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Evaluación de compuestos organosulfurados derivados de cebolla y microorganismos de control biológico en olivo (*Olea europaea*): Investigación de la capacidad antimicrobiana y bioestimulante

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RESUMEN

Esta tesis doctoral aborda la caracterización y evaluación de compuestos organosulfurados (OSCs) derivados de cebolla (*Allium cepa*) y cepas bacterianas como *Bacillus sp.* GG-22 y *Pseudomonas sp.* PV8, con el objetivo de controlar fitopatógenos y bioestimular cultivos de olivo (*Olea europaea*). El trabajo explora tanto la capacidad antimicrobiana como su aplicación práctica en ensayos *in vitro*, *in planta* y bajo condiciones reales de campo, proporcionando alternativas sostenibles para el manejo integrado de plagas y enfermedades en el olivo. Los análisis mediante GC/MS y HPLC confirmaron la alta concentración de OSCs, especialmente propil propano tiosulfinato (PTS) y propil propano tiosulfonato (PTSO) en el extracto de cebolla, que demostraron una destacada actividad antimicrobiana frente a patógenos claves como *Xylella fastidiosa* y *Verticillium dahliae*. Los OSCs no solo inhibieron el crecimiento de estos patógenos en estudios *in vitro*, sino que mostraron una reducción significativa de los síntomas de verticilosis en plantones infectados con *V. dahliae*. Asimismo, en condiciones controladas, se observó un efecto positivo sobre la fisiología de los plantones de la variedad Picual, estimulando el crecimiento radicular y mejorando la resistencia al estrés oxidativo en plantas no estresadas. Este doble rol, como agentes protectores y bioestimulantes, reforzaría su potencial en el manejo integrado de cultivos. En los ensayos de campo realizados en dos fincas comerciales en las provincias de Jaén y Córdoba, los tratamientos con OSCs redujeron la severidad de la verticilosis, disminuyendo el número de copias del hongo (evaluado por qPCR) y aumentando el contenido graso en los frutos de olivos infectados. Además, la aplicación repetida de estos OSCs en dosis bajas incrementó su eficacia, debido a su rápida degradación en el suelo y baja persistencia, sugiriendo que la frecuencia de aplicación es un factor crítico para su aplicación práctica. A nivel de seguridad ambiental, los estudios confirmaron la ausencia de toxicidad por contacto y oral en polinizadores como las abejas melíferas (*Apis mellifera*), lo que refuerza su viabilidad en sistemas agrícolas sostenibles. Por otro lado, el análisis genómico y los ensayos con *Bacillus altitudinis* GG-22 confirmaron su capacidad para actuar como agente de biocontrol, inhibiendo el desarrollo de hongos fitopatógenos mediante la producción de lipopéptidos antifúngicos y activando respuestas de defensa sistémica en las plantas. Asimismo, los estudios del transcriptoma demostraron que GG-22 mejora la absorción de nutrientes como el hierro y la solubilización de fosfatos, lo que subraya su valor en suelos empobrecidos. En conclusión, esta investigación demuestra el gran potencial de los OSCs y los microorganismos de biocontrol en el manejo de enfermedades y la bioestimulación del olivo, tanto en condiciones controladas como en escenarios de campo. Los resultados avalan su uso como una alternativa eficaz y sostenible, ofreciendo un enfoque prometedor para reducir el uso de productos químicos convencionales. No obstante, es necesario continuar con investigaciones adicionales que permitan optimizar su aplicación en diferentes contextos agronómicos, con el objetivo de maximizar su efectividad y asegurar su adopción a gran escala.

Palabras clave: Olivo, OSCs, cebolla, *Xylella, Verticillium, Bacillus altitudinis.*

ABSTRACT

This doctoral thesis focuses on the characterization and evaluation of organosulfur compounds (OSCs) derived from onion (*Allium cepa*) and bacterial strains such as *Bacillus* sp. GG-22 and *Pseudomonas* sp. PV8, with the aim of controlling phytopathogens and bio-stimulating olive trees (*Olea europaea*). The study explores both the antimicrobial activity and practical application of these agents through in vitro, *in planta*, and field trials under real conditions, providing sustainable alternatives for integrated pest and disease management in olive tree cultivation. Analyses conducted via GC/MS and HPLC confirmed the high concentration of OSCs, particularly propyl propane thiosulfinate (PTS) and propyl propane thiosulfonate (PTSO), in onion extracts, which exhibited significant antimicrobial activity against key pathogens such as *Xylella fastidiosa* and *Verticillium dahliae.* The OSCs not only inhibited the growth of these pathogens in vitro but also significantly reduced verticillium wilt symptoms in olive tree seedlings infected with *V. dahliae*. Furthermore, under controlled conditions, a positive effect on the physiology of Picual olive tree seedlings was observed, stimulating root growth and enhancing oxidative stress resistance in unstressed plants. This dual role, as protective and bio-stimulant agents, reinforces their potential in integrated crop management. Field trials conducted at two commercial farms in the provinces of Jaén and Córdoba (Andalucía, Spain) showed that OSC treatments reduced the severity of verticillium wilt, decreased fungal load (as evaluated by qPCR), and increased the oil content in the fruit of infected olive trees. Additionally, repeated applications of OSCs at low doses increased their efficacy, owing to their rapid degradation in the soil and low persistence, suggesting that application frequency is a critical factor for practical use. In terms of environmental safety, studies confirmed the absence of contact and oral toxicity in pollinators such as honey bees (*Apis mellifera*), reinforcing the feasibility of their use in sustainable agricultural systems. Moreover, genomic analyses and experiments with *Bacillus altitudinis* GG-22 confirmed its capacity as a biocontrol agent, inhibiting the development of fungal phytopathogens through the production of antifungal lipopeptides and activating systemic defence responses in olive trees. Transcriptomic studies also demonstrated that GG-22 strain enhances nutrient absorption, such as iron, and solubilizes phosphates, underscoring its value in nutrient-deprived soils. In conclusion, this research highlights the substantial potential of OSCs and biocontrol microorganisms for managing diseases and bio-stimulating olive trees, both in controlled environments and under field conditions. The results validate their use as an effective and sustainable alternative, offering a promising approach to reducing the reliance on conventional chemical products. However, further research is needed to optimize their application under various agronomic contexts, with the aim of maximizing their efficacy and ensuring widespread adoption.

Keywords: Olive trees, OSCs, Onion, *Xylella, Verticillium, Bacillus altitudinis*.

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INTRODUCCIÓN

1. Caracterización y ciclo biológico del olivo

El olivo (*Olea europaea subsp. europaea var europaea*) es un árbol perennifolio, que suele medir entre 4 y 8 metros de altura, aunque puede alcanzar hasta 15 m. Tiene un crecimiento lento, por lo que es una especie muy longeva, y puede permanecer productivo durante cientos de años. Sus hojas son opuestas y elípticas (Kaniewski et al. 2012). El haz se caracteriza por una densa cutícula, mientras que el envés está cubierto por pelos aparasolados. Los estomas solo se forman en el envés, por lo que la pérdida de agua no solo se regula por su apertura y cierre, sino también por su ubicación y por la protección de los pelos aparasolados. Las flores son pequeñas y blancas, y se agrupan in inflorescencias. Las inflorescencias suelen tener entre 10 y 40 flores. Las flores pueden ser hermafroditas, con estambres y pistilo, que al ser polinizadas dan lugar a frutos, o masculinas, que únicamente tienen estambres y no forman fruto. La polinización es predominantemente anemófila. El fruto es una drupa carnosa formada por epicarpo (piel), mesocarpo (pulpa) y endocarpo (hueso) (Green 2002; Barranco Navero, Fernández Escobar, and Luis 2017).

El ciclo biológico del olivo se caracteriza por dos procesos cíclicos: el crecimiento vegetativo o desarrollo de los brotes, y el ciclo reproductivo, que da lugar al desarrollo de frutos (Orlandi et al. 2013). El ciclo reproductivo se caracteriza por la alternancia de la fructificación, que requiere de dos estaciones consecutivas. Esta alternancia, conocida como vecería o bienalidad, se debe principalmente a la competencia entre los órganos vegetativos y reproductivos por los nutrientes (Salimonti et al. 2021). El año de alta producción de frutos o año de carga se caracteriza por un crecimiento vegetativo reducido, lo que resulta en un menor número de ramas nuevas y flores. En contraste, durante el año de baja producción o año de descarga tiene lugar un fuerte crecimiento vegetativo, observándose un incremento en la producción de ramas y flores (Kour et al. 2018).

El crecimiento vegetativo está controlado por la temperatura (Fabbri et al. 2023). Este ciclo comienza con una etapa conocida como dormancia invernal en la que el olivo, expuesto a bajas temperaturas, reduce su actividad metabólica. Esta etapa es fundamental para el posterior desarrollo y diferenciación de las yemas en florales, que dan lugar a frutos, o vegetativas, a partir de las cuales se desarrollan hojas y ramas (Garrido et al. 2021). El crecimiento de las yemas comienza en primavera y se extiende hasta julio en el hemisferio norte. Bajo condiciones favorables de lluvias o en cultivos de regadío puede ocurrir un segundo periodo de crecimiento vegetativo en septiembre y octubre (Orlandi et al. 2013).

El ciclo reproductivo comienza con la aparición de las yemas florales. La inducción floral será mayor en los años de descarga, marcando así la alternancia de la fructificación. Las yemas florales permanecerán en latencia hasta mayo del año siguiente, cuando comience la floración. Durante la floración tiene lugar la polinización cruzada y fecundación (Benlloch-González et al. 2018). El fruto se desarrolla durante los meses de

verano y madura durante el otoño. El contenido en aceite aumenta gradualmente y alcanza su máximo cuando los frutos se vuelven completamente negros (Haggag et al. 2013).

2. Historia y relevancia del cultivo del olivo

El olivo es considerado el árbol más emblemático de la cuenca mediterránea, donde tiene un gran impacto económico, ambiental y social (Bizos et al. 2020). El antecesor silvestre del olivo, el acebuche (*Olea europaea subsp. europaea var. sylvestris*), es nativo del Mediterráneo oriental, concretamente de la región del Levante que incluye la actual Jordania, Siria, Líbano, y la parte sur de Turquía. Existen evidencias arqueológicas de que el olivo salvaje podría haber sido explotado desde hace más de 10.000 años (Bullones et al. 2023). A pesar de que el origen geográfico y el momento de su domesticación aún son objeto de debate, las evidencias actuales sitúan la domesticación del olivo en esta misma región en torno al año 5000 a.C. (Barazani, Dag, and Dunseth 2023) El proceso de domesticación implicó la selección de variedades con características agronómicas favorables, tales como una mayor producción de frutos y un mayor contenido de aceite, y su propagación por medios vegetativos (Besnard, Terral, and Cornille 2018).

Gracias a la propagación vegetativa, y a través de las rutas comerciales y las migraciones de civilizaciones, el olivo se extendió rápidamente a otras regiones del Mediterráneo (Vossen 2007). La expansión del olivo a medida que avanzaba la civilización se debió en gran medida a sus múltiples usos como fuentes de combustible, medicina, madera, forraje y alimento, así como en rituales religiosos (Langgut et al. 2019; Besnard, Terral, and Cornille 2018). En las obras botánicas de Teofrasto, De Causis Plantarum y De Historia Plantarum, el olivo es descrito en detalle debido a su importancia económica y cultural en la Grecia clásica. Estas obras constituyen la primera sistematización del mundo botánico y una contribución fundamental a la ciencia botánica actual (Kaniewski et al. 2012).

Los procesos de domesticación que tuvieron lugar tras el evento primario dieron lugar a un árbol altamente adaptable y a un gran número de variedades característicos de cada región (Fanelli et al. 2022). Desde entonces, sus múltiples usos y omnipresencia en los agrosistemas tradicionales han hecho de esta especie el pilar económico de la agricultura mediterránea (Lo Giudice et al. 2021; Zabaniotou, Rovas, and Monteleone 2015).

De acuerdo con los datos de la FAO (La Organización de las Naciones Unidas para la Alimentación y la Agricultura) recopilados entre los años 2021 y 2022, el cultivo del olivo está presente en 67 países y abarca un área de 10,328,666 hectáreas (FAO 2022). En el área mediterránea hay 9 millones de hectáreas dedicadas al olivar, que concentran el 98% de la producción mundial de aceite de oliva y el 80% de la de aceitunas de mesa (Cardoni and Mercado-Blanco 2023). De los 9 millones de hectáreas, 5 millones se encuentran en la Unión Europea (Arenas-Castro et al. 2020).

España es el país con mayor superficie de territorio destinado al cultivo del olivo a nivel mundial, con 2.75 millones de hectáreas, lo que supone el 55% del territorio de la UE. De acuerdo con la Encuesta de Superficies y Rendimientos de cultivos de España (ESYRCE), elaborada por el Ministerio de Agricultura, Pesca y Alimentación (MAPA), la superficie del olivar representa el 16.1 % de la superficie de cultivo total del territorio nacional. Este cultivo se encuentra distribuido de manera desigual en las diferentes comunidades autónomas. Andalucía es la comunidad autónoma con mayor superficie de olivar. Abarca 1.67 millones de hectáreas, que se corresponden con el 60.5% de la superficie nacional. Le siguen Castilla-La Mancha con un 15.9% y Extremadura con un 10.5%. El olivar representa el 46.7% de las tierras de cultivo de Andalucía (Subsecretaría de Agricultura Pesca y Alimentación and Subdirección General de Análisis Coordinación y Estadística 2019). Jaén y Córdoba son las provincias españolas con mayor proporción de olivar respecto a la superficie total, con el 44.04% y el 27.24%, respectivamente (Figura 1) (Ministerio de Agricultura Pesca y Alimentación 2023). La variedad Picual es la más importante de España. Ocupa más de 850,000 hectáreas en Andalucía, siendo la variedad predominante en las provincias de Jaén (97%), Córdoba (38%) y Granada (40%). La variedad Picual es la base de las nuevas plantaciones en todo el país. Se caracteriza por su elevada tolerancia al frío, salinidad y exceso de humedad del suelo. Por el contrario, es sensible a la sequía y altamente susceptible a la verticilosis (MAPA 2007).

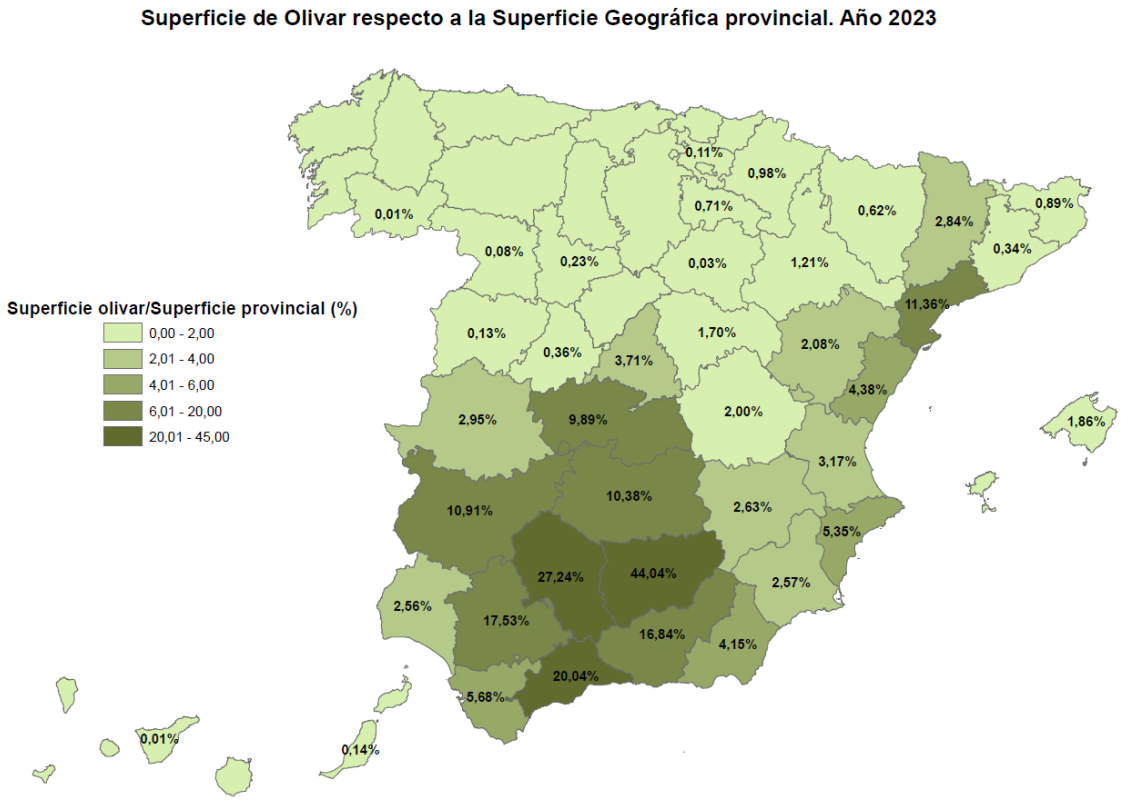

Figura 1. Distribución provincial de la superficie de olivar respecto a la superficie geográfica en el año 2023. Fuente: ESYRCE, MAPA (Ministerio de Agricultura Pesca y Alimentación 2023).

De la producción total de aceitunas en España, el 94% se destina a la producción de aceite de oliva, mientras que el 6% restante se destina a la producción de aceitunas de mesa (Honorio et al. 2024). España es el primer productor mundial de aceite de oliva y aceituna de mesa (Figuras 2 y 3). Representa el 63% de la producción de la UE y el 45% de la producción mundial de aceite de oliva. Respecto a la producción de aceituna de mesa, es responsable del 48% de la producción de la UE y del 28% de la producción mundial (FAO 2022). Los productos derivados del olivar son consumidos en un total de 179 países. Esto pone de manifiesto que el sector oleícola se basa en una producción altamente localizada y en una demanda que se extiende ampliamente a nivel internacional (Yamani and Cordovilla 2024).

El sector del olivar no sólo es fundamental desde el punto de vista económico, sino que también tiene un impacto significativo en los ámbitos social, ambiental y territorial. Previene la erosión del suelo. Esto tiene una gran repercusión en terrenos áridos y montañosos, donde las raíces ayudan a mantener la integridad del terreno. Además, los olivares apoyan la biodiversidad local al proporcionar hábitats para diversas especies de flora y fauna, contribuyendo así al equilibrio ecológico de la región (Giourga and Loumou 2003). En el aspecto socioeconómico, el cultivo del olivo es una fuente vital de ingresos para las comunidades rurales, fortaleciendo la economía local y mitigando el abandono de estas áreas. Más de 350.000 agricultores se dedican al cultivo del olivo. Este sector sostiene aproximadamente 15.000 puestos de trabajo y genera más de 32 millones de jornales cada temporada (MAPA 2020).

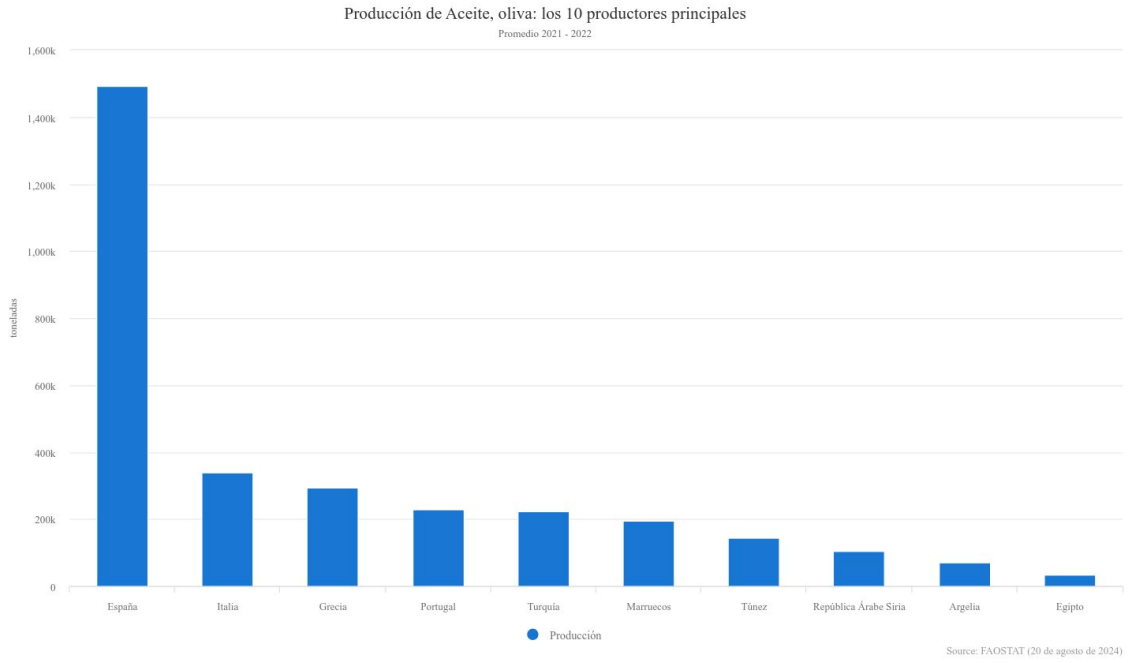

Figura 2. Principales países productores de aceite de oliva en los años 2021 y 2022. Fuente: FAOSTAT (FAO 2022).

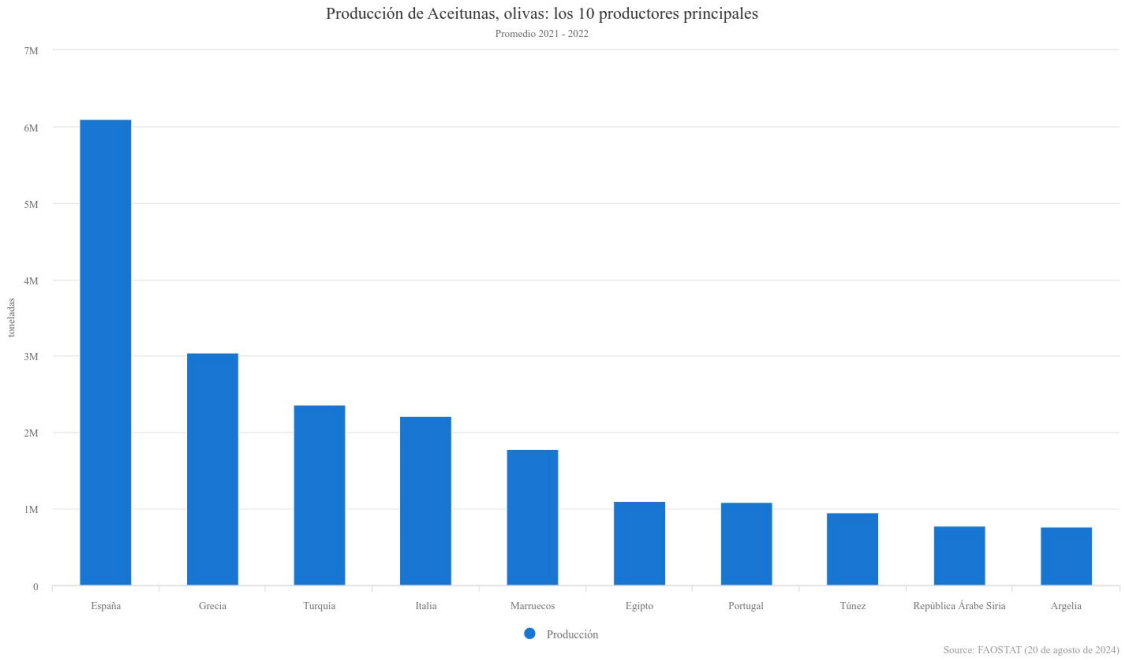

Figura 3. Principales países productores de aceituna de mesa en los años 2021 y 2022. Fuente: FAOSTAT (FAO 2022).

