be cheaper than agrochemicals, there are still certain restrictions on their production [64]. Arora and colleagues [65] have identified some production and marketing constraints with developing efficient application of bioformulations, including: (i) the high cost of production; (ii) shelf life; and (iii) inconsistent performance in open field. Producing bioinoculants/bioformulations require certain biotechnological equipment and hi-tech instrumentation to large-scale production, and non-efficient handling procedures are a major cause of underperformance in open field application. Additionally, shelf life of the products depends on several factors, such as the culture medium, the physiological traits of the microbial species, the use of protective materials, the type of drying and rate of dehydration technology used. Finally, it is proposed that the inconsistent field results are the major constrain associated with the bioformulation marketing [65]. Therefore, it is important to avoid cell death of the microbial agents and to maintain good colony forming units of the inoculant. Additionally, to obtain better results in field, it is necessary to check out all the microbial agent specificities and see under what environmental circumstances the inoculum works better. Figure 2 summarizes the process of isolating bacteria as biocontrol agents and plant growth promoters, the necessary screening to identify the best strains and the appropriate tests to achieve the formulation of the bioinoculant.

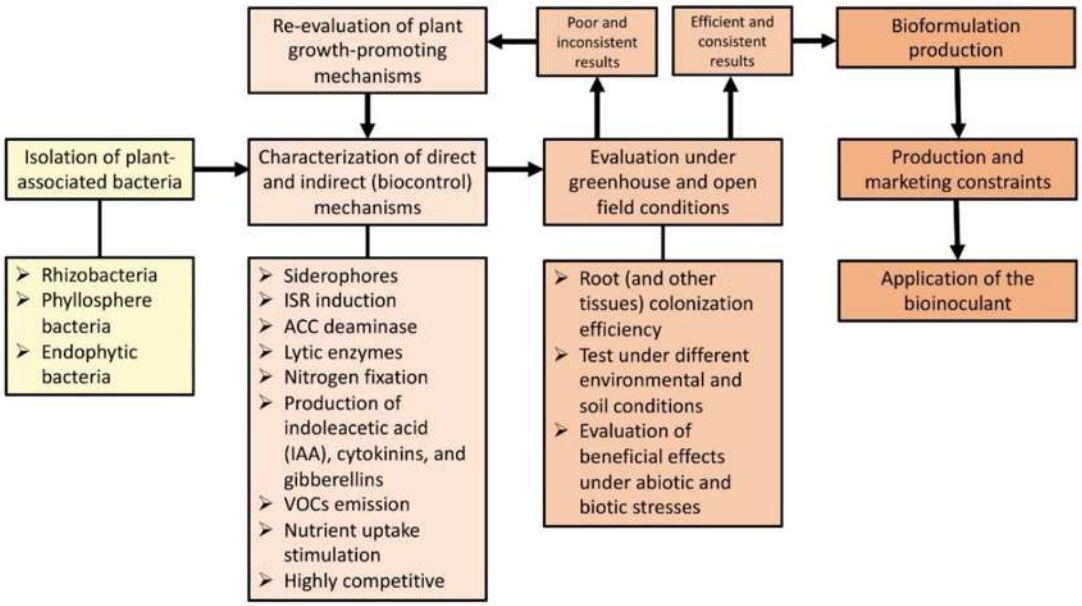

Figure 2. A summary of the processes to isolate and characterize bacteria as biocontrol agents and plant growth promoters, the preliminary screening to identify the best strains and the appropriate tests to achieve the formulation of the bioinoculant. Before successful application in open field conditions, bioinoculants should have passed through certain constraints (see text for details).

5. Challenges in the Application of Bioinoculants

At a global level, the application of PGPB in the open field still requires breaking certain barriers that allow a broader use to stimulate the production and improvements of agricultural crops, as well as counteract the negative effects of potential phytopathogens [11]. There are still certain inconsistent results with some bioinoculants, whether they are biostimulants of plant growth or biopesticides, when applied in the field. The factors can be diverse, but a challenge is the lifetime that PGPB have as part of a bioinoculant. Some strains, such as *Bacillus* spp., can be applied in the form of spores, which prolongs their shelf life [27]. However, other non-sporulating strains require novel formulations that allow them to survive and maintain efficient viability until inoculation.

Once they have been inoculated in the field, it is desirable that the inoculated bacterial agents persist in the soil and colonize spaces such as the rhizosphere. Therefore, it is required to select and apply those strains that are highly competitive in the rhizosphere of plants, that are efficient colonizers of spaces and can exert their beneficial activities in such microenvironment [12,13]. The application of inoculants in powder or liquid form to the plant phyllosphere also presents certain challenges. For example, the weather conditions can be changeable and lower the efficiency if there is no adequate bacterial colonization and attachment to the leaves [17]. Therefore, some of these aspects can be technical and therefore, the development of suitable protocols can increase the effectiveness of bioinoculants.

On the other hand, the challenges faced by bioinoculants may be different around the world, for the simple fact that edaphic, climatic and geographical conditions are enormously variable [3,9]. For this reason, for decades it has been tried to isolate native strains that allow to improve the crops of the same localities from which they were isolated, which would suggest a better efficiency to exercise their beneficial actions when associated with plants in the same types of agricultural soils. For this reason, more research is required to associate abiotic aspects with the beneficial properties of each bioinoculant.

6. Other Challenges of PGPB Application: The Case of Latin America

With respect to the development and commercialization of bioinoculants around the world have occurred unevenly worldwide. While some European countries have taken important measures to limit the use of agrochemicals and stimulate the generation of bioinoculants, other countries, such as Mexico and some in Latin America (and other regions of the world) have not had the same response [66–68]. This situation persists despite international markets such as the United States of America, Canada, and other Asian and European countries having implemented restrictive measures on the importation of products produced using agrochemicals. Thus, questions arise as to why the development of bioinoculants has not been promoted in countries such as Mexico and others with similar conditions in Latin America.

Farmers in Mexico and several countries in Latin America own few hectares of orchards or farmland; they are called small-scale producers if they have up to five hectares. Some other definitions by the Food and Agriculture Organization of the United Nations suggest that landowners are small-scale producers if they have up to two hectares [69,70]. These small-scale producers require economic support to operate their farmland, for example, the provision of credit that would allow them to access seeds suitable for cultivation in the region where they live, as well as biofertilizers that would allow them to sustainably increase their production. Likewise, it is important that government policies promote the reduction of agrochemicals, so that small owners do not see their market limited locally or regionally, which would allow them to market their product at national and international levels. The Secretary of Agriculture and Rural Development of Mexico (https://www.gob.mx/agricultura, accessed on 1 March 2021) has various programs to support farmers in need of assistance; however, the bureaucracy and delay in the delivery of agricultural supplements can cause inefficient production systems.

In the case of large-scale fruit producers, such as avocado producers, knowledge of the use of biofertilizers that improve the nutrition and production of crops should be promoted. If necessary, the prophylactic application of antifungal or biocidal microbial agents should be more widely used in orchards [71]. For example, 77% of Mexico's avocado crop is produced in the State of Michoacán, with this country being the world's largest producer of this fruit. In the marketing year of 2016–2017, Mexico exported almost 895,000 tonnes of avocados to 26 countries, bringing in revenues of USD 2.5 billion [72]. This is an important income source for the families of Michoacán and Mexico. Therefore, the development of government policies that promote the use of bioinoculants (either biofertilizers or biocides) during fruit production is essential, especially given the high demand for organic products. In addition, the reduction and, ideally, the elimination of toxic chemicals would cause less damage to the garden workers themselves [73]. Such policies would also offer an alternative for small producers, who would see a higher income when marketing their products in other countries, without restrictions due to the use of agrochemicals.

Latin American countries share diverse historical, social, and cultural components. These countries originated from a mixture of millennial races that inhabited America long before the arrival of colonizers, who mainly came from Europe, Spain, Portugal, and France. These historical events have left these countries with a genetic footprint and a fairly similar cultural heritage [74]. In Mexico, there is no culture of prevention; only when a problem is visible, are steps taken to solve it. This is also the case for some farmers; they consider that it is not necessary to apply products in the field that could prevent potential infection by pathogens that could reduce crop yield. It is very difficult to "convince" farmers that bioinoculants can work preventatively in a similar way to a chemical fertilizer or fungicide. Therefore, it is necessary to work actively to change the mentality of small-scale producers. Both educational institutions and bioinoculant producing companies have an opportunity to provide education to farmers, making them aware of the ecological and economic benefits of using microbial inoculants.

The gross domestic product of Mexico and some Latin American countries is less than 1% that of countries such as Germany, Japan, or the United States, with these differences being significant [75]. This makes it more difficult to receive scientific research support in a country such as Mexico. In Mexico, research projects are mainly financed by the National Science and Technology Council and partly by internal funds from universities and higher education institutions; however, these are limited. This is not from a lack of will, but rather the fact that the universities themselves (the vast majority of which are public) depend on the federal government for financing. Furthermore, there are significant differences in the budgets for supporting research projects between universities in the capital and provinces. In some cases, there are universities and private institutions that generate high-quality science and make an effort to compete in generating knowledge. This affects not only the development of research on microbial bioinoculants, but all research from different fields of knowledge. An important factor that has not been widely explored is that of farmer inclusion in academic research projects [76,77].

Bureaucracy in Mexico and Latin America is an obstacle to development [78]. For Mexico, the creation of a new company is subject to endless requirements that can delay its inception. Likewise, the application and commercialization of products, including bioinoculants, are faced with various obstacles. There are some cases of ephemeral success in the production of bioinoculants, such as the case of a biofertilizer based on the application of the *Azospirillum* bacterium. The development of this biofertilizer (one of the first ones in our country) was undertaken by Dr. Jesús Caballero-Mellado, who recently passed away [79]; however, it was a project that was abandoned after a few years, due to lack of support. A more recent example is that of the first biofungicide based on the *Bacillus* sp. strain 83, Fungifree AB®, which was developed in Mexico by researchers from the National Autonomous University of Mexico and the Center for Food and Development Research, Culiacán Unit. The success of this product required the researchers themselves to create their own company to market their biofungicide [80]. This joint effort of institutions

and researchers is highly commendable; however, not all institutions have the established statutes or internal policies to promote the development and generation of patents, or to promote the necessary agreements between the academy and the company. Additionally, scientists often lack experience beyond their laboratories and lack knowledge in areas such as high-scale production, marketing, and advertising [80]. This is where agreements with companies can promote strategies for the production and marketing of bioinoculants to achieve long-term success.

In general, in some countries, researchers isolate, characterize, and perform in vitro, greenhouse, and/or field research to test the capabilities of new microorganisms. Once their effectiveness has been proven, and the potential of specific activities to promote growth or biocontrol certain pathogens is known, the task of the researcher is complete. The entrepreneur can take over and produce and market the product, until it reaches the farmer. This process prevents researchers from spending time on tasks that are not their specialty, leaving the subject of marketing to the experts [81].

7. Conclusions and Remarks

Sustainable agriculture makes rational use of resources, in particular, soil, water, and agricultural inputs, for the production of vegetables, seeds, and fruit. Its objective is to achieve higher production in a small area to satisfy basic food needs, but it also involves the social and economic aspects of a society. One of the main focuses is on the reduction or elimination of agrochemical use through changes in management. These must ensure adequate nutrition, growth, production, and protection of plant crops [82]. To achieve this sustainable objective, various changes must be made to conventional strategies of food production, such as bridging basic and applied research [76]. It is important to change our way of thinking to be more eco-friendly and to open spaces for the discussion of proposals and public policies that promote the use of environmentally friendly strategies, such as the use of inoculants based on beneficial microorganisms, such as plant growth-promoting bacteria, including rhizosphere, phyllosphere and bacterial endophyte communities [7,83,84].

Finally, for thousands of years, civilizations such as that of the Mayans have valued the use of microorganisms (mainly mixtures of algae and cyanobacteria) to increase soil nutrients [85]. Today, we must continue the development of these technologies to improve crops via the application of microorganisms such as PGPB—knowledge that was already appreciated by the ancient civilizations of Mesoamerica, and that must continue to benefit future generations.

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Review

Bioencapsulation of Microbial Inoculants: Mechanisms, Formulation Types and Application Techniques

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Abstract: The excessive use of agrochemicals in the field to increase production and counteract the negative effects caused by biotic and abiotic factors has led to a deterioration in soil fertility, plus an increment in negative impacts on the environment and human health. Therefore, the application of beneficial microorganisms as bioinoculants is an eco-friendly alternative to agrochemicals. Plant growth-promoting bacteria and fungi have been effective in promoting plant growth and production, as well as reducing the action of pathogens in multiple crops. However, successful application of such beneficial microorganisms in the agricultural field has faced several difficulties, such as survival, colonization efficiency and short periods of shelf storage. Therefore, it is essential to explore novel ways to encapsulate, formulate and apply bioinoculants. To obtain the expected quality in bioencapsulated products, it is essential to determine the type of polymer, capsule size, encapsulation technique and use the correct chemical and physical cofactors involved in the production process. Thus, this review highlights the various formulation types and application techniques, as well as discussing the multiple advantages of using microbial encapsulates to have better results in agricultural production.

Keywords: bioinoculants; beneficial fungi; microbial bioencapsulation; PGPR; pellet; agriculture

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1. Introduction

The continuous increase in the world population is accompanied by a high demand for agricultural products that must be satisfied in quantity and quality. In recent decades, and particularly since the advent of the green revolution, excessive use of chemical fertilizers has taken place to maximize production and improve the quality of crops and try to increase the productivity of nutritionally poor soils [1]. However, the benefits initially observed by the use of agrochemicals on crop productivity have been overshadowed by studies that show the adverse effects of excessive use of these products on the environment [2].

Among the damages caused to the soil are the deterioration in its structure and texture and a reduction in the populations of microflora and microfauna, which together trigger a nutritional imbalance within the soil. In addition, chemical inputs are the main source of contamination in soils used for agricultural production; they contribute significantly to the contamination of water and the atmosphere, triggering diseases in living beings [2].

In addition to the above, it has been shown that the use of chemical fertilizers by plants is inefficient, since they only take advantage of ~50% or less of the chemical doses

applied, regardless of the nitrogen source with which they are formulated [3], causing an accumulation of the products used in the soil.

The damages that have been caused over time by the excessive use of agrochemicals require the development and implementation of technologies that have a minimal impact on the environment and that are designed in such a way that they can maintain and preserve the productivity of the soil. In addition, agricultural products intended for human consumption that are free of chemicals gives added value to these products [4].

One of the technological options to decrease the amount of chemicals in the soil environment is to use beneficial microorganisms that can promote plant growth and reduce synthetic fertilizers without negatively affecting crop productivity [5,6]. The use of these microorganisms, also known as biofertilizers, is one of the most important contributions of biotechnology and microbiology to modern agriculture, and it is an alternative for the reduction in production costs and the environmental impact caused by the excessive use of agrochemicals [7,8].

Therefore, the inoculation of microorganisms that promote plant growth in crops is a practical alternative to agrochemicals and can be applied directly to the soil, sprayed on plants or used as a coating for seeds [9].

2. The Role of Beneficial Microorganisms as Inoculants

The interaction of plants with microbial communities results from co-evolution over millions of years, contributing to the adaptation of plants on earth [10]. The interactions between microorganisms and plants occurs mainly in the portion of the soil that is in close contact with the plant root, known as the rhizosphere. This zone is defined as the volume of soil associated with and influenced by plant roots [11], constituting a favorable environment for the development of microorganisms in quantities that are much higher than those found in the rest of the soil. These high microorganism concentrations are a consequence of the fact that plants provide the necessary nutrients for the development of these microorganisms, which in turn provide the plants with substances that promote their growth, establishing a mutualistic relationship between both organisms [12].

The interactions between plants and beneficial microorganisms have been the subject of various scientific investigations, since this relationship provides a viable alternative for sustainable plant development and the conservation of the environment [4]. Some microorganisms that promote plant growth include mycorrhizal fungi, beneficial fungi or promoters of plant growth and certain rhizobacteria [13–15].

Mycorrhizal fungi are a group of root biotrophs that exchange mutual benefits with approximately 80% of plants and include arbuscular mycorrhizae and ectomycorrhizae from multiple fungal clades, such as *Glomeromycota*, *Ascomycota* and *Basidiomycota* [16]. Among the benefits of this mutualism is the supply of soil nutrients to plants in exchange for carbon from the host plants. This relationship results in an increase in the absorption of nutrients, the production of bioactive compounds and an increase in the production of fruits and tubers. In addition, this relationship has been highly effective as a nematicide, in addition to increasing the uptake of water to plants in certain environmental conditions [16,17].

Beneficial fungi or plant growth-promoting fungi (PGPF) have taken on great importance since it has been proven that they promote plant growth and, in turn, control numerous foliar and root pathogens by activating induced systemic resistance (ISR) in the host plant through various signaling pathways [8,18]. This group of organisms includes species of genera, such as *Trichoderma*, *Aspergillus* and *Phoma* [19,20].

Plant growth-promoting bacteria (PGPB) are a set of bacteria that inhabit the rhizosphere. Through different mechanisms, they promote plant growth and provide them with tolerance to both biotic and abiotic stress conditions [21]. Within this group we find species belonging to the genera *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Klebsiella* and *Pseudomonas*, as well as some endophytic species, such as *Axoarcus*, *Gluconacetobacter* and *Herbaspirillum* [22–24].

The role of inoculants, such as PGPF and PGPB, on plants is to improve plant growth, production and resistance against several phytopathogens. These microorganisms are used as different types of formulations, prepared accordingly to the desired function or effect of the microorganism to be used. These formulations contain live or latent microorganisms (bacteria or fungi, alone or in combination) and, depending on the mechanism they use to promote plant growth (direct, indirect or both), are classified into one of three categories, i.e., biofertilizers, biostimulants or biopesticides [4,25]. The mechanisms of action of beneficial microorganisms are discussed in the next section.

Biofertilizers are formulations with one or several microorganisms that provide and improve the bioavailability of nutrients when applied to crops, biostimulants include microorganisms that promote plant growth directly through the production of hormones and biopesticides include microorganisms that are used to control phytopathogenic agents [26–28].

3. Mechanisms of Action of Beneficial Microorganisms

Certain microorganisms can promote plant growth through direct mechanisms or indirect mechanisms, although some mechanisms can work both directly and indirectly [29]. These mechanisms of action are briefly described below. We recommend the reader to other excellent recent reviews in this area [30,31].

3.1. Direct Mechanisms of Action

Direct mechanisms refer to the promotion of plant growth in two ways: microorganisms make it easier for plants to acquire the nutrients they need or they help to modulate the levels of plant hormones involved in the development and growth of plants [32–34].

With the increased use of chemicals in agriculture, much of the nutrients, such as soluble inorganic phosphorus used as a chemical fertilizer, becomes immobilized soon after its application, making it unavailable to plants [35]. Naturally, in the soil, insoluble phosphorus is found as apatite or in some organic forms, such as inositol phosphate (phytate), phosphomonoesters and phosphodiesters [36], forms which plants cannot directly assimilate. However, some microorganisms are capable of solubilizing inorganic phosphates through the production of low molecular weight organic acids that act on the inorganic phosphates making them available so that they can be used by plants [35]. Other microorganisms contain enzymes that can break down organic phosphates into a plant usable form [33].