3. Principales problemáticas que amenazan el cultivo del olivo

El cultivo del olivo se enfrenta a una serie de desafíos que ponen en riesgo tanto su productividad como la calidad de los productos obtenidos. Tradicionalmente, el olivo se ha cultivado de forma extensiva en condiciones de secano. A pesar de que la mayor parte de los olivares mediterráneos se cultivan bajo este sistema, los nuevos olivares son en su mayoría de regadío (Villalobos et al. 2006). En las últimas décadas la creciente demanda de aceite de oliva está impulsando la transición hacia sistemas de cultivo intensivos y de regadío (Fernández-Escobar et al. 2013). De acuerdo con la Encuesta sobre Superficies y Rendimientos de Cultivos realizados por el MAPA, la tendencia del cultivo de regadío en España durante el período 2010-19 es al alza. Aunque la superficie de secano disminuye respecto al total, continúa representando el 69,4% de la superficie total de olivar en España, mientras que la superficie de regadío representa el 30.6% (Subsecretaría de Agricultura Pesca y Alimentación and Subdirección General de Análisis Coordinación y Estadística 2019). La disponibilidad de agua se ve comprometida no solo por el aumento de olivares irrigados, que generan un mayor consumo de las reservas, sino también por el impacto del cambio climático. El escenario actual de cambio climático está alterando patrones del clima como la temperatura y las precipitaciones, lo que limita la disponibilidad de recursos hídricos y altera al crecimiento y a la fenología de la planta. Por otra parte, la intensificación del cultivo y los cambios de las condiciones climáticas modifican la distribución y virulencia de diversas plagas y enfermedades (Tous, Romero, and Hermoso 2010). Estas problemáticas afectan de manera directa al desarrollo y sostenibilidad del olivar.

3.1. Estrés abiótico

Globalmente, el cultivo del olivo está limitado aproximadamente por los paralelos 30° y 45°, lo que sugiere que las condiciones climáticas son un factor clave para su ciclo de desarrollo. El olivo es una de las especies mejor adaptadas al clima mediterráneo, caracterizado por inviernos suaves y húmedos y veranos cálidos, secos y con altos niveles de radiación solar (Fraga et al. 2021).

El olivo ha desarrollado diversas estrategias que le permiten adaptarse a diferentes regímenes de temperatura, así como tolerar cierto grado de estrés hídrico y salino. Para resistir al estrés hídrico, el olivo emplea estrategias fisiológicas, como el control estomático, y adaptaciones morfológicas que minimizan la evaporación. El tamaño reducido de las hojas y su estructura gruesa disminuyen la superficie expuesta para la transpiración. La cutícula cerosa que cubre el haz de la hoja actúa como una barrera, reduciendo significativamente la pérdida de agua. Por su parte, el envés está cubierto por pelos aparasolados, tricomas con forma similar a un parasol que forman una capa protectora y contribuyen a la regulación de la transpiración (Sebastiani et al. 2016; Barranco Navero, Fernández Escobar, and Luis 2017). La tolerancia a la salinidad está relacionada principalmente con mecanismos eficientes de exclusión y retención de iones Na^+ y Cl^-, así como de absorción de K^+, que ayuda a mantener un balance osmótico favorable (Chartzoulakis 2005).

A pesar de su notable adaptabilidad, cambios climáticos severos pueden afectar adversamente al olivo, alterando sus condiciones óptimas de crecimiento y productividad, y reduciendo significativamente la superficie apta para su cultivo (Fanelli et al. 2022). La región mediterránea, que ha experimentado cambios climáticos significativos en el pasado, ha sido reconocida como una de las áreas más vulnerables al impacto del cambio climático (Giorgi and Lionello 2008). En la última década, la región mediterránea ha experimentado un incremento de las temperaturas medias y la alteración de la distribución de las precipitaciones, así como un aumento en la frecuencia de eventos climáticos extremos, como las olas de calor (Maesano et al. 2021).

El rango óptimo de temperatura para el crecimiento vegetativo del olivo se sitúa entre los 10°C y 30°C, siendo entre 20°C y 30°C el rango ideal para la síntesis de carbohidratos en las hojas (Honorio et al. 2024). Estos carbohidratos desempeñan un papel esencial en el crecimiento vegetativo, así como en los procesos de floración en primavera y de fructificación en verano, al actuar como una fuente de energía y ser compuestos estructurales necesarios para el desarrollo de nuevos tejidos (Tombesi et al. 2007). La transición de la etapa de crecimiento a la de dormancia tiene lugar por debajo de los 14°C. Durante el invierno, los olivos requieren temperaturas bajas, entre 0 °C y 7 °C, para que las yemas se diferencien adecuadamente (López-Bernal et al. 2020). Por otro lado, la cantidad de precipitaciones del área mediterránea suele oscilar entre 150 y 800 mm anuales, siendo necesario un mínimo de 500 mm/año para obtener una producción de aceitunas comercial aceptable. Además, las lluvias deben distribuirse de forma que se eviten periodos secos de más de 45 días (Tombesi et al. 2007; Maesano et al. 2021).

El incremento de la temperatura media tiene efectos directos sobre la fenología del olivo, entre ellos el adelanto de la floración. También acorta la etapa de dormancia invernal y el aumento de la etapa de crecimiento vegetativo. Esto provoca la inhibición de la diferenciación de las yemas, lo que también tiene un efecto perjudicial en la floración y, por consiguiente, en la producción de aceitunas (Pérez-López et al. 2008; Aguilera et al. 2015; Ayerza and Steven Sibbett 2001). Asimismo, las altas temperaturas afectan a la maduración de los frutos, generando cosechas tempranas con una composición de ácidos grasos alterada, lo que afecta a la acidez y la estabilidad oxidativa del aceite. En concreto, estas condiciones se han relacionado con la disminución del contenido de ácido oleico y el incremento de ácido linoleico (Tombesi et al. 2007; Dag et al. 2014). El ácido oleico es un ácido graso monoinsaturado que contribuye significativamente a la calidad del aceite de oliva, al aportar estabilidad oxidativa y beneficios nutricionales. Por el contrario, el incremento de ácido linoleico, ácido graso poliinsaturado, reduce la estabilidad del aceite y afecta negativamente a su perfil organoléptico (Hernández et al. 2021). La disponibilidad de agua es un factor determinante para el rendimiento del olivo, puesto que se relaciona con la reducción del crecimiento vegetativo y una menor producción de frutos. Al igual que las altas temperaturas, el déficit hídrico acelera la maduración, y tiene un efecto negativo sobre la masa seca del fruto y la acumulación de ácidos grasos. Los aceites obtenidos de árboles que han experimentado un estrés hídrico acusado se caracterizan por ser excesivamente amargos (Brito et al. 2019; Dias et al. 2022).

Además, la escasez de precipitaciones resulta en una mayor demanda del uso de agua para riego. El aumento de los olivares de regadío, impulsado por la disminución de las precipitaciones y la consiguiente reducción en la producción, se ha relacionado con el incremento de la salinización del suelo. El uso de agua de baja calidad, con alto contenido en sales, agrava la situación de estrés causada por la salinización primaria en las regiones mediterráneas que presentan características de climas semiáridos (Tadić et al. 2021).

3.2. Estrés biótico

El estrés biótico abarca un amplio espectro de organismos patógenos, entre los cuales se encuentran insectos, ácaros, nematodos, virus, hongos y bacterias. En los últimos años, el impacto de las enfermedades y plagas en el olivar ha aumentado debido a las variaciones climáticas, la intensificación agrícola y la globalización del comercio, que conlleva un incremento en el intercambio de mercancías. Estos factores han creado condiciones más favorables para la proliferación de patógenos y han facilitado su establecimiento en regiones previamente no afectadas (EIP-AGRI Focus Group 2020).

Más de 255 especies, incluyendo ácaros, insectos y microorganismos patógenos, han sido identificadas como dañinas para el olivo. De estas, aproximadamente la mitad son plagas y la otra mitad patógenos, y solo unas doce son responsables de daños económicos significativos (Haniotakis 2005).

En la región mediterránea se han identificado tres plagas principales del olivo: la polilla del olivo (*Prays oleae*) y la cochinilla negra (*Saissetia oleae*), cuyo impacto ha disminuido considerablemente en los últimos años gracias a los avances en el manejo de plagas, y la mosca del olivo (*Bactrocera oleae*), la plaga más importante que afecta a este cultivo y que sigue causando daños significativos en la producción de aceitunas (Nobre 2019).

Las bacterias patógenas de mayor relevancia en el olivar son *Xylella fastidiosa* y *Pseudomonas savastanoi*. *X. fastidiosa* es una bacteria emergente en la cuenca mediterránea causante del síndrome de decaimiento rápido del olivo. Esta bacteria ha generado una gran preocupación debido a su rápida diseminación, y es considerada una de las mayores amenazas para los olivares europeos. *P. savastanoi* es un patógeno endémico en muchas regiones olivareras. Es el agente causante de la tuberculosis del olivo, y aunque su impacto es menos severo que el de *X. fastidiosa*, reduce la productividad y la calidad del aceite (Moretti et al. 2008). Provoca hiperplasia, que se manifiesta en forma de agallas o tumores en los órganos aéreos del olivo, principalmente tallos y ramas. Esta enfermedad tiene un carácter crónico, por lo que los síntomas perduran a lo largo de los años (Cardoni and Mercado-Blanco 2023). *P. savastanoi* no tiene la capacidad para sobrevivir en suelo de forma prolongada. Permanece en la superficie de los tejidos aéreos del olivo en fase epífita, donde se multiplica sin causar daños visibles, hasta que penetra en el árbol a través de heridas causadas en la recolección, poda o por heladas o granizos, entre otras causas, y coloniza tejidos superficiales cercanos. Esta bacteria es diseminada eficazmente por la lluvia, el viento, insectos o prácticas culturales, pudiendo colonizar todo el olivar en un corto periodo de tiempo (Ramos et al. 2012).

Entre los hongos fitopatógenos más significativos que afectan al olivo destacan *Verticillium dahliae* y *Fusicladium oleagineum*. *V. dahliae*, causante de la verticilosis del olivo, es considerado un hongo endémico en diversas regiones mediterráneas, aunque su incidencia ha aumentado en las últimas décadas (EIP-AGRI Focus Group 2020). *F. oleagineum*, responsable del repilo del olivo, es también un hongo endémico en la cuenca mediterránea. Se desarrolla en la superficie de las hojas, penetra a través de la cutícula y se establece en los tejidos epidérmicos, donde puede sobrevivir durante largos periodos formando estructuras de esporulación sin provocar síntomas visibles (Jaber et al. 2020). Cuando las esporas germinan, provoca la aparición de manchas circulares necróticas en las hojas y, en casos de infección severa, una intensa defoliación. Esto reduce la capacidad fotosintética del árbol y afecta negativamente al cuajado de los frutos. Las esporas del hongo se dispersan principalmente a través de las gotas de agua, aunque el viento también puede contribuir a su diseminación. Las hojas caídas no tienen importancia epidemiológica, ya que el hongo no genera inóculo viable en tejido muerto. Se ha estimado una pérdida de rendimiento de 20-30% en áreas donde este hongo es recurrente (Buonaurio et al. 2023; Roca et al. 2010).

3.2.1. Mosca del olivo

La mosca del olivo (*B. oleae*) es la plaga más importante en el cultivo del olivar. Infesta el fruto del olivo, afectando tanto la cantidad como la calidad de la producción. Las hembras depositan sus huevos en las aceitunas realizando una incisión en la piel del fruto, que deja una marca característica (Figura 4a). En la naturaleza, una sola hembra puede ovipositar unos 12 huevos al día y unos 200-250 huevos a lo largo de su vida (Malheiro et al. 2015). La larva nace y se alimenta de la pulpa, pudiendo consumir entre el 10 y el 30% del peso de la aceituna. Es común que durante la etapa de desarrollo larvario el fruto caiga prematuramente. Al finalizar su desarrollo, la larva puede o bien pupar dentro del fruto o escapar para completar su ciclo en el suelo. En ambos casos, los frutos infestados presentan un orificio de salida por donde la pupa o la mosca adulta emerge (Figura 4b), lo que contribuye a la propagación de la plaga. Además de la pérdida de calidad de las aceitunas debido al daño directo de las larvas, las cavidades generadas por las larvas dentro de la aceituna favorecen la proliferación de hongos, lo que acelera el deterioro del fruto (Martín Gil and Ruíz Torres 2014).

Los daños indirectos se reflejan en la pérdida de calidad del aceite obtenido de aceitunas infestadas. En el caso de las aceitunas de mesa, los frutos dañados suelen ser descartados. Las pérdidas potenciales en la cosecha debidas a esta plaga actualmente varían entre el 5% y el 40%. Esto se traduce en un daño medio anual de 800 millones de euros en la producción mundial (Alonso Muñoz and García Marí 2012).

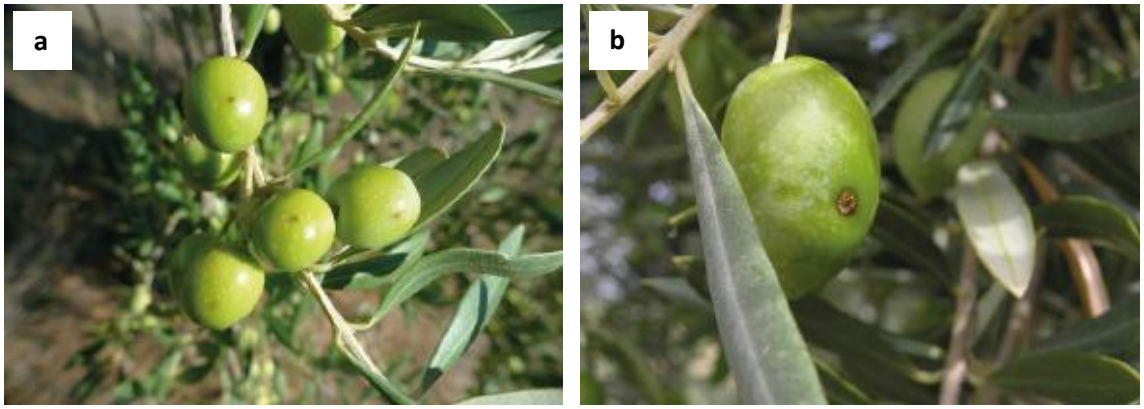

Figura 4. Mosca del olivo. (a) Frutos con marcas de puesta del huevo. (b) Fruto con orificio de salida. Fuente: Guía de Gestión Integrada de Plagas - Olivar, MAPA (Martín Gil and Ruíz Torres 2014).

3.2.2. *Xylella fastidiosa*

Xylella fastidiosa es una bacteria Gram-negativa limitada al xilema y con un amplio rango de hospedantes, capaz de infectar a más de 500 especies vegetales entre las que se incluyen cultivos de gran importancia económica. *X. fastidiosa* presenta una interacción variable con sus hospedantes, actuando como comensal en la mayoría de ellos, mientras que causa infecciones perjudiciales en un número limitado de especies susceptibles. Es responsable de la enfermedad de *Pierce* en la vid, la clorosis variegada de los cítricos, la quemadura de las hojas de los almendros y del síndrome de

decaimiento rápido del olivo (OQDS) (White et al. 2020; Kubaa et al. 2019). Históricamente, la distribución de *X. fastidiosa* se limitaba al territorio de los Estados Unidos, donde la bacteria ha sido asociada con importantes pérdidas económicas en cultivos de vid y cítricos. En 2013, *X. fastidiosa* subsp. *pauca* ST53 se detectó por primera vez en Europa, en olivos de la región de Puglia, situada en el sur de Italia. Esta identificación no solo expandió el espectro conocido de hospedantes y la distribución geográfica del patógeno, sino que también subrayó su capacidad para adaptarse a ecosistemas previamente no afectados. Su rápida propagación en la región y los extensos daños provocados han desencadenado una emergencia fitosanitaria (Strona et al. 2020).

El síndrome de decaimiento rápido del olivo se caracteriza por el quemado de las hojas y la desecación de las ramas. Los síntomas suelen comenzar en la parte superior, y se extienden progresivamente al resto de la copa. La infección confiere un aspecto quemado, y culmina con la muerte del olivo unos pocos años después de la aparición de los primeros síntomas (Figura 5) (Saponari et al. 2019).

X. fastidiosa se propaga principalmente a través de insectos vectores que se alimentan del xilema de las plantas. Estos insectos, al alimentarse de plantas infectadas, adquieren la bacteria y luego la transmiten a otras plantas sanas, facilitando la rápida dispersión del patógeno. En el caso del olivo, el principal insecto vector es *Philaenus spumarius*, que está presente en todos los olivares de la región. Un aspecto crucial en la transmisión de *X. fastidiosa* es su capacidad para formar biopelículas dentro del intestino anterior del vector, que son esenciales para la retención y transmisión eficaz de la bacteria a través del mecanismo combinado de egestión y salivación (Rapicavoli et al. 2018). La formación de biopelículas en las paredes del xilema también es un proceso crucial en la colonización del sistema vascular del olivo por parte de *X. fastidiosa*. Estas biopelículas son estructuras complejas compuestas por las células bacterianas y una matriz de sustancias poliméricas extracelulares. Las biopelículas permiten a las bacterias tener un comportamiento coordinado y regular colectivamente funciones vitales, confiriendo una mayor resistencia frente a respuestas inmunitarias o tratamientos antimicrobianos, lo cual es fundamental para la persistencia y virulencia de la infección. La formación de las biopelículas resulta en la obstrucción de los vasos xilemáticos, interrumpiendo el flujo de agua y nutrientes esenciales. Esto genera un déficit hídrico significativo dentro de la planta, comprometiendo su capacidad para realizar funciones vitales y conduciendo progresivamente a la muerte del olivo (Cattò et al. 2019).

Desde la detección inicial de *X. fastidiosa* en 2013 en Apulia, la Unión Europea ha implementado múltiples medidas enfocadas en prevenir su diseminación. La Comisión Europea adoptó la Decisión de Ejecución (UE) 2015/789 en mayo de 2015, que obliga a delimitar las zonas infectadas y zonas tampón adyacentes y prohíbe la plantación de variedades de olivo susceptibles, dado que estas aceleran la diseminación de la bacteria y complican los esfuerzos de erradicación. También establece protocolos rigurosos de vigilancia y erradicación, impone restricciones severas sobre el traslado de especies de plantas susceptibles y material vegetal desde las zonas afectadas, y define estrategias para el control del vector (European Union 2016). Recientemente, el Reglamento de

Ejecución (UE) 2020/1201 de la Comisión Europea ha incorporado la replantación de cultivares de olivo parcialmente resistentes a *X. fastidiosa* como una medida de control (European Union 2020).

Figura 5. Olivos afectados por el síndrome de decaimiento rápido del olivo (OQDS), causado por *Xylella fastidiosa*. Fuente: imagen propia.

El cultivo del olivo en la región de Puglia representa aproximadamente el 40% de la producción de aceite de oliva de Italia, y es de gran importancia para la economía y el patrimonio regional y natural regional. La mayoría de los olivares de la región de Puglia albergan huertos tradicionales con los cultivares locales Ogliarola salentina y Cellina di Nardò, que son altamente susceptibles a *X. fastidiosa*, exacerbando así la situación fitosanitaria en la región (B. M. Ali, van der Welf, and Lansink 2021). En el año 2019, se estimó que en Italia había aproximadamente 4 millones de olivos inactivos, gravemente infectados o muertos debido a *X. fastidiosa*. Esto se traduce en una reducción del 10% en la producción de aceite de oliva, que equivale a una pérdida económica de 390 millones de euros (White et al. 2020).

Se han documentado brotes de *X. fastidiosa* en varios países europeos. En España hay actualmente dos brotes activos: en las Islas Baleares, que afecta a olivos, algarrobos y almendros y donde se aplica una estrategia de contención; y en Alicante, donde infecta a almendros y está sujeto a erradicación. En Francia, la presencia de la bacteria está confirmada tanto en Córcega como en la región sur de Cote d'Azur. Los brotes en España y Francia evidencian múltiples introducciones independientes de *X. fastidiosa*, ya que las cepas identificadas en cada ubicación son distintas. Aunque *X. fastidiosa* ha sido detectada en otros países europeos, estos brotes han ocurrido en viveros y no en cultivos al aire libre, limitándose a eventos de detección aislados (Sicard et al. 2018; RAIF 2024).

De acuerdo con la estimación realizada por el JRC (Joint Research Centre), la propagación de *X. fastidiosa* por todo el territorio de la Unión Europea podría afectar a

más del 70% del valor de la producción de olivos que tienen más de 30 años y al 35% de los olivos más jóvenes (EIP-AGRI Focus Group 2020).

3.2.3. *Verticillium dahliae*

Verticillium dahliae es un hongo ascomiceto que infecta a un elevado número de especies de plantas, incluyendo importantes cultivos como algodón, tomate, patata y girasol (Luo et al. 2014; Acharya et al. 2020). Este patógeno es responsable de la verticilosis del olivo, la enfermedad más relevante transmitida por el suelo que afecta a este cultivo en la región mediterránea (Calderón et al. 2014). La severidad de la infección de *V. dahliae* en olivo depende en gran medida del patotipo que infecta a los árboles, distinguiéndose entre el patotipo defoliante (D) y no defoliante (ND) en función de su capacidad para provocar la defoliación de la planta (Figura 6) (Jiménez-Díaz et al. 2011). El patotipo D es altamente virulento y puede ser letal para la planta. Causa la caída temprana de hojas verdes y deriva en la defoliación completa y necrosis del olivo. Por otro lado, el patotipo ND, aunque menos agresivo, está asociado con un síndrome de decaimiento lento del olivo. El decaimiento lento del olivo se caracteriza por la muerte regresiva de ramas y brotes sin desprendimiento de hojas. Los árboles infectados por el patotipo ND pueden mostrar una remisión completa de los síntomas (Gramaje et al. 2013; Jiménez-Fernández et al. 2016).

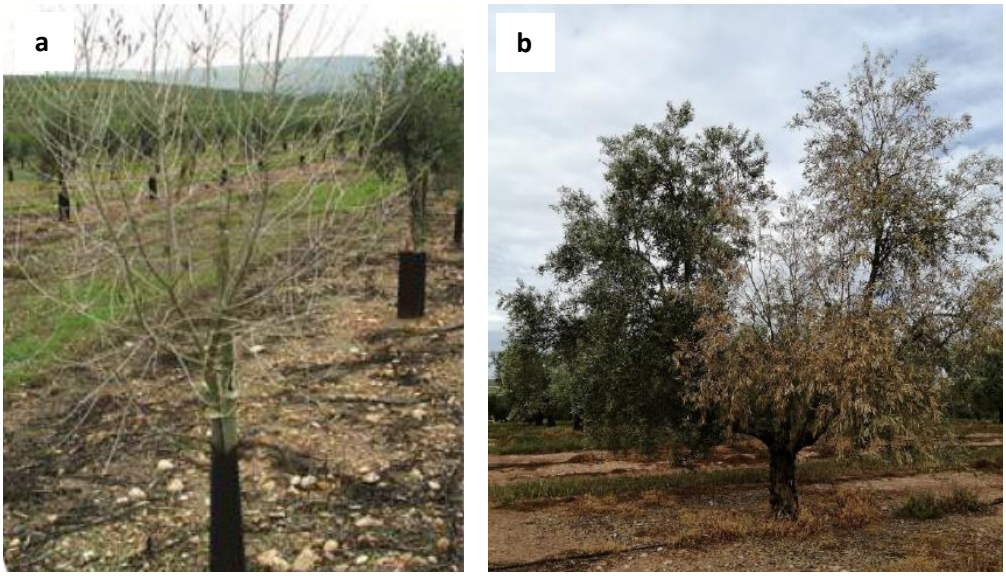

Figura 6. *Verticillium dahliae.* (a) Síntomas del patotipo defoliante. Fuente: Jiménez-Díaz et al. 2012 (b) Síntomas del patotipo no defoliante. Imagen propia.

La incidencia de verticilosis en el olivo está estrechamente vinculada a la densidad del hongo en el suelo, la cual es significativamente influenciada por las condiciones de humedad del mismo (Requena-Mullor et al. 2020). *V. dahliae* se reproduce de forma estrictamente asexual y se caracteriza por la producción de microesclerocios, estructuras de resistencia melanizadas que desempeñan un papel crucial en la supervivencia, diseminación y epidemiología del patógeno. Gracias al papel protector de la melanina, los microesclerocios pueden sobrevivir en el suelo sin hospedante

durante más de diez años bajo condiciones adversas (Milgroom et al. 2014; Harting et al. 2020). La germinación de los microesclerocios tiene lugar en respuesta a exudados radiculares tanto de plantas hospedadoras como no hospedadoras, y es altamente dependiente de la disponibilidad de agua en el suelo (Hu et al. 2014). En estudios previos llevados a cabo en suelos secos se ha observado que los microesclerocios se multiplican y reactivan su actividad metabólica al rehumedecerse. Por lo tanto, la humedad del suelo promueve tanto la producción de nuevas estructuras de resistencia, lo que aumenta la población del hongo en el suelo, como su germinación (Ben-Yephet and Pinkas 1977). La respuesta de estas estructuras a los exudados da lugar a la formación de hifas, que penetran las raíces y colonizan el xilema. Esto causa una disrupción significativa en la conducción de agua y nutrientes dentro de la planta, provocando estrés hídrico y, en casos severos, la muerte del árbol (Calderón et al. 2014). Cuando el olivo o partes específicas como hojas o flores entran en senescencia, *V. dahliae* produce grandes cantidades de microesclerocios dentro de los tejidos necróticos. Estos microesclerocios, que regresan al suelo junto con los restos vegetales, constituyen una nueva fuente de inóculo e inician un nuevo ciclo de infección (Duressa et al. 2013). Un microesclerocio puede experimentar múltiples procesos de germinación, debido a que no todas las células melanizadas que lo componen germinan al mismo tiempo (Hu et al. 2014).

Debido a su amplia gama de plantas hospedadoras, a la viabilidad de los microesclerocios en suelo y a su capacidad para crecer confinado en el xilema de la planta, *V. dahliae* es un hongo muy difícil de controlar. El desplazamiento de hojas, restos de poda y suelo infestado, ya sea por maquinaria o por agua de riego, contribuye a su propagación. Además, el olivo es un cultivo que se reproduce principalmente por esquejes, por lo que el uso de material de plantación infectado facilita la diseminación del patógeno y complica aún más su control en nuevas áreas (Jiménez Días, et al. 2009).

V. dahliae ocasiona la pérdida de olivos y tiene un impacto ecológico considerable en los ecosistemas locales. La alta incidencia de esta enfermedad en las regiones olivícolas de la cuenca mediterránea no solo reduce significativamente el rendimiento del cultivo, generando importantes pérdidas económicas, también altera las propiedades organolépticas de las aceitunas, lo que repercute negativamente en el valor comercial del aceite de oliva (Rhouma et al. 2023). En las últimas décadas, la verticilosis se ha extendido por las principales zonas olivícolas españolas (Mercado-Blanco et al. 2004; Ostos et al. 2020). El hongo prospera especialmente en olivares intensivos en Andalucía, donde el regadío y la alta densidad de árboles crean condiciones óptimas para su proliferación. En Andalucía occidental, *V. dahliae* afecta a aproximadamente un 38% de los cultivos de olivo, mientras que en la provincia de Granada (Andalucía oriental) afecta al 14%. Además, en Andalucía se ha observado una prevalencia creciente del patotipo D, que está desplazando progresivamente al patotipo ND (Requena-Mullor et al. 2020).

4. Problemáticas asociadas al control de fitopatógenos y normativa

El control de fitopatógenos en la agricultura ha sido un desafío persistente, abordado tradicionalmente mediante el uso de agroquímicos sintéticos. Estos incluyen

fungicidas, bactericidas e insecticidas, que han demostrado ser fundamentales en la protección de los cultivos frente a estrés biótico y para mejorar los rendimientos agrícolas (Rongai, Milano, and Sciò 2012). No obstante, el uso intensivo de estos productos conlleva importantes consecuencias ecológicas, entre las que se encuentran la contaminación medioambiental, el impacto en la biodiversidad y el desarrollo de resistencias (Park et al. 2008).

La aplicación continuada de productos biocidas puede conducir al desarrollo de resistencia en las poblaciones de fitopatógenos (Egamberdieva et al. 2023). Estos organismos patógenos tienen la capacidad de adaptarse y evolucionar rápidamente frente a la presión selectiva impuesta por el uso repetitivo de estos productos (Miller, Ferreira, and LeJeune 2022). Los microorganismos patógenos, así como los ácaros e insectos, pueden desarrollar resistencia a través de distintos mecanismos, entre los que se incluyen la alteración de los blancos de los biocidas mediante mutaciones o alteraciones en la pared celular, la adquisición de determinantes de resistencia de otras cepas por transferencia horizontal de genes, la descomposición metabólica del biocida llevada a cabo por enzimas y bombas de eflujo para expulsar el pesticida (Farooq et al. 2022; Hahn 2014). Este fenómeno disminuye la eficacia de los tratamientos, lo que compromete el control de enfermedades agrícolas y obliga a los agricultores a emplear dosis más altas o recurrir a productos más tóxicos, exacerbando los problemas ambientales y de salud asociados (Corkley, Fraaije, and Hawkins 2021).

La intensificación del uso de biocidas puede dañar significativamente la biodiversidad de los agroecosistemas, afectando negativamente a organismos no objetivo, como aves, insectos beneficiosos y otros animales (Pires Ribeiro 2009). La reducción de la biodiversidad en los ecosistemas agrícolas puede perturbar el equilibrio ecológico, alterando servicios ecosistémicos fundamentales como la polinización y el control biológico de plagas por enemigos naturales. La polinización participa en la preservación de la biodiversidad de plantas silvestres y mejora el rendimiento los principales cultivos comestibles, contribuyendo al 35% de la producción agrícola mundial (Papa et al. 2022; Gallai and Vaissière 2009). Aproximadamente 5 millones de euros de la producción agrícola anual de la Unión Europea se atribuyen a los polinizadores (European Commission 2018). La abeja melífera occidental (*Apis mellifera* Linnaeus; Hymenoptera: Apidae) es el polinizador con mayor relevancia ecológica y económica, que contribuye a la polinización de 71 de las 100 especies que proporcionan el 90% de los alimentos a nivel mundial (Paudel et al. 2015). Diversos estudios sugieren que los agroquímicos sintéticos son responsables de la disminución de las poblaciones de abejas y otros polinizadores (Fernandes et al. 2022; Halvorson et al. 2021). Asimismo, los enemigos naturales, que incluyen microorganismos, insectos y arácnidos beneficiosos, aves, mamíferos y anélidos, desempeñan un papel vital en la regulación de plagas y la preservación de la salud del ecosistema. Son responsables de que numerosos organismos no se conviertan en plagas de importancia económica. A menudo se ha relacionado el control de una plaga con el brote de otra, debido a la reducción de la población de su enemigo natural. Los pesticidas generalmente afectan a las poblaciones

de organismos al aumentar la mortalidad por contacto o por toxicidad oral. Aunque se ha argumentado que los biocidas sistémicos son más seguros que los no sistémicos debido a que la exposición solo ocurre cuando los organismos se alimentan de tejido vegetal, numerosos estudios han demostrado que los residuos pueden permanecer en los tejidos durante semanas y contaminar el néctar, afectando a organismos polinizadores (El-Wakeil et al. 2013). El efecto de los biocidas no debe medirse únicamente por sus efectos letales sobre los organismos en contacto directo, sino que se deben de tener en cuenta otros factores, como las interacciones entre especies debido a depredación o competencia, que aumentan el radio de acción de los agroquímicos. La exposición a dosis subletales favorece la dispersión de los residuos en el ecosistema y reduce la fertilidad de los individuos (Sánchez-Bayo 2021). Además de los problemas asociados a la persistencia y la movilidad de los agroquímicos en el entorno natural, estos también pueden acumularse en los productos agrícolas y llegar a la cadena alimentaria, planteando riegos potenciales para la salud humana (Ahmad et al. 2024).

La aplicación extensiva de agroquímicos sintéticos tiene como consecuencia la contaminación del suelo y de las fuentes de agua cercanas (Yadav et al. 2015). Los residuos pueden infiltrar el suelo y afectar gravemente el microbioma, compuesto por bacterias, hongos, protozoos y algas. El microbioma del suelo juega un papel crucial en la fertilidad del mismo y en el ciclo y la disponibilidad de nutrientes. También mejora las propiedades físicas del suelo y contribuye a procesos ecológicos como la biorremediación y el biocontrol (Chaudhary et al. 2022). Los microorganismos del suelo establecen asociaciones simbióticas con las raíces de las plantas, facilitando procesos vitales para su crecimiento y desarrollo. Producen hormonas que estimulan el sistema inmunológico de las plantas, fomentan el crecimiento y activan las respuestas al estrés (Mitter et al. 2013). Son fijadores de elementos esenciales para las plantas, como nitrógeno, fósforo y potasio, por lo que facilitan la disponibilidad y absorción de estos nutrientes. Ciertos microorganismos tienen la capacidad de descomponer materia orgánica e inorgánica, convirtiéndola en nutrientes que las plantas pueden utilizar. Este proceso de descomposición es crucial para el reciclaje de nutrientes en el suelo, asegurando una fertilidad continua y el mantenimiento de la salud del ecosistema (Meena et al. 2020). Estas asociaciones también influyen en la estructura del suelo, al formar agregados que mejoran la porosidad y la aireación, lo que facilita la infiltración de agua y reduce la erosión. Los microorganismos del suelo también juegan un papel importante en la regulación de enfermedades microbianas al competir con patógenos potenciales y producir compuestos antimicrobianos. Además, contribuyen a la biorremediación de suelos contaminados al descomponer sustancias tóxicas y reducir la contaminación. En conjunto, estas interacciones no solo promueven el crecimiento saludable de las plantas, sino que también apoyan la estabilidad y la funcionalidad del ecosistema en general, asegurando la provisión de servicios ecosistémicos vitales. El uso prolongado de biocidas altera el equilibrio y la funcionalidad del microbioma del suelo, comprometiendo su capacidad para realizar funciones esenciales. Esta alteración deteriora la salud del suelo y su capacidad para sostener una agricultura sostenible (Tripathi et al. 2020).

Los agroquímicos pueden ser arrastrados por la lluvia hacia grandes masas de agua, como ríos, lagos y acuíferos. La contaminación de reservorios de agua plantea serios riesgos para el medio ambiente y la salud humana. La presencia de estos residuos en fuentes de agua puede afectar a las comunidades acuáticas al alterar los ecosistemas y reducir la biodiversidad. También puede acelerar la eutrofización de cuerpos de agua que hayan recibido un aporte excesivo de nutrientes. Estos nutrientes provocan un crecimiento desmedido de algas, que agotan el oxígeno en el agua y pueden provocar la muerte masiva de peces y otras especies acuáticas. Además, los residuos pueden entrar en la cadena alimentaria a través del agua potable y afectar a la salud humana al provocar enfermedades gastrointestinales, trastornos hormonales y efectos tóxicos a largo plazo (Srivastav 2020).

A pesar de que los agroquímicos sintéticos han contribuido a aumentar la producción de alimentos mediante el control de fitopatógenos, los problemas derivados de su aplicación continuada han conducido a una revisión crítica y a la implementación de normativas restrictivas. Estas restricciones incluyen la prohibición o limitación de ciertos productos químicos, la regulación de las dosis aplicadas y la promoción de métodos más sostenibles (Feijao et al. 2022).

Desde el año 2011, las sustancias activas fitosanitarias están reguladas en la Unión Europea por el Reglamento (CE) n.º 1107/2009, que define los requisitos para su autorización y regulación, incluyendo la evaluación de riesgos para la salud humana, animal y el medio ambiente. El Reglamento (CE) n.º 1107/2009 engloba bajo el término fitosanitario a cualquier producto que contenga una o más sustancias activas destinadas a proteger a los vegetales de todos los organismos nocivos o evitar la acción de estos, y a influir en los procesos vitales de los vegetales de forma distinta a los nutrientes. Por tanto, los productos fitosanitarios incluyen bactericidas, fungicidas, insecticidas, acaricidas, repelentes y bioestimulantes (European Union 2009). Este Reglamento es complementario al Reglamento (CE) n.º 396/2005, que regula los límites máximos de residuos de plaguicidas en alimentos y piensos, y a la Directiva (CE) n.º 128/2009, que proporciona un marco para la acción comunitaria orientada a reducir el impacto ambiental de los plaguicidas.

El Reglamento de Ejecución (UE) n.º 540/2011 recoge la lista oficial de sustancias activas aprobadas, así como aquellas sustancias que están pendientes de reevaluación. Desde la implementación del Reglamento (CE) n.º 1107/2009, el número de sustancias activas aprobadas ha disminuido drásticamente. La pérdida neta ha sido de 95 sustancias activas en los últimos 4 años, y 118 desde 2011. La fluctuación de las sustancias activas aprobadas durante este tiempo esconde hechos relevantes. La reducción de sustancias activas ha afectado principalmente a aquellas de naturaleza química (Figura 7), mientras que el número de agentes de control biológico y sustancias básicas aprobadas ha aumentado (Figura 8) (Marchand 2023).

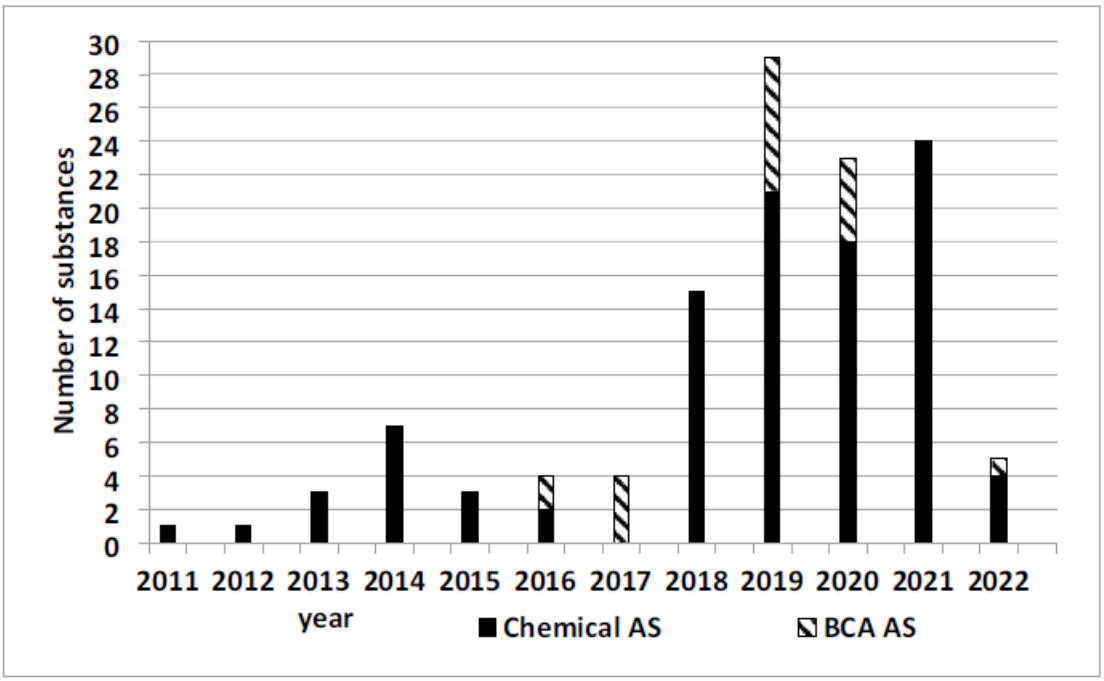

Figura 7. Número de sustancias activas (AS) eliminadas desde el año 2011. BCA: Agente de control biológico. Fuente: (Marchand 2023).

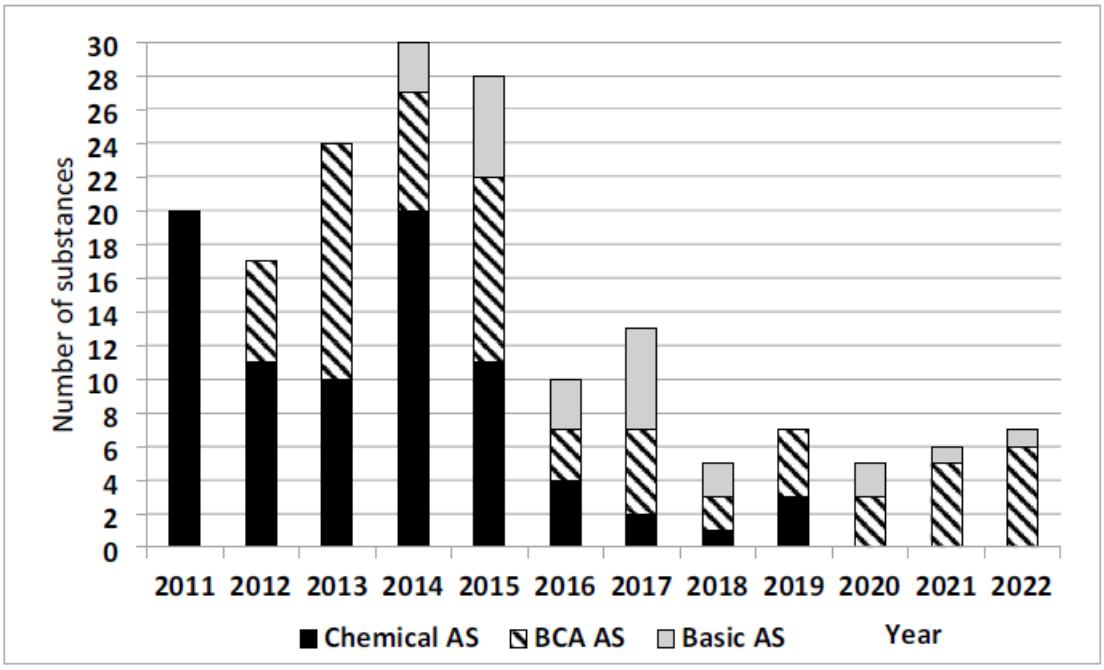

Figura 8. Número de sustancias activas (AS) aprobadas desde el año 2011. BCA: Agente de control biológico. Fuente: (Marchand 2023).

En el marco del Pacto Verde Europeo, iniciativa estratégica de la Unión Europea presentada en diciembre de 2019, se busca transformar la economía del continente hacia un modelo más sostenible y respetuoso con el medio ambiente. Dentro de esta iniciativa, la Estrategia "De la Granja a la Mesa" subraya la necesidad urgente de reducir la dependencia de los pesticidas. Esta Estrategia, junto con el Plan de Acción de

Contaminación Cero y la Estrategia de Biodiversidad 2030, establece como objetivos clave para 2030 reducir en un 50% el uso y el riesgo de pesticidas químicos, disminuir en un 50% los más peligrosos y dedicar al menos el 25% de la superficie agrícola de la UE a la agricultura ecológica (European Environment Agency 2023). Estos objetivos se alinean con la Iniciativa de la UE sobre polinizadores, presentada por la Comisión Europea en 2018, que constituye el primer marco normativo de la UE por el que se establecen acciones a corto y medio plazo, así como objetivos a largo plazo para la protección de los polinizadores (European Commission 2018). Esta iniciativa ha sido revisada en 2023 y, entre otras medidas, establece la reducción del uso de los pesticidas químicos más peligrosos para los polinizadores en un 50% para 2030 y su sustitución progresiva por métodos más sostenibles de gestión de plagas que ayuden a preservar las abejas melíferas (European Commission 2023). En las últimas décadas, la Organización para la Cooperación y el Desarrollo Económicos (OCDE) y la Organización Europea y Mediterránea de Protección de las Plantas (OEPP), entre otros, han desarrollado métodos de evaluación de riesgos específicos para las especies del género Apis (Barascou et al. 2021), que han sido validados por la EFSA (Autoridad Europea de Seguridad Alimentaria) de acuerdo con el reglamento (UE) 1107/2009 y aceptados internacionalmente (EFSA 2023).

Esta tendencia hacia la disminución de los productos químicos tradicionales y su sustitución por sustancias activas más sostenibles en la UE tiene implicaciones para la protección de cultivos y la gestión de residuos. La normativa europea ha limitado las opciones inmediatas disponibles para los agricultores. Sin embargo, también ha fomentado la búsqueda de métodos alternativos de protección de cultivos que cumplan con los estándares de sostenibilidad. A pesar de este impulso, la transición hacia nuevas alternativas presenta dificultades. La necesidad de altos rendimientos, así como de mantener los precios de los alimentos bajos, complican la adopción de métodos sostenibles a corto plazo (*European Union* 2021).

En el caso del olivar, la alta demanda de aceite de oliva y la presión por no elevar los precios hace que los agricultores sean reticentes a adoptar nuevas prácticas. La transición hacia métodos sostenibles en el cultivo de olivo debe equilibrar las necesidades del mercado con el objetivo de reducir el impacto ambiental. En respuesta a estos desafíos, la guía de Gestión Integrada de Plagas en el Olivar publicada por el Ministerio de Agricultura, Alimentación y Medio Ambiente en 2014 establece una serie de alternativas y estrategias destinadas a minimizar el uso de productos químicos y a fomentar prácticas agrícolas más respetuosas con el medio ambiente. La guía promueve el manejo integrado de plagas como una estrategia integral que combina métodos biológicos, físicos y culturales. Además de enfatizar la importancia del monitoreo constante y prácticas culturales adecuadas, tales como la poda y la gestión de restos vegetales para reducir los hábitats de patógenos y plagas, la aplicación de microorganismos beneficiosos y extractos vegetales se destaca como una forma eficaz de apoyar la salud del olivo y mejorar su resistencia natural a enfermedades y plagas. La adopción de estas prácticas es crucial no solo para cumplir con las normativas europeas

de sostenibilidad, sino también para que los agricultores puedan adaptar sus métodos a las demandas del mercado y a los requisitos ambientales, garantizando al mismo tiempo la viabilidad económica del cultivo (Martín Gil and Ruíz Torres 2014).

5. Alternativas sostenibles para el control de fitopatógenos

La convergencia de los desafíos a los que se enfrenta el olivar, junto con las consecuencias medioambientales derivadas de la aplicación de agroquímicos de síntesis, subraya la necesidad de adoptar estrategias más sostenibles y adaptativas. Esto implica reducir la dependencia de productos químicos sintéticos que tradicionalmente se han utilizado en agricultura, y sustituirlos por soluciones alineadas con el enfoque de la agricultura sostenible, con el objetivo de asegurar la viabilidad económica y ecológica de los olivares a largo plazo. Este enfoque se refleja claramente en los cambios adoptados por la normativa europea en relación con las sustancias activas aprobadas detallados anteriormente. Además de establecer un marco general que promueve el uso de prácticas sostenibles, como la mejora de la conservación, la eliminación de partes dañadas de las plantas y el control del crecimiento indeseado, la normativa promueve la aplicación de microorganismos y extractos vegetales para el control de poblaciones de plagas y enfermedades, así como su uso como bioestimulantes (Jindo et al. 2022; Asif et al. 2023). Los bioestimulantes se definen como sustancias o microorganismos que mejoran la tolerancia al estrés, la eficiencia del uso de nutrientes y las características de calidad de los cultivos, utilizando mecanismos que no implican el aporte directo de nutrientes al suelo (du Jardin 2015).