Another important nutrient for plants is iron. The predominant form of iron in nature is Fe^{2+}, which is not assimilable by plants. Some microorganisms can synthesize complex peptide molecules with a high affinity for Fe^{3+}; these peptides are known as siderophores. The siderophores trap iron forming a complex; this complex may be taken up by membrane receptors of microorganisms and thus facilitates its acquisition. These iron–siderophore complexes can also be assimilated by plants and subsequently broken down inside of the plant, thus providing plants with the iron they need [37].

Nitrogen is one of the nutrients that plants require in larger concentrations, and it is found primarily in organic form in the soil. Nonetheless, plants take up inorganic nitrogen as ammonium and nitrates, rather than the organic form; thus, nitrogen mineralization from organic to inorganic form is crucial for plant growth and crop production [38,39]. Nitrogen fixing bacteria have gained attention in this regard, due to their capability to convert atmospheric nitrogen (N_2) into ammonia (NH_3), which plants can use, in a process called biological nitrogen fixation. Bacteria capable of such conversion encode the enzyme nitrogenase (a highly conserved enzyme complex), which catalyzes the conversion of N_2 to NH_3 [40–42].

Biological mechanisms, such as nitrogen, sulfur or phosphorous fixation [43], production of siderophores to increase iron bioavailability [44,45], phosphate and sulfates solubilization [46,47] and iron sequestration [33] help to incorporate or increase nutrients in the soil, along with their bioavailability to the plants. This increased provision

of nutrients is provided by the following organisms: *Rhizobium* spp., *Sinorhizobium* spp., *Mesorhizobium* spp., *Azotobacter* spp., *Azospirillum* spp., *Pseudomonas* spp., *Bacillus* spp., *Aureobasidium pullulans*, *Epicoccum nigrum*, *Scolecobasidium constrictum*, *Myrothecium cinctum* and *Acidianus* spp., among others [43,44,46,47].

Some rhizospheric and endophytic microorganisms can produce plant hormones or induce their synthesis in plants. Many soil bacteria can produce hormones, such as cytokinins, gibberellins or auxins. In addition, some rhizosphere microorganisms produce the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, where ACC is the immediate precursor of ethylene, a hormone related to the senescence of plants and the ripening of fruits and that very high levels of this hormone inhibit plant growth. The enzyme ACC deaminase converts ACC into α-ketobutyrate and ammonia, thus ethylene is no longer produced, and ammonia and α-ketobutyrate are compounds that can be assimilated by plants [42,48,49]. ACC deaminase production has been observed in plant beneficial organisms, such as *B. subtilis*, *P. fluorescens*, *B. amyloliquefaciens*, *Enterobacter cloacae* and *Trichoderma* sp., among others [50–52].

3.2. Indirect Mechanisms of Action

Other microorganisms can promote plant growth indirectly. A common indirect mechanism is competition for space and nutrients, where the beneficial microorganism competes with a pathogen and the one with the greatest capacity to take up nutrients and the fastest growth rate proliferating in the soil displaces the pathogen and prevents it from colonizing and infecting plants [34,53,54]. The successful competition of PGPB with pathogens provides plants with a greater opportunity to grow and develop. It is worth mentioning that the production of siderophores can also be classified as an indirect mechanism, since the microorganisms with the capacity to produce these molecules and take up siderophore–iron complexes will limit the growth of the pathogen competing for this nutrient [45,55,56].

The production of antibiotics or antimicrobial compounds is a common and often studied indirect mechanism of plant growth promotion. For example, pyrrolnitrin produced by *Pseudomanas* spp. [57], iturine produced by *Bacillus* spp. [58] and syringomycin, produced by *P. syringae* [59] are some of the most common antipathogen antibiotics.

In addition to antibiotics, various microorganisms produce volatile organic compounds (VOCs) that can also be toxic to pathogens, preventing their growth or leading to their death, such as dimethyl disulfide produced by *Bacillus* sp. E25 [60] and *B. thuringiensis* CR71 [61]. Some of these VOCs, in addition to having antimicrobial action, can also promote plant growth directly, as is the case of dimethyl-hexa-decylamine [62]. Therefore, the production of VOCs can be classified as both a direct and an indirect mechanism.

Another indirect mechanism of plant growth promotion is the production of lytic enzymes related to the ability of the microorganism to parasitize and destroy the pathogen, as is the case of mycoparasitism carried out by fungi of the genus *Trichoderma*, adhering to the hyphae of the pathogen, where it secretes enzymes that degrade the cell wall, resulting in the death of the pathogen, or the destruction of its structure, preventing its development [50,63–65].

Finally, induced systemic resistance, initiated by the PGPB, is an indirect mechanism that involves activating the biochemical and molecular defense responses of the host plant, these may include the production of reactive oxygen species (ROS), phytoalexins, synthesis of proteins related to pathogenesis (PR), lignin accumulation at the site of infection, among others [66]. Some microorganisms that promote plant growth induce this defense response, preventing the colonization or the development of infection by phytopathogens [45,67,68].

4. Formulation of Microbial Inoculants

To enable plant growth-promoting microorganisms to be used and applied in agricultural practices, it is necessary to develop formulations based on these bioinoculants. Formulating a bioinoculant includes the entire series of procedures and technologies after

the growth in culture of the microorganisms that promote plant growth. Bioinoculant formulation includes the mixture of a selected beneficial strain with a suitable vehicle that preserves the viability of the microorganism in either a dormant or metabolically active state during transport, storage and application [69]. To obtain a successful formulation, the microorganism must overcome the conditions of temperature, humidity, salinity, UV radiation and water stress present in the soil and during its formulation, in addition to being effective and competitive against the native microbial populations of the soil [1,70].

The compatibility of the physical form of the bioinoculant (solids in the form of powder, granules or capsules and liquids) and its incorporation through agricultural practices is a key factor determining the durability of the product and its ability to colonize plant roots [71]. According to the physical form of the inoculant, it is classified as a liquid formulation, a solid formulation or bioencapsulated [21,71].

4.1. Liquid Formulation

Liquid formulations use culture broths or formulations based mainly on water, mineral or organic oils. Seeds and seedlings can be immersed in the inoculant before sowing or transplanting [70,72], and for the biocontrol of pathogens or physiological stimulation, they can be sprinkled on the foliage of already established plants or applied directly to the soil [73]. This formulation method is the most commonly used. It is directly applied to crops without going through other processes following fermentation; most microorganisms can survive for more than one year if the containers are kept at ~4 °C, they are easy to inoculate and their application is very practical when implemented in irrigation or sprinkler systems. Liquid formulations are relatively low cost. However, even when its efficacy has been proven, its stability during storage is often limited due to its susceptibility to contamination with other microorganisms [69,74].

Liquid formulations of different microorganisms, or even in microbial consortia, and with the use of various additives, have managed to increase yields in agricultural fields. For example, when a liquid bioinoculant based on sugar and coconut water, including *Pseudomonas* spp., *Bacillus* spp., *Klebsiella* spp., *Aspergillus* spp., and *Azotobacter* spp., was used to inoculate soybean plants, the result was improved nutrient solubility and increased crop yield [75].

It has also been shown that the phosphate solubilization capacity and the survival rate of *Pseudomonas* and *Pantonea* strains increases and are preserved when they are used in liquid formulations up to three months after their formulation containing diluted concentrations of phosphate buffer and nutrient broth with glycerol [76]. In addition, Camelo-Rusinque et al. [77] assessed the population dynamics of the *Azotobacter chrocoocum* strain AC1 in MBR culture medium under bioreactor conditions after 105 days and found that both the cell viability and the biological activity of the strain was maintained, regardless of the storage temperature. This indicates that some liquid formulations can be used for a specific time, with the organisms retaining their activity and continuing to be viable for use as bioinoculants.

4.2. Solid Formulation

Solid formulations are used widely in the agricultural industry because of the advantages they offer during storage and transportation. A simple technique used for the preparation of solid formulations is adsorption, which consists of mixing the microorganisms with a solid support, such as vermiculite, perlite, sepiolite, kaolin, diatomaceous earth, natural zeolite, peat or clay, the latter being of great interest in agriculture thanks to its ability to act as a desiccant and provide excellent storage conditions for various inocula, as it has a good ability to adsorb agents dispersed or suspended in it [78,79].

Peat is one of the supports most used worldwide in commercial crops due to its low cost. However, being a complex organic matter, different batches present great chemical variability and, consequently, it is difficult to maintain the same quality in all batches. In addition, its storage is very susceptible to humidity, which decreases the inoculum cell

survival [78]. According to Rose et al. [80], it is essential to be able to quantify the number of viable cells of each microorganism per unit weight of inoculant, to determine the inoculum potential at different application doses and for field results to be properly interpreted.

In a study by Quiroz Sarmiento et al. [81], the effectiveness of peat was evaluated with the following bacteria: *Serratia liquefaciens* CPAC53, *S. plymuthica* CPPC55, *P. tolaasii* P61 and *P. yamanorum* OLsSf5, in comparison with the encapsulation of the strains using alginate beads. Following a storage period of 150 days, the results showed that the encapsulated strains maintained the highest population. The effect of both types of bioinoculants on poblano chili seedlings (*Capsicum annum* L.) was also evaluated. In this case, the best results were observed with the encapsulated strains [81]. This suggests that the success in using peat as a support material for solid formulations depends on the conditions in which the bioinoculum will be used and the availability of other strategies.

A common technique of solid formulation is spraying or lyophilization. This technique allows for the realization of high microbial survival rates without the need to use any support, allowing for easy inoculum storage for long periods, at room temperature, without the need for refrigeration. One of the disadvantages of lyophilization is that it is necessary to protect the cell membrane and cytoplasm against dehydration during the storage period, using a cryoprotectant, such as mannitol and microcrystalline cellulose [82]. In this way, the cells remain viable and can be used long after lyophilization, for at least a year [83].

Lyophilized microorganisms may be mixed with a solid support or used directly. For example, in the laboratory, Grzegorczyk et al. [84] studied the survival and storage stability of a strain of *Trichoderma hariazum*, four strains of *Trichoderma atroviride* and two strains of *Trichoderma virens*, after culture lyophilization in solid wheat straw medium with and without the addition of maltodextrin. It was observed that the strains had a higher survival capacity (except for strain *T. atroviride* TRS40), compared to the addition of distilled water only, and in comparison with the bioformulation containing just maltodextrin. Three months after lyophilization, the strains remained stable and most still showed cellulolytic and xylanolytic activity.

Wessman et al. [85] studied the survival of the bacterial strains *P. putida* KT2440 and *A. chlorophenolicus* A6 after lyophilization in four different formulations, including (i) sucrose, (ii) Ficoll PM400 a sucrose polymer, (iii) hydroxyethylcellulose (HEC), and (iv) hydroxypropylmethylcellulose (HPMC). The polymers were chosen to obtain a monomeric structure, such as sucrose. The results of this study indicated that a key factor to help cell survival is the ability of the added ingredients to replace water during dehydration, thereby maintaining the structure of proteins and cell membranes in a dry state. Disaccharides, such as sucrose, show this property, while polymers, such as starch-based polysaccharide, do not. Thus, some polymers can facilitate cell survival to the same extent as disaccharides provided that certain physical properties of the formulation are controlled [85].

In fact, one of the techniques that have gained great importance in recent years thanks to its advantages, is the solid formulations developed based on polymers. These polymers, in the presence of ions or changes in chemical conditions, form complex matrices so that microorganisms become immobilized and encapsulated in the matrix and are gradually released as the polymer degrades. The technique of microorganism immobilization ultimately creates barriers between the microbes and the environment, improving their bioavailability and preserving their biological stability [86].

5. Bioencapsulation of Plant Growth-Promoting Microorganisms

The growing demand for the use of microorganisms as bioinoculants for use in agriculture has facilitated the use of technological tools that allow compliance with the need to develop products that can promulgate agricultural sustainability. When plants are commercially inoculated with plant growth-promoting microbes, the formulation process generates certain problems when applied in the field, given that the main physical form in which the formulation is presented as a liquid or as powders that fails to protect the survival of

the microbial strains in the face of abiotic conditions (temperature, humidity, salinity, UV radiation, pH) present in the application process [87].

The use of polymers through the encapsulation technique has been proven as a highly effective alternative to increase the viability of microorganisms and, in turn, provide protection against the environmental conditions present [88,89]. Figure 1 represents the various biotic and abiotic factors that bioencapsulated microbes face when used in the field.

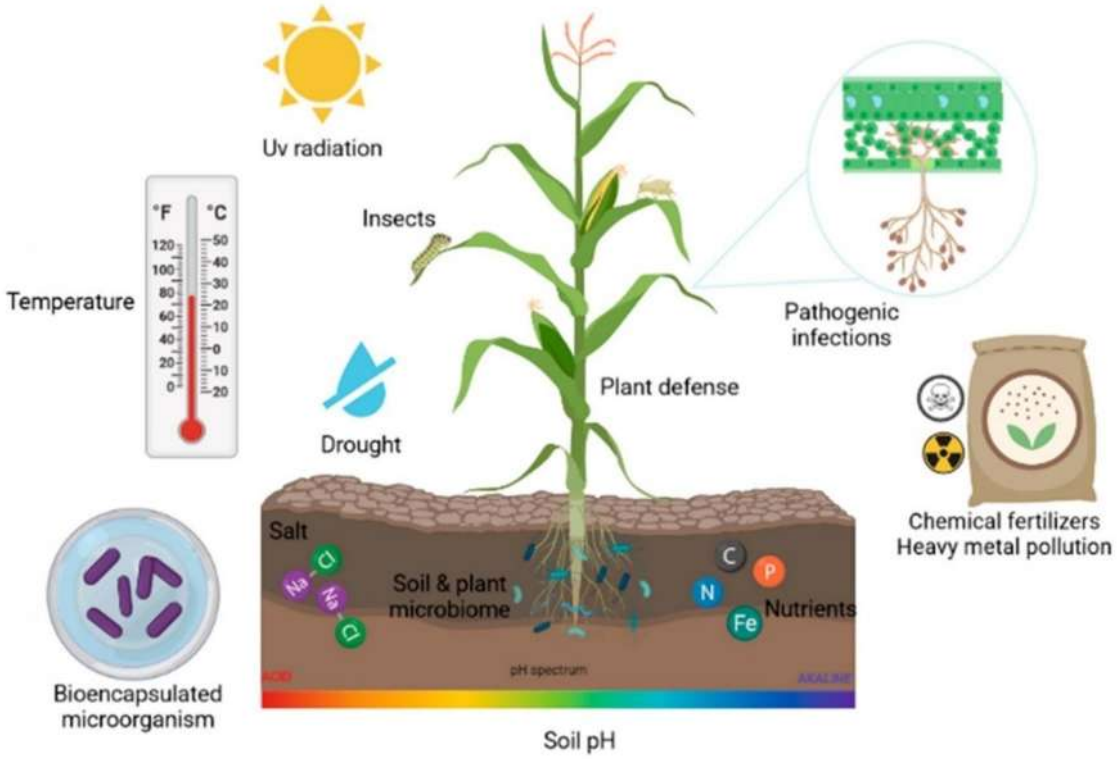

Figure 1. Biotic and abiotic factors that cause stress in plants and affect bioencapsulated microorganisms.

6. Classification of the Well-Encapsulated

Bioencapsulated microbes can be classified according to the type of mechanism used by the microorganism to promote plant growth or according to the type of microorganisms used in their formulation, whether they are bacteria, fungi or a combination of both [69,86].

6.1. Bioencapsulated Bacteria

Bacteria, particularly PGPB, have been widely used in agricultural research and have proven to be a tool for improving plant health and growth without causing environmental pollution [89,90]. PGPB manage to mitigate abiotic stress in the soil through the production of phytohormones and associated metabolites, as well as through significant morphological changes in the roots [91–93]. These changes result in a better nutritional status for plants and, in turn, stimulate plant defense mechanisms to overcome unfavorable environmental conditions [10,94].

Some bacteria can migrate from the rhizosphere to the internal tissues of the plant. They do this through cracks that form in the roots as a consequence of growth, they can also enter through lenticels, due to the emergence of lateral roots or by the cells of the root hairs, among other ways. Once inside the plant, they can exert their action of promoting plant growth either by direct or indirect mechanisms and, in turn, the bacteria are protected

from abiotic stress and are exempt from competing for resources and space with other soil microorganisms. The bacteria capable of colonizing the internal tissues of a plant without causing damage are known as endophytes [30,95,96].

Some bacteria can form highly stable dormant spores that can germinate, forming active bacteria when conditions are favorable [97]. Fully formed spores are recognized as one of the most resistant life forms on the planet, they protect the bacterial genome against heat, desiccation, radiation and oxidation, as well as being an efficient way to escape predation by higher organisms. Spore germination is triggered by the presence of nutrients in the environment, these are detected by membrane receptors and in a matter of minutes, the nucleus of the spores is activated, the spore rehydrates, the cortex hydrolyzes and its surface cover changes [98–100]. This naturally occurring process is considered to be an effective technique for the development of inoculants as it allows greater survival of the strains during the storage process, making it possible to develop encapsulation on a large scale and, in turn, is more profitable [101].

Among the bacteria that form spores are *Clostridium*, *Sporosarcina*, *Thermoactinomyces* and *Bacillus*; there are numerous reports of the beneficial effects of *Bacillus* strains on crops of agricultural interest. *Bacillus* strains promote plant growth, increase phosphorus solubilization and increase the production of growth regulators, and they are highly efficient for pest control. Within this group is the *B. thuringensis* species, which is widely applied worldwide as a consequence of its activity as a biological insecticide [102,103].

Many strains of bacteria have been used to make bioencapsulated bacteria based on various polymers, and their effect as biostimulants and/or biopesticides has been studied in several field or greenhouse experiments (Table 1). The bioencapsulation of spore-forming bacteria provides them with additional resistance to environmental factors; however, this type of formulation represents a greater advantage for those organisms that do not form spores, also preserving their viability, which opens the possibility of using a greater diversity of organisms that promote plant growth or biocontrol in the field.

Table 1. Encapsulated microorganisms of agricultural importance, their beneficial effect on plant crop and application technique.