5.1. Microorganismos

El uso de microrganismos con capacidad antagonista para combatir fitopatógenos, conocidos como agentes de control biológico o de biocontrol, ha cobrado relevancia en los últimos años, siendo considerado uno de los métodos alternativos más efectivos que contribuye a la reducción del uso de pesticidas químicos (Iftikhar et al. 2020). Los microorganismos de biocontrol inhiben el crecimiento de microorganismos fitopatógenos o interrumpen el desarrollo normal de la enfermedad mediante una variedad de mecanismos de acción, tales como la competencia por nutrientes y por el espacio, el parasitismo, la inducción de resistencia sistémica y la producción de compuestos antimicrobianos (Dukare et al. 2019).

En comparación con los agroquímicos sintéticos, el uso de microorganismos de biocontrol ofrece ciertas ventajas. Son más respetuosos con el medio ambiente y con organismos no diana, como polinizadores y enemigos naturales, ya que presentan una menor toxicidad y persistencia. Esto reduce la acumulación de residuos en suelos, cuerpos de agua y en los cultivos, lo que contribuye a la seguridad alimentaria y a la preservación de la biodiversidad. Además, reducen el riesgo de que los microorganismos fitopatógenos generen resistencias (Tilocca, Cao, and Migheli 2020; Mayo-Prieto et al. 2020). El empleo de agentes de control biológico garantiza una mayor seguridad en su aplicación, ya que no implican un riesgo para los trabajadores agrícolas ni para los consumidores. También destacan por ser una alternativa económicamente

viable en términos de producción, lo que los convierte en una opción accesible para comunidades con recursos limitados (Bonaterra et al. 2012).

Sin embargo, la aplicación de agentes de control biológico enfrenta limitaciones bajo diversas condiciones ambientales que afectan a su eficacia. Es esencial seleccionar cepas con alta adaptabilidad y supervivencia en el campo, así como desarrollar formulaciones mejoradas para optimizar el rendimiento de los microorganismos y facilitar su comercialización. A pesar de los avances realizados en esta área, sigue siendo una necesidad profundizar en el desarrollo de formulaciones innovadoras, que integren el uso de nuevas tecnologías y consideren el impacto ambiental y la producción en masa (Iftikhar et al. 2020).

Entre los microorganismos utilizados en la agricultura como agentes de biocontrol, destacan varios géneros con capacidades antagonistas. *Pantoea* spp. es eficaz en la inhibición de fitopatógenos bacterianos y fúngicos. *Trichoderma* spp. es muy utilizado para el control de hongos del suelo debido a su capacidad de micoparasitismo y la producción de enzimas que degradan las paredes celulares de los patógenos (Harman et al. 2004). Asimismo, *Clonostachys rosea* es otro hongo que se ha utilizado en el control de esclerocios y compite con otros patógenos fúngicos (Sun et al. 2023). Otros hongos como *Beauveria bassiana*, aunque más conocido como un agente de control biológico de insectos, también puede reducir indirectamente algunas enfermedades fúngicas (Russo et al. 2024). Finalmente, las micorrizas arbusculares no solo mejoran la absorción de nutrientes, sino que también inducen resistencia sistémica en las plantas, protegiéndolas contra diversos patógenos (Molinari 2024).

Sin lugar a dudas, entre los microorganismos que actúan como agentes de biocontrol frente a bacterias y hongos fitopatógenos destacan los de los géneros *Bacillus* spp. y *Pseudomonas* spp. (Boro et al. 2022). Además, numerosas cepas de estos géneros bacterianos han demostrado su capacidad para promover el crecimiento de las plantas (Saranraj 2014).

5.1.1. *Bacillus* spp.

Las bacterias del género *Bacillus* colonizan una amplia variedad de nichos ecológicos, como la filosfera, la rizosfera y el suelo de las plantas. Su capacidad para formar endosporas es esencial para su supervivencia y adaptación a diferentes entornos (Fira et al. 2018; Pérez-García, Romero, and de Vicente 2011). Diversos estudios han demostrado que las cepas de *Bacillus* sintetizan metabolitos secundarios con capacidad antagonista de distinta naturaleza, tales como péptidos ribosomales (bacteriocinas) y moléculas no ribosomales (péptidos, lipopéptidos y policétidos) (Li et al. 2020). No obstante, también pueden ejercer su capacidad de biocontrol mediante la secreción de enzimas líticas que hidrolizan componentes de las paredes celulares de los fitopatógenos, así como compuestos orgánicos volátiles (VOC) que inhiben el desarrollo micelial y la germinación de esporas (Miljaković, Marinković, and Balešević-Tubić 2020; Fessia et al. 2022).

Por otro lado, estas bacterias promueven el desarrollo vegetal a través de la fijación de nitrógeno, la solubilización de fósforo y la producción de sideróforos, que solubilizan el hierro presente en formas no disponible para las plantas (Fe^{3+}), facilitando así su absorción (Sawant, Song, and Seo 2022). Las cepas bacterianas que producen grandes cantidades de sideróforos no solo estimulan el crecimiento de la planta aumentando el hierro disponible, sino que reducen las poblaciones de fitopatógenos en la rizosfera mediante la competencia por hierro (Köhl, Kolnaar, and Ravensberg 2019).

Asimismo, estas bacterias producen fitohormonas, como ácido indolacético (IAA), giberelinas (GA), citoquininas y ácido abscísico (ABA), que intervienen en distintos procesos de desarrollo de las plantas. IAA tiene un papel crucial en procesos clave del crecimiento vegetal, tales como la elongación celular y el desarrollo de tejido vascular. Las citoquininas también están implicadas en procesos de crecimiento como la regulación de la división celular, la diferenciación de los brotes o el desarrollo fotomorfogénico. GA interviene en la germinación de semillas, el inicio de la floración y el desarrollo de flores y frutos. ABA está implicada en las respuestas de las plantas a estrés abiótico (Poveda and González-Andrés 2021). Los VOCs producidos por las cepas de *Bacillus* también son determinantes en el desarrollo de la planta. Por ejemplo, VOCs como el albuterol y el 1,3-propanodiol inducen la expresión de genes relacionados con la biosíntesis de fitohormonas (Tsotetsi et al. 2022). Otro mecanismo mediante el cual las cepas de *Bacillus* estimulan el crecimiento de plantas en situaciones de estrés es a través de la síntesis de la enzima 1-aminociclopropano-1-carboxilato (ACC) desaminasa, que reduce los niveles de ACC, precursor del etileno (Miljaković, Marinković, and Balešević-Tubić 2020). Niveles elevados de esta hormona están asociados con la inhibición de la división celular, de la síntesis de ADN y, en consecuencia, con la disminución del crecimiento vegetal (Gamalero, Lingua, and Glick 2023).

Además de los mecanismos mencionados, estudios previos han relacionado la aplicación de *Bacillus* en plantas con un aumento en la producción de enzimas antioxidantes como la superóxido dismutasa y la peroxidasa, que eliminan especies reactivas de oxígeno generadas en respuesta a estrés biótico (Tsotetsi et al. 2022).

5.1.2. *Pseudomonas* spp.

Las bacterias del género *Pseudomonas* se encuentran en una alta densidad en suelo, especialmente en la rizosfera. Emplean una amplia variedad de exudados como fuente de nutrientes, y tienen una elevada tasa de crecimiento. Las cepas beneficiosas y patógenas colonizan los mismos nichos ecológicos y comparten mecanismos de colonización. Ambas pueden pertenecer a una misma especie, siendo las cepas de biocontrol y promotoras del crecimiento vegetal predominantes en las especies *P. fluorescens* y *P. putida* (Höfte and Altier 2010).

Las bacterias del género *Bacillus* y *Pseudomonas* emplean mecanismos similares de biocontrol y estimulación del crecimiento vegetal, tales como la síntesis de compuestos antimicrobianos, fitohormonas (IAA, GA y citoquininas), sideróforos y VOCs como el cianuro de hidrógeno, compuesto volátil que inhibe la respiración celular

(Saranraj 2014). Al igual que las cepas de *Bacillus*, las *Pseudomonas* reducen los niveles de etileno en los tejidos vegetales mediante la síntesis de ACC desaminasa (Patten and Glick 2002).

Entre los compuestos antimicrobianos producidos por cepas de *Pseudomonas* se encuentran los antibióticos policétidos pioluteorina y el 2,4-diacetilfloroglucinol, el antibiótico orgánico pirrolnitrina, y enzimas hidrolíticas como quitinasas, proteasas y β-glucanasas (Cesa-Luna et al. 2020; Liu et al. 2022). Además, las especies de *Pseudomonas* son las principales productoras de fenazinas. Las fenazinas son compuestos nitrogenados altamente estables en ambientes naturales con actividad antimicrobiana de amplio espectro, debido a su capacidad para reducir el oxígeno molecular y producir especies reactivas de oxígeno (ROS), lo que genera estrés oxidativo en los microorganismos fitopatógenos (Serafim, Bernardino, and Freitas 2023).

5.2. Extractos vegetales

Los extractos vegetales están captando cada vez más atención como una opción sostenible y efectiva para mejorar la resistencia al estrés en cultivos. Estos extractos, ricos en metabolitos, han demostrado una efectividad significativa como bioestimulantes y agentes antimicrobianos en una gran variedad de cultivos (Soriano et al. 2022). A diferencia de los productos químicos sintéticos, los extractos vegetales se consideran seguros debido a su baja toxicidad, alta biodegradabilidad y menor riesgo de generar resistencia en patógenos (Deresa and Diriba 2023; Walia et al. 2017).

La obtención de extractos vegetales no se limita a plantas medicinales y aromáticas, sino que también abarca el uso de subproductos de la industria alimentaria y materiales vegetales de segunda clase. Esta estrategia innovadora incluye el aprovechamiento de partes no comestibles de plantas cultivadas, tales como hojas de plantas de patata o berenjena, cáscara de castaño o desechos de uva, lo que no solo contribuye a la revalorización de los desechos agrícolas, sino que también promueve la economía circular y reduce la dependencia de insumos externos (Rayne, Karacabey, and Mazza 2008; Gahukar 2012; Pane et al. 2020).

El uso de extractos vegetales como bioestimulantes y antimicrobianos naturales ha mostrado resultados prometedores en la mejora del rendimiento de los cultivos y la resistencia a enfermedades (Sciubba et al. 2020). Numerosos estudios han evidenciado la capacidad de diversos extractos vegetales para mejorar la resiliencia de los cultivos antes distintos tipos de estrés abiótico, como la sequía, el calor, y la salinidad, activando rutas metabólicas y fortaleciendo mecanismos de defensa naturales en las plantas. Por ejemplo, extractos de algas marinas y moringa han sido utilizados para incrementar la tolerancia al estrés hídrico en cultivos leñosos y frutales, mejorando la eficiencia en el uso del agua y la estabilidad de los rendimientos bajo condiciones adversas (Salvi et al. 2019; Bakhsh et al. 2020; Van Oosten et al. 2017). Asimismo, numerosos extractos han revelado propiedades antimicrobianas frente a fitopatógenos tanto *in vitro* como *in vivo*, así como sus propiedades repelentes y biocidas frente a plagas. De acuerdo con estudios previos, extractos de plantas como el tomillo, el eucalipto y vegetales del género *Brassica*

contienen compuestos con propiedades antimicrobianas que actúan de manera sinérgica para controlar enfermedades microbianas que afectan a cultivos, sin causar daño al medio ambiente ni a la salud humana (Mohd Israfi et al. 2022; He et al. 2024).

5.2.1. Extractos del género *Allium*

Dentro de los extractos vegetales, los derivados del género *Allium* han sido objeto de numerosos estudios debido a sus propiedades bioactivas. El género *Allium* incluye más de 900 especies, siendo las más estudiadas el ajo (*Allium sativum*), la cebolla (*Allium cepa*), el puerro (*Allium ampeloprasum var. porrum*) y la chalota (*Allium ascalonicum*) (Guillamón et al. 2021). Los compuestos organosulfurados (OSC) son los principales compuestos bioactivos presentes en las especies del género *Allium*, que se generan como un mecanismo de defensa tras el daño tisular. Los bulbos intactos son ricos en S-alqu(en)il-cisteína sulfóxidos (ACSO), que funcionan como precursores de diversas moléculas bioactivas (Guillamón 2018). En el ajo, el principal ACSO es la aliina (S-(2-propenil)-L-cisteína sulfóxido), que tras la acción de la enzima aliinasa se convierte en alicina, un compuesto con potentes propiedades antimicrobianas y antioxidantes. En la cebolla, los ACSO predominantes son la isoaliina, que se convierte en el factor lacrimógeno, metiina (S-metil-L-cisteína sulfóxido), también presente en el ajo, y propiina (S-propil-L-cisteína sulfóxido) (Keusgen et al. 2002). Cuando es procesada por la aliinasa, la propiina da lugar a tiosulfinato de dipropilo (PTS), que a su vez se transforma en propil-propano tiosulfonato (PTSO) a través de reacciones de dismutación o desproporción.

Estos compuestos organosulfurados son conocidos por sus propiedades antimicrobianas y antioxidantes (Guillamón et al. 2021). Los estudios sobre las distintas aplicaciones de extractos de ajo, así como de sus principales compuestos, son mucho más exhaustivos que los de la cebolla. La alicina ha sido ampliamente investigada en diversos campos, desde el tratamiento de enfermedades humanas hasta su uso en el manejo integrado de plagas (Corzo-Martínez, Corzo, and Villamiel 2007; Curtis et al. 2004). A pesar de sus propiedades, la alicina es altamente inestable, lo que representa una desventaja significativa, ya que se descompone rápidamente en otros compuestos menos activos, lo que puede limitar su efectividad en aplicaciones a largo plazo o en condiciones ambientales desfavorables (Borlinghaus et al. 2014). En el ámbito agrícola, la actividad antifúngica de los derivados del ajo está bien documentada contra patógenos como *Botrytis cinerea*, *Phytophthora infestans y Fusarium* spp. (Portz, Koch, and Slusarenko 2008; Kutawa, Danladi, and Haruna 2018; Sarfraz et al. 2020). También se ha demostrado que los extractos de ajo ricos en alicina mejoran el crecimiento, rendimiento, calidad de los frutos y tolerancia al estrés en cultivos como pepino (*Cucumis sativus*), tomate (*Solanum lycopersicum*), berenjena (*Solanum melongena*) y pimiento (*Capsicum annuum*) (Hayat et al. 2018; M. Ali et al. 2019; Hayat et al. 2016).

En comparación, los estudios sobre las propiedades bioactivas de los compuestos derivados de la cebolla son más limitados, y se ha centrado en el potencial de los tiosulfinatos y tiosulfonatos contra infecciones en humanos y animales. Estudios

recientes han mostrado que PTS y PTSO tienen una actividad antibacteriana de amplio espectro y son efectivos contra aislados clínicos de bacterias y especies de *Candida* resistentes a antibióticos (Sorlozano-Puerto et al. 2021; 2018). También presentan propiedades antiparasíticas, antiinflamatorias e inmunomoduladoras (Guillamón et al. 2023; Cabello-Gómez et al. 2022). Sin embargo, las aplicaciones agrícolas de estos compuestos han sido escasamente investigada. El extracto de cebolla ha demostrado ser efectivo como insecticida en ensayos realizados con larvas de *Spodoptera littoralis*, una plaga que causa daños severos en plantas de algodón, mostrando una reducción significativa en el tamaño de las larvas (Ahmed et al. 2021). Además, estudios recientes han demostrado que un extracto de cebolla utilizado como bioestimulante en el cultivo de microalgas mejora la productividad de la biomasa y el contenido de lípidos (Suparmaniam et al. 2024; 2023). Además de la variedad de propiedades funcionales, los tiosulfinatos y tiosulfonatos derivados de cebolla también han mostrado una mayor estabilidad y biodisponibilidad que otros compuestos organosulfurados de aliáceas como la alicina (Aguinaga-Casañas et al. 2022). Todos estos datos respaldan la idoneidad de investigar las aplicaciones agrícolas de tiosulfinatos y tiosulfonatos.

Diversos estudios han descrito múltiples mecanismos mediante los cuales los compuestos organosulfurados derivados de especies del género *Allium* ejercen su efecto antimicrobiano. Los compuestos organosulfurados reaccionan con los grupos tiol presentes en enzimas esenciales del metabolismo microbiano, afectando a funciones críticas (Rahman 2007). Entre las enzimas con las que pueden reaccionar estos compuestos se incluyen la succinato deshidrogenasa, involucrada en el ciclo de Krebs y en la cadena de transporte de electrones (Yorke 2016), la alcohol deshidrogenasa y la tiorredoxina reductasa, que son clave en el mantenimiento del equilibrio redox y la defensa antioxidante (Ankri and Mirelman 1999). Estos compuestos también interfieren en la síntesis de ácidos grasos y en la formación de fosfolípidos de la membrana celular, afectando a enzimas como la acetato quinasa, la fosfotransacetilasa y la acetil-CoA sintetasa. Asimismo, alteran los niveles de glutatión intracelular, desencadenando estrés oxidativo que deriva en apoptosis celular (Salehi et al. 2019). Finalmente, su acción inhibidora sobre la ARN polimerasa detiene la síntesis de ARN (Batiha et al. 2020).

6. Aspectos de seguridad de las nuevas soluciones

A pesar de que los extractos naturales y los microorganismos de biocontrol se presentan como alternativas atractivas y sostenibles a los pesticidas químicos en la agricultura, su implementación comercial debe someterse a una rigurosa evaluación regulatoria para garantizar su seguridad. Muchas de estas soluciones de origen natural se consideran más ecológicas y menos perjudiciales para la salud humana y el medio ambiente; sin embargo, eso no significa que estén exentas de riesgo. Es crucial que se realicen estudios detallados sobre sus posibles efectos tóxicos y ecológicos, a fin de asegurar que su uso sea seguro y efectivo.

Un ejemplo destacado son los extractos de plantas de la familia Aliaceae, que contienen compuestos como la alicina, conocidos por sus propiedades antimicrobianas

y fungicidas (Nakamoto et al. 2020). Si bien estos compuestos tienen un estatus GRAS (*Generally Recognized As Safe*) por parte de la FDA en ciertos contextos, como su uso en alimentos, este reconocimiento solo se refiere a su seguridad en aplicaciones alimentarias y no es suficiente para su uso en productos fitosanitarios o biopesticidas. El estatus GRAS implica que una sustancia ha sido aprobada con base en un consenso entre expertos científicos de que es segura bajo las condiciones previstas de uso, lo cual exime a los productores de realizar pruebas toxicológicas exhaustivas en el ámbito alimentario (Jackson-Davis et al. 2023). Sin embargo, en el contexto de la protección de cultivos, la situación es más compleja.

Para que un extracto natural sea aprobado como biopesticida, es necesario presentar un dossier toxicológico completo. Este debe incluir estudios sobre toxicidad aguda y crónica, para evaluar cualquier posible efecto adverso a corto o largo plazo, y genotoxicidad, que asegure que la sustancia no cause daño al material genético de los organismos expuestos. Además, se deben llevar a cabo pruebas de eco-toxicidad, que evalúen el impacto del producto en organismos no objetivo como insectos polinizadores, organismos acuáticos y la microbiota del suelo. Estos estudios son especialmente relevantes en el caso de compuestos organosulfurados. Según estudios recientes, PTSO presenta baja toxicidad aguda, subcrónica y no muestra efectos genotóxicos o mutagénicos (Mellado-García et al. 2017). Cascajosa-Lira, en un estudio de toxicidad subcrónica de 90 días en ratas, no encontró efectos adversos significativos, concluyendo que el PTSO es seguro para su uso en productos alimentarios y como aditivo para piensos (Cascajosa-Lira et al. 2020).

Otro aspecto importante a considerar es la persistencia de estos compuestos en el medio ambiente. Aunque los productos naturales suelen considerarse biodegradables, es necesario confirmar su degradación rápida y segura en diferentes condiciones ambientales, para evitar problemas de acumulación que podrían afectar a ecosistemas sensibles. Estudios realizados han indicado que compuestos como el PTSO y otros derivados del ajo y cebolla no tienden a bioacumularse, lo que reduce su riesgo a largo plazo para el ecosistema acuático y terrestre (Verdú et al. 2023).

En cuanto a los microorganismos utilizados como agentes de biocontrol, tanto la EFSA en la Unión Europea como la FDA (Administración de Alimentos y Medicamentos) en Estados Unidos imponen estrictas regulaciones para garantizar su seguridad. En la UE, uno de los criterios más importantes es que el microorganismo en cuestión debe estar presente en la QPS List (*Qualified Presumption of Safety*). Esta lista incluye microorganismos que se han considerado seguros con base en estudios previos o una larga historia de uso sin efectos adversos. Para que un microorganismo sea incluido en la QPS List, debe cumplir una serie de requisitos, como no ser patógeno para humanos, animales o plantas, no tener determinantes de virulencia, y no portar genes que confieran resistencia a antibióticos clínicamente importantes. Estos criterios son esenciales para evitar la diseminación de resistencias bacterianas que puedan comprometer el uso de antibióticos en la medicina humana y veterinaria, un aspecto crítico dado el aumento global de la resistencia a antibióticos (EFSA 2024).

Además, los microorganismos de biocontrol deben someterse a un análisis exhaustivo de su genoma para identificar posibles riesgos. Este análisis permite detectar genes que puedan estar relacionados con la virulencia o la resistencia a antibióticos (Schroeder, Brooks, and Brooks 2017).

En Estados Unidos, la EPA (Agencia de Protección Ambiental) regula los biopesticidas, y exige un proceso riguroso de registro que incluye pruebas de seguridad para humanos y el medio ambiente. La FDA, por su parte, supervisa los microorganismos que se usan en productos que pueden tener contacto con alimentos. Ambas agencias exigen que los microorganismos de biocontrol no sean patógenos para los humanos ni los animales y que no representen un riesgo para los consumidores o los trabajadores agrícolas (EPA 2024).

Dos de los microorganismos más ampliamente utilizados como agentes de biocontrol son *Bacillus* spp. y *Pseudomonas* spp., debido a sus propiedades antagonistas contra una amplia gama de fitopatógenos y su bajo riesgo para la salud humana y ambiental. Estos microorganismos han sido estudiados extensamente y aprobados bajo normativas regulatorias en muchas partes del mundo.

Bacillus subtilis y Bacillus amyloliquefaciens son ejemplos clave dentro del grupo de *Bacillus* catalogados como biopesticidas y promotores del crecimiento de plantas seguros. Estos microorganismos incluidos dentro de la QPS List son capaces de inhibir el crecimiento de hongos patógenos a través de la producción de antibióticos como surfactina, iturina y fengicina (Arguelles-Arias et al. 2009). Además, estudios toxicológicos y eco-toxicológicos han demostrado que estos microorganismos no afectan negativamente a organismos no objetivo, como insectos beneficiosos y microorganismos del suelo.

Pseudomonas fluorescens es otro agente de biocontrol ampliamente utilizado debido a su capacidad para producir metabolitos secundarios como sideróforos y antimicrobianos que suprimen el crecimiento de fitopatógenos (A. O. Ali, Awla, and Rashid 2024). Sin embargo, no está incluida en la lista QPS debido a preocupaciones sobre su potencial patogenicidad en ciertos contextos (Biaggini et al. 2015). Algunas cepas de *P. fluorescens* pueden producir toxinas o hemolisinas, lo que hace necesario una evaluación caso por caso antes de su uso en productos alimentarios o agrícolas (Sperandio et al. 2012).

En resumen, a pesar de tener un origen natural, tanto los extractos botánicos como los microorganismos de biocontrol deben cumplir con estrictos requisitos regulatorios para garantizar su seguridad y efectividad. Es necesario un enfoque integral que considere no solo los efectos tóxicos directos sobre el organismo objetivo, sino también el impacto en la salud humana, el medio ambiente, y la biodiversidad.

6.1. Productos comerciales basados en extractos vegetales y micoorganismos

El auge de soluciones más sostenibles ha llevado al desarrollo de una amplia gama de productos comerciales, adoptados por agricultores que buscan prácticas

respetuosas con el medio ambiente. Entre los productos comerciales más notables se encuentra BIO 125 Ajo® de IDAI NATURE, que ha sido extensamente aplicado en cultivos hortofrutícolas para el control de enfermedades fúngicas, nematodos y artrópodos. A nivel internacional, Garlic Barrier AG®, desarrollado en Estados Unidos, ha demostrado ser eficaz como repelente de insectos, mientras que Envirepel® aprovecha los compuestos organosulfurados del ajo para reducir plagas en cultivos hortícolas. En Europa, NemGuard® se ha posicionado como un nematicida basado en ajo, especialmente útil en cultivos de hortalizas, destacando por su capacidad de proteger los sistemas radiculares sin dañar el medio ambiente.

A pesar de que los extractos de cebolla poseen un gran potencial, los productos comerciales derivados de este vegetal son menos comunes que los de ajo. Aunque la cebolla comparte propiedades antifúngicas y bactericidas con el ajo, su desarrollo en la industria fitosanitaria sigue siendo limitado. El aceite de cebolla ha sido aprobado en la Unión Europea para controlar patógenos como *Fusarium* spp. y *Rhizoctonia* spp., pero su uso comercial es escaso. Esto resalta la necesidad de investigar más a fondo sus propiedades biocidas, que podrían ofrecer nuevas oportunidades para soluciones sostenibles en la protección de cultivos. Su potencial en el mercado agrícola aún no ha sido plenamente aprovechado, lo que indica un prometedor campo de investigación.

En el ámbito del biocontrol, especies de bacterias *Bacillus* spp. y *Pseudomonas* spp. han emergido como alternativas sólidas en el control de patógenos. Serenade®, basado en *B. subtilis*, y BlightBan® A506, que utiliza *P. fluorescens*, son ejemplos de productos microbianos que no solo controlan enfermedades, sino que también promueven el crecimiento de las plantas. Además, Serifel®, desarrollado por BASF, emplea *B. amyloliquefaciens* (cepa MBI 600) para controlar enfermedades fúngicas como *Sclerotinia* spp. y *Rhizoctonia* spp., mostrando gran eficacia en el manejo de enfermedades foliares y radiculares en cultivos tanto en invernaderos como a campo abierto. Este tipo de soluciones microbianas, junto con los extractos vegetales, forman parte de un enfoque integrado que busca reducir el uso de pesticidas químicos.

En este contexto, es crucial continuar investigando y desarrollando nuevas biosoluciones que no solo aprovechen el potencial de los extractos vegetales y los microorganismos, sino que también faciliten la transferencia de conocimiento hacia el sector biotecnológico, que se encuentra en pleno auge. Este sector juega un papel clave en la creación de productos innovadores, sostenibles y de alta eficacia, diseñados para satisfacer las necesidades de una agricultura más ecológica y resiliente.

Aunque el sector hortofrutícola está más familiarizado con estas soluciones biológicas, sectores como el del olivar aún muestran cierta resistencia a adoptar estas tecnologías. Sin embargo, las regulaciones normativas cada vez más estrictas, junto con el aumento de la superficie de cultivo de olivar en producción ecológica, indican que el desarrollo de biosoluciones adaptadas a este sector tendría un impacto significativo. La introducción de productos basados en extractos vegetales y microorganismos para el control de plagas y enfermedades en el olivar no solo contribuiría a mejorar la

sostenibilidad de la producción, sino que también respondería a la creciente demanda de productos más saludables y respetuosos con el medio ambiente en los mercados internacionales.

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JUSTIFICACIÓN Y OBJETIVOS

El olivo ha sido un pilar en la agricultura mediterránea desde tiempos ancestrales, no solo por su valor económico, sino también por su arraigo cultural y ambiental. Sin embargo, el sector olivarero enfrenta hoy una serie de desafíos sin precedentes, derivados de factores como la emergencia climática, la incidencia de nuevas enfermedades y la degradación de los recursos naturales. Estos retos requieren un replanteamiento de las estrategias productivas, promoviendo un enfoque más sostenible, eficiente y adaptado a las nuevas exigencias del mercado y del medio ambiente.

La emergencia climática ha amplificado los fenómenos de estrés hídrico, afectando la fisiología de los cultivos y comprometiendo la productividad y calidad del aceite de oliva. La irregularidad en las precipitaciones y el aumento de temperaturas han desencadenado procesos de estrés oxidativo en las plantas, reduciendo su capacidad para resistir a condiciones adversas y haciéndolas más vulnerables a patógenos. En este contexto, surge la necesidad urgente de incorporar soluciones innovadoras que refuercen la resistencia del olivo ante estos cambios climáticos extremos.

Además, la proliferación de enfermedades fitosanitarias, tanto endémicas como emergentes, añade una complejidad adicional al manejo de los cultivos. En particular, la verticilosis del olivo, causada por el hongo *Verticillium dahliae*, es una de las enfermedades endémicas más devastadoras en las zonas olivareras españolas. Este patógeno del suelo puede infectar a las plantas a través de las raíces, bloqueando los vasos conductores y provocando la muerte de ramas y, en casos severos, de todo el árbol. A pesar de los esfuerzos por controlar su propagación, la verticilosis sigue siendo una amenaza grave, sobre todo en plantaciones jóvenes y en zonas de regadío. Su manejo tradicional, basado en fitosanitarios químicos, no ha sido eficaz en la erradicación del patógeno y ha generado resistencia en algunos casos.

A esta amenaza endémica se suma la reciente propagación de la bacteria *Xylella fastidiosa*, responsable del síndrome del decaimiento rápido del olivo. Este patógeno emergente, detectado por primera vez en Europa en 2013, ha causado grandes pérdidas en olivares de Italia y España. La dificultad para controlar su diseminación a través de vectores, sumada a su virulencia, ha impulsado la búsqueda de nuevas estrategias de control, entre las que destacan el uso de bioestimulantes y de agentes de control biológico.

Frente a este escenario, el sector biotecnológico ha asumido un papel decisivo en el desarrollo de soluciones que permitan a los agricultores enfrentar estos retos mediante tecnologías más sostenibles. Los bioestimulantes y los agentes de control biológico han demostrado ser herramientas clave para mejorar la resiliencia de los cultivos frente a los estreses abióticos y bióticos, promoviendo un crecimiento saludable de las plantas y minimizando el uso de fitosanitarios químicos. La apuesta por estas tecnologías no solo responde a la creciente demanda de productos más respetuosos con el medio ambiente,

sino también a la necesidad de garantizar la sostenibilidad a largo plazo de las explotaciones olivareras.

Además, la apertura del mercado hacia estas tecnologías sostenibles está respaldada por el marco regulatorio europeo, como el REGLAMENTO (UE) 2019/1009, que establece directrices para la comercialización de productos fertilizantes y bioestimulantes. Este reglamento fomenta la entrada de nuevos productos basados en principios activos naturales que no solo mejoran la productividad, sino que también cumplen con altos estándares de seguridad y eficiencia, aportando valor tanto al agricultor como al consumidor. En este contexto, los principios activos de origen vegetal y los microorganismos juegan un papel clave en la creación de productos innovadores con aplicaciones en el control de patógenos y la mejora del rendimiento de los cultivos.

En este marco, la investigación y desarrollo (I+D) orientados a la transferencia tecnológica hacia el sector industrial cobran una relevancia fundamental, especialmente cuando se establecen alianzas estratégicas con expertos y centros especializados en la resolución de problemáticas emergentes que afectan a la sostenibilidad agrícola. Un ejemplo destacado es la amenaza creciente de *X. fastidiosa*, un patógeno que ha tenido un impacto devastador en cultivos clave como el olivar. Para abordar este desafío, se ha establecido una colaboración con el equipo del Dr. Pasquale Saldarelli, del *Institute for Sustainable Plant Protection, National Research Council* (CNR), Bari, Italia, quienes son referentes mundiales en la investigación de este patógeno. La doctoranda ha llevado a cabo una estancia en este centro con el objetivo de ampliar su conocimiento sobre la biología de *Xylella*, y contribuir al desarrollo de biosoluciones innovadoras que puedan implementarse de manera efectiva en los sistemas agrícolas afectados. En la misma línea, se ha establecido una colaboración con el grupo del Dr. Juan A. Navas Cortés del Instituto de Agricultura Sostenible (IAS-CSIC), Córdoba, referente mundial en la verticilosis del olivar. Estas colaboraciones han permitido abordar de manera integral las principales enfermedades que afectan a este cultivo.

Asimismo, garantizar la sostenibilidad de estas innovaciones no solo implica su eficacia en términos de productividad, sino también la protección del ecosistema circundante, particularmente de los polinizadores, que desempeñan un rol crucial en la biodiversidad y en la estabilidad de los ecosistemas agrícolas. En este sentido, se ha trabajado en estrecha colaboración con el Centro de Investigación Apícola Marchamalo de Guadalajara, donde la doctoranda ha realizado estancias de investigación en el marco de este doctorado, con el objetivo de evaluar rigurosamente la seguridad de las biosoluciones para polinizadores. Estas evaluaciones son esenciales para asegurar que los tratamientos no generen efectos negativos en las poblaciones de insectos polinizadores, lo que garantiza un enfoque integral y sostenible en la protección del olivar.

Este enfoque colaborativo y multidisciplinario, que integra la investigación aplicada, la cooperación con centros de excelencia, y la participación de actores clave del sector como la cooperativa DCOOP, el mayor productor de aceite de oliva del mundo,

resulta esencial para que las innovaciones biotecnológicas lleguen al mercado de manera eficiente y eficaz. La interacción entre centros de investigación, empresas y agricultores permite no solo validar los resultados científicos en condiciones reales de producción, sino también adaptar las soluciones a las necesidades concretas del sector olivarero, asegurando su implementación inmediata y sostenible.

Como empresa de base tecnológica, DMC Research se especializa en la investigación y desarrollo de compuestos bioactivos de origen natural, orientados a mejorar la protección y el rendimiento de los cultivos agrícolas, con un enfoque particular en productos compatibles con la agricultura ecológica. Esta empresa ha sido clave en la búsqueda de nuevas soluciones sostenibles para el sector agrícola, integrando el uso de extractos vegetales y microorganismos antagonistas de patógenos. En el marco de este proyecto de tesis, DMC Research colabora estrechamente en la formación de doctores industriales, como parte del programa de Ayudas para la Formación de Doctores en Empresas "Doctorados Industriales", lo que permite una transferencia directa del conocimiento científico al ámbito empresarial.

El Doctorado Industrial adquiere un papel crucial, ya que tiene como objetivo principal fomentar la investigación aplicada y su transferencia directa al sector productivo. Este tipo de doctorado permite que los conocimientos científicos generados en el ámbito académico se integren en las empresas, facilitando la resolución de problemas específicos de la industria y acelerando la innovación tecnológica en sectores clave como el olivarero. Además, la orientación hacia la transferencia tecnológica, que caracteriza a los doctorados industriales, asegura que los resultados no solo queden en el ámbito de la investigación, sino que sean implementados de manera efectiva en condiciones reales de cultivo. Esta transferencia no solo aumenta la capacidad de innovación de las empresas, sino que también asegura que los avances científicos se traduzcan en mejoras concretas en la producción, haciendo a las empresas más competitivas y sostenibles.

Este proyecto de tesis, desarrollado en colaboración con el Departamento de Fisiología Vegetal de la Universidad de Granada, se ha alineado con grandes proyectos de investigación europeos y nacionales, como el proyecto BIOVEXO, dentro del programa Horizonte 2020, que tiene como objetivo el biocontrol de *Xylella fastidiosa* y su vector en olivos, y el proyecto SALUDOLIVAR, de la convocatoria de Grupos Operativos Supraautonómicos del MAPAMA, que busca desarrollar estrategias innovadoras para el control de plagas y enfermedades endémicas, como la verticilosis, y emergentes, como *Xylella fastidiosa*. Ambos proyectos refuerzan la importancia de abordar estos retos desde una perspectiva multidisciplinar, integrando la investigación de nuevos compuestos bioactivos con la validación de su eficacia en campo, y promoviendo soluciones que sean transferibles y comercializables, garantizando así la sostenibilidad del sector olivarero en un entorno de creciente presión climática y sanitaria.

La presente hipótesis plantea que la aplicación de extractos organosulfurados de cebolla (*Allium cepa*) y cepas bacterianas seleccionadas por su capacidad bioestimulante

y de control biológico, incrementará la resistencia fisiológica del olivo frente a condiciones de estrés abiótico, como la sequía, así como frente a agresiones bióticas provocadas por patógenos como *X. fastidiosa* y *V. dahliae*. Se postula que estos compuestos organosulfurados, ricos en principios bioactivos con propiedades funcionales como tiosulfinatos y tiosulfonatos, serán capaces de modular respuestas fisiológicas y bioquímicas clave en el olivo, mejorando su capacidad de respuesta ante el estrés inducido por factores externos. Asimismo, se espera que los extractos de *Allium* ejerzan un efecto sinérgico sobre la inhibición del crecimiento y propagación de los fitopatógenos, reduciendo así la dependencia de fitosanitarios de síntesis química. Se anticipa, además, que la utilización de estos compuestos naturales no tendrá un impacto negativo sobre los polinizadores, garantizando la seguridad ambiental y la compatibilidad con los principios de la agricultura ecológica. Por tanto, la hipótesis plantea que la implementación de estas alternativas de origen natural no solo potenciará la resiliencia del olivo ante el estrés climático y las enfermedades, sino que también contribuirá a un modelo agrícola más sostenible, alineado con las regulaciones medioambientales y las exigencias del mercado actual.

En este contexto, se establece como objetivo general de la presente tesis la "***Caracterización y evaluación de la capacidad protectora y bioestimulante compuestos organosulfurados de Allium cepa y cepas bacterianas en cultivos de olivo***". Este objetivo se alinea con la necesidad de desarrollar soluciones sostenibles que permitan a los agricultores enfrentar los retos fitosanitarios y climáticos que actualmente amenazan la rentabilidad y la sostenibilidad del sector olivarero.

Para alcanzar el objetivo general planteado, se han definido los siguientes objetivos específicos:

1. Caracterización inicial de extractos de *Allium cepa*, evaluando su composición química detallada con un enfoque en los compuestos organosulfurados y su potencial bioactivo, destacando sus propiedades antifúngicas y antibacterianas.
2. Evaluación funcional del efecto de los extractos sobre la fisiología de la planta en condiciones controladas, utilizando plantones de olivo de la variedad Picual.
3. Evaluación de la eficacia antimicrobiana de compuestos organosulfurados de aliáceas en modelos experimentales para el control de *Xylella fastidiosa*, *Verticillium dahliae* y otros patógenos relevantes del olivo, analizando su capacidad para inhibir el crecimiento y la propagación de estos patógenos tanto en laboratorio como en invernaderos experimentales.
4. Estudio de la toxicidad de los extractos de *Allium cepa* en un modelo con abejas melíferas (*Apis mellifera*), para garantizar la seguridad de los compuestos y evaluar su impacto en polinizadores, asegurando que no afecten negativamente a estos insectos beneficiosos para el ecosistema agrícola.

5. Desarrollo y optimización de formulaciones prototipo de los principios activos seleccionados en productos adecuados para su aplicación foliar o mediante fertiirrigación.

6. Ensayos de eficacia en campo, donde se probarán las formulaciones seleccionadas en fincas comerciales, evaluando su efectividad en olivos adultos bajo situaciones de estrés abiótico y biótico, en condiciones reales de cultivo.

7. Caracterización genómica, transcriptómica y funcional de cepas microbianas con potencial bioestimulante y de control biológico, evaluando su capacidad para inhibir fitopatógenos del olivo y mejorar su desarrollo. Se analizarán el genoma y el transcriptoma para identificar los mecanismos moleculares clave implicados en la resistencia del olivo a enfermedades y en la promoción de su crecimiento bajo condiciones de estrés.

METODOLOGÍA GENERAL

1. Compuestos organosulfurados y extracto de cebolla

Se obtuvieron fracciones estandarizadas de PTS y PTSO a partir de cebollas no aptas para consumo de acuerdo con la metodología descrita por Hu et al., 2002. Estos compuestos organosulfurados (OSCs) se emplearon para los ensayos de evaluación de actividad antimicrobiana in vitro, higienización de sustrato y toxicidad en abejas. Para los ensayos realizados en olivos crecidos en cámara climática, en fincas experimentales y olivares comerciales se utilizó un formulado que contenía un 85% del extracto de bulbo de cebolla con un 50% de tiosulfinatos y tiosulfonatos derivados de la propiina. El producto se diluyó en agua a una concentración de 500 mg/L para su aplicación.

El extracto de cebolla rico en OSCs se obtuvo a partir de bulbos de cebolla de baja calidad. Las cebollas se pelaron, se lavaron en una solución acuosa de ácido acético al 1%, y se trituraron. La pasta resultante se maceró en una solución acuosa de etanol al 30% a temperatura ambiente durante 24 horas y posteriormente se filtró para eliminar los sólidos. La solución filtrada se sometió a hidrodestilación para concentrar los OSCs. El destilado fue extraído utilizando acetato de etilo en proporción (1:1). La fase orgánica, que contenía los OSCs de interés, se secó con sal de sulfato anhidro y luego se filtró. El solvente se eliminó bajo presión reducida a 40°C, obteniendo el extracto final de cebolla, el cual fue analizado por cromatografía de gases-espectrometría de masas (GC/MS) para confirmar su composición en OSCs.

1.1. Caracterización del extracto mediante GC/MS

Se utilizó un Cromatógrafo de Gases 7820A acoplado a un Espectrómetro de Masas 5977B. El método se basó en un procedimiento publicado recientemente (Pastor-Belda et al. 2020). La columna seleccionada fue una Agilent ultra-inerte DB-5MS UI con un liner de trampa de ID de 4 mm. Los parámetros del método fueron los siguientes: se utilizó helio (99,99%) como gas portador a un caudal de 1,0 mL/min; el volumen de inyección fue de 1 μL en modo split-less; la temperatura del inyector fue de 280°C. El programa de temperatura del horno consistió en una etapa isotérmica inicial a 50°C durante 1 minuto, seguida de un aumento a 160°C a 25°C/min, y luego a 250°C a 30°C/min, donde la temperatura se mantuvo durante 1,3 minutos adicionales. Los parámetros del espectrómetro de masas fueron los siguientes: voltaje de ionización de 70 eV, temperatura de 230°C, temperatura del cuadrupolo de 150°C, temperatura de la línea de transferencia de 300°C, rango de escaneo de 50 a 550 amu (unidades de masa atómica), y un retardo de solvente de 4 minutos. Los compuestos del cromatograma de iones totales se identificaron comparándolos con la biblioteca de espectros de masas NIST.

1.2. Cuantificación de OSCs por HPLC-UV

Se empleó un equipo HPLC Agilent Infinity 1260. El método cromatográfico se basó en un procedimiento previamente publicado (Abad et al. 2015). Se usó una columna Agilent Zorbax Eclipse Plus y todos los análisis se realizaron a 25°C. Las fases móviles

utilizadas fueron MeCN (A) y una solución acuosa de ácido perclórico 30 mM (B). El volumen de inyección fue de 10 µL y el caudal de 0,85 mL/min. La longitud de onda del detector se fijó en 200 nm. Las muestras se inyectaron directamente sin dilución, después de ser filtradas a través de un filtro de nailon de 0,22 µm. Se utilizó un estándar comercial para la curva de calibración con cinco niveles de concentración diferentes, que variaban entre 80 y 500 mg/L, cada uno preparado por duplicado e inyectado por triplicado.

2. Microorganismos de control biológico

Se utilizó la cepa de *Bacillus* sp. GG-22, aislada de la filosfera de una planta de tomate de una finca ecológica de Adra (Almería), y la cepa endófita y psicrófila de *Pseudomonas lactis* PV8 aislada de plantas cultivadas del Parque Natural de Sierra Nevada (Granada).

3. Organismos fitopatógenos

Los microorganismos fitopatógenos fueron obtenidos de la Colección Española de Cultivos Tipo (CECT), la Colección Alemana de Microorganismos y Cultivos Celulares (DSMZ), la colección de fitopatógenos de DMC Research y la colección de cultivos del Departamento de Protección de Cultivos del Instituto de Agricultura Sostenible, Consejo Superior de Investigaciones Científicas (Córdoba, España). Las cepas, enumeradas en la Tabla 1, se cultivaron bajo las condiciones específicas indicadas por la colección de origen. Los individuos adultos del pulgón del algodón (*Aphis gossypii* Glover, Hemiptera: Aphididae) fueron proporcionados por el Centro Tecnológico TECNOVA (Almería, España).

4. Estudios *in vitro*

4.1. Evaluación de la actividad antimicrobiana de los OSCs

La actividad antimicrobiana de PTS y PTSO se evaluó mediante diferentes procedimientos experimentales. Para la evaluación de la actividad de PTS y PTSO en medio sólido, se utilizó el método de difusión en disco (A. W. Bauer et al. 1966; Calvo and Asensio 1999). Se inocularon placas de agar con una suspensión microbiana ajustada a 10^6 UFC/mL, de forma que el crecimiento tras la incubación fuera confluente. En el centro de la placa de agar se colocaron discos de celulosa estériles de 6 mm impregnados con 20 µl de PTS o PTSO a concentraciones de 2,5 a 50 µg/µL. Tras el periodo de incubación se midió el diámetro de la zona de inhibición del crecimiento. La actividad antimicrobiana de la fase gaseosa de estos compuestos se evaluó utilizando el mismo método con modificaciones. En este caso, los discos de celulosa impregnados se colocaron en el centro de las tapas de las placas de Petri. Las placas se colocaron boca abajo, evitando el contacto directo entre los compuestos organosulfurados y los microorganismos (Sorlozano-Puerto et al. 2021). La determinación de la Concentración Mínima Bactericida/Fungicida (CMB/F) se llevó a cabo mediante el método de microdilución en caldo, conforme a las directrices del CLSI (*Clinical and Laboratory Standards Institute*) (CLSI 2017; 2018). Para cada compuesto, se prepararon diluciones seriadas en caldo nutritivo con un factor 1:2, desde una concentración inicial de 10.000

μg/mL hasta llegar a 9,76 μg/mL. Cada dilución fue inoculada con una suspensión microbiana ajustada a 10^5 UFC/mL, y se incubó durante 24h a temperatura ambiente. El crecimiento microbiano se evaluó sembrando las diluciones en placas de agar, y la CMB/F se definió como la concentración más baja de PTS/PTSO en la que no se observó crecimiento. Como control de crecimiento se empleó caldo nutritivo inoculado con el microorganismo. Finalmente, en el caso de los hongos y oomicetos, se llevó a cabo un estudio adicional para evaluar la influencia de los compuestos en el desarrollo micelial. Para ello, se prepararon placas de agar suplementado con PTS y PTSO, donde se cultivaron los hongos u oomicetos patógenos. Como control de crecimiento, se emplearon placas de agar sin suplementar. El desarrollo micelial de las cepas patógenas en las placas suplementadas se comparó con el control, y se calculó el porcentaje de inhibición del crecimiento (Ma et al. 2020; Liu et al. 2017).