Encapsulated Microorganism	Encapsulation Polymer	Crop	Effect	Type of Assay	Reference
***Streptomyces fulvissimus* Uts22**	Chitosan + gellan gum	*Triticum* sp.	Biocontrol against *Gaeumannomyces graminis*; Growth promotion of root and branches systems in wheat plants.	Greenhouse assay	[104]
***Pseudomonas fluorescens* Ms-01 (Pf) *Azospirillum brasilense* DSM1690 (Ab)**	Montmorillonite+alginate [Mt-Ag] y hallosite+alginate [Ha-Ag]	*Triticum* sp.	Increase in root and branches biomass; Increase in root nitrogen adsorption	Greenhouse assay	[105]
***Azotobacter chroococcum* C26**	Carrageenan	-	Cell preservation	-	[106]
***Bacillus subtilis* SL-13**	Alginate-bentonite-polyvinyl alcohol-SDS	*Gossypium hirsutum*	Increase in seed germination and plant biomass	Greenhouse assay	[107]
***Pseudomonas putida* Rs-198**	Alginate/bentonite/starch	*Gossypium hirsutum*	Plant growth promotion under saline stress; increase in IAA and gibberellin production	Greenhouse assay	[108]
***Ensifer fredii* LP2/20**	Agar and alginate	-	Bacterial community structure modifications	Field assay	[109]
Pseudomonas putida	Alginate	*Capsicum annuum* L.	Plant growth promotion	Greenhouse assay	[110]
***Pantonea agglomerans* KL**	Alginate	*Oryza sativa*	Reduction in saline stress	Greenhouse assay	[111]
***Paenibacillus polymyxa* MSRH5, *Bacillus nakamurai* MSRH1 and *Bacillus pacificus* MSR H3**	Alginate	*Triticum aestivum* L.	Reduction in saline stress and increase in plant growth	Field assay	[112]
***Pseudomonas fluorescens* strains VUPF5 y T17-4**	Alginate-gelatin	*Solanum tuberosum*	Biocontrol against *Fusarium solani*; Plant growth promotion	Greenhouse assay	[113]
***Bacillus subtilis* cbrf24**	Carboxymethylcellulose Xanthan	*Solanum lycopersicum*	Nematicide against *Meloidogyne incognita*	Greenhouse assay	[114]
***Pseudomonas fluorescens* (KY823007), *P. taiwanensis* (KY823006), *P. monteilii* (KY823008), *P. rhodesiae* (KY823010), *P. putida* (KY823009).**	Laponite	*Vigna unguiculata*	Overall plant growth promotion	Greenhouse assay	[115]
***Pseudomonas libanensis* TR1**	Alginate	*Vigna unguiculata*	Plant growth promotion and reduction in drought stress	Greenhouse assay	[116]
***Pseudomonas* sp. DN18**	Alginate supplemented with salicylic acid and zinc oxide	*Oryza sativa*	Plant growth promotion and biocontrol against *S. rolfsii*	Greenhouse assay	[117]
Bacillus licheniformis	Alginate supplemented with chitosan	*Capsicum annuum* L.	Plant growth promotion and biocontrol against *S. rolfsii*	Greenhouse assay	[118]

Table 1. *Cont.*

Encapsulated Microorganism	Encapsulation Polymer	Crop	Effect	Type of Assay	Reference
***Azospirillum brasilense*, *Burkholderia cepacia*, *Bacillus thuringiensis*, *B. megaterium*, *B. cereus*, *B. subtilis*, *B. subtilis* 1411 and *Trichoderma* sp.**	Alginate Clay	*Eugenia stipitata*	Increase in nitrate and phosphorous concentration *in planta*	Field assay	[119]
***Bacillus megaterium* MTCC 2412, *Azotobacter chroococcum* MTCC 3853 *Pseudomonas flourescens* MTCC*Trichoderma viride* MTCC 793**	Alginate	*Cajanus cajan*	Plant growth promotion	Greenhouse assay	[120]
***Glomus* sp. y *Acaulospora* sp.**	Alginate	*Zea mays*	Increase in root colonization of maize plants and increase resistance to drought stress	Greenhouse assay	[121]
***Mesorhizobium ciceri* ST-282 *Bradyrhizobium japonicum* M8**	Alginate + gelatin/pectin/ kaolin/bentonite	*Cicer arietinum* and *Glycine max*	Increase in plant nodules	Field assay	[122]
***Metarhizium brunneum* CB15**	Pectin/starch, cellulose, and yeast	*Solanum tuberosum*	Plant growth promotion; increase in nitrogen and phosphorous content	Greenhouse assay	[123]
***Metarhizium brunneum* BIPESCO5**	Alginate	*Solanum lycopersicum* L. cv. Ruthje	Increase in endophytic behavior	Greenhouse effect	[124]
Trichoderma viride	Alginate in combination with calcium or copper	*Lactuca sativa* L.	Increase in yield and secondary metabolite production	Field assay	[125]
***Candida tropicals* CY4, *Cryptococcus tephrensis* TY17 and *Saccharomyces cerevisiae* CuY10**	Alginate	Cucumber cv. *Beta alpha*	Biocontrol againts *F. oxysporum*; Increase in plant growth	Greenhouse assay	[126]

6.2. Bioencapsulated Fungi

Among the fungi that have been bioencapsulated, the entomopathogenic fungi, mycorrhizal fungi and fungi that promote plant growth and biocontrol stand out.

Entomopathogenic fungi (HEP) are part of the most important biological formulations in the microbial control of pest insects; they have also gained importance in their use as plant growth promoters and even as good plant tissue colonizers. For example, the endophyte, *Metarhizum brunneum*, encapsulated in alginate, was able to preserve its viability, in addition to being able to efficiently colonize tomato plants [124].

Arbuscular mycorrhizal fungi (AMF) are plant growth-promoting organisms that, following the process of absorbing mineral nutrients from the soil, transfer these nutrients to the plant. Arbuscular mycorrhizal hyphae spread widely in the soil and function as an extension of the roots, increasing the ability of plants to absorb water and nutrients from the soil [127]. Among the beneficial effects they offer are stability to the soil structure, greater host plant tolerance to water stress [128] and, as a consequence of its mycelium increasing the absorption range of the roots, it mitigates aluminum toxicity stress generated in the soil [129].

The use of encapsulation for AMF spores improves the efficiency and stability of fungal bioinocula and, therefore, it is possible to meet the optimal mechanical properties for their handling, transport and stability, following their formulation [121].

Trichoderma and *Metarhizum* are among the genera of fungi that promote plant growth of agronomic importance, which, through their encapsulation based on polymers, have managed to promote the plant growth of crops, such as *Cajanus cajan*, *Lactuca sativa* and *Solanum tuberosum* [120,123,125].

7. Bioencapsulation Process

Bioencapsulation is a cell immobilization process that consists of trapping microorganisms in a polymeric material, capable of allowing the passage of metabolites and gases to preserve cell viability and forming small capsules [86,130]. The bioencapsulation process uses different techniques, depending on the purpose and the type of microorganism being used. However, for each bioencapsulation, it is necessary to take into account: the selection of the material or polymer used, the desired size of the capsule, and the most appropriate technique to use [86]. Figure 2 illustrates the general steps of the bioencapsulation process of plant growth-promoting microorganisms (PGPM), and the most relevant points to consider for bioencapsulation are described below.

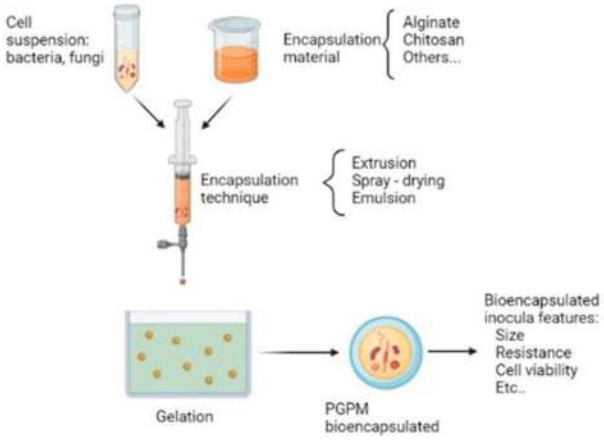

Figure 2. General bioencapsulation process of plant growth-promoting microorganisms (PGPM).

7.1. Coating Materials in Encapsulation

The materials used for the encapsulation of the microbial cells are a key part of the formulation process. It is necessary that these materials have certain properties or characteristics, such as the ability to (i) protect cells against environmental conditions, (ii) disperse with the material to be encapsulated, (iii) release their contents under specific conditions, and (iv) cover and maintain the encapsulated organisms within its structure [78,86,131,132]. These coating materials include hydrogenated oils, waxes, maltodextrins, celluloses, starches, gums and various polymers, the latter of which play a dominant role in determining and forming capsules [69,86].

Polymers in general are chemical substances made up of many repeating units called monomers, with multiple bonds chemically linked or polarized together to build the polymer chain. Natural polymers (biopolymers) are compounds typically formed by polysaccharides, such as cellulose, chitosan or starch, and proteins, such as keratin or collagen [133]. These biopolymers have gained importance in the manufacture of bioencapsulated microorganisms because, compared to synthetic polymers, they have greater benefits for the microorganisms that are encapsulated within them. For example, greater resistance to environmental factors and increased cell viability was observed with biopolymers, compared to synthetic polymers [78,86]. Among the main polymers used in agricultural bacterial encapsulation are alginate and chitosan. In addition, other biopolymers, such as carrageenan, gelatin and laponite, have been used [106,115,118], although their use is less frequent, or they are used in combination with other polymers.

7.1.1. Alginate

Alginate is a linear polysaccharide of D-mannuronic and L-guluronic acids that is naturally present in various species of algae and some bacteria [134,135]. Alginate is the most widely used polysaccharide in the encapsulation process because it is a non-toxic, biocompatible, inexpensive and readily available material that allows the encapsulation of microorganisms in a simple way [136]. Among its main advantages is the fact that it can gel easily, has good solubility and low viscosity and can be used in mild conditions, allowing cells to be trapped with minimal losses of viability [134,137].

Calcium alginate capsules (pearls) are structured as a flexible network, and if they have been used for encapsulation, they are filled with a large amount of water (97–98% by weight). Using only alginate for cell bioencapsulation does not adequately protect the cells during the drying or solidification process and, consequently, the pearls are slightly deformed, so it is generally used in combination with other organic compounds. These compounds include starch, glycerol, chitin, skim milk or humic acids [69,131,138]. Alternatively, alginate may be mixed with clays, such as montmorillonite or halloysite, thereby increasing the mechanical resistance and improving the quality of the capsule, in addition to the survival of microorganisms [105].

Biocapsules made with alginate have many advantages for microorganisms. *Glomus* sp. and *Acaulospora* sp. alginate biocapsules increase water adsorption, thereby favoring the germination of these mycorrhizal fungi; increase colonization of plant roots; and plant resistance to water stress [121]. The concentration of nitrate and phosphorus in *Eugenia stipitata* plants, as well as plant biomass, increases when plants are treated with encapsulated inocula (with alginate or clay) of *Azospirillum brasilense*, *Burkholderia cepacia*, *B. thuringiensis*, *B. megaterium*, *B. cereus*, *B. subtilis*, *B. subtilis* strain 1411 and *Trichoderma* sp. [139]. Using alginate with gelatin, pectin, kaolin or bentonite to encapsulate *Mesorhizobium ciceri* and *Bradyrhizobium japonicum* increased the number of nodules formed in plants of *Cicer arietinum* (chickpea) and *Glycine max* (soybean), in comparison with non-inoculated plants [122].

The use of alginate to encapsulate microorganisms of agricultural interest improves their characteristics as biocontrol agents and as growth promoters, promoting endophytism and the content of nitrogen and phosphorus in plants [112,113,116,123,140].

7.1.2. Chitosan

Chitosan is made from glucosamine and N-acetyl-glucosamine units, is biodegradable, abundant and easy to obtain from the deacetylation of chitin, a component of the exoskeleton of crustaceans, mollusks, insects and fungi [141].

In the presence of anions and polyanions, such as alginate, chitosan can polymerize through cross-links, reducing the porosity of the alginate, and, therefore, improving its protective effect of the encapsulated microbe [141]. By combining both biopolymers to encapsulate the bacterium *Bacillus licheniformis*, the promotion of plant growth of *Capsicum annum* L. plants was improved, as well as its capacity to act as a biocontrol agent of the pathogen *S. rolfsii* [118]. *Streptomyces fulvissimus* Uts22 encapsulated in a mixture of chitosan with gellan gum promotes the development of roots and lateral shoots in wheat plants, in addition to controlling the growth of the pathogen *Gaeumannomyces graminis* [104] (Table 1).

In general, the use of chitosan, whether applied alone or for the introduction of other particles or microorganisms in agriculture, is highly efficient in controlling both biotic and abiotic stress, in addition to promoting growth of various plant species, thus, when used to encapsulate microbes, chitosan can have an additive effect to the benefits of the microbe [142].

7.1.3. Other Biopolymers

In addition to alginate and chitosan, there are other polymers that can be used to improve the stability and/or quality of capsules. Among these polymers is gum Arabic, which is obtained from *Acacia senegal* and *A. seyal* trees and is made up of a mixture of complex polysaccharides. In addition to oligosaccharides and glycoproteins, gum Arabic is rich in essential elements and trace elements, such as aluminum, phosphorus, magnesium, copper, zinc and iron, and acts as an emulsifier, stabilizer and protector from chemical decomposition [143–145].

Starch is one of the most widely used accompanying polymers with alginate, as it provides good protection to bacterial cells and allows optimal diffusion of micronutrients and metabolites in various formulations [146]. The starch is mainly obtained from corn, potato, barley and oats, and its amylose and amylopectin units are linked by glycosidic bonds [147]. In combination with maltodextrin and sodium alginate, the encapsulation of *B. subtilis* with these materials maintain cell viability and is an efficient way to control the pathogen *Fusarium oxysporum* f. sp. *lycopersici* [148].

Maltodextrin, which is obtained from starch, is a linear polysaccharide of glucosamine and N-acetyl-D-glucosamine units. It is used at low concentrations as a coating material for the production of microparticles [148].

Gelatin, which is derived from collagen and consists of glycine, proline and 4-hydroxyproline residues, is very useful as a thermoreversible gelling agent for encapsulation, either alone or in combination with other polymers. Due to its amphoteric nature, it can also form a strong interaction with anionic polymers, providing greater stability to the capsules. Gelatin, in combination with gum Arabic, has been used to encapsulate *Metarhizium anisopliae*, which is used to biocontrol fire ants (*Solenopsis invicta)* [145] or in combination with alginate to encapsulate *B. subtilis* SL-13, greatly increasing cell viability [74].

Carrageenan, which is extracted from red seaweed (Irish moss) and some bacteria, are polymers that have a linear structure made up of D-galactose units alternately linked by α-(1,3) and β-(1,4) bonds. These polymers can form a gel that traps microbial cells and can be used in combination with alginate. However, the gelation of carrageenan is induced by changes in temperature, which should be considered when using it to make bioencapsulated microbes, especially when it comes to organisms that may be sensitive to temperature [149,150].

Another polymer that has been used is agar/agarose. It is mainly extracted from marine red algae and is composed of alternating β-D-galactopyranosyl and 3,6-anhydro-α-L-galactopyranosyl units. One of its important characteristics is that it is resistant to degradation by most known microorganisms and is a thermosetting hydrogel, gelling

in response to a reduction in temperature. However, it has the disadvantages of low mechanical strength and high cost [151,152], which should be considered when using it as a material to produce bioencapsulated microbes.

An important factor for the success of the bioencapsulated microbe formulation is the correct selection of the appropriate carrier for the microorganism of interest, providing stability and protection against environmental factors, such as UV radiation, dryness and high temperature [152]. Therefore, when choosing the appropriate polymer, factors, such as availability of the polymer, resistance to environmental factors, ability to allow cell viability, whether it will be used alone or in combination with other polymers and costs, should be considered. Table 1 shows examples of studies where various biopolymers have been used for microbial encapsulations.

7.2. Capsule Size Selection

According to the size of the capsule that is formed, capsules can be classified as macrocapsules or microcapsules. Macrocapsules range in size from millimeters to centimeters, while microcapsules range in size from 1 to 1000 μm [86]. For the preparation of bioencapsulated microorganisms of agricultural interest, the use of microcapsules is preferred, because the smaller size increases the cell concentration, is more resistant and can be better dispersed in the soil or in pots [86,131].

The size of the capsule is important at the time of application in an agricultural field; normally the most common formulation is 1–4 mm in size. However, when freely mixed with seeds and sown together, the spheres can fall far from the seed and these distances can be restrictive for many beneficial bacteria, even though their mobility in the soil has been proven. To produce smaller spheres with sizes ranging from 50 to 200 μm, it is necessary to use the appropriate technology that ensures the concentration of the biomass of the microorganisms to be encapsulated [88,131], Therefore, a small capsule size is preferred to favor its dispersion close to the plant and to ensure the interaction of beneficial microorganisms with the plants (Figure 2).

7.3. Encapsulation Techniques

There are various encapsulation techniques that allow microcapsules to be produced. However, their choice depends on various factors, such as the microorganism, to be encapsulated, the temperature, humidity, and agitation (all of which affect the microorganism's survival), the polymer to be used and the purpose of the bioencapsulation. The most commonly used techniques for the formulation of bioencapsulated microbes for use in agriculture is extrusion, although there are other techniques that could also be used, such as the spray or emulsification technique.

7.3.1. Extrusion Encapsulation

Extrusion encapsulation is the most studied and oldest technique for producing capsules with polysaccharides, such as alginate, as it has the advantages of low cost and ease of implementation. This technique consists of mixing microbial cells in an aqueous solution of biopolymer that has gelling capabilities, and then this mixture is extruded in a gelling environment through a small nozzle or syringe to create small droplets of biopolymer containing microbial cells [86]. This technique also incorporates some methods to coat or gel the capsules, thus stabilizing the biopolymer droplets to prevent their dissociation or aggregation and provides them with better quality [133].

There are two mechanisms to carry out the gelation of capsules. The first is called external gelation and is obtained when the solution of the compound to be encapsulated is added together with the selected coating material, the mixture is forced through nozzles generating drops, and these fall into a bath of calcium ions, thereby forming a gel capsule. This mechanism is quite common and is simple to perform; however, it produces heterogeneous gels because surface gelation often occurs before core gelation, resulting in a rigid surface and a soft core [153,154]. The second mechanism, internal gelation, consists of the

preparation of a solution of calcium ions and the compound of interest to be encapsulated, this mixture is forced through a nozzle and poured into a bath of sodium alginate. To carry out the release of calcium ions, the medium is acidified by adding an organic acid, such as acetic, adipic or glucono δ-lactone. In addition, a sequestering agent is added, which binds with free calcium, thus slowing down the gelling process [155–157].

External gelation provides larger capsule sizes (>2000 μm) and better encapsulation efficiency, therefore, it is used for the encapsulation of essential oils, bioactive compounds and plant-derived extracts [158,159]. On the other hand, internal gelation is used more frequently for the encapsulation of microorganisms due to the fact that a uniform capsule size and a smooth surface are obtained, through which agglutinations, cracks and pores are less likely to occur [154,160], resulting in better quality and more resistant capsules, without the need to use organic solvents or high temperatures to harden or coat the capsules.

The extrusion technique is gentle on microorganisms and does not require toxic solvents, and, therefore, does not cause cell damage, ensuring greater cell viability. However, this technique has the disadvantages that the production capacity is low if it is required on an industrial scale, and the size of the particles can be too large for some uses, such as agriculture [161]. However, other techniques can be adapted, along with extrusion, to obtain the desired particle size, such as the precision particle manufacturing (PPF) technique, and rotary atomization discs, among others [86,161].