4.2. Evaluación de la actividad antimicrobiana de los OSCs frente a *X. fastidiosa*

El efecto de OSCs en el crecimiento de *X. fastidiosa* se evaluó mediante el método de difusión en pocillos de agar con algunas modificaciones (Zicca et al. 2020). Las concentraciones evaluadas fueron 50; 25; 10; 5 y 2,5 μg/μL. Se colocaron 3 gotas de 20 μL de suspensión bacteriana a 10^8 UFC/mL en la parte superior de la placa de Petri, que se dejaron fluir hacia el lado opuesto de la placa para generar 3 filas paralelas de cultivo. Tras 24 h de incubación, se realizó un pocillo de 8 mm en la parte superior de las filas de *X. fastidiosa*, donde se depositaron 200 μL del compuesto. Tras el periodo de incubación se midió el área de inhibición.

4.3. Evaluación de la actividad frente a áfidos

Se evaluó la toxicidad por contacto y capacidad repelente de PTS y PTSO a 5; 2,5; 1 y 0,5 μg/μL. En ambos ensayos se empleó agua como control negativo. Como estándar de toxicidad se empleó Decis® Protech en el ensayo de toxicidad por contacto según las indicaciones en la ficha técnica, y DEET a 2.000 μg/mL en el ensayo de repelencia (Jiang et al. 2016).

La toxicidad por contacto de PTS y PTSO se evaluó mediante el método de inmersión de hojas (Sayed et al. 2022). Se sumergieron recortes circulares de hojas de calabacín en las soluciones de tratamiento y control durante 5 segundos, se secaron al aire y se colocaron en placas de Petri. Se transfirieron 24 pulgones a cada recorte de hoja tratada utilizando un pincel. Las placas se mantuvieron en una cámara climática a 25 ± 1°C, 75 ± 5% de humedad relativa y un fotoperiodo luz:oscuridad de 14:10, y se registró la mortalidad a las 24 horas (Zhou et al. 2016). La capacidad repelente se evaluó mediante un ensayo de elección en placa de Petri (Semerdjieva et al. 2021). Se sumergieron recortes circulares de hojas de calabacín en las soluciones durante 5 segundos y se dejaron secar a temperatura ambiente, como en el ensayo anterior. En cada placa de Petri se colocaron una hoja tratada y una hoja sumergida en agua sobre un disco de papel filtro húmedo (Zheljazkov et al. 2021). Se depositaron 24 adultos en cada placa de Petri utilizando un pincel. Las placas se mantuvieron en las condiciones previamente indicadas. El efecto se observó después de 24 horas y se expresó como porcentaje de repelencia.

Tabla 1. Bacterias, hongos y oomicetos fitopatógenos utilizados en el estudio, junto con sus referencias y fuente de aislamiento.

	Especie	**Referencia**	**Fuente de aislamiento**
Bacterias	*Pseudomonas savastanoi*	CECT 5023	Olivo
	Agrobacterium tumefaciens	DMC 47	Olivo
	Xylella fastidiosa ST53 subsp *pauca*	CFBP 8402	Olivo
	Erwinia persicina	DSM 19328	Tomate
	Xanthomonas campestris	CECT 97	Col de Bruselas
	Pseudomonas syringae	DMC 15	Melocotonero
	Clavibacter michiganensis	CECT 790	Tomate
	Agrobacterium tumefaciens	CECT 4119	Manzano
Hongos /oomicetos	*Verticillium dahliae* [1]	V136I	Olivo
	Verticillium dahliae [2]	V1235I	Olivo
	Verticillium dahliae [2]	V1266I	Olivo
	Fusarium oxysporum	DMC 03	Olivo
	Pythium spp.	DMC 11	Olivo
	Phyllosticta spp	DMC 10	Olivo
	Geotrichum candidum	DSM 1240	Tomate
	Alternaria alternata	CECT 2662	*Lycopersicon* spp
	Fusarium oxysporum f. sp. *Cubense*	DMC 02	Platanero
	Fusarium graminearum	DSM 1095	Maíz
	Phytophthora cinnamomi	CECT 20186	Raíz de aguacate
	Penicillium expansum	DMC 01	Manzano
	Penicillium digitatum	DMC 07	Naranjo dulce

[1] Patotipo defoliante, [2] patotipo no defoliante.

4.4. Evaluación de la capacidad de desinfección de sustrato de los OSCs

Se llevaron a cabo dos ensayos para evaluar la capacidad de una mezcla de los compuestos organosulfurados PTS y PTSO, en proporción 1:1 (w/w), de reducir la densidad de patógenos en suelo. En el primer estudio, se utilizó un sustrato universal esterilizado en autoclave a 121°C durante 20 minutos, que se inoculó con una suspensión de *V. dahliae* V136I ajustada a 10^7 CFU/mL, de forma que la concentración final en suelo fuese 10^5 CFU/g. El tratamiento se aplicó 72 horas después de la inoculación a concentraciones de 100, 500 y 1.000 μg/g. La densidad de *V. dahliae* en suelo se cuantificó 1, 2, 3 y 4 días después del tratamiento. Para ello, se diluyeron 25 g de sustrato en 225 mL de agua de peptona tamponada, se prepararon diluciones 1:10 y se sembraron en medio sólido (Deberdt et al. 2012; Falade et al. 2016).

El segundo ensayo se realizó en sistema de microcosmos (Del Papa et al. 2003) con suelo proveniente de un olivar localizado en Linares (Jaén, España), propiedad de la cooperativa DCOOP (Antequera, Málaga, España), donde no se habían aplicado productos antimicrobianos durante los últimos dos años. El suelo se caracterizó como franco arenoso, con un pH moderadamente básico de 8,71. Su composición incluyó 67,1 % de arena, 21,5 % de arcilla y 11,4 % de limo. Contenía un 1,13 % de materia orgánica y presentó una Capacidad Máxima de Retención de Agua de 0,414 g H_2O por g de materia seca. El suelo fue esterilizado mediante autoclave (Kelsey et al. 2010), y se inoculó con

suspensiones de *A. tumefaciens* y *F. oxysporum* a 10^9 CFU/mL, de forma que la concentración final en suelo fuese 10^7 CFU/g. Se evaluaron dos concentraciones de la mezcla de PTS y PTSO, 100 μg/g (50 μg/g cada uno) y 500 μg/g (250 μg/g cada uno); y se comparó la eficacia de un tratamiento basado en una única aplicación frente a la eficacia de un tratamiento consistente en 3 aplicaciones de la misma dosis separadas en el tiempo. Como estándar de toxicidad se empleó Metam sodium a 60 μg/g (Li et al. 2017). El tratamiento comenzó a aplicarse 4 días después de la inoculación. En los grupos experimentales que recibieron 3 aplicaciones, las aplicaciones restantes se realizaron 10 y 30 días después de la primera aplicación. La población microbiana se cuantificó 1, 2, 4, 7, 11, 15, 31 y 45 después de la primera aplicación. Para ello, se diluyeron 25 g de sustrato en 225 ml de agua de peptona tamponada, se prepararon diluciones 1:10 y se sembraron en medio sólido (Deberdt et al. 2012). Finalmente, la concentración de PTS y PTSO en el suelo se determinó mediante Cromatografía Líquida de Alta Eficiencia con detección ultravioleta (HPLC-UV), utilizando un sistema Agilent 1260 Infinity, una columna Zorbax Eclipse Plus RRHD (50 x 2.1 mm, 1.8 μm) operando a 25°C, y el gradiente y fase móvil descrito por Sorlozano-Puerto et al. 2021. El método incluyó la extracción de PTS y PTSO de 50 g de suelo mediante la adición de 100 mL de acetona, seguida de homogeneización en vórtex durante 1 minuto y sonicación durante 10 minutos. El sobrenadante fue filtrado, y se repitió el proceso de extracción con 20 mL de acetona adicionales. El sobrenadante de ambas extracciones fue evaporado hasta sequedad en un rotavapor al vacío y reconstituido en 10 mL de metanol, antes de ser filtrado y analizado por HPLC. Las concentraciones de PTS y PTSO se determinaron utilizando una curva de calibración basada en estándares de ambos compuestos.

4.5. Estudio de la capacidad antagonista de microorganismos de control biológico

La capacidad antagonista de las cepas de control biológico frente a bacterias fitopatógenas se evaluó mediante el método descrito en el apartado 3.4.1. En este caso, en el pocillo se depositaron 200 μL de suspensión de la cepa de biocontrol a 10^8 UFC/mL. La actividad frente a hongos y oomicetos se evaluó mediante cultivo dual. La cepa de biocontrol se sembró en un lado de la placa de Petri, mientras que en el lado opuesto se colocó un tapón de agar de 6 mm de diámetro obtenido de un cultivo del microorganismo patógeno en medio sólido. Como control se emplearon placas con tapón de agar, pero sin la cepa de biocontrol. Las placas se incubaron durante 14 días. Durante este periodo de tiempo se midió el crecimiento micelial de las placas control y co-cultivo.

5. Caracterización genómica de la cepa *Bacillus* sp. GG-22

El ADN genómico fue extraído de un cultivo líquido puro de una metodología previamente descrita (Martín-Platero et al. 2007). La secuenciación del genoma se realizó con la plataforma Illumina HiSeq4000. Para el ensamblaje y la anotación se empleó Bacflux, un flujo de trabajo desarrollado en el Austrian Institute of Technology (AIT) (Antonielli et al. 2024). Con Bacflux se realizó el preprocesamiento de lecturas, ensamblaje, control de calidad, evaluación de contaminación y completitud, análisis

taxonómico, anotación, búsqueda de genes de resistencia antimicrobiana, y la detección de plásmidos y profagos. El análisis de Identidad Promedio de Nucleótidos (ANI) se realizó comparando todos los genomas disponibles en NCBI GenBank hasta enero de 2024. Bacflux integra varias plataformas para la anotación y el análisis funcional. La predicción de genes y la identificación de secuencias codificantes se realizaron con Prokka y Bakta, mientras que los genes fueron asignados a categorías funcionales COG utilizando eggNOG. Los *clusters* de genes relacionados con la producción de metabolitos secundarios se predijeron mediante la herramienta en línea antiSMASH. Para la predicción de resistencia antimicrobiana (AMR), las lecturas filtradas se mapearon contra la base de datos CARD usando BBMap, y los *contigs* se examinaron en busca de genes de resistencia antimicrobiana y virulencia con ABRicate. La presencia de plásmidos se investigó con Platon y los *contigs* fueron analizados en busca de secuencias virales utilizando VirSorter2. Finalmente, el archivo FASTA de secuencias proteicas generado fue cargado en PGPT-Pre en PlaBAse, una herramienta de predicción de rasgos promotores del crecimiento vegetal en bacterias (Patz et al. 2021).

6. Estudios *in planta*

6.1. Evaluación de la actividad bioestimulante de los OSCs

Se evaluó la capacidad bioestimulante del extracto de cebolla en olivos de la variedad Picual en tres entornos experimentales: cámara climática, finca experimental y olivares comerciales.

6.1.1. Ensayo en cámara climática

En el ensayo en cámara climática, 60 plantones de olivo de 1 año de edad y aproximadamente 50 cm de altura, se dividieron en 3 grupos experimentales: control, tratamiento por aspersión foliar y tratamiento por riego. Las plántulas se mantuvieron bajo condiciones no estresantes, con un fotoperiodo de 8 horas de luz y 16 horas de oscuridad, y temperaturas de 25/12°C (máxima/mínima). El extracto de cebolla se diluyó a 500 mg/L y se aplicó 150 mL por planta vía riego, mientras que para la aspersión foliar se utilizaron 3 mL por planta, cubriendo toda la parte aérea. Se realizaron tres aplicaciones: al inicio del ensayo, a los 15 días y a los 30 días. Se realizaron muestreos destructivos de hojas y raíces de 5 plántulas de olivo en 4 momentos diferentes: 24 horas después de la primera aplicación del tratamiento (T1), a los 15 días (T2), a los 30 días (T3) y a los 60 días (T4). En los días en que coincidían el muestreo y la aplicación del tratamiento (T2 y T3), primero se realizó el muestreo y luego se aplicó el tratamiento al resto de las plántulas. Las hojas y raíces se congelaron en nitrógeno líquido, se pulverizaron utilizando un molinillo y se almacenaron inmediatamente a -80 °C hasta su análisis. Para cada tiempo de muestreo, se determinó el contenido de MDA (malondialdehído) y se realizó el análisis FRAP (capacidad antioxidante de reducción férrica). Además, después del último muestreo, se evaluaron la biomasa foliar y la longitud de las raíces. El contenido en MDA se determinó siguiendo el procedimiento TBARS (Heath and Packer 1968). Se maceraron 300 mg de cada muestra foliar con 2 mL de TCA (ácido tricloroacético) 20% (p/v) y 0,4 mL de BTH (butilhidroxitolueno) de 4%

(p/v), centrifugando a 12,000 rpm durante 10 minutos. El sobrenadante se mezcló con un 0.5% (p/v) de TBA (ácido tiobarbitúrico) en TCA al 20% (1:3) y la mezcla se incubó a 95°C durante 30 minutos en un baño de agua. Las muestras se enfriaron en hielo para detener la reacción y luego se centrifugaron nuevamente a 12.000 rpm durante 10 minutos. La absorbancia del sobrenadante se midió a 532 nm y 600 nm. Se generó una curva estándar utilizando concentraciones conocidas de MDA, y los resultados se expresaron en nmol de MDA/g de muestra. La determinación de MDA en raíces se realizó siguiendo el mismo método, utilizando en este caso 30 mg de muestra liofilizada. Para el ensayo FRAP, se maceraron 300 mg de cada muestra de hoja con acetona al 80% (v/v) y se agitaron durante 15 minutos a 4°C en oscuridad. Después de centrifugar a 5.000 rpm a 4°C durante 15 minutos, se añadió el reactivo FRAP al sobrenadante. Las muestras se incubaron durante 30 minutos a 37°C en oscuridad y se midió la absorbancia a 595 nm. La cantidad de Fe^{2+} en las muestras se calculó a partir de una curva estándar con concentraciones conocidas. Los resultados se expresaron en μg de Fe^{2+}/mg de muestra. El mismo protocolo se utilizó para el ensayo FRAP en tejidos de raíz, empleando 20 mg de muestras liofilizadas (Benzie and Strain 1996).

6.1.2. Ensayo en finca experimental

Este ensayo se llevó a cabo entre marzo y junio de 2021 en colaboración con Neval S.L., en una finca experimental ubicada en Xilxes, Valencia, España (30N 740759.99 4406967.05 UTM WGS84). El suelo fue clasificado como franco, con un contenido de 45% de arena, 34% de limo, 21% de arcilla y 1,21% de materia orgánica. La actividad bioestimulante se evaluó en olivos de 4 años completamente defoliados debido al estrés abiótico por trasplante. Los olivos fueron trasplantados desde macetas al suelo, que no había recibido tratamiento previo, con un marco de plantación de 3.5 × 2 m. Tras un mes de aclimatación, se aplicó el extracto de cebolla a 500 mg/L mediante pulverización foliar con mochila (700 L/ha) y riego (10 L/árbol), mientras que el grupo control recibió únicamente agua. Se realizaron tres aplicaciones a intervalos de 21 días, siguiendo un diseño aleatorizado de 3 réplicas por cada grupo experimental con 3 olivos cada una. El régimen de riego semanal se mantuvo constante para evitar estrés hídrico. La longitud y el número de brotes se midieron 20 días después de la segunda y tercera aplicación.

6.1.3. Ensayos en olivares comerciales

El tercer ensayo se realizó en dos olivares comerciales ubicados en Linares, Jaén (30N 444908.38 4209274.77 UTM WGS84) y Santaella, Córdoba (30N 335550.44 4153847.83 UTM WGS84), propiedad de la cooperativa DCOOP. El suelo del olivar de Linares era franco arenoso (68% arena, 18% limo y 14% arcilla) con 1,13% de materia orgánica, mientras que el de Santaella era arenoso franco (78% arena, 13% limo y 9% arcilla) con 0,8% de materia orgánica. Ambos olivares eran intensivos con riego. Los olivos de Linares, bicentenarios, tenían un marco de plantación de 10 × 10 m y recibían 12 horas de riego cada 4 días, mientras que los olivos de 25 años en Santaella, con un marco de 7 × 7 m, recibían 3 horas de riego diario de abril a octubre. Ambos olivares presentaban una alta incidencia de la verticilosis y síntomas de síndrome de

decaimiento. El ensayo, de 14 meses de duración, se realizó entre abril de 2021 y junio de 2022. Los olivares se dividieron en dos áreas: tratamiento y control. El tratamiento se aplicó en primavera a través del sistema de riego (5 L/ha) con tres aplicaciones a intervalos de un mes, comenzando en abril de 2021 y repitiendo en la primavera de 2022. Se realizaron muestreos de frutos en noviembre de 2021 y de hojas en junio de 2022, dos semanas después de la última aplicación. En cada área se muestrearon 20 olivos seleccionados al azar, ubicados en la zona central. Se recolectaron 20 aceitunas por árbol, 5 de cada punto cardinal, tanto de las ramas superficiales como de la zona interna, obteniendo un total de 400 aceitunas por área y olivar. Para las hojas, se muestrearon 30 olivos en cada área. Se recogieron al menos 100 hojas por árbol, de los 4 puntos cardinales y de ramas externas e internas.

6.1.3.1. Análisis de fruto

Los frutos de 5 olivos se analizaron en conjunto, agrupados según la proximidad entre los árboles. Se determinó el peso y volumen de cada grupo de 100 aceitunas. El volumen se midió utilizando el principio de Arquímedes, colocando las aceitunas en 500 ml de agua y midiendo el desplazamiento. El contenido de agua se determinó gravimétricamente a partir de 30 g de aceitunas molidas, tras secarlas en un horno de aire a 105°C durante 24 horas, según la norma UNE 55031:1973 (Asociación Española de Normalización y Racionalización (AENOR) 1973). El contenido graso se midió utilizando la muestra seca de la determinación de humedad, siguiendo el método Soxhlet, conforme a la norma UNE 55030:1961 (Asociación Española de Normalización y Racionalización (AENOR) 1961). La grasa se extrajo con hexano en un extractor Soxhlet durante 6 h. Posteriormente, se eliminó el solvente con un evaporador rotatorio a 40°C, y el aceite residual se secó en un horno a 60°C hasta obtener un peso constante.

6.1.3.2. Análisis foliar

Las muestras de hojas de cada árbol se numeraron y analizaron por separado. Las hojas se congelaron en nitrógeno líquido, se pulverizaron con un molinillo y se almacenaron a -80°C. La determinación de MDA y el FRAP se realizaron siguiendo los protocolos descritos en la sección 3.6.1. La prolina se analizó a partir de un extracto de aminoácidos, obtenido empleando una mezcla de etanol/cloroformo/agua (12/5/1; v/v/v). Se usaron norvalina y sarcosina como estándares internos. El extracto fue centrifugado a 3.500 x g durante 10 minutos a 4°C, y el sobrenadante se separó en fases acuosa y de cloroformo añadiendo HCl 0.1 N y cloroformo. Tras una segunda centrifugación a 3.500 x g durante 5 minutos, la fase acuosa se secó con flujo de nitrógeno y se almacenó a -20°C en atmósfera inerte. Las muestras secas se resuspendieron en 0,9 mL de HCl 0,1 N, se sonicaron y se filtraron en filtro de nylon (0,22 μm). La derivatización de los aminoácidos se realizó utilizando quimiotipos de o-ftalaldehído (OPA) y cloroformato de fluorenilmetilo (FMOC). La prolina, junto con el resto de aminoácidos se cuantificaron por HPLC (Agilent 1260 Infinity) con una columna ACE 5 C18-PFP de 4.6 mm x 250 mm, utilizando un fluorómetro con longitudes de excitación y emisión de 340 y 450 nm (0–15 min), y de 260 y 325 nm (16–33 min) (Palma et al. 2019).

Se eluyeron a un flujo de 1 mL/min usando un gradiente de elución con tampón de acetato de sodio 25 mM pH 6.8 (A) y una mezcla de acetonitrilo/metanol/agua (45/45/10, v/v/v) (B). El perfil del gradiente, expresado como (t [min]; %A), fue: (0; 80%), (20; 40%), (24; 40%), (26; 0%), (31; 0%) y (33; 80%). Los resultados se expresaron en pg de prolina/g de muestra.

6.2. Eficacia en la supresión de la verticilosis en olivos

La evaluación de capacidad de los OSCs de cebolla para controlar la verticilosis se realizó en olivos de la variedad Picual a través de ensayos llevados a cabo en cámara climática y olivares comerciales.

6.2.1. Ensayo en cámara climática

En el ensayo en cámara climática se utilizaron plantones de olivo de 7 meses cultivados en suelo artificialmente infestado con el patotipo defoliante de *V. dahliae* V136I (Jiménez-Díaz et al. 2011; Jiménez-Fernández et al. 2016). El suelo infestado fue tratado con dos dosis de la mezcla PTS/PTSO (250 y 500 μg/mL) y almacenado en bolsas de plástico selladas durante 2 días. Posteriormente, las plantas se trasplantaron a macetas de 1.500 mL con este suelo. Las plantas control se trasplantaron a suelo no infestado. Una semana después del trasplante, la mitad de los olivos sembrados en suelo tratado no recibieron tratamiento, mientras que la otra mitad recibió 100 mL de PTS/PTSO (250 y 500 μg/mL) por riego. Se utilizaron 10 plantas por grupo experimental, con diseño completamente randomizado. Las plantas e incubaron en una cámara de crecimiento a 22 ± 2°C, 60-80% de humedad relativa y un fotoperiodo de 14 h de luz fluorescente (360 μmol m^{-2} s^{-1}) durante 3 meses. Se determinó la incidencia y severidad de la enfermedad a partir del número de plantas y ramas con síntomas, respectivamente. Además, se determinó la colonización vascular aislando el hongo a partir de trozos de tallo, que se sembraron en CWA (chlortetracycline-amended water agar) en placas de Petri, y la densidad de *V. dahliae* en suelo, mediante el procedimiento descrito en el apartado 3.4.4.

6.2.2. Ensayos en olivares comerciales

El estudio en olivares comerciales se llevó a cabo como parte del ensayo de evaluación de la capacidad bioestimulante del extracto de cebolla en los olivares localizados en Linares, Jaén, y Santaella, Córdoba, propiedad de la cooperativa DCOOP. Se detectó y cuantificó *V. dahliae* en las muestras foliares mediante RT-qPCR. La extracción de ADN de las hojas se realizó utilizando el kit Plant/Fungi DNA Isolation Kit de Norgen Biotek, siguiendo las instrucciones del fabricante. Este mismo kit se empleó para extraer ADN de un cultivo puro de *V. dahliae* V136I. El ADN se almacenó a -20°C hasta su uso. Para generar una curva estándar, el ADN de *V. dahliae* V136I se amplificó mediante PCR convencional en un termociclador, utilizando cebadores dirigidos a la región de espaciador transcrito inverso, ITS (Keykhasaber et al. 2017): VerDITS-F 5'-CCGGTCCATCAGTCTCTCTG -3' y VerDITS-R 5'- CACACTACATATCGCGTTTCG -3', que amplifican un fragmento de 132 pb. La amplificación se realizó en un volumen final de reacción de 25 μL, que contenía 5 μL de ADN, 2 μL de cebadores (10 μM), 12.5 μL de

AmpliTaq Gold 360 Master Mix y agua ultrapura libre de nucleasas hasta completar el volumen de reacción. Las condiciones consistieron en 5 minutos a 95°C, seguidos de 40 ciclos de 5 segundos a 95°C, 15 segundos a 55°C y 30 segundos a 72°C, con una extensión final de 7 minutos a 72°C. La concentración de ADN se midió con un fluorómetro Qubit 4 y se calculó el número de copias. Para la cuantificación absoluta, se prepararon diluciones seriadas de ADN en agua libre de nucleasas, con concentraciones de 10^8 a 10^1 copias/µL. La identificación de árboles infectados y cuantificación de *V. dahliae* se realizó mediante RT-qPCR en el sistema de detección Bio-Rad CFX Connect Real-Time PCR. El volumen de reacción (20 µL) contenía 5 µL de ADN, 2 µL de cebadores (10 µM), 10 µL de iTaq Universal SYBR Green Supermix y 3 µL de agua ultrapura libre de nucleasas. Se incluyó un control positivo de ADN de *V. dahliae* V136I y un control sin plantilla (NTC), en el que el ADN fue reemplazado por agua libre de nucleasas. El programa de RT-qPCR consistió en una etapa inicial de desnaturalización de 5 minutos a 95°C, seguida de 40 ciclos de 5 segundos a 95°C, 45 segundos a 63°C y 3 segundos a 77°C. Se realizó una curva de disociación desde 65°C hasta 95°C, con una velocidad de calentamiento de 0.5°C/s (Serrano et al. 2023). El número inicial de copias de cada muestra se calculó utilizando el software CFX Manager v.3.1 de Bio-Rad.

6.3. Análisis transcriptómico de olivos tratados con *Bacillus* spp. GG-22

Se estudió el efecto de la cepa GG-22 sobre la expresión génica de olivo en plantas de 1 año de edad de la variedad Ogliarola salentina. Las plantas se cultivaron en macetas en condiciones controladas de invernadero, a una temperatura de 25°C y una humedad relativa del 65%. Se tomaron muestras de tejidos de ramas a tiempo 0. Ese mismo día, tras el muestreo, las plantas fueron tratadas con GG-22 mediante pulverización (15 g/L). Las plantas se muestrearon a las 12 horas y a los 15 días de la aplicación de GG-22. Inmediatamente después del muestreo del día 15, las plantas fueron inoculadas con *X. fastidiosa* utilizando la técnica de inoculación por punción con aguja. El muestreo final se realizó 10 días después de la inoculación con *X. fastidiosa*. La inoculación con *X. fastidiosa* siguió el método de inoculación por pinchazo descrito en la norma EPPO PM7/24 (EPPO 2018), utilizando suspensiones ajustadas a 10^9 UFC/mL en PBS (NaCl 137 mM, KCl 2,7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1,8 mM, pH 7,4). Se inocularon cinco ramitas por planta, colocando gotas de la suspensión en tres nodos consecutivos de las hojas y perforándolos cinco veces con una aguja entomológica estéril. Para favorecer la absorción, las plantas se mantuvieron en posición horizontal durante unos minutos. Se monitoreó regularmente la aparición de síntomas de desecación durante los 36 meses posteriores a la inoculación.

El transcriptoma se analizó mediante la secuenciación de ARNm. Se utilizó 1 g de tejido de xilema pulverizado en nitrógeno líquido para extraer el ARN total de cada planta. El ARN se cuantificó con Nanodrop y su integridad se evaluó mediante visualización en gel de agarosa al 1%. Se construyeron bibliotecas de cDNA y se secuenciaron utilizando tecnología de Illumina en la Instalación de Secuenciación de Nueva Generación de las Instalaciones del Centro de BioCiencias de Viena. Los datos de RNAseq se obtuvieron como archivos *fastq*, que incluían datos de *fastqc* y multiQC. Las

lecturas crudas se mapearon en el ensamblaje del genoma de referencia del olivo Olea europaea variedad Farga (Cruz et al. 2016). El conjunto de datos obtenido se analizó primero mediante un análisis de componentes principales (PCA) utilizando el software PCA *explorer* (Marini and Binder 2019). Posteriormente, se realizó un análisis exploratorio de datos (EDA) y un estudio de los genes enriquecidos en los grupos, con el fin de obtener información sobre las funciones de los genes co-expresados utilizando el paquete iDEP *explorer* (de Pascali et al. 2019). Se llevó a cabo un análisis de expresión diferencial con DESeq2 (Love, Huber, and Anders 2014) para identificar genes que se expresaron diferencialmente de manera significativa (DEGs) en los tiempos postratamiento en comparación con el T0 (antes del tratamiento con GG-22). El análisis de enriquecimiento de ontología génica se realizó en las listas de DEGs obtenidas mediante la plataforma ShinyGo (Ge, Jung, and Yao 2020).

7. Evaluación toxicológica de OSCs en polinizadores

En colaboración con el Centro de Investigación Apícola y Agroambiental (CIAPA) en Marchamalo (Castilla-La Mancha, España), se evaluó la toxicidad de los compuestos organosulfurados PTS y PTSO utilizando como modelo abejas melíferas adultas (*Apis mellifera* Linnaeus; Hymenoptera: Apidae) de invierno y de primavera. Para facilitar la manipulación, las abejas fueron anestesiadas con dióxido de carbono. Se distribuyeron aleatoriamente en grupos de 15 dentro de jaulas cilíndricas de malla de acero (175 mm de largo, 45 mm de diámetro), que se mantuvieron en una incubadora a 25 ± 1°C. Estas jaulas tenían un orificio de 9,5 mm de diámetro en una de las bases para introducir un tubo cónico con el alimento. En los ensayos se utilizó dimetoato a 0,3 μg/μL en acetona como estándar tóxico. También se evaluó la toxicidad de la acetona y del polisorbato, empleados como diluyentes para el dimetoato y PTS y PTSO, respectivamente, para verificar que afectaban negativamente a las abejas (R. Martín-Hernández et al. 2007; Urbieta-Magro et al. 2019).

La toxicidad aguda por contacto de PTS y PTSO se evaluó mediante una prueba límite de acuerdo con la Guía de la OCDE para la Prueba de Químicos en Abejas (OECD 1998b). Se evaluó la toxicidad de cada compuesto a 100 μg/μL, según lo establecido por la guía, y 1 μg/μL, concentración máxima a la que se aplican en campo. Asimismo, se evaluó la sinergia entre PTS y PTSO. Se aplicó un volumen de 1 μL de la dilución correspondiente, preparada en agua bidestilada, en el tórax de abejas anestesiadas. La toxicidad oral aguda también se evaluó mediante una prueba límite de acuerdo con la Guía de la OCDE con algunas modificaciones (OECD 1998a). Se evaluaron las mismas concentraciones, que en este caso se prepararon en solución de sacarosa. Las abejas fueron privadas de alimento durante 2 horas para que el contenido intestinal al inicio de la prueba fuera similar. Empleando una micropipeta, se administró 1 μL de la dilución correspondiente a cada abeja, desorientada por la acción del dióxido de carbono, directamente en la boca. En ambos ensayos, las jaulas se mantuvieron en incubadora en oscuridad, y se registró la mortalidad a las 24 y 48 h. Las abejas supervivientes se observaron para detectar posibles daños subletales, tales como dificultad para volar, movimientos musculares anormales y letargo. Durante ese tiempo, las abejas se

alimentaron *ad libitum* con solución de sacarosa al 50% en agua (w/v) (Raquel Martín-Hernández et al. 2011). Cuando la aplicación oral o por contacto de PTS o PTSO a la concentración de 100 μg/μL causó mortalidad, se realizó un estudio para determinar la dosis letal media (LD_{50}). Para la determinación de la LD_{50} por contacto y oral de cada compuesto, se prepararon diluciones comprendidas entre 100 y 1 μg/μL en agua y solución de sacarosa, respectivamente. Las dosis se administraron siguiendo los procedimientos descritos para las pruebas límite.

Además, para determinar si las abejas melíferas consumen voluntariamente los productos, se realizó un estudio de ingesta voluntaria de diferentes concentraciones de PTS y PTSO según la Guía de la OCDE (OECD 1998a). Las abejas fueron privadas de alimento durante 2 h. Las diluciones de cada compuesto, preparadas en solución de sacarosa, se depositaron en los comederos. Se registró el peso de los comederos antes y después de depositar 200 μL de la dilución correspondiente. Los comederos se colocaron en las bases de las jaulas, y tras 6 h en el caso de las abejas de invierno y de 24 h en el caso de las abejas de primavera, los comederos se retiraron de las jaulas y se pesaron para evaluar la cantidad de dieta tratada consumida (mg de alimento/abeja). Posteriormente se proporcionó solución de sacarosa *ad libitum*, registrándose la mortalidad a las 24 y 48 h. Se estableció una relación entre la dieta consumida y la mortalidad.

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CAPÍTULO 1. POTENCIAL DE COMPUESTOS ORGANOSULFURADOS DERIVADOS DE LA CEBOLLA Y MICROORGANISMOS EN EL CONTROL DE FITOPATÓGENOS

1.1. PTS and PTSO, two Organosulfur Compounds from onion by-products as a novel solution for plant disease and pest management.

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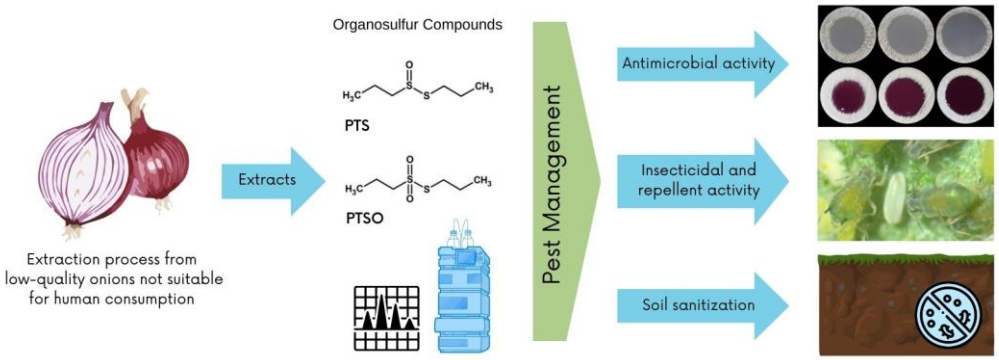
Abstract

Background Over the past decade, the great impact of agricultural crop diseases has generated considerable economic losses and has compromised the production of edible crops at a time when the world population is only expected to rise, leading to the search for new pest management strategies. Besides that, the environmental impact resulting from the continued use of chemical pesticides has led to the search for natural and sustainable alternatives. One of the existing solutions that currently stands out for its effectiveness is the use of bioactive plant extracts. This study aims to evaluate the antimicrobial activity of propyl propane thiosulfinate (PTS) and propyl propane thiosulfonate (PTSO), two organosulfur compounds (OSCs) derived from *Allium cepa*, against a wide range of target bacteria and fungi. To this end, various *in vitro* procedures were conducted as well as soil sanitization tests using sterile substrate inoculated with soil-borne pathogens. In addition, this study also evaluates the pesticidal activity of both compounds through *in vitro* mortality and repellence tests.

Results PTS and PTSO revealed inhibition activity on all the pathogens tested, belonging to different taxonomic groups. Moreover, both significatively reduced the population of bacteria and fungi in soil. The quantification of active substances in soil carried out in parallel to the microbial quantification showed that their use reduces the risk of residue accumulation since they break down quickly when applied. The set of antimicrobial tests performed demonstrated that the antifungal effect of both compounds is higher than the bactericidal effect. Lastly, PTS and PTSO showed a concentration-dependent significant biocidal and repellent effect against aphids.

Conclusions The results presented in this work demonstrate that both PTS and PTSO have a significant antimicrobial and pesticidal activity against the great majority of phytopathogens tested, being a promising tool to improve pest management in crops.

Keywords Propyl-propane-thiosulfinate; propyl-propane-thiosulfonate; *Allium cepa*; phytopathogens; antimicrobial; soil sanitization; botanical pesticide; insecticidal activity; integrated pest management.



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1. Introduction

The global human population is predicted to number between 9.4 and 10.1 billion in 2050, an increase by about 1.5 billion compared to 2020 (United Nations, 2019). Around 80% of agricultural production is dedicated to human nutrition (FAO, 2018). This includes not only the direct use of agricultural products as food, but also the use of crops and other vegetal matter to feed animals, which are in turn intended for human consumption. Keeping in mind the estimated population growth, the production of edible crops might need to increase by up to 119% (Davies et al., 2021). This imposes a serious challenge, which involves adopting changes to ensure the transformation of agricultural and food systems toward greater sustainability, and to reduce waste and spoilage (Nelson, 2020).

Plant diseases caused by biotic factors are the main responsible for the decrease in crop productivity; in fact, pests and pathogens bring about 40 billion dollars losses a year worldwide (Syed Ab Rahman et al., 2018), which means reductions between 21 and 30% globally in major crops (Savary et al., 2019). Plant pathogen control will be even more challenging as climate change conditions progress (Burdon & Zhan, 2020). Since the environment has a great impact on plant pathogenesis (Scholthof, 2007), global warming is directly related to disease incidence and severity (Velásquez et al., 2018). Higher temperatures are correlated with soil degradation and less water availability, and foster the emergence of new pathogens and a changeable geographic distribution (Rossati, 2017).

Over the last several decades, synthetic agrochemicals have contributed to increase food production worldwide through controlling crop diseases, but with a severe environmental impact (Rongai et al., 2012). Their application has not only gradually disrupted biological control by natural enemies, but also caused disease outbreaks and the development of resistance (Park et al., 2008). Moreover, synthetic pesticides severely damage non-target organisms, such as pollinators, and human health (Soriano et al., 2022).

In this regard, plant-derived secondary metabolites are receiving increasing attention and gradually replacing synthetic biocides and soil disinfectants from disease management protocols (Zefzoufi et al., 2020). Many products based on antimicrobial phytochemicals isolated from plants have been developed over the past few years as novel eco-friendly non-synthetic plant protection measures (Pane et al., 2020). The extraction of phytochemicals from medicinal and fragrant plants is quite common (Kalemba & Kunicka, 2003; Pavela, 2016); however, the extraction of bioactive compounds from by-products from the food industry or second-class plant material, such as grape cane waste (Rayne et al., 2008) and pepper leaves (Pane et al., 2016), through clean extraction methodologies has become an innovative strategy that contributes to the revaluation of agricultural waste and support circular economy (Sciubba et al., 2020).

In recent years, the functional properties of organosulfur compounds (OSCs) obtained from onion (*Allium cepa*) and garlic (*Allium sativum*), such as antioxidative, immunomodulatory and antimicrobial activity, have been deeply studied (Putnik et al., 2019). OSCs are secondary metabolites that are biosynthesized by the plant as a defence mechanism against biotic and abiotic stressors (Putnik et al., 2019). Garlic bulbs are rich in alliin (S-allyl cysteine sulfoxide) and in a lower degree methiin (S-methyl-L-cysteine sulfoxide), while onion bulbs contain methiin but also isoalliin (S-propenyl-L-cysteine sulfoxide) and propiin (S-propyl-L-cysteine sulfoxide) (Putnik et al., 2019). Cysteine sulfoxides are natural constituents of fresh bulb tissue, non-volatile and odourless (Breu, 1996; Rose et al., 2005). The disruption of the bulb tissue triggers an enzymatic reaction carried out by alliinase, that catalyses the conversion of these precursors to thiosulfinates (Ramirez et al., 2017), volatile compounds to which the antimicrobial activity of *Allium* genus plants are mainly attributed and bear the primary responsibility for their organoleptic properties (Kyung, 2012; Poojary et al., 2017).

According to the existing literature, the antimicrobial effect of thiosulfinates is primarily due to their ability to inhibit thiol-containing enzymes by oxidizing protein cysteine or glutathione residues (Beshbishy et al., 2020). Enzymes containing thiol include the main enzymes of microbial metabolism as well as bacterial enzymes of the acetyl-CoA-forming system, and RNA polymerase (Beshbishy et al., 2020). In onion, propiin turns into propyl-propane thiosulfinate (PTS), a labile compound that changes into dipropyl disulphide and propyl-propane thiosulfonate (PTSO) through dismutation or disproportionation reactions (Guillamón et al., 2021).

Whereas bioactive properties of Allicin—that represent about 75% of thiosulfinates in garlic—has been thoroughly investigated in several crops of study, from antimicrobial therapy for human infections to integrated pest management (Choo et al., 2020; Curtis et al., 2004), information regarding the antimicrobial activity of PTS and PTSO from onion is limited and focused on the potential of thiosulfinates against human and animal infections. Recent studies have shown broad-spectrum antibacterial activity of PTS and PTSO and its gaseous form against clinical isolates of bacteria and *Candida* species that are resistant to at least one group of antibiotics (Sorlozano-Puerto et al., 2018, 2021). In a previous study we demonstrated the *in vitro* and *in planta* antifungal activity of volatile organosulfur compounds PTS and PTSO from onion against *Verticillium dahliae* (Falcón-Piñeiro et al., 2021), the most devastating soil-borne pathogenic fungi affecting olive trees (Montes-Osuna & Mercado-Blanco, 2020). Moreover, the same study showed the potential of both compounds as soil sanitizers, as they reduced *V. dahliae* population in an artificially infested substrate. As previously mentioned, PTS and PTSO are volatile compounds (Lanzotti, 2006). Owing to their low molecular weight (< 300 g/mol), they can diffuse through plant cell membranes and soil, playing a key role in the functioning of the whole ecosystem (Kaddes et al., 2019). The study of the active properties of their gaseous phase is thus of interest to back up their use against phytopathogens and pest management, especially in the present context in which the

search for alternatives to conventional pesticides has become one of the main focuses of modern agriculture research (Jiao et al., 2021).

Within this context, the aim of the present study was to evaluate the bactericidal, fungicidal, pesticidal and repellent activity of PTS and PTSO obtained from low-quality onions not suitable for human consumption, through *in vitro* methodologies and performing soil sanitization trials.

2. Materials and Methods

2.1. Compounds and Reagents

Standardized fractions of PTS and PTSO at 20% were supplied by DOMCA SA (Granada, Spain). Both compounds were obtained from onions that had been discarded as they were not suitable for human consumption, following the methodology described by Hu et al. (2002) to obtain the allyl derivatives form garlic. Summarizing, onions were chopped and immersed in a solution of Ethanol (70%) in a percentage equivalent to four times their weight. The extraction was carried out for 2 weeks at room temperature, then the mixture was filtered, and the solution was concentrated and extracted with Ethyl acetate (EtOAc). The EtOAc extract were concentrated and fractionated by 2 sequential column chromatography's, taking the trichloromethane ($CHCl_3$) fraction from the first column, and then using EtOAc/hexane as mobile phase in the second column to obtain purified PTS and PTSO. All reagents were purchased from Sigma-Aldrich Química S.L. (Spain), unless otherwise stated.

2.2. Phytopathogens strains and growth media used

Bacteria and fungus used in this study were obtained from the Spanish Collection of Type Cultures (CECT), the German Collection of Microorganisms and Cell Cultures (DSMZ), the plant pathogen collection of DMC Research and the culture collection of the Department of Crop Protection, Institute for Sustainable Agriculture, Spanish National Research Council (Córdoba, Spain), which are listed in Table 1. Each phytopathogen grew on a specific culture medium and time, indicated by the corresponding culture collection. For the antimicrobial activity tests against pathogenic bacteria, Mueller-Hinton Agar and Mueller-Hinton broth supplied by Scharlau (Barcelona, Spain) were used as culture media (CLSI, 2018); for the in vitro antimycotic test, Rose-Bengal agar supplied by Scharlau and RPMI-1640 medium with L-glutamine (CLSI, 2017) supplied by Labclinics (Barcelona, Spain) were used.

2.3. Insects

Adult individuals of the cotton aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae) were supplied by TECNOVA Technological Center (Almería, Spain). The individuals were reared on courgette leaves at the TECNOVA Experimental Center greenhouse for future experiments.

2.4. Soil

The soil used in this study was superficially collected from an olive grove in Linares, Jaen (30U 444908.38 4209274.77 UTM WGS84), owned by the cooperative DCOOP, the world's largest producer of olive oil. This soil was chosen because no biocide product had been applied on the farm in the last two years, as it was the control farm of an experimental field trial.

The soil was dried in an oven at 50 °C and passed through a 2 mm pore sieve to remove plant material, soil macrofauna and stones (Bhavya et al., 2018). Then, it was stored in polyethylene bags for future analysis and characterized according to the procedures previously described. Soil pH, which was determined in a 1:1 water suspension according to the international standard (International Society of Soil Science, ISSS), was 8.71 ± 0.01, that is to say, moderately basic according to the criteria established by the United States Department of Agriculture (Marañes Corbacho et al., 1994). Moreover, the soil, classified as sandy loam, contained 6.5 ± 0.22 % fine silt, 4.9 ± 0.85 % coarse silt, 21.5 ± 0.07 % clay, 67.1 ± 0.72 % sand (determined through Robinson pipette method (Robinson, 1992)), and 1.13 % organic matter (soil organic matter fractionation was measured according to Tyurin I.V., (1951)). Lastly, this soil presented a maximum Water Holding Capacity (mWHC) of 0.414 g H2O per g soil dry matter (determined by the Keen - box method (Keen & Raczkowski, 1921)). Based on this parameter, it was determined that the soil had 95.46% dry matter of field-moist soil and a water content of natural moist soil of 0.04 g water/g dry matter.

Table 1. Bacterial and fungal strains used along with their references and source of isolation.

Group	Species	Strain Ref.	Host plant
Bacteria	*Erwinia persicina*	DSM 19328	Tomato plant (*Lycopersicon esculentum*)
	Xanthomonas campestris	CECT 97	Brussels sprout (*Brassica oleracea* var.
	Pseudomonas savastanoi	CECT 5023	Olive tree (*Olea europaea*)
	Pseudomonas syringae	DMC 15	Peach (*Prunus persica*)
	Clavibacter michiganensis	CECT 790	Tomato plant (*Lycopersicon esculentum*)
	Agrobacterium tumefaciens	CECT 4119	Crown gall of apple seedling (*Malus* spp)
Fungi	*Geotrichum candidum*	DSM 1240	Tomato plant (*Lycopersicon esculentum*)
	Alternaria alternata	CECT 2662	*Lycopersicon* spp
	Fusarium oxysporum f. sp.	DMC 02	Banana tree (*Musa paradisiaca*)
	Fusarium graminearum	DSM 1095	Maize (*Zea mays*)
	Phytophthora cinnamomi	CECT 20186	Avocado pear root (*Persea americana*)
	Penicillium expansum	DMC 01	Apple (*Malus domestica*)
	Penicillium digitatum	DMC 07	Sweet orange (*Citrus sinensis*)
	Phyllosticta spp.	DMC 10	Olive tree (*Olea europaea*)

2.5. *In vitro* antimicrobial activity against pathogenic bacteria

The antibacterial activity of organosulfur compounds PTS and PTSO was evaluated by performing different testing procedures. The disk diffusion method proposed by A. W. Bauer et al. (1966) and modified by Calvo & Asensio (1999) was used to evaluate the antibacterial activity. Agar plates were inoculated using bacterial suspension adjusted to 10^6 CFU/mL, so that the growth after incubation was confluent.