Other bioencapsulation techniques have been used, such as spray-drying or emulsification, to obtain capsules of beneficial microorganisms for the food industry; however, the use and study of these techniques for the encapsulation of microorganisms of agricultural interest is not well studied.

7.3.2. Spray Drying

Spray drying is a technique that has been employed mainly in the encapsulation of microorganisms for the food industry [86,161]. It is a technique that consists of atomizing a suspension that contains the microorganism to be encapsulated and a polymeric material inside a chamber with hot gas (>100 °C up to 170 °C), generally air, which promotes the evaporation of water, causing the microorganisms to remain trapped inside the encapsulating material, giving rise to the formation of microparticles [161]. However, because this technique simultaneously dehydrates and raises the temperature of microorganisms, various microorganisms, such as non-spore-forming bacteria can suffer high mortality [69]. In addition to temperature, other variables that need to be controlled are feed flow and air flow, being key to determining the viability of microorganisms and the success of this technique for the production of bioencapsulated microbes [162,163].

7.3.3. Emulsification

Emulsification is a technique used to encapsulate different microorganisms, and it consists of mixing a disperse phase, formed by the cells and the encapsulating polymer, in a continuous phase, which is generally oil or an organic solvent. In this way, an emulsion of water in oil is obtained that is homogenized using a surfactant, such as Tween, and with constant agitation, which promotes the stability of the capsules. Alginate, carrageenan and pectin are ideal for use in this technique as encapsulating materials. Then a solidifying agent, such as calcium chloride, is added to the emulsion to form the capsules that will later be filtered. The capsules obtained can vary in size between 25 μm and 2 mm, depending on the speed of agitation, so this technique can be used to make microcapsules. However, one of the main disadvantages of this technique is the use of organic solvents that could be toxic in the subsequent use of the encapsulated microbes and removing the oil from the capsules can be difficult [86,164]. On the other hand, with this technique other types of low molecular weight materials can also be used to coat the capsules, however, the microorganisms tend to be rapidly released through the gel [133].

8. Benefits of Bioencapsulation in the Field

One of the greatest challenges in the use of plant growth promoting microorganisms in the field is their distribution in the soil and how to apply them. By directly applying microorganisms to the soil, they are exposed to environmental conditions that can be harmful to them, such as lack of water, changes in pH and temperature, in addition to the fact that their distribution area may be limited, all of which can result in the mortality of organisms and a decrease in their ability to benefit crops [69]. The use of techniques to immobilize and distribute microorganisms, such as the production of bioencapsulated microbes, has become essential to overcome the limitations encountered with the direct use of these in the field. Thus, bioencapsulation has many advantages for and the deliberate release of microorganisms to the soil. These advantages include being able to provide protection against environmental factors, increasing cell viability in the soil, favoring cell dispersion and facilitating microbial cell contact with plants, thereby increasing their effectiveness [69,86].

Among the microorganisms evaluated in the field or in greenhouse experiments, there are fungi, such as *Trichoderma* and *Metarhizum*, and a variety of bacteria, including *Bacillus*, *Pseudomonas*, *Azotobacter*, among others, which have been widely used as biofertilizers, biostimulators or as biopesticides and insecticides in crops of interest, such as *Zea mays*, *Triticum* sp., *Solanum lycopersicum*, *Oryza sativa*, etc., [90,105,114,121].

In a greenhouse experiment, the inoculation of wheat seeds with *Streptomyces fulvissimus* Uts22 microcapsules, prepared with chitosan and gellan gum, promoted the growth of plants and increased their resistance to the pathogen *Gaeumannomyces graminis* var. *tritici*, to a greater extent than inoculation with the free bacteria [104]. In a separate greenhouse experiment, with bell pepper plants, the inoculation of *P. putida* microcapsules significantly promoted plant growth, compared to uninoculated plants or plants inoculated with unencapsulated bacterial cultures [110]. These examples are consistent with the benefits of encapsulated microbes, compared to the application of liquid or unencapsulated cultures.

In the field, the inoculation of capsules of different bacteria of the genus *Bacillus*, *Azospirillum* and *Burkholderia* increased the concentration of nutrients, such as nitrogen and phosphorus, in *Eugenia stipitata* plants [119]. Microcapsules containing *Mesorhizobium ciceri* ST-282 and *Bradyrhizobium japonicum* M8 increased the number of nodules in roots of chickpea and soybean plants in a field experiment [122]. The encapsulation of *T. viride* increased the content of secondary metabolites in lettuce plants, when grown in the field and also in hydroponic culture [125], highlighting the importance of encapsulating beneficial microorganisms, including both fungi bacteria in different cultivation techniques.

The encapsulation of *Ensifer fredii* LP2/20 applied to soil cultivated with kale significantly modified the composition of the microbial community, also increasing the biomass of plants [109], which suggests an interaction of the encapsulated microorganisms with the soil microbiota, resulting in a positive effect for crops in general.

Beneficial microorganisms can also protect plants against different types of stress. The encapsulation of *Paenibacillus polymyxa* MSRH5, *B. nakamurai* MSRH1 and *B. pacificus* MSR H3 reduce the effects caused by salt stress in wheat plants, in addition to increasing plant biomass [112]. The inoculation of microcapsules of *P. putida* Rs-198 promotes plant growth of cotton plants when they are subjected to salt stress [108], providing resistance to this type of abiotic stress, and suggesting that encapsulated microorganisms can resist environmental factors, such as salt stress.

It is worth mentioning that most of the bioencapsulation experiments have been carried out under greenhouse conditions (Table 1) with favorable results. However, the application of bioencapsulated microbes in field experiments poses other challenges, such as greater exposure to environmental factors, that cannot be controlled. The studies mentioned above and others referring to organisms of agricultural interest that have been encapsulated and used in greenhouse or field experiments are summarized in Table 1.

9. Conclusions and Perspectives

Today the growing world population is accompanied by an increased demand for agricultural products. Thus, it is necessary to maintain food security for humans and animals without neglecting the conservation and improvement of ecosystems. The excessive use of agrochemicals to increase agrarian production has caused great damage to human health and ecosystems, deteriorating soil and water quality and in general, altering the environment. However, one of the alternatives to agrochemicals in the field is the use of beneficial microorganisms. Its use favorably contributes to increased crop yields, increased tolerance to water and saline stress conditions, and increased resistance to phytopathogens, thus potentially replacing the excessive use of chemical fertilizers and pesticides. However, the employment of microorganisms in the soil poses various challenges, such as maintaining cell viability and microbial resistance to different environmental conditions. Therefore, it is important to study tools and techniques, such as bioencapsulation, which allow these difficulties to be overcome.

Microbial encapsulation is one of the bioinoculum formulation methods that has attracted great interest in the agricultural area, thanks to the benefits it offers by safeguarding microbial cell viability during its formulation and later in its application and release in the field. To hasten the adoption of this technology, it is important to carry out in-depth studies on the appropriate bioencapsulation technique to be used, which is adjusted to the needs of the crop, the microorganism used and the specific environmental conditions. This approach should help to achieve the preservation of beneficial microorganisms and their efficient distribution in the soil, thus guaranteeing their efficacy as biostimulants, biofertilizers and biopesticides.

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Outstanding biocontrol and plant growth promotion traits of *Pseudomonas fluorescens* UM270 and other plant-associated *Pseudomonas*

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Plant pathogens

ABSTRACT

Plant-beneficial *Pseudomonas* spp. is a group of soil bacteria with a broad metabolic and functional repertoire that has been exploited as bioinoculants to enhance crop health and production. Among them, *Pseudomonas fluorescens* strain UM270 stands out as a biocontrol agent and plant growth promoter, which was isolated from the rhizosphere of *Medicago truncatula* plants in Morelia, Mexico. Its genome contains genes with direct and indirect beneficial functions for plants, such as the production of siderophores, 2,4-diacetylphloroglucinol, phosphate solubilization, phenazines, cyanogens, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, proteases, indole-3-acetic acid, or antimicrobial volatiles like dimethyl disulfide and dimethylhexadecylamine, among others. Its antagonistic properties have been tested against major fungal pathogens such as *Botrytis cinerea*, *Rhizoctonia solani*, *Diaporthe phaseolorum*, *Fusarium* spp., and *Colletotrichum lindemuthianum*. The UM270 strain has been shown to be beneficial (either in greenhouse or open-field conditions) for crops such as maize, common bean, husk tomato, blueberry, tomato and squash. In this review, we analyze the phylogenetic, genomic, functional and ecological interactions traits of the UM270 strain in the context of other *Pseudomonas* spp. strains, highlighting its potential as a bioinoculant to address the challenges of sustainable agriculture.

1. Introduction

Different agricultural production systems still rely on synthetic chemical inputs such as pesticides, fungicides, and nematicides, which help control soil pests or microorganisms that damage crops and cause severe economic losses. Additionally, nitrogen-based fertilizers or phytostimulators, among others, are necessary in some low-fertility soils to improve plant growth [1]. However, overwhelming evidence has documented the risks to human and animal health, as well as the harmful environmental impact, of continued agrochemical use. Therefore, in recent decades, efforts have been made in different regions of the world to pursue sustainable agriculture, aiming to fulfill the global demand for food in a growing population [2].

In this context, plant growth-promoting bacteria (PGPB) emerge as a viable, economical, and sustainable alternative to enhance crop production and protect plants from pathogen attacks. Among PGPB, a group of bacteria inhabiting the rhizospheric soil (the narrow soil zone influenced by plant roots) has a significant influence on plant metabolism and physiology [3,4]. These plant growth-promoting rhizobacteria (PGPR) are free-living in the rhizosphere and have mechanisms for promoting plant growth directly and indirectly. Direct mechanisms include the production of phytohormones such as auxins, gibberellins, and cytokinins, which not only stimulate plant growth but also promote development through the formation of new organs, particularly in the root system. The production of siderophores, phosphate solubilization, or other essential microelements is also considered a direct pathway for growth promotion [5,6]. On the other hand, indirect mechanisms include the inhibition of phytopathogens through various actions, such as antibiosis or the production of diffusible or volatile antimicrobial compounds. PGPR can also produce elicitors of the plant immune

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system, helping plants to prepare and better protect themselves against potential pathogen attacks [7]. Furthermore, plants face not only pathogen attacks but also abiotic factors such as drought, flooding, soil salinity, and the presence of heavy metals, among others, which increase ethylene production (the stress hormone) and can limit their growth [8]. To regulate or reduce ethylene levels, PGPR assist plants by acting on the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which degrades the ACC precursor, reducing ethylene levels and increasing plant fitness under stress conditions. Different types of stress may be exacerbated by global climate change, and PGPR can also support plants in addressing these challenges [9].

One bacterial genus commonly found in soils and plant root environments, recognized for its direct and indirect properties to stimulate plant growth, is *Pseudomonas*, also known as pseudomonads. *Pseudomonas* species (aerobic, Gram-negative bacteria) such as *P. chlororaphis* [10] and *P. protegens* [11,12] stand out for their beneficial properties in promoting plant growth and health. On the other hand, species like *P. palleroniana* [13] or *P. syringae* [14] can be pathogenic. Notably, *Pseudomonas* spp. are also responsible for the disease suppressiveness of diverse soils, making their colonization and abundance in agricultural soils essential for improving fertility [15].

Among the fluorescent group of *Pseudomonas* species, *P. fluorescens* stands out [16–18]. Strains of *Pseudomonas fluorescens* produce antibiotics such as pyrrolnitrin, phenazines, 2-hexyl-5-propyl resorcinol, cyanogens, siderophores like pyoverdine and achromobactin, cyclic lipopeptides, 2,4-diacetylphloroglucinol (DAPG), as well as hydrolytic enzymes such as proteases, cellulase, chitinase, and β-glucanase. They also release a complex blend of volatile organic compounds (VOCs) like dimethyl disulfide and dimethylhexadecylamine, which effectively contribute to controlling several plant pathogens. Additionally, they produce phytohormones such as indole-3-acetic acid (IAA), ACC deaminase, degrade multiple complex compounds and produce biofilms as determinants of excellent rhizosphere colonization and interactions with other beneficial soil microbes [19,20].

In this study, we explore the beneficial capacities of various pseudomonads, using the rhizobacterium *P. fluorescens* strain UM270 as a reference. This strain stands out for its direct and indirect mechanisms of plant growth promotion. The capabilities of strain UM270 as an excellent bioinoculant under in vitro, greenhouse, or open-field conditions have remained robust over time in multiple studies by different laboratories, making it a great biofertilizer and biocontrol agent in crops.

2. Phylogeny of *Pseudomonas* spp. And *P. fluorescens* UM270

Bacteria belonging to the genus *Pseudomonas* are part of the γ subclass of Proteobacteria. They are rod-shaped, polar-flagellated, and Gram-negative. One of the defining features of this group of microorganisms is their remarkable metabolic versatility and ability to colonize diverse environments, making them ubiquitous in both aquatic and terrestrial ecosystems [21]. To date, over 200 species have been identified, and hundreds of genomes have been sequenced, most of them available as draft assemblies in the GenBank and JGI-IMG portals.

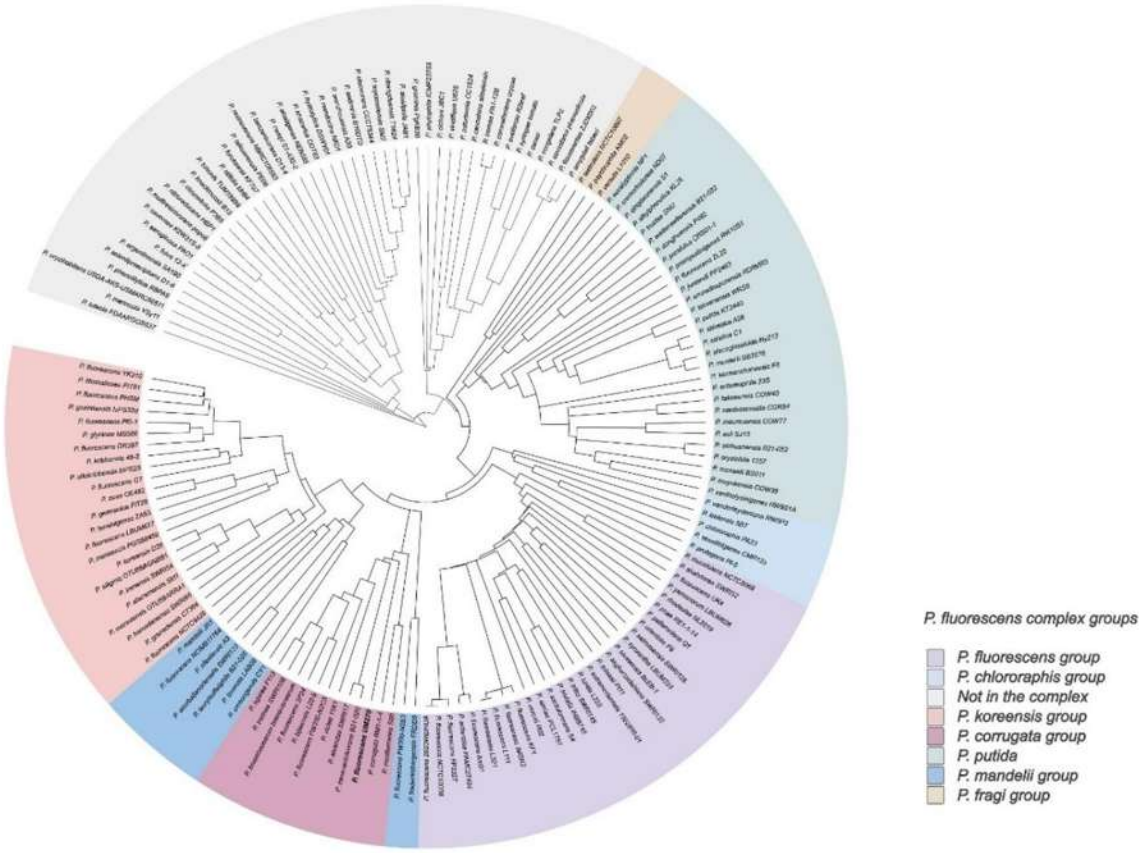

Fig. 1. FastANI-based hierarchical clustering displaying genomic distances among representatives of all available *Pseudomonas* species.

Among these, *P. aeruginosa* stands out due to its medical relevance in humans. Additionally, more than 50 genomes of *Pseudomonas* species have been reported as beneficial to plants [22].

Fig. 1 illustrates the phylogeny of a group of *Pseudomonas* spp., highlighting the genomic diversity within the genus and the numerous species it encompasses. Notably, certain *P. fluorescens* strains exhibit close relationships with other species that are not classified as *P. fluorescens*, forming clades with these non-*P. fluorescens* species. In the case of strain UM270 (Fig. 1, dashed lines), it clusters with species such as *P. mediterranea* S58, *P. corrugata* RM1-1-4, *P. canavaninivorans* B21-020, *P. alvandae* SWRI17, *P. viciae* 11K1, *P. fluorescens* FW300-N2C3, *P. bijieensis* L22-9, *P. fluorescens* 2P24, *P. brassicacearum*, *P. zarinae* SWRI108, and *P. ogarae* F113. ANI (Average Nucleotide Identity) analyses suggest that strain UM270 shares 99.13 % similarity with *P. chlororaphis*, while its highest similarity with *P. fluorescens* strains is only 89 %. However, its genome is being re-sequenced, in addition to a polyphasic analysis, which includes biochemical tests and membrane fatty acid analysis, that will help us obtain a more accurate taxonomic affiliation for UM270.

Fig. 2 presents a phylogeny based solely on complete genomes of various *P. fluorescens* strains. UM270 clusters in a clade with strains 2P24, et76, FW300-N2C3, DSM11579, and others. However, other plant-associated strains, such as *P. fluorescens* Pf0-1, are phylogenetically distant from UM270. The phylogenies were constructed as follows: For ANI calculations, a total of 155 genomic sequences were downloaded from the NCBI RefSeq database [23], including 133 representatives of various *Pseudomonas* species and 22 *P. fluorescens* genomes. Genomic distances with *P. fluorescens* UM270 were calculated via pairwise comparisons with reference genomes using FastANI v1.32 [24], which computes ANI [25]. Hierarchical clustering trees (Figs. 1 and 2) were generated based on these distances using FastANI results and the divisive (diana) method implemented in R [26]. The representative tree was visualized using iTOL [27].

3. Genomic features of *P. fluorescens* UM270

The *P. fluorescens* strain UM270 was isolated from rhizospheric soil of alfalfa plants in an agricultural field located in Morelia, Mexico. It is a Gram-negative, non-sporulating, motile, rod-shaped bacterium, considered mesophilic, with optimal growth in a pH range of 6–8.5 (preferably 7–8) and at a temperature of 28 °C [28,29].

Its genome contains 5509 genes, of which 5396 encode proteins, making 97 % of the genome coding. The identified genes include those involved in carbohydrate and protein metabolism, cell growth and division, as well as genes related to colonization and survival in soil environments. Comparative analysis of the *P. fluorescens* UM270 genome with seven complete genomes of *Pseudomonas* species (strains UM270, Pf0-1, A506, F113, SBW25, PICF-7, UK4, and UW4) revealed 599 unique genes in UM270. These include numerous flagellar protein-coding genes, such as biosynthetic (FlhABPQR), regulatory (FleQ), motor switch (FliN), and basal-body rod proteins (FlgCDFG). Additionally, 15 transporters (11 ABC-type), 14 regulatory proteins (8 transcriptional regulators), and a variety of proteases, hydrolases, secretion factors, activators, reductases, and biosynthetic proteins were identified [30].

Other plant-interaction-related genes found in the UM270 genome include 192 coding sequences involved in the synthesis of indole-3-acetic acid and phenylacetic acid, signaling, rhizosphere colonization, and competition [29,30]. It is worth noting that more genomes are now

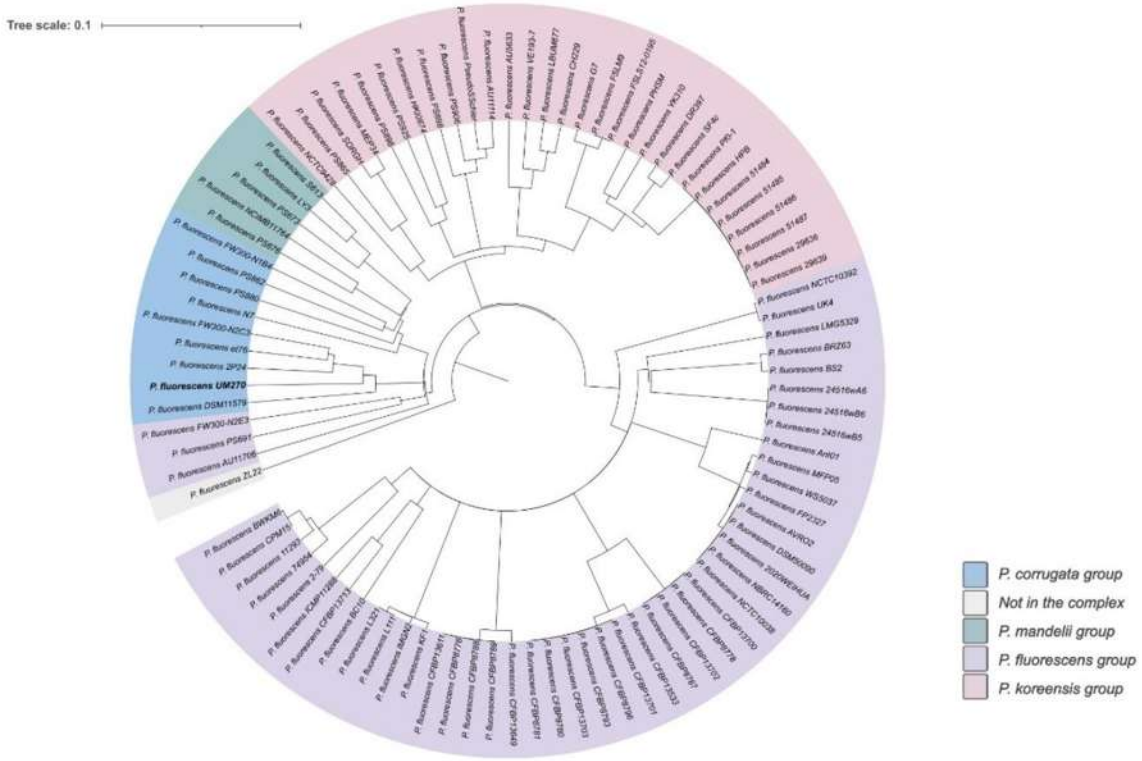

Fig. 2. FastANI-based hierarchical clustering showing genomic distances among representatives of available *P. fluorescens* strains. The type strains of *P. fluorescens* are highlighted in gray and strain UM270 in bold.

available for comparison with UM270, and additional comparative analyses are underway to update this published information.

4. Biocontrol activities of *P. fluorescens* UM270

The strain was characterized in a study published by Hernández-León et al. (2015) [28]. In this pioneering work, the antifungal effects of strain UM270 were evaluated through the production of diffusible and volatile compounds in a dual Petri dish system. In this system, strain UM270 inhibited the pathogen *Botrytis cinerea* by 86 % and 53 %, respectively. Other fungal pathogens inhibited by UM270 included agronomically important species such as *Rhizoctonia solani*, *Diaporthe phaseolorum*, *Fusarium* spp., and *Colletotrichum lindemuthianum*. Additionally, this study assessed the effect of UM270 inoculation on *Medicago truncatula* plants exposed to *B. cinerea* in vitro, reducing disease symptoms and root necrosis caused by the pathogen. This system functionally identified the role of strain UM270 as a biocontrol agent for fungal pathogens in a tripartite plant-bacteria-pathogen interaction system.

Exploring the direct and indirect mechanisms of strain UM270 revealed its production of sulfur-based volatile compounds, including methanethiol, dimethyl sulfide, methyl thiolacetate, dimethyl disulfide, and dimethyl trisulfide. Other relevant VOCs included 1-undecanol, hydrogen cyanide (HCN), and dimethylhexadecylamine, known for their antagonistic effects against pathogens. In a recent review, the production of various VOCs by strains of the *Pseudomonas fluorescens* complex with multiple beneficial roles in plant interactions was highlighted. Some of these VOCs are shared with the UM270 strain and include alkenes (e.g., 1-decene); sulfur compounds (e.g., dimethyl disulfide, dimethyl trisulfide); alcohols (e.g., 3-methyl-1-butanol); ketones (e.g., 2-butanone); organic acids (e.g., acetic acid); and inorganic compounds (e.g., ammonia) [31]. Furthermore, diffusible metabolites such as siderophores and genes encoding the antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG), ACC deaminase, and robust biofilm production (Fig. 3) were reported [28]. These features are also represented in various *Pseudomonas* spp. genomes. For example, antiSMASH analysis of ten *Pseudomonas* strains identified clusters encoding metabolites such as 2,4-DAPG, pyoverdine, pyrrolnitrin, and phenazine [32]. Clusters encoding rhizoxin A were exclusively identified in the genome of *Pseudomonas protegens* Pf-5, a strain widely studied for its biocontrol capabilities (Fig. 3). These findings highlight the metabolic and functional diversity of different *Pseudomonas* species and strains, suggesting that comparative studies could reveal multiple antagonistic capabilities against various pathogens.

In another study focused on biocontrol, the antagonistic effect of strain UM270 against the phytopathogenic fungi *B. cinerea*, *Fusarium oxysporum*, *Fusarium solani*, and *R. solani* was evaluated. The study found that strain UM270 inhibited mycelial growth of *B. cinerea* (45 %), *F. solani* (25 %), and *R. solani* (24 %), while no significant inhibition was observed for *F. oxysporum* (1 %). Additionally, the expression of the genes *phlD* (a key gene in the 2,4-DAPG biosynthetic operon) and *hcnC* (encoding hydrogen cyanide production) in strain UM270 was modulated in the presence of phytopathogens during in vitro antagonism assays. Interestingly, *B. cinerea* induced *phlD* expression, while the other pathogens repressed or did not affect it. Regarding *hcnC*, *B. cinerea* and *F. oxysporum* had no effect on its expression, whereas *F. solani* and *R. solani* inhibited it. These results suggest that the expression of genes crucial for antimicrobial compound synthesis in *P. fluorescens* UM270 can be modulated by the presence of phytopathogens [33].

In a comparative study with *Bacillus* species, strain UM270 was evaluated for its antifungal capabilities against post-harvest fungi. It demonstrated biocontrol effects, inhibiting mycelial growth by over 35 % for *Botrytis* sp., *B. cinerea*, *Geotrichum candidum*, *Cladosporium* sp., *Geotrichum phurueaensis*, *Fusarium brachygibbosum*, *Penicillium crustosum*, *Penicillium expansum*, and *Alternaria* spp. The fungi *F. brachygibbosum*, *B. cinerea*, and *A. alternata* were selected for further evaluation on strawberries and grapes previously inoculated with strain UM270. Results showed that UM270 reduced the incidence of *F. brachygibbosum*, *B. cinerea*, and *A. alternata* on strawberries and grapes by 60 %, 55 %, and 65 %, respectively [34].

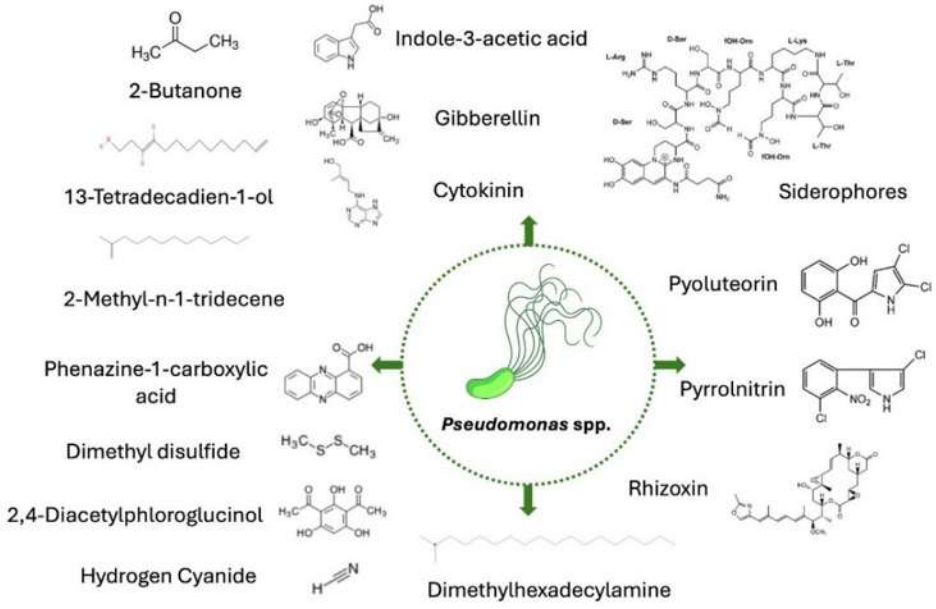

Fig. 3. Diversity of the metabolite arsenal produced by *Pseudomonas* species and *P. fluorescens* UM270 strain, as detected by various evaluation methods, including biochemical assays, enzymatic activity, PCR amplification of genes, High-Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS), functional and genomic analyses, and the AntiSMASH program, among others.

5. Plant growth-stimulating activities of *P. fluorescens* UM270

The relationship between plants and bacteria has existed for millions of years. The effects of this plant-bacteria interaction can be positive, negative, or neutral. In recent years, there has been significant interest in elucidating the positive relationship between plants and plant growth-promoting rhizobacteria (PGPR), with particular emphasis on the species *Pseudomonas fluorescens* due to its potential application in agriculture [35–37]. Table 1 highlights a list of *P. fluorescens* strains providing various services to their associated plant hosts, including crops such as barley (*Hordeum vulgare* L.) [38], melon (*Cucumis melo* L.) [39], rapeseed (*Brassica napus* L.) [40], lemon balm (*Melissa officinalis* L.) [41], tomato (*Solanum lycopersicum* cv. CaljN3) [42], rice (*Oryza sativa* L.) [43], cucumber (*Cucumis sativus*) [44], pea (*Pisum sativum*) [45], wheat (*Triticum aestivum* L.) [46,47], and peanuts (*Arachis hypogaea* L.) [48], among others.

Plants recruit their rhizosphere microbiome through the root exudation of various substances that act as chemoattractants. These include sugars, polysaccharides, amino acids, aromatic acids, aliphatic acids, fatty acids, sterols, phenols, secondary metabolites, proteins, and diverse enzymes [49,50]. The composition of exudates changes as the plant develops or responds to external stimuli, as well as depending on the plant genotype, collectively shaping the rhizosphere microbiome [51,52]. Some PGPR colonizing the rhizosphere can enter plant tissues through wounds, lateral root emergence zones, nodules, or root fissures. Moreover, PGPR can actively colonize endosphere spaces by producing

Table 1
Recent works evaluating biocontrol and plant growth-promoting activities of diverse *Pseudomonas fluorescens* strains.

Strains	Mechanism(s) of action	Beneficiated plant host (Common name/ Species)	Biocontrol and Plant Growth Promotion Benefits	Reference
P. fluorescens B-10, B2-10, B2-11 and B4-6	ACC deaminase, production of siderophore, IAA and solubilization of phosphate	Barley (*Hordeum vulgare* L.)	Increase in plant height, spike length, weight and number, peduncle length, number of grains per spike, 1000-grain weight and grain yield	[38]
P. fluorescens	Increase in availability and release of Na, Mg, K, Mn, Zn, Fe and P	Melon (*Cucumis melo* L.)	Improved fruit size and weight	[39]
P. fluorescens ALEB7B	Modulation of GA and JA signaling pathways	Atractylis (*Atractylodes macrocephala*)	Enhance sesquiterpenoid content	[87]
P. fluorescens P1 and P8	Improves the availability of nutrients in the soil (P and Fe)	Maize (*Zea mays* L. var saccharate)	Increased tolerance to water deficit, in addition to higher yield	[88]
P. fluorescens BRZ63	Production of biosurfactants, siderophores, IAA), ACC deaminase, and ammonia as well as phosphate solubilization.	Rape (*Brassica napus* L.)	Inhibition of mycelial growth of *Rhizoctonia solani* W70, *Colletotrichum dematium Sclerotinia sclerotiorum* K2291, and *Fusarium avenaceum*, in addition to stimulating germination and growth	[40]
P. fluorescens PF-135	Production of IAA and ACC deaminase	Lemon balm (*Melissa officinalis* L.)	Greater tolerance to water stress, increase in relative water content (RWC) and antioxidant activity	[41]
P. fluorescens CHA0	Inducción de enzimas de defensa POX, PPO and accumulation of H2O2	Tomato (*Solanum lycopersicum* cv. CaljN3)	Reduction in the rate of disease caused by the nematode *Meloidogyne javanica* (No. of galls and egg masses/plant and No. of eggs/individual egg mass)	[42]
P. fluorescens Pf-8	Activation of ISR	Rice (*Oryza sativa* L.)	Reduced the incidence of blight disease caused by *Magnaporthe oryzae* and increased vegetative and yield parameters	[89]
P. fluorescnces P60	Production of chitinase and protease	Maize (*Zea mays* L.)	Reduction of disease and infection rate *Rhizoctonia solani*, improved plant fresh weight and root fresh weight	[90]
P. fluorescens ZX	Volatile organic compounds such as DMDS and DMTS	Citrus (*Citrus sinensis* Osbeck)	Lower incidence of the disease in fruits by inhibiting mycelial growth and germination of conidia *Penicillium italicum*	[91]
P. fluorescens G20-18	Production of cytokinin	Tomato (*Solanum lycopersicum* L.)	Higher content of chlorophyll, ABA and stomatal closure, increased activity of antioxidant enzymes	[92]
P. fluorescens NK4	Production of siderophores supplemented with nanoparticles of ZnO-NP	Cucumber (*Cucumis sativus*)	Increased root length and yield, in addition to controlling *Pseudomonas viridiflava* NK2	[44]
P. fluorescens DR397	Genes related to the synthesis of compatible solutes, exopolysaccharides and plant growth promotion (IAA, transketolase, and thiamine phosphate synthesis)	Pea (*Pisum sativum*) and Bean (*Phaseolus vulgaris*)	Increased growth of shoots and roots	[45]
P. fluorescens PFTT4	Production of IAA, HCN, ammonia nd phosphate solubilization	Okra (*Abelmoschus esculentus* L.)	Higher germination percentage, shoot length, root length and dry weight	[93]
P. fluorescens 2137	Activation of ISR	Barley (*Hordeum vulgare* L.)	Induction of defense genes LOX, PAL, PR4, and PR1 that reduced the incidence of damage caused by *Fusarium culmorum*	[94]
P. fluorescens RB5	Production of siderophores, proteases and chitinases	Wheat (*Triticum aestivum* L.)	Reduction in the rate of disease caused by *Rhizoctonia cerealis*, through the alteration of mycelial morphology and enzymatic inhibition	[46]
P. fluorescens SBW25	Production of CLPs	Wheat (*Triticum aestivum* Heerup and Sheriff)	Improved root colonization with beneficial microbial communities, reducing the abundance of *Phytophthora* sp.	[47]
P. fluorescens Pf10	Production of siderophore and cell wall degrading enzymes such as chitinase and β-1,3 glucanase	Tomato (*Solanum licopersicum* L.)	Inhibition of mycelial development of *Rhizoctonia solani*	[95]
P. fluorescens ATCC 17386	IAA production, phosphorus and potassium solubilization	Trebol (*Melilotus officinalis*)	Improved germination rate and vigor index, greater root and shoot length and weight	[96]
P. fluorescens (PF)	Activation of ISR and IAA production	Peanuts (*Arachis hypogaea* L.)	Stimulation of root development, improved antioxidant activity, lignin biosynthesis was promoted, resistance to damage caused by *Fusarium oxysporum* and finally a higher pod yield.	[48]

Abbreviations: ACC (1-aminocyclopropane-1-carboxylate), IAA (indole-3-acetic acid), Gibberellic acid (GA), Jasmonic acid (JA), Induction of System Resistance (ISR), Peroxidase (POX), Polifenol oxidase (PPO), Dimethyl disulfide (DMDS), Dimethyl trisulfide (DMTS), Abscisic acid (ABA), Hydrogen cyanide (HCN), Cyclic lipopeptides (CLPs).

hydrolytic enzymes capable of degrading the plant cell wall [53]. Consequently, the rhizosphere has been suggested as a repository of potential plant growth-promoting bacterial endophytes (PGPBEs). For strain UM270, this capacity as an endophyte has not been explored; however, its rhizospheric inoculation is known to modulate the endophytic microbiome of plants [54].

One of the initial steps in selecting PGPR is establishing in vitro cultures interacting with plants. Hernández-León et al. (2015) [55] demonstrated that strain UM270 can stimulate the growth of *Medicago truncatula* plants by promoting shoot and root growth, as well as chlorophyll content [55]. The stimulation of *M. truncatula* growth was evaluated through direct interaction (diffusible compounds production) and indirect interaction (VOCs production). In both cases, the plant-bacteria interaction effect was positive. One proposed mechanism for plant growth promotion was the production of auxins, such as indole-3-acetic acid (IAA), which UM270 produces at concentrations of ~10 μg/mL.

In a study by Rojas-Solis et al. (2016) [56], the interaction of four *Pseudomonas fluorescens* strains, including UM270, with a biocontrol strain of *Bacillus thuringiensis* (UM96) was evaluated for their synergistic effect on rhizosphere colonization in maize (*Zea mays* L.) and their growth-promoting effect on green tomato (*Physalis ixocarpa* Brot. ex Horm.) seedlings. The results confirmed that all five strains were competent rhizosphere colonizers in maize, whether individually or in consortium. When assessing the combined effect, only the UM96-UM16 consortium significantly improved the total fresh weight, hypocotyl length, and root length of the seedlings. Individually, the *P. fluorescens* strains were the only ones with a positive effect on seedling development. No synergistic effects were observed between UM270 and the UM96 strain of *Bacillus*, indicating that individual inoculation of UM270 was more effective in stimulating plant growth.