Sterile 6 mm cellulose disks (Whatman® antibiotic test discs, Buckinghamshire, UK) impregnated with 20 μL of PTS or PTSO at 5, 10 and 25 μg/μL were placed in the centre of inoculated agar plates. The inhibition zone of bacterial growth was measured after 48 h incubation.

Determination of the Minimum Bactericidal Concentration (MBC) was performed by the broth microdilution method, following the guidelines of the *Clinical and Laboratory Standards Institute* (CLSI) collected in the standard Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically (CLSI, 2018), in order to establish the lowest concentration of each antimicrobial agent that reduces the viability of the initial inoculum by 99.9%. 1:2 decreasing dilution were prepared from an initial solution of each compound at 10,000 μg/mL so that the following concentrations were obtained: 5,000; 2,500; 1,250; 625; 312.5; 156.25; 78.125; 39.06; 19.53; and 9.76 μg/mL. Each dilution was inoculated with bacterial suspension so that the final concentration in each well was 10^5 CFU/mL, and incubated overnight at room temperature. As positive control, a mix of ampicillin and streptomycin (100,000 and 25,000 μg/mL, resp.) was used. As negative control, liquid media without antimicrobial agent was inoculated with bacteria. Bacterial growth was tested by culturing in agar plates, and the lowest concentration of PTS/PTSO in which no growth was observed was established as the MBC.

The antibacterial activity of the gaseous phase of PTS and PTSO was assessed through a previously described procedure (Falcón-Piñeiro et al., 2021). Bacterial suspensions adjusted to 10^6 CFU/mL were spread on agar plates. Sterile 6 mm cellulose disks were placed, not in the centre of the plate, but in the centre of the lid of the petri dish, and they were impregnated with 20 μL of PTS or PTSO solutions. The same PTS and PTSO concentrations as in the disk diffusion assay were used, i.e., 5, 10 and 25 μg/μL. Plates were incubated for 48 h and subsequently growth inhibition zones were measured. All *in vitro* assays were performed in duplicate.

2.6. *In vitro* antimicrobial activity against pathogenic fungi

The antifungal activity of PTS and PTSO was evaluated following the same methodology described for bacteria, using the appropriate liquid and solid media indicated in section "Phytopathogens strains and growth media used". The disk diffusion method and the gas phase activity test were carried out with no modifications, with the exception of the incubation time, which was 5 days. Regarding the determination of the Minimum Fungicidal Concentration (MFC), the broth microdilution method was also carried out according to the standard reference method for broth dilution antifungal susceptibility testing of filamentous fungi of the CLSI, which does not differ from that described for the determination of MBC (CLSI, 2017). For the positive control, natamycin (50,000 μg/mL) was used instead of ampicillin and streptomycin.

Moreover, in a fourth trial, the influence of both organosulfur compounds on mycelial growth was determined. Different volumes of PTS and PTSO at 20% were

added to Rose-Bengal medium to obtain supplemented agar plates at 25, 50, 100, 250 and 500 μg/mL. Each fungal strain was grown on Rose-Bengal agar for 3 days. From these cultures, agar plugs of 5 mm diameter were obtained, which were distributed among the supplemented agar plates (Ma et al., 2020). In addition, non-supplemented plates with 5 mm agar plugs from each strain tested were incubated as control of fungal growth. For 17 days of incubation the diameter of the mycelium over time was measured, compared to the mycelial growth of each fungus when growing on non-supplemented Rose Bengal agar plates. Each experiment was repeated twice.

2.7 *In vitro* activity against aphids

In this study, the contact toxicity and repellent activity were evaluated for PTS and PTSO at different concentrations (5,000; 2,500; 1,000 and 500 μg/mL). Since the treatments were prepared in water, blank control included only water.

The contact toxicity of PTS and PTSO was assessed by the leaf immersion method, as previously reported (Sayed et al., 2022). Circular cuttings of courgette leaves of 55 mm diameter were immersed in the treatment and control solutions for 5 s., air-dried and placed on 60 mm diameter petri dishes. Twenty-four adults were transferred to each treated leave cutting in petri dish using a brush. Decis® Protech, a deltamethrin-based pyrethroid insecticide purchased from Bayer CropScience S.L. (Barcelona, Spain) (Ref 84942464) was used as positive control at the dose indicated on the label. The plates were wrapped with Parafilm® purchased from Amcor (Valencia, Spain) to prevent the aphids to scape, and maintained in a climate chamber at 25 ± 1°C, 75 ± 5 relative humidity and Light:Dark photoperiod of 14:10 (Zhou et al., 2016). Mortality was recorded after 24 h. An aphid was considered dead if it did not move its legs when touching its abdomen with a brush and if the body turned black (C. Zhao et al., 2021).

The repellent activity was assessed by a choice assay in 90 mm diameter petri dishes (Semerdjieva et al., 2021). N, N-diethyl-meta-toluamide (DEET), an active ingredient used in many repellent products, was purchased from Sigma-Aldrich at 97% (Ref. D100951) and diluted to 2,000 μg/mL as positive control (Jiang et al., 2016). Circular cuttings of courgette leaves of 25 mm diameter were immersed in the treatment and control solutions for 5 s and dried at room temperature, as in the previous trial. A treated leaf and a negative control leaf were placed in each petri dish on a moist filter paper disk to maintain humidity (Zheljazkov et al., 2021). Then, 24 adults were introduced into each petri dish using a brush. The parafilm-sealed petri dishes were maintained in the conditions previously indicated. The repellent effect was observed after 24 h and expressed as percentage of repellence according to the following formula (Pavela et al., 2009):

$$\%\, repellence = [(C - T)/(C + T)]\, x\, 100$$

where C is the number of aphids on the control leaf, and T is the number of aphids on the treated leaf. Each experiment was performed in triplicate.

2.8. Soil sanitization

The study of the persistence of phytopathogenic microorganisms in soil was carried out by microcosm systems (Del Papa et al., 2003). The ability of a powder blend of PTS and PTSO in proportion 1:1 (w/w) to reduce the population of a pathogenic microorganism artificially inoculated in soil was determined against the bacterium *A. tumefaciens* and the fungus *F. oxysporum*, both pathogens that inhabit the soil, where they can survive for long periods (Michielse et al., 2009; Zupan et al., 2000). Two different concentrations of active substances, 100 μg/g (50 μg/g of each one) and 500 μg/g (250 μg/g of each one), were tested; and the efficacy of a treatment based on a single application was compared with the efficacy of a treatment consisting of 3 applications of the same dose separated in time. In addition, non-inoculated soil was used as sterility control, while untreated inoculated soil was used to follow up microbial growth. Finally, as positive control, the assay included a study group of inoculated soil that was treated with soil fumigant Metam sodium ($C_2H_4NNaS_2$) (EPA Reg. No. 45728-16) (Stephen B. Pruett, L. Peyton Myers, 2001). Metam sodium 42.1% aqueous solution was purchased from Eastman Chemical Company (Madrid, Spain), diluted in sterile water and set to 60 μg/g, in accordance with the recommended application rates (Li et al., 2017). Each study group consisted of four replicates. Table 2 shows the experimental design of the assay and details of the different groups of study.

Table 2. Experimental design of soil sanitization assay through microcosm system.

Group	Treatment dose	Number of treatment applications	Microorganism concentration
Negative control	-	-	-
Positive control	-	-	10^7 CFU/g soil
Metam Sodium	60 μg/g	1 application: 4 days after inoculation	10^7 CFU/g soil
PTS/PTSO	100 μg/g	1 application: 4 days after inoculation	10^7 CFU/g soil
PTS/PTSO	100 μg/g	3 applications: 4 days after inoculation 10 days after 1st application 30 days after 1st application	10^7 CFU/g soil
PTS/PTSO	500 μg/g	1 application: 4 days after inoculation	10^7 CFU/g soil
PTS/PTSO	500 μg/g	3 applications: 4 days after inoculation 10 days after 1st application 30 days after 1st application	10^7 CFU/g soil

The experimental microcosm unit consisted of a polypropylene box with a drainage system, of 28 cm length x 5 cm width x 17 cm height and 1.75 l capacity. The soil was autoclaved through 4 cycles of 20 min at 121°C in a steam sterilizer (Raypa, Terrasa, Spain) for 4 successive days (Kelsey et al., 2010), interspersed with incubations

at 4°C, in order to eliminate vegetative forms by heat shock (Louie et al., 2012). After drying in an oven at 50°C, 700 gr of sterile soil were introduced into each microcosm unit on a bed of sterile gravel to facilitate drainage and prevent soil compaction. Subsequently, soil was inoculated with 140 mL of microbial suspension previously adjusted to 10^9 CFU/mL, so that the soil moisture was adjusted to 60% of the water holding capacity (WHC) (Vischetti et al., 2002). The negative control group was inoculated with 140 mL of distilled water. Microcosms were then placed in a room at 25 °C (optimal growth temperature of the two phytopathogens used), where they were kept until the end of the trial. Four days after inoculation, every study group was sampled to establish the starting microbial population. Next, the treatments were applied to the corresponding group at the appropriate dose. Microbial population was quantified 1, 2, 4, 7, 11, 15, 31 and 45 days after treatment. The second and third application of the PTS/PTSO powder treatment was added to the corresponding microcosm units 10 and 30 days after the first application. At each enumerated date, 25 g of soil were diluted in 225 mL of buffered peptone water (Scharlau). A lab paddle blender (MASTICATOR, UIL, Barcelona, Spain) was used to homogenize the samples. Serial dilutions were prepared from the supernatant, cultured in the appropriate solid medium, and incubated at 25°C for 3 days (Deberdt et al., 2012). In the cases in which no microbial growth was observed on the plate, to confirm the absence of microorganisms a pre-enrichment step was carried out in a non-selective nutrient medium. Microorganism population was expressed as Log 10 CFU/g soil.

PTS and PTSO concentrations achieved by each application protocol were assessed by High-Performance Liquid Chromatography using a UV detector (HPLC-UV). Fifty grams of soil were mixed with 100 mL of acetone, homogenized with vortex for 1 min and extracted in a sonication bath for 10 min. Supernatant was separated from the soil by filtration and the process of extraction was repeated adding 20 millilitres of acetone to the solid residue. Then, the supernatant from both extractions was evaporated until dryness in a vacuum rotator and reconstituted with 10 mL of methanol (MeOH) vortexing for 30s. Finally, the extract was filtered through a nylon filter of 0.2 μm (Sigma Aldrich, Darmstadt, Germany) and injected into the HPLC system. For the PTS and PTSO determination, an Agilent 1260 Infinity LC (Agilent Technologies, Santa Clara, CA, USA) system was used. The separation of the compounds was accomplished using a Zorbax Eclipse Plus RRHD (50 x 2.1 mm, 1.8 mm) column at 25ºC, and the gradient and mobile phases described by Sorlozano-Puerto et al. (2021). Wavelength of detection was set at 200 nm. A calibration curve using PTS and PTSO standards was made for the quantification.

2.9. Statistical treatment

GraphPad prism 8.0 software (GraphPad Software Inc., San Diego, California) was used for statistical analysis. The data obtained in the *in vitro* antimicrobial activity assays were analysed using descriptive statistics. Shapiro–Wilk normality tests were used to determine normal distribution of all data subjected to ANOVA. A one-way ANOVA test supplemented with Tukey's post hoc test was used to compare every

treatment and control of the *in vitro* assays against aphids with each other. Repeated measures two-way ANOVA test supplemented with Dunnett's post-hoc test was used for evaluation of statistically significant inhibition of mycelial growth and to establish significant differences between microorganism survival in treated soil and the positive control, considering different treatments and time points. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. *In vitro* assessment of antibacterial activity

The antibacterial activity of PTS and PTSO was tested against different bacteria involved in infectious processes of agricultural crops. As shown in Table 3, both compounds displayed antimicrobial activity against all the bacterial strains included in the study in various degrees. Moreover, in most cases the bacteriostatic effect rises as the concentration of the product increases, being considerably more modest in the case of *P. syringae*. Regarding the volatility-linked activity assay, PTS and PTSO inhibited growth of all bacteria tested without coming into direct contact with either the medium or the microorganism, but rather by diffusion of their gas phase, as shown in Figure 1. *Xanthomonas campestris*, *C. m. michiganensis* and *A. tumefaciens* were the most sensitive, showing growth inhibition zones of at least 40 mm in all cases (Table 3 and 4). *Erwinia persicina* and specially *P. syringae* were, on the other hand, the most resistant strains to PTS and PTSO in both tests, with the smallest inhibition zones among the strains studied. Furthermore, in both agar tests PTSO displayed a greater capacity to inhibit the growth of *P. savastanoi*, *P. syringae*, *C. m. michiganensis* and *A. tumefaciens* than PTS. Whereas the results of the diffusion and volatility tests in agar appear to suggest that PTSO may have higher antibacterial capacity, the results obtained in the MBC test indicated that it was significantly more active than PTS. As shown in Table 5, the lower MBC of PTS is 156.25 μg/mL (Me = 312.5 μg/mL), while MBC data for PTSO ranges from 156.25 to 19.53 μg/mL (Me = 78.125 μg/mL).

Table 3. Antimicrobial activity of PTS and PTSO against phytopathogenic bacteria by disk-diffusion method, expressed as the average diameter ± standard deviation of inhibition zone (mm).

Species	PTS (μg/μL)			PTSO (μg/μL)		
	5	10	25	5	10	25
E. persicina	18.0 ± 0.71	23.0 ± 1.87	29.5 ± 1.12	17.5 ± 2.06	20.3 ± 1.79	29.0 ± 0.71
X. campestris	31.5 ± 1.12	36.3 ± 1.09	47.3 ± 1.48	41.0 ± 1.58	48.5 ± 2.06	61.3 ± 1.48
P. savastanoi	12.8 ± 1.30	22.0 ± 1.58	25.3 ± 1.48	25.5 ± 2.06	38.0 ± 1.87	45.5 ± 1.66
P. syringae	11.3 ± 1.09	11.8 ± 1.48	14.5 ± 1.12	19.3 ± 1.09	19.5 ± 1.50	29.8 ± 1.09
C. m. michiganensis	38.0 ± 1.58	46.3 ± 0.83	59.3 ± 1.09	51.5 ± 1.12	56.0 ± 1.58	75.0 ± 1.41
A. tumefaciens	27.5 ± 1.12	36.5 ± 2.18	48.8 ± 2.38	54.3 ± 0.83	64.0 ± 1.58	75.3 ± 2.68

Table 4. *In vitro* antimicrobial activity of PTS and PTSO against phytopathogenic bacteria via the gas phase, expressed as the average diameter ± standard deviation of inhibition zone (mm).

Species	PTS (μg/μL)			PTSO (μg/μL)		
	5	10	25	5	10	25
E. persicina	19.0 ± 1.41	25.0 ± 3.16	40.8 ± 2.59	15.8 ± 2.59	22.3 ± 2.17	28.5 ± 2.69
X. campestris	42.8 ± 3.96	50.8 ± 2.59	59.3 ± 3.67	44.3 ± 3.11	54.5 ± 3.84	67.3 ± 3.03
P. savastanoi	16.3 ± 0.83	19.5 ± 1.66	26.8 ± 1.09	26.5 ± 1.12	36.3 ± 1.92	47.0 ± 1.41
P. syringae	0.0 ± 0.00	7.5 ± 1.66	13.3 ± 1.48	20.0 ± 1.41	23.5 ± 1.12	27.8 ± 1.48
C. m. michiganensis	15.0 ± 3.36	25.0 ± 1.41	40.5 ± 1.12	44.8 ± 2.86	55.0 ± 3.39	65.8 ± 1.92
A. tumefaciens	15.5 ± 2.18	26.5 ± 1.12	41.0 ± 2.55	49.5 ± 1.80	57.5 ± 2.69	60.5 ± 2.69

Table 5. Minimum bactericidal concentration (MBC) of PTS and PTSO against phytopathogenic bacteria.

Species	MBC (μg/mL)	
	PTS	**PTSO**
E. persicina	312.5	156.25
X. campestris	156.25	19.53
P. savastanoi	312.5	39.06
P. syringae	156.25	78.125
C. m. michiganensis	312.5	78.125
A. tumefaciens	625	78.125

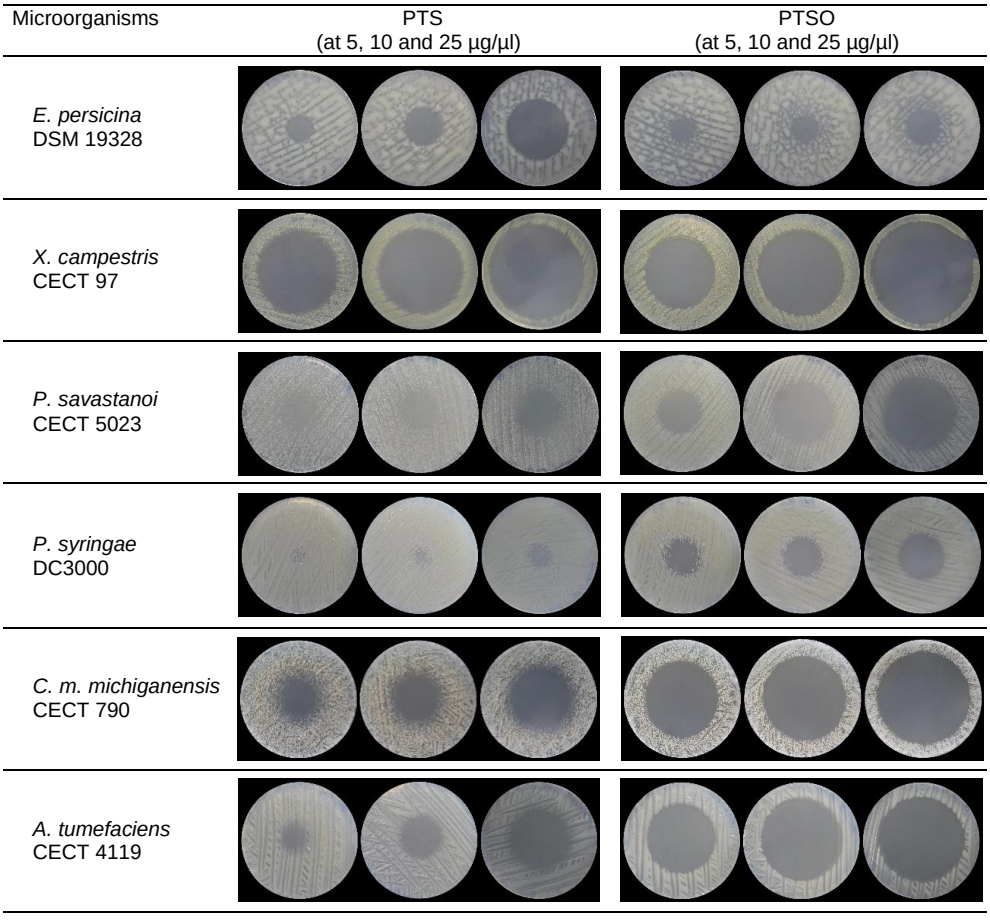

Figure 1. Antibacterial activity of the gaseous phase of PTS and PTSO against phytopathogenic bacteria.

3.2. *In vitro* antimycotic activity

As for antifungal activity, all phytopathogenic fungi used in this study were sensitive to both organosulfur compounds in a dose dependent manner according to the results of the disk-diffusion method, presented in Table 6. Table 7 details the results obtained in the MFC determination. In all cases, the MFC of PTSO for each strain was at least one dilution lower than the corresponding concentration of PTS. The highest values obtained, which ranges from 625 to 156.25 μg/mL, correspond to the two *Penicillium* species used. On the other hand, *A. alternata*, *F. oxysporum* and *F. graminearum* were the most sensitive, displaying PTSO MFC values of 39.06, 19.53 and 9.76 μg/mL, respectively. The fungicidal activity of the gas phase of the compounds was also demonstrated, since the volatility test generated growth inhibition halos whose diameters were similar to the halos obtained by the agar diffusion test (Table 8 and Figure 2).

To complete the *in vitro* assessment of antimycotic activity, the influence of both organosulfur compounds on the mycelial growth was studied. The results of the mycelial growth inhibition test, represented in Figure 3 and 4, also indicates that most fungal species were sensitive to at least the two highest concentrations evaluated (250 and 500 μg/mL), showing significant inhibition ($p < 0.05$). In the case of *F. graminearum*, which, as in the MFC test, turned out to be the most sensitive, all concentrations of both products completely inhibited the mycelial growth ($p < 0.0001$), with the exception of PTSO at 25 μg/mL. Moreover, the treatments of 50 and 100 μg/mL of PTS and PTSO successfully controlled the development of *P. cinnamomi*, *Phyllosticta* spp and *F. graminearum* in agar plates. On the other hand, only the exposure to PTS and PTSO at 500 μg/mL achieved a significant reduction of *P. digitatum* and *G. candidum* growth ($p < 0.05$). *Penicillium expansum* was the most resistant fungal strain, since none of the evaluated concentrations of PTS and PTSO significantly inhibited mycelial development. Although in the case of the other fungal strains no differences were observed between compounds, the effectiveness of PTSO was lower against *Phyllosticta* spp, *F. oxysporum* and *F. graminearum*.

Table 6. Antimicrobial activity of PTS and PTSO against phytopathogenic fungus by disk-diffusion method, expressed as the average diameter ± standard deviation of inhibition zone (mm).

Species	PTS (μg/μL)			PTSO (μg/μL)		
	5	10	25	5	10	25
G. candidum	15.5 ± 1.80	26.0 ± 3.16	36.8 ± 3.34	27.3 ± 2.86	34.8 ± 3.11	44.8 ± 2.59
A. alternata	38.5 ± 3.35	44.8 ± 2.17	53.3 ± 2.05	30.8 ± 0.83	42.0 ± 2.12	56.3 ± 1.64
Phyllosticta spp	64.0 ± 1.41	68.8 ± 0.83	75.5 ± 1.12	55.0 ± 1.63	64.5 ± 2.05	79.5 ± 1.25
P. cinnamomi	28.5 ± 1.12	42.5 ± 3.64	54.0 ± 1.41	39.5 ± 2.69	52.0 ± 1.41	66.3 ± 2.59
F. oxysporum	26.5 ± 2.18	46.5 ± 1.66	60.5 ± 2.69	41.8 ± 3.03	51.3 ± 2.59	54.0 ± 1.41
F. graminearum	51.5 ± 2.06	58.0 ± 1.22	65.3 ± 2.17	50.8 ± 2.59	57.8 ± 0.83	69.0 ± 2.12
P. expansum	34.3 ± 3.03	39.8 ± 1.64	48.5 ± 1.50	35.8 ± 3.34	46.3 ± 0.83	54.8 ± 1.48
P. digitatum	15.8 ± 0.83	20.5 ± 0.50	34.0 ± 1.22	12.0 ± 2.55	18.5 ± 1.12	26.0 ± 1.22

Table 7. Minimum fungicidal concentration (MFC) of PTS and PTSO against phytopathogenic fungi.

Species	MFC (μg/mL)	
	PTS	**PTSO**
G. candidum	156.25	78.125
Alternaria spp	78.125	39.06
Phyllosticta spp	156.25	39.06
P. cinnamomi	156.25	78.125
F. oxysporum	78.125	19.53
F. graminearum	39.06	9.76
P. expansum	625	312.5
P. digitatum	312.5	156.25

Table 8. *In vitro* antimicrobial activity of PTS and PTSO against phytopathogenic fungus via the gas phase, expressed as the average diameter ± standard deviation of inhibition zone (mm).

Species	PTS (μg/μL