6. Plant growth promotion by UM270 strain under stress conditions

Different types of abiotic stress can limit the growth and productivity of agricultural crops, including salinity. Salinity causes ionic imbalances that hinder water absorption, affecting photosynthesis and other metabolic processes, ultimately resulting in reduced seed germination and delayed plant growth. This directly impacts crop productivity and plant-associated microbiomes [45,57].

In a recent study, Rojas-Solis and colleagues (2023) [58]evaluated the role of two genes involved in cardiolipin synthesis by generating mutants (Δ*clsA* and Δ*clsB*). Cardiolipin (CL) is a membrane phospholipid that plays a crucial role in bacterial adaptation to stress, including salt stress. Their findings revealed that both mutations significantly reduced CL synthesis (58 % and 53 %, respectively). Although reduced CL slightly affected cell growth under saline conditions, it was not critical for survival. Regarding plant growth promotion in tomato plants, the mutant strains showed reduced production of indole-3-acetic acid (IAA) but maintained siderophore excretion and increased biofilm formation, even under saline stress. These results highlight the role of CL in the adaptation and growth-promoting function of UM270, although it is not indispensable under extreme conditions (Rojas-Solis et al., 2023) [58]. Additionally, inoculation of the wild-type UM270 strain in tomato (*Solanum lycopersicum*) plants grown under saline stress conditions (100 and 200 mM NaCl) resulted in increased root and shoot length, chlorophyll content, and total dry weight. In contrast, plants inoculated with the mutant strains showed reduced root length at 200 mM NaCl, while shoot length, chlorophyll content, and total dry weight were significantly reduced under both normal and saline conditions (100 and 200 mM NaCl) compared to plants inoculated with the wild-type UM270 strain. Thus, the *clsA* and *clsB* genes play a fundamental role in promoting the growth of *Solanum lycopersicum* plants under saline stress.

Another stress condition where the role of UM270 inoculation has been evaluated is in plants exposed to heavy metals, such as mercury, and metalloids, such as arsenic. Agricultural soils near mining sites may become contaminated with heavy metals released during the extraction and processing of minerals. Therefore, the role of PGPRs in processes that stimulate phytoremediation and enhance tolerance to heavy metal stress can help improve crop growth and productivity [59–61]. In this context, Rojas-Solis et al. (2023) [58]evaluated the individual and combined effects of *P. fluorescens* UM270 and *Bacillus paralicheniformis* ZAP17 on the growth of maize (*Zea mays* L.) plants subjected to arsenic and mercury stress. Co-inoculation of both bacterial strains in maize plants exposed to different concentrations of metal salts enhanced stem growth and increased plant biomass compared to uninoculated plants. Furthermore, key PGPR mechanisms, such as phosphate solubilization, siderophore production, and the emission of plant growth-promoting VOCs (e.g., 2-butanone, 2,3-butanediol, dimethyl disulfide, nonanal, hexadecanal, 2-tetradecanone, and 2-tridecanone), were altered under Hg and As stress but remained active. Thus, these two PGPR strains represent potential plant growth promoters under stress conditions as a synthetic community.

7. Evaluation of beneficial traits with PLaBAse

In silico analyses of *Pseudomonas* genomes and other plant-associated bacteria have demonstrated their enormous potential for detecting direct and indirect mechanisms of plant growth promotion. In some cases, these types of predictions can corroborate experimental results and vice versa, where biocontrol activities are observed (e.g., production of antimicrobial compounds that displace pathogens competing for nutrients and space in the rhizosphere) and/or plant growth promotion (e.g., production of phytohormones such as auxins, gibberellins, or cytokinins, as well as nutrient solubilization that facilitates their uptake by the plant) [53]. This is the case with the PlaBAse database, which was recently launched by Patz et al. (2021) [62]. This database is a web resource for genome analysis and predicting plant growth-promoting traits (PGPTs). For example, in the case of strain UM270, the PLaBA-db (The Plant-associated Bacteria Database) service has identified functions such as plant colonization, competition, biocontrol, stress control, biofertilization, phytohormone production and plant signaling, bioremediation, and stimulation of the plant immune system. Fig. 4 illustrates these functions, categorized into direct and indirect mechanisms.

8. Synergistic interactions with *Trichoderma*

Trichoderma is a beneficial fungus widely applied in the field due to its biocontrol abilities against pathogens and its capacity to enhance plant health. One of the primary biocontrol mechanisms is mycoparasitism, in which *Trichoderma* coils around the pathogen's hyphae, penetrates, and parasitizes it. To achieve this, *Trichoderma* produces cell wall-degrading enzymes that assist in the mycoparasitism process [63].

However, *Trichoderma* can also produce a variety of antimicrobial or plant growth-promoting metabolites (e.g., phytohormones such as auxins, abscisic acid, gibberellins, salicylic acid, and cytokinins), making it an excellent plant symbiont [64]. Although the molecular mechanisms of interaction between *Trichoderma* species and plants are still under investigation, it has been proposed that effector-like proteins could mediate the beneficial communication between the fungus and its plant host [65].

In this context, Guzmán-Guzmán and colleagues (2024) [66] evaluated the role of four PGPRs (*Pseudomonas fluorescens* UM270, *Bacillus velezensis* AF12, *B. halotolerans* AF23, and *Rouxiella badensis* SER3) in interaction with *Trichoderma* to determine whether these bacterial agents could stimulate the expression of effector-like protein genes (*epl1*, *tatrx2*, and *tacfem1*). The results showed that the consortium of *T. atroviride* with *R. badensis* SER3 was the most effective in inhibiting the growth of pathogens such as *Fusarium brachygibbosum* and stimulating *A. thaliana* PR1:GUS and LOX2:GUS for SA- and JA-mediated

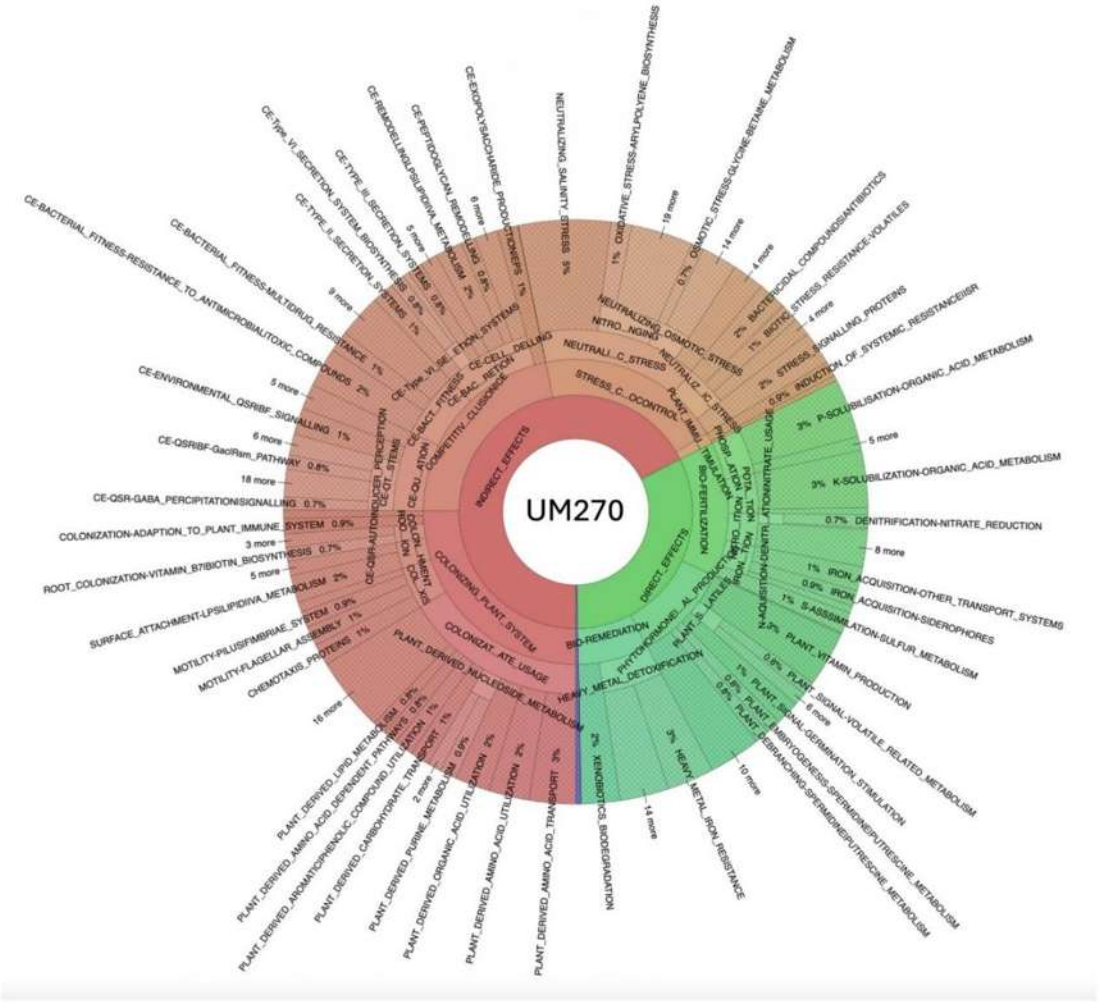

Fig. 4. Graph representing the direct and indirect functions of *Pseudomonas fluorescens* strain UM270 predicted using the PlaBAse database, and specifically the PLaBA-db service (https://plabase.cs.uni-tuebingen.de/pb/plabase.php). Some of the direct plant growth promotion mechanisms predicted by this database include phytohormone production, bioremediation, and biofertilization, which includes phosphate and potassium solubilization mechanisms that enhance nutrient acquisition in plants. Among the indirect mechanisms, functions such as biocontrol, colonization, and competition for spaces are predicted, all of which are fundamental in environments like the rhizosphere.

defense responses. Finally, the consortium of *T. atroviride* with *P. fluorescens* UM270 was the most effective in promoting biomass (dry weight) and root development (primary root length and lateral roots) in *Arabidopsis thaliana* plants. Notably, during the interaction between the UM270 strain, *T. atroviride,* and the plant, the expression of the *epl1* and *tatrx2* genes increased, particularly in the consortium with *Pseudomonas fluorescens* UM270. This demonstrated that this rhizobacterium forms synergistic interactions with other beneficial microorganisms applied in agriculture.

9. Modulation of plant microbiomes by UM270 inoculation

Numerous studies have highlighted the biocontrol, growth-promoting, and abiotic stress amelioration roles of microbial-based bioinoculants in plants. However, the in situ impact of microbial inoculants on rhizosphere microbiota, particularly their influence on indigenous plant microbiomes and the types of interactions they form, remains poorly understood [67,68] proposed that various molecules (not only sugars as nutrients) secreted into the rhizosphere can modulate and recruit a beneficial microbiome to promote plant health, trigger induced systemic resistance (ISR), and enhance host fitness under challenging environmental conditions [69].

Specific recruitment of certain microbial groups, such as the phyla *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, and *Firmicutes*, by agronomically relevant plant species like barley, has been shown to be highly consistent [70]. More recently, Song et al. (2021) [71] demonstrated that genetic factors such as the *FERONIA (FER)* receptor kinase (*fer-8*) in *Arabidopsis* play a key role in regulating the abundance of *Pseudomonas fluorescens*, revealing a specific plant-*Pseudomonas* crosstalk.

Once this beneficial microbial community is "assembled" in the root ecosystem, it is expected to modulate gene expression and plant metabolism, increasing the arsenal of defense metabolites against pathogens

and other protective responses, particularly under potential pathogen infection. This selective recruitment of defensive soil microorganisms by plants is referred to as the "cry for help" mechanism [72].

Although studies on specific inoculation with *Pseudomonas* spp. are limited, Yin et al. (2013) [73]evaluated the impact of the biocontrol agents *Pseudomonas fluorescens* 2P24 and CPF10 on the native rhizosphere bacterial community of cucumber. They observed a decrease in groups such as *Cyanobacterium*, *Beta-proteobacterium*, and *Staphylococcus*, alongside a slight increase in *Bacillus* populations.

More recently, Jiménez et al. (2020) [74], using next-generation sequencing targeting the 16S rDNA V4 region, characterized the microbial communities associated with three different oilseed crops inoculated with the PGPR *P. fluorescens* LBUM677. This study revealed differential abundance of 29 bacterial taxa (e.g., *Actinobacteria*, *Proteobacteria*, *Bacteroidetes*, *Planctomycetes*, and *Armatimonadetes* phyla), in treatments inoculated with strain LBUM677. Functional analyses using PICRUSt showed increases in pathways related to the tricarboxylic acid (TCA) cycle and menaquinone biosynthesis, including menaquinone-6, menaquinone-9, and menaquinone-10.

In the case of strain UM270, its inoculation was evaluated in maize plants grown under greenhouse conditions in three different soil types (clay, sandy loam, and loam) from distinct origins, each with diverse microbial communities. Results showed that strain UM270 significantly increased the populations of *Proteobacteria* and *Acidobacteria* while decreasing *Actinobacteria* and *Bacteroidetes*. Furthermore, UM270 enhanced maize growth by increasing root and shoot weight, chlorophyll content, and total biomass across all soil types. However, no specific correlation with particular bacterial groups was observed, suggesting that the modulation and possible synergistic interactions with the microbiota depend on the native diversity of each soil [75].

The impact of PGPR *P. fluorescens* UM270 on the plant microbiome extends beyond the rhizosphere to endosphere communities. In a field study, UM270 was inoculated into maize plants cultivated in a milpa system over two seasons (2021 and 2023). Inoculation with UM270 significantly altered the root endophytic microbiome of maize plants by stimulating the presence of genera such as *Burkholderia* and *Pseudomonas* (based on operational taxonomic unit analysis). In the Mesoamerican triad, inoculation enhanced endophytic diversity, including genera such as *Burkholderia* and *Variovorax*. Interestingly, nitrogen-fixing rhizobia, including *Rhizobium*, *Mesorhizobium*, and *Bradyrhizobium*, were stimulated in the milpa model where legumes such as common beans (*Phaseolus vulgaris* L.) were co-cultivated with maize but were absent in uninoculated plants [76]. In contrast, fungal diversity analysis using the ITS region did not show an impact from UM270 inoculation. Finally, network analyses revealed unique interactions with species such as *Stenotrophomonas* sp., *Burkholderia xenovorans*, and

Table 2
Biocontrol and plant growth promotion studies on *P. fluorescens* UM270. Some studies show the interaction with other plant growth-promoting bacteria or fungi.

Contribution of the Work	Plant Beneficiated	Pathogen Antagonized	Interaction with Other Microorganisms	Reference
Increase in plant promotion of *Medicago truncatula*in vitro through the emission of volatile and diffusible organic compounds by the inoculation of *P. fluorescens* UM270	*Medicago truncatula*	*B. cinerea*	*P. fluorescens* strains UM16, UM240, UM256	[28]
Synergistic effect between *P. fluorescens* UM270 and *B. thuringiensis* to promote plant growth of Mexican husk tomato and rhizosphere colonization of *Zea mays* roots	*Zea mays* L. *Physalis ixocarpa* Brot ex Horm	–	*Bacillus thuringiensis* UM96	[56]
Genome sequencing of the *P. fluorescens* rhizobacterium strain UM270; Detection of genes involved in biocontrol and plant promotion	–	–	*P. fluorescens* UM270	[29]
Genomic comparison between *Pseudomonas* strains for the analysis of unique genes in *P. fluorescens* UM270; Detection of enzymes and metabolites associated with competition and rhizosphere colonization present in the strain *P. fluorescens*	–	–	*P. fluorescens* strains Pf0-1, A506, F113, SBW25, PICF 7, UK4, and UW4	[30]
Overexpression of the *phlD* and *hcnC* genes of *P. fluorescens* UM270 in the presence of phytopathogenic fungi	–	*B. cinerea F. oxysporum F. solani R. solani*	–	[33]
Comparison of plant growth promotion mechanisms (IAA, biofilm, siderophore production, proteases) of the UM270 strain with endophytic isolates from *Vaccinium corymbosum* L. cv. Biloxi	–	–	–	[97]
Analysis of metabolites secreted by *P. fluorescens* UM270 that promote plant growth and affect the motility of other bacteria	–	–	*Bacillus* sp. ZAP018 *P. aeruginosa* PA01 *A. agilis* UMCV2	[98]
Plant growth promotion and increase in *Physalis ixocarpa* fruit production in field conditions inoculated with *P. fluorescens* UM270	*Physalis ixocarpa* Brot. ex Horm	–	*P. fluorescens* UM270	[79]
Plant growth promotion of blueberry plants in a greenhouse inoculated with *P. fluorescens* UM270	*Vaccinium* sp., cv. Biloxi	–	*P. fluorescens* strains UM16, UM240, UM256	[77]
Biocontrol of postharvest phytopathogens through the emission of volatile and diffusible organic compounds by *P. fluorescens* UM270; Biocontrol in grape and strawberry fruit models against *F. brachygibbosum*, *B. cinerea*, and *A. alternata*	*Vitis vinifera Fragaria x ananassa* fruits	*F. brachygibbosum B. cinerea A. alternata*	*Bacillus* spp.	[34]
Induction of gene expression of effector like proteins in *T. atroviride* during interaction with *P. fluorescens* UM270 and *F. brachygibbosum*	*Arabidopsis thaliana*	*F. brachygibbosum*	*B. velezensis* AF12 *B. halotolerans* AF23 *T. atroviride R. badensis* SER3	[66]
Plant growth promotion of tomato by *P. fluorescens* UM270 under saline stress and analysis of cardiolipin synthesis mutants	*Solanum lycopersicum*	–	–	[58]
Plant growth promotion of maize subjected to stress due to exposure to mercury (Hg) and arsenic (As); analysis of plant growth promoting mechanisms under the presence of Hg and As	*Zea mays* L.	–	*B. paralicheniformis* ZAP17	[99]
Increase in plant growth of maize growing in three different soil types; analysis of the rhizosphere bacteriome associated with each soil type inoculated or not with UM270 strain	*Zea mays* L.	–	*Rhizomicrobium*	[75]
Modulation of the endophytic microbiome of maize roots obtained from the milpa field model by UM270	*Zea mays* L.	–	*Burkholderia, Variovorax, Rhizobium, Mesorhizobium*, and *Bradyrhizobium*	[54]
Increase in plant growth promotion and maize production under the milpa field model with the inoculation of *P. fluorescens* UM270; increase in common bean and squash yield in plants inoculated with UM270	*Zea mays* L. *Cucurbita pepo Phaseolus vulgaris*	–	–	[76]

Sphingobium yanoikuyae, which may play beneficial roles in plants but remain a topic for further in situ investigation.

10. Greenhouse and field evaluations of UM270 as a bioinoculant

The research path of strain UM270 since 2015 has included its isolation and in vitro characterization, genome sequencing, and subsequent functional studies as a PGPR. Table 2 summarizes the contributions made with strain UM270. However, the work with strain UM270 extends beyond the laboratory. Recently, Cortes-Solis and colleagues (2023) [77]evaluated its role as a plant growth promoter in *Vaccinium* sp. (var. Bilox) blueberry plants under greenhouse conditions. Blueberry cultivation in Mexico has increased in recent years, driven by its global demand due to its health benefits and high antioxidant content. The results showed that blueberry plants inoculated with strain UM270 (and other strains of *Pseudomonas fluorescens*) exhibited significant increases in shoot length and fresh weight, as well as root length and dry weight, compared to uninoculated plants.

A vegetable crop that also demonstrates production benefits of the UM270 strain under open-field irrigation conditions is the Mexican husk tomato or tomatillo (*Physalis ixocarpa* Brot. ex Horm). The tomatillo or husk tomato (*Physalis* spp.) is a crop of forage, medicinal, ornamental, industrial, and human consumption importance. It includes around 100 species distributed across the Americas, with Mexico considered the center of domestication for this genus. In Mexico, 70 wild species have been identified, although only two, *Physalis ixocarpa* Brot. ex Horm (*P. philadelphica* Lam.) and *P. angulata*, are cultivated for edible purposes [78].

In a field inoculation trial, Villaseñor-Tulais (2023) [79]inoculated the UM270 strain during a production cycle of *Physalis ixocarpa*, showing that plants inoculated with the rhizobacterium increased their height by 14.64 %, stem diameter by 17.74 %, biovolume index by 35.14 %, and fruit production by 65.54 % (compared to uninoculated plants). This suggests that the *Pseudomonas fluorescens* UM270 strain is an excellent bioinoculant that enhances husk tomato production under field conditions.

More recently, Rojas-Sánchez et al. (2024) [54] evaluated the biofertilizer effect of strain UM270 on maize plants in a milpa system over two cycles. The milpa is a polyculture system where fertilizers are not typically used. In this study, the effect of inoculating strain UM270 on maize growth and total grain production was assessed. Various phytoparameters in maize plants revealed improvements in plant height, root length, chlorophyll content, and total dry weight in inoculated plants. When maize grain production was analyzed, a remarkable increase of up to 40 % was observed in the monoculture inoculated with UM270 compared to uninoculated plants. In addition, inoculation with strain UM270 was evaluated in co-fertilization with diammonium phosphate (DAP), yielding up to a 50 % increase in maize production. These results underscore that inoculation with strain UM270 alone can replace the application of chemical fertilizers such as DAP.

Another notable finding of this study was that maize cobs from plants inoculated with UM270 showed improved nitrogen and phosphorus content. The milpa system where strain UM270 was evaluated as a biofertilizer involves the co-cultivation of other species, such as common beans and squash. For common beans, the yield of plants inoculated with strain UM270 increased by 12.5 % and 13.32 % in the 2021 and 2023 cycles, respectively. Similarly, biofertilization with strain UM270 boosted squash yield, showing increases of 30.27 % and 20.90 % in the two evaluated cycles, respectively. These results were compared to plants without the PGPR inoculation.

Fig. 5 illustrates a summary of the journey of strain UM270, from its isolation and first publication to the latest results presented in the field. It is worth noting that work is currently being done on the formulation and evaluation, also in the field, of a bioinoculant using this strain as the active agent.

11. Conclusions and perspectives

Pseudomonas fluorescens species are a promising alternative to the use and application of polluting chemical fertilizers. Their benefits are broad and have been recognized in numerous studies [80–84]. Their advantages include extensive metabolic versatility, which enables them to colonize spaces, outcompete potential phytopathogens, and produce metabolites that stimulate crosstalk with plants, thereby enhancing plant fitness [85,86].

Regarding strain UM270, efforts are currently underway to develop a bioinoculant based on a combination of alginate and UM270 cells, focusing on its long-term effects on maize crops under open-field conditions. During the first cycle, an increase in maize cob production has been observed, along with greater cell survival in this type of encapsulation throughout the complete crop cycle (Rojas-Sánchez, unpublished results). These results are promising for the short-term development of a new bioinoculant that is agro-sustainable, cost-effective, and delivers robust field results. However, the biocontrol effect of UM270 against fungal pathogens still needs to be evaluated in field studies to gain a

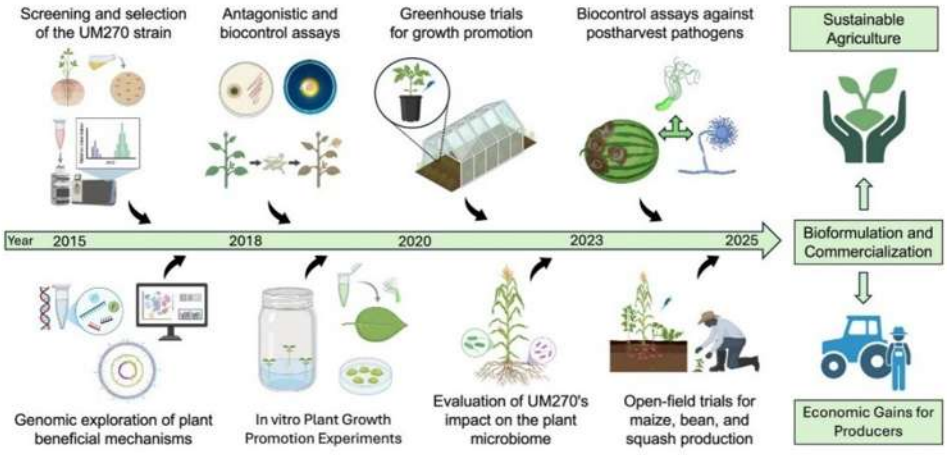

Fig. 5. Timeline of the main research conducted with strain UM270 since its discovery ten years ago.

broader understanding of its benefits, not only as a biofertilizer but also as a biofungicide. Another perspective is to assess the beneficial effects of UM270 in co-inoculation with other beneficial microorganisms, such as *Bacillus* spp. or *Trichoderma* spp., and to evaluate its performance under different stress conditions.

Finally, this approach of inoculating PGPR UM270 offers significant economic and agroecological benefits for local farmers who practice polycultures or the milpa system as a production option. It also provides an alternative to reduce reliance on expensive synthetic fertilizers while supporting sustainable agricultural practices.

CRediT authorship contribution statement

Gustavo Santoyo: Writing – review & editing, Writing – original draft, Supervision, Investigation, Formal analysis, Conceptualization. **Blanca Rojas-Sánchez:** Writing – original draft, Investigation, Formal analysis. **Julie Hernández-Salmerón:** Investigation, Formal analysis. **Rocío Hernández-León:** Investigation, Formal analysis. **Daniel Rojas-Solis:** Investigation, Formal analysis. **Gabriel Moreno-Hagelsieb:** Writing – review & editing, Investigation, Formal analysis. **Ma del Carmen Orozco-Mosqueda:** Writing – review & editing, Investigation, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Discover Applied Sciences

Review

Optimizing milpa agrosystems with beneficial microbes and their ecological interactions: a review

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Abstract
Ensuring food security through sustainable systems remains a key goal for the agricultural sector. However, poor crop management practices in recent decades have caused significant ecological harm, evidenced by climate change impacts, soil degradation, and water scarcity. Biotic and abiotic stresses during crop development further reduce yield and quality. Reviving traditional farming practices, such as the milpa system, offers a solution to boost production sustainably while repairing past damage. This comprehensive polyculture system centers on maize, intercropped with beans, squash, chili, fava beans, and other crops. Ecologically, milpas enhance biodiversity, improve soil physicochemical properties, and mitigate environmental harm through beneficial interactions among plants, insects, and microorganisms. This work examines these interactions, with a focus on the role of beneficial microorganisms in reversing environmental damage and revitalizing milpa systems. Adopting these tools can strengthen traditional practices, promoting sustainability and ensuring food security.

Article Highlights

- Milpa systems enhance product diversity and support the cultural and economic well-being of small producers.
- The ecological interactions within milpa systems help mitigate climate change and benefit the environment.
- Plant growth-promoting microorganisms provide a sustainable approach to improving milpa production.

Keywords Sustainable agriculture · Plant growth-promoting microorganisms · Biological control · Intercropping · Agroecology

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1 Introduction

The milpa system is an ancestral model of economic, social, and cultural importance. It serves as a foundation for designing sustainable crop production systems, particularly in Mexico, where it is regarded as a traditional agricultural system characterized by the ancient, long-standing model of various types of polycropping in the cultivating field, creating a dynamic space of genetic resources [1, 2]. Recent data suggest that small producers account for 30% of global food production, yet they are the group most affected by food insecurity. The decline in this system could have negative consequences for the food security of subsistence farmers [3]. As a self-consumption system, its establishment has decreased in recent years, negatively impacting the conservation of native varieties and biodiversity. The area dedicated to this type of system has been replaced over time by agricultural systems based on planting hybrid seeds in large monoculture areas using heavy agricultural machinery [4, 5].

To achieve high yields, high doses of synthetic sources are applied as nutrients for plants, acting as stimulants, fungicides, pesticides, and herbicides. However, the benefits provided by these products have caused toxic effects on soils, water, and even living organisms, contributing to global warming [6–9]. Reviving traditional agricultural systems like the milpa is an option to safeguard food security at a lower ecological and economic cost, although it needs to be redesigned to ensure its effectiveness in the field due to low soil fertility biotic and abiotic factors affecting crops [10, 11]. One option is the addition of microbial bioinoculants, which have been proven to act as biofertilizers, biofungicides, and biopesticides, providing crop protection and improving the physicochemical conditions of saline or metal contaminated soils. These bioinoculants also participate in the efficient use of soil and thus in the recovery of its biodiversity and richness [12–14].

Some of the microorganisms used in bioinoculant formulations, that enhance plant growth and protect plants from biotic and abiotic stress, are called plant growth-promoting microorganisms (PGPMs); through various mechanisms, PGPMs control pathogenic microorganisms of agricultural interest, stimulate plants under extreme conditions of salinity, drought, and frost, and promote plant growth through nutrient acquisition, among other benefits. Consequently, they increase crop production and thus safeguard food security [15–18]. Some genera reported to have these effects include *Azospirillum*, *Trichoderma*, *Bacillus*, *Pseudomonas* and *Burkholderia*, among others [19–23]. In a field experiment in milpa systems, it has been shown that inoculation with *Pseudomonas fluorescens* UM270 increases maize production and modulates the endophytic biodiversity in the plant's roots, promoting the abundance of species known as plant growth promoters [24, 25]. Based on these results and previous studies on the role of bioinoculants, this work analyzes various studies that highlight the milpa as a sustainable production system. It is proposed that the milpa can be redesigned through the application of bioinoculants, which would increase its production while also serving as a research model in various fields, helping to better understand ecological interactions in these systems and promoting food self-sufficiency for small-scale farmers[26].

2 Origin of the milpa

The milpa system is an ancestral practice integrated since the domestication of maize ~ 2400 years ago. The earliest known evidence of its origin was found in the Guilá Naquitz Cave in Oaxaca (central-southern Mexico) and they later appeared in the south and north of the country (Tehuacán, Puebla, and Ocampo, Tamaulipas). In Nahuatl, it is called "milpan," meaning "on top of the sown plot." [27, 28]. The core of this system is maize, with associated crops such as beans and squash, forming the "Mesoamerican triad." This association played a crucial role in the development of Mesoamerican culture, giving direction and civilization to the peoples, creating the basis of the local economy and social organization, and forming a connection between agrobiodiversity and Mayan culture. This led to the description of the milpa system as a biocultural food system [29, 30]. Some authors suggest that the milpa, as an agricultural production system, can be a model of resistance against industrial agriculture, where the use of transgenics and excessive application of toxic agrochemicals prevail [31].

3 The milpa as a polyculture

To achieve successful open-field production systems, it is necessary to revisit traditional systems like the milpa, which originally do not use agrochemicals.The milpa has played a crucial role in the subsistence and nutrition of Mesoamerican indigenous populations for over 5000 years. Indigenous peoples domesticated, adapted, and managed the biological diversity of crops in their regions for decades, creating a worldview, beliefs, knowledge, and traditions that have made the milpa a complex yet highly productive biocultural system. In the coevolution process, inhabitants select seeds resistant to environmental conditions and biotic factors, preserving the germplasm and increasing soil fertility and microbial

richness by establishing various types of crops [32–34]. The importance of the milpa transcends agriculture, connecting multiple sectors. In the cultural sphere, it encompasses a series of traditions carried out by farmers who, from planting to harvest, perform traditional rituals as a symbol of respect and connection with nature. Socially, the milpa strengthens family bonds, as the work involved in cultivation engages entire families [35, 36]. From a nutritional perspective, it serves as the foundation of Mexican cuisine and the diets of the regions where it is cultivated, providing a greater variety of nutrients for both people and animals by producing diverse products through crop rotation systems [37, 38]. Although there is limited information on the effect of combined crops on their nutritional and phytochemical composition, it has been proven that polycultures increase maize protein and produce phenolic antioxidants, potentially reducing diseases associated with oxidative stress and hypertension [39–41]. For these reasons, the milpa is positioned as an exploratory model with great potential for various fields of research, such as biomedicine, nanotechnology, nutrigenomics, and metagenomics, among others. This highlights the importance of its preservation and redesign to contribute to sustainability and scientific innovation (Fig. 1) [42, 43].

Originally, milpa systems were established with native maize seeds (*Zea mays* L.), which are indigenous to specific regions. Maize, the core crop of the system, was accompanied by associated crops such as beans (*Phaseolus vulgaris*) and squash (*Cucurbita pepo*). Over time, the biophysical and cultural conditions of each region have integrated new species into the milpa models, including chilacayote (*C. ficifolia*), ayote (*C. argyrosperma*), güicoy (*C. pepo*), fava bean (*Vicia faba*), tomato (*Lycopersicon esculentum*), miltomate (*Solanum lycopersicum*), macuy (*Solanum nigra*), white herb (*Brassica* sp.), güisquil (*Sechium edule*), epazote (*Chenopodium ambrosioides*), amaranth (*Amaranthus* spp.), purslane (*Portulaca oleracea*), chipilín (*Crotalaria longirostrata*), chili (*Capsicum* spp.), chaya (*Cnidoscolus chayamansa*), jicama (*Pachyrhizus erosus*), sweet potato (*Ipomoea batatas*), and cassava (*Manihot esculenta*) [44–47].

4 Milpa system intercropped with fruit trees (MIAF)

A proposed change to the traditional milpa concept is the establishment of polycultures under the MIAF system, characterized by being an agroecological multiple-cropping system where maize, beans or other preferred edible legumes, and fruit trees with fresh fruit market demand interact agronomically in alternate strips perpendicular to the slope of the land [48, 49]. In the MIAF system, trees are the main source of income, serve as living walls for controlling soil water erosion, and are key elements for carbon capture and retention in terms of environmental services. This system includes fruit trees like papaya (*Carica papaya*), banana (*Musa paradisiaca*), avocado (*Persea americana*), guava (*Psidium guajjava*), citrus (*Citrus* spp.), peach (*Prunus persica*), elderberry (*Sambucus mexicana*), izote (*Yucca gigantean*) and trees like alder (*Alnus* spp.), oaks (*Quercus* spp.), and pine (*Pinus* spp.), among others [27, 50–52].

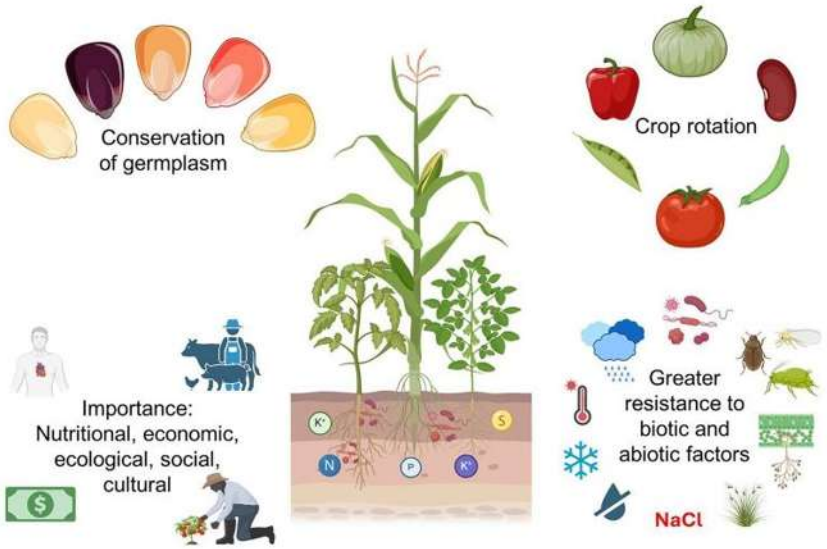

Fig. 1 Some benefits of the Milpa system. The milpa is an agro-sustainable production system primarily composed of maize, along with co-crops such as beans, squash, tomatoes, and others, depending on the region where it is cultivated. Some of the mentioned benefits include germplasm conservation, biodiversity generation, increased resistance to biotic and abiotic factors, and recruitment of beneficial PGPRs (plant growth-promoting rhizobacteria). Finally, the milpa can provide economic benefits to farmers

5 Ecological interactions in milpa systems

Throughout history, the milpa has been a place where farmers of each region experiment, select, and domesticate plants that provide them with the most significant nutritional benefits, ensuring production for the next crop cycle and achieving unparalleled inter- and intra-species diversity [53, 54]. Interactions between plants, plants-insects, plants-microorganisms, or microorganisms-microorganisms are fundamental for the success of milpa systems, and this communication between species occurs mainly through the production of secondary metabolites and phytohormones (Fig. 2) [55–57].

5.1 Plant–plant interaction

The Mesoamerican triadsystem is well-known for the benefits provided between plants. Maize, the core of the system, provides support for beans, which increase nitrogen fixation through root nodule production. This nitrogen is then utilized by maize and squash. Squash, with its creeping habit and broad leaves, covers and protects the soil, reducing weed growth and maintaining soil moisture [33, 44]. A key requirement for increasing yield in such systems is the spatial arrangement of plants, allowing better exploration of lateral roots, which communicate with neighboring plants through exudates. The variety and quantity of exudates are determined by environmental factors, nutrient availability, plant age, and genotype [58, 59]. Root exudates and plant communication are essential vehicles for the cycling of materials and energy exchange. The fact that exudation profiles differ in each plant creates changes in the rhizosphere microbiota, which in turn exerts selective pressure on the endophytic microorganisms that each plant possesses [60, 61]. By generating different allelochemical signals through root exudation, plants gain the advantage of adapting and resisting unfavorable conditions present within the rhizosphere or their surrounding environment [62, 63]. This improves phosphorus acquisition, enhances plant robustness, reduces competition with neighboring plants or weeds, and increases yield [64, 65]. In milpa systems, there are countless metabolic pathways through which plants communicate. Even though there is limited information on this, recent studies have demonstrated the importance of establishing associations, such as the maize-bean interaction [66]. Maize, being a C4 plant, exudes more photosynthates than beans, and its roots secrete

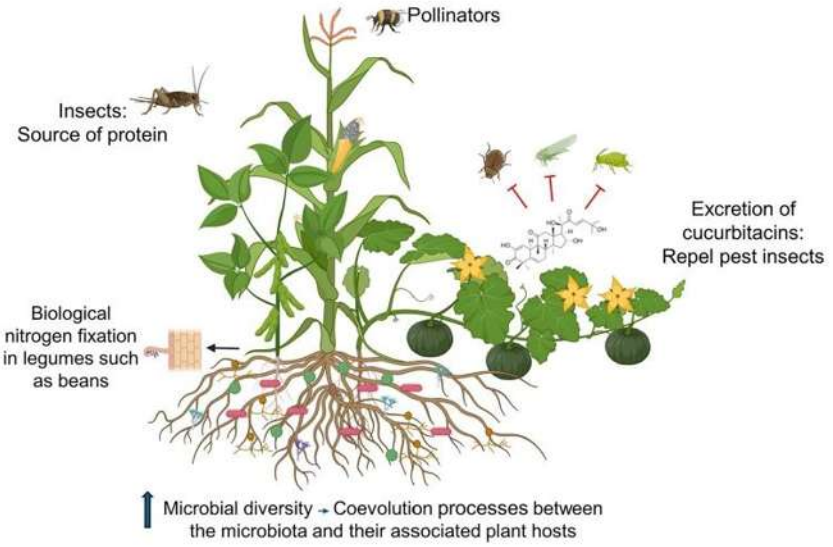

Fig. 2 Symbiotic associations in the Milpa model. The milpa is an agro-sustainable model that relies on symbiotic associations among plants, microorganisms, and pollinators. Beans (*Phaseolus spp.*) fix atmospheric nitrogen through rhizobia, enriching the soil, while mycorrhizal fungi in maize (*Zea mays*) roots enhance nutrient absorption and stress tolerance. Plant growth-promoting rhizobacteria (PGPRs) protect plants from pathogens and stimulate their growth. Pollinators, such as bees (*Apis spp.*, Meliponini) and butterflies, play a crucial role in co-crops like squash (*Cucurbita spp.*), tomatoes, and beans, boosting their productivity. Moreover, crop interactions promote sustainability: beans enrich the soil with nitrogen, maize provides structural support, and squash acts as ground cover, conserving moisture and suppressing weeds. These interactions improve biodiversity, soil quality, and system resilience, benefiting both farmers and the ecosystem

glucose, melibiose, maltose, and fructose. In turn, bean exudates induce rhizobial genes for nodulation and the degradation of aromatic compounds, highlighting the importance of neighboring plants in changing exudation profiles [67, 68]. Furthermore, intercropping *Triticum aestivum* L. (wheat) and *Vicia faba* L. (broad beans) under different fertilizer doses has been shown to modulate metabolite secretion, increasing the secretion of genistein, hesperetin, and naringenin from broad bean roots [69].

5.2 Plant–insect interaction

The type of agricultural systems is crucial in determining plant-pollinator interactions. In diverse milpa models, there is a significant advantage in these interactions because plants produce volatile compounds that facilitate attraction processes. With a greater variety of crops, there is an increased variety of secondary metabolites, which in turn enhances pollination and ultimately increases crop yield. One group of secondary metabolites highly associated with this type of interaction is the terpenoids [70, 71]. Another advantage of plant–insect attraction processes in milpa models is the high population of grasshoppers (*Sphenarium purpurascens*), which are endemic herbivorous insects in Mexico with a protein content even higher than that of meat, making them a valuable food source [72, 73]. Additionally, squash plants in milpa systems exude allelopathic compounds called cucurbitacins, along with tetraterpenes primarily represented by carotenoids and some sesquiterpenes. These compounds repel pest insects that are present during crop development in milpa models. They are released through leaching by rain and act as biopesticides [29, 74, 75].

5.3 Plant–microbe interactions

Symbiotic interactions between plants and microorganisms are important both ecologically and economically. Their effectiveness and complexity make them a significant niche for various research studies [76, 77]. In milpa systems, one of the interactions with great economic and nutritional importance is that of maize with the fungus huitlacoche (*Ustilago maydis*), also known as "black mold" or "Mexican truffle". Although *U. maydis* is a parasite that infects maize kernels and can cause significant production losses under certain conditions, it is considered a culinary delicacy in Mexico. Known for its unique flavor, huitlacoche is highly valued in traditional Mexican cuisine and offers substantial nutritional benefits. Its nutritional composition includes adequate soluble and isoluble dietary fiber, protein, amino acids, fatty acids, monosaccharides, oligosaccharides, and minerals, as well as nutraceutic compounds like β-glucans, which are considered prebiotics with antidiabetic properties [78]. The infection mechanism of *U. maydis* on maize involves the production of effectors and secreted proteins, as well as metabolites like surfactants. Additionally, the production of melanin and iron carriers is associated with its pathogenicity [79–81].

On the other hand, even though microbial diversity in traditional systems receives little attention because it is not part of global agriculture, it is known that there is a coevolution between plants and microorganisms that is important to evaluate for future biotechnological applications. The most complex interaction arises in the rhizosphere, where communication is driven and modulated both by the host plant through root exudates and by microorganisms [55]. Together, they determine the diversity and balance of the soil's ecological network.

The assembly of the microbial community is closely related to rhizodepositions, creating a symbiotic relationship between plants and microorganisms [82, 83]. Phytohormones, which modulate plant growth, play a crucial role in the adaptation and survival of plants under environmental stress conditions [72]. It is important to highlight that both plants and plant growth-promoting microorganisms (PGPM) can produce phytohormone [84, 85]. Furthermore, microorganisms produce, volatile organic compounds (VOCs), siderophores, and secondary metabolites and induce systemic resistance (ISR) [86–88]. They play an important role in nutrient absorption and, by competing for space and nutrients, reduce soil pathogen populations [89–91]. Understanding the dynamics of the microbial community on crop plants is crucial for building more efficient agroecosystems. In agricultural systems, the interaction between microorganisms has gained greater relevance in recent years. Through the formulation of microbial consortia, the effects of stress under adverse conditions have been mitigated, and the presence of pathogens or insect pests in crops has been controlled [92–97]. This application is common in monoculture fields; however, since these microorganisms are allochthonous, their adaptation may be limited. In milpa systems, various studies suggest isolating microbial strains that promote plant growth to facilitate better adaptation and efficiency [43]. By inoculating different microbial species and promoting their interaction with other microorganisms, it is possible to improve the physical and chemical conditions of the soil and even recover beneficial organisms lost in infertile soils [98]. Several studies have isolated microorganisms associated with maize cultivation that show positive effects of

both promotion and biocontrol, such as *Bacillus subtilis*, *Pseudomonas koreensis*, and *Aeromonas spp.*, among others [99–101]. Beneficial strains that promote resistance to water or saline stress and improve production in crops such as maize include *Bacillus cereus*, *Pseudomonas putida*, *Azotobacter chroococcum*, *Pseudomonas koreensis*, and *Azotobacter nigricans* [102–104].

6 Inoculation with PGPMs in current milpa models

Modern agricultural production is increasingly based on sustainable techniques supported by biotechnology. The goal is to achieve higher yields with high quality standards. One valuable tool is the application of PGPMs. These microorganisms utilize multisite mechanisms that enable biocontrol against pathogens, promote plant growth, and act as biostimulants under extreme conditions such as drought, salinity, and water stress, among others. Additionally, they function as soil phytoremediators and nutrient solubilizers, contributing to the balance of plant–plant rhizospheric interactions and/or plant–microbiome interactions [10, 105, 106]. Enhancing the effectiveness and proliferation of bioinoculants depends on the formulation type and understanding their role within the microbial community in inoculated areas, such as the chemically complex rhizosphere where species interactions occur. Today, metagenomic tools such as massive 16S sequencing, depth range metagenome-assembled genomes, or shotgun sequencing, allow the assessment of alterations in the microbial community, resulting from natural or anthropogenic disturbances; whether they are changes in the diversity of the soil microbiome dependent on forest wildfires, deforestation, loss of vegetation cover, or the recovery of forests; as well as providing current insights into the effects of bioinoculant applications on rhizospheric microbiome changes. However, deeper research is needed to determine the communication pathways between species [107–109]. In maize monocultures, strains like *Amycolatopsis* BX17, *Burkholderia* sp. y *Pseudomonas psychrotolerans* CS51 have demonstrated biocontrol effects against *Fusarium graminearum* RH, improved germination, reduced saline stress, and counteracted silicon absorption while promoting plant growth [110–112]. *Streptomyces* sp. and *Rhizophagus irregularis*, on the other hand, reduce petroleum hydrocarbons in contaminated soils, enhance maize plant development, and improve phosphorus translocation [113, 114]. Other crops benefiting from these practices in milpa models include chickpea (*Cicer arietinum* L.), where under monoculture systems, bacteria like *Ensifer adhaerens* MSN12 and *Bacillus cereus* promote growth, yield, and soil fertility [115]. In field experiments, *Pseudomonas fluorescens* strain UM270 altered the endophytic root microbiome of maize plants, stimulating the presence of bacterial operational taxonomic units (OTUs) from genera *Burkholderia* and *Pseudomonas* (in monoculture). In the milpa system, PGPMs promoted greater endophytic diversity and presence of genera such as *Burkholderia*, *Variovorax*, nitrogen-fixing rhizobial genera including *Rhizobium*, *Mesorhizobium*, and *Bradyrhizobium*, as well as beneficial fungi like *Rhizophagus irregularis* and *Exophiala pisciphila*. Unique species were identified in specific endobiomes, such as *Stenotrophomonas* sp., *Burkholderia xenovorans*, and *Sphingobium yanoikuyae*, potentially promoting plant growth, development, and health [24].

One of the main limitations in the effectiveness of plant growth-promoting microorganisms is their formulation, as it is crucial to ensure both their effectiveness and shelf life. Various environmental factors can compromise the cellular viability of these microorganisms, such as bacterial stress, variations in pressure and temperature, dehydration/rehydration, ultraviolet radiation, pH fluctuations, and nutrient concentration [116]. However, there are different methods to mitigate these effects, such as the encapsulation of beneficial microorganisms, which provides protection, especially for non-spore-forming bacteria, for long periods [109, 117, 118]. Furthermore, encapsulation allows for the progressive release of microorganisms once inoculated in the field. Several studies have shown that microorganisms such as *Azospirillum brasilense*, *Burkholderia cepacia*, *Bacillus thuringiensis*, *Bacillus megaterium*, *Bacillus cereus*, *Bacillus subtilis*, *Bacillus subtilis 1411*, *Trichoderma sp.*, *Trichoderma viride*, *Mesorhizobium ciceri* ST-282, and *Bradyrhizobium japonicum* M8, when encapsulated in alginate, increase nutrient acquisition and metabolite production. This has shown positive results in the inoculation and production of crops such as *Eugenia stipitata*, *Cicer arietinum*, *Glycine max*, and *Lactuca sativa* in the field [119–121].

Thanks to the action mechanisms provided by plant growth-promoting microorganisms, such as the production of phytohormones, nutrient acquisition, and biological control of pathogens, their application can be proposed for traditional agricultural systems, such as milpa, including agroforestry systems. Through different bioformulations, field success can be ensured while adhering to sustainable cultivation principles (Fig. 3).

7 The milpa: a model of sustainable production

Milpa systems reflect various cultures, and despite their transformation and adaptation, elements of culture are embedded in the historical memory of adults, youth, and children who shape their identity, such as the Mazahua people [122]. As comprehensive models with a multifunctional approach, they become highly valuable resources for safeguarding food security and conserving biodiversity through germplasm conservation. The communal and indigenous agricultural values play a crucial role in this strategy, as they have access to materials that enable sustainable conservation and utilization. The adoption of biotechnological innovations has led to improved yields in production units [4, 123, 124]. Recent research focuses on social, cultural, economic, and ecological sectors, demonstrating that polyculture establishment increases crop yields and achieves higher values in equivalent land use compared to monoculture. This is evident in polyculture systems such as maize-bean and maize-bean-squash, which also enhance photosynthetically active radiation capture and reduce infestations of pests like *S. fungipera* and predator populations from orders *Coleoptera*, *Hemiptera*, *Neuroptera*, *Hymenoptera*, and *Dermaptera*. [28, 125], particularly in maize-squash intercropping, there is an increase in parasitoid presence such as *Archytas piliventris* and *Lespesia* [126]. Biotechnological tools have shown that intercropping *C. cajan* with maize increases myo-inositol and proline production, while intercropping *Z. mays* enhances galactose, D-glucopyranoside, and arginine in the root exudates, highlighting new molecular candidates likely involved in rhizobial fitness in associated cropping systems (Table 1) [127].

8 Conclusions and future perspectives

As an integrated model, milpa systems emerge as a viable and profitable option for sustainable food production. By establishing these systems and generating a greater variety of agricultural products, new health and nutrition programs can be designed to benefit both animal and human health. The adoption of new agricultural practices in various polycultures will facilitate the recruitment of microorganisms and thereby modulate the soil microbiome. This makes it a promising avenue to increase the quantity of beneficial soil microorganisms that can be utilized for future biotechnological applications. The use of bioinoculants can enhance the effectiveness of such systems. Evaluating their impact on soil population modulation or behavior and uncovering the ecological foundations that govern the assembly of a healthy microbiota in

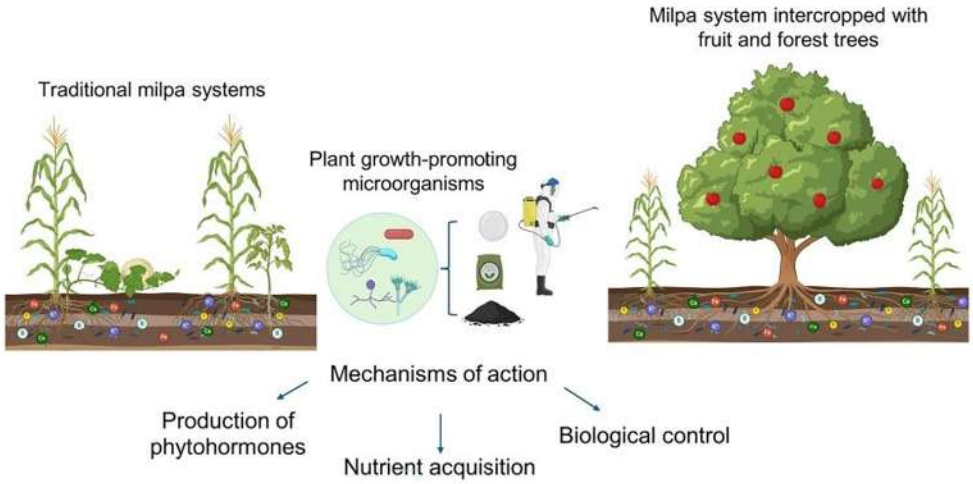

Fig. 3 Applications and benefits of plant growth-promoting microorganisms (PGPM) in the Milpa agroecosystem. PGPMs (Plant Growth-Promoting Microorganisms) provide the milpa model with the advantage of delivering pathogen biocontrol services through various mechanisms, including the production of antimicrobial compounds (lytic enzymes, lipopeptides, antibiotics, etc.), space occupation, nutrient restriction (e.g., Fe chelation), among others. Additionally, PGPMs produce phytohormones that stimulate plant growth, such as indole acetic acid, gibberellins, and cytokinins. They also produce elicitors that activate the plant's immune system and enhance resistance to pathogens. All these services are provided by PGPMs without causing toxic adverse effects on the environment or human health

Table 1 Research works with the milpa, where most of them had maize as a core crop plant

Intercropped species/crops	Beneficial effects	References
Maize, bean, squash	Greater land use efficiency when establishing maize-squash and maize-bean-squash polycultures compared to monocultures of maize, bean, or squash	[125]
Maize, bean	Increased maize yield when intercropped with beans. Greater capture of photosynthetically active radiation throughout the plant canopy. The decrease in bean yield intercropped with maize depends on the topological arrangement with both species	[58]
Maize, bean, squash	Maize-bean association increases total production. Maize-bean greater land equivalent ratio	[28]
Maize-squash, maize-sesame, maize-bean, maize-sunflower	Lower infestation of *S. frugiperda* in maize-squash and maize-bean polycultures. Maize-squash with greater representativeness of predators and parasitoids, *Archytas piliventris* (Lin) and *Lespesia archyppivora* (Riley) were the main parasitoids	[126]
Common Bean-Maize, Common Bean, Bean Cultivar Cariocа, Bean Cultivar Rio Tibagi	Intercropping increased nodulation and biomass of common bean, mainly with *Rhizobium*, but mineral N was detrimental to nodulation. Better results in common bean inoculated with *Rhizobium*	[128]
Helianthus annuus L *Trifolium pratense* *Trifolium repens* *Lotus corniculatus*	Contribution of residue by legumes. Increase in sustainability indices. Promotes biodiversity conservation	[129]
Cajanus cajan Maize	Intercropped *C. cajan* showed an increase in the production of myo-inositol and proline and greater biofilm formation. Intercropped maize showed an increase in galactose, D-glucopyranoside, and arginine, and accelerated bacterial growth	[127]
Maize *Vicia faba* L	Associated cropping of beans and maize improves productivity, nodulation, and N_2 fixation of beans through interspecific interactions with maize roots. Maize exudates increase the exudation of flavonoids and positively regulate the expression of a chalcone	[66]
Maize *Vigna unguiculata* *Stizolobium deeringianum* **L** *Canavalia ensiformis* **L** *Crotalaria Juncea* **L** *Sesbania rostrata* **L**	Increased bean production. Improvement of soil physical fertility	[130]
Maize-Squash-Bean	Beneficial ecological interactions were generated between the UM270 strain of *Pseudomonas fluorescens* and species of the rhizospheric and endophytic microbiome ofmaize. Total maize production increased by 26% to 45% when inoculated with the UM270 strain. Squash and bean production also reflected increases in production	[24]

plants established within milpa systems can be achieved through metagenomic strategies. Determining communication pathways between different species such as microorganisms, insects, and plants would enable the establishment of new techniques for milpa or agroforestry model (MIAF) establishment. This could promote their antimicrobial effects, growth promotion abilities, among other benefits.

In conclusion, the establishment and management of monocultures have caused disruptions in soil microbial communities by reducing some populations and, in extreme cases, rendering soils completely infertile. By adopting milpa models, it becomes possible to recover bacterial communities lost in monocultures. These systems exert different selective pressures on soil microorganisms. Within the microbial communities of milpa models, there is potential to harness beneficial microorganisms for future biotechnological applications. Moreover, the application of microbial bioinoculants can improve soil quality and conditions, paving the way for new research focused on various areas to enhance production systems.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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Blanca Rojas Sánchez

Impacto de la rizobacteria Pseudomonas fluorescens UM270 en el crecimiento, producción y modulación

Universidad Michoacana de San Nicolás de Hidalgo

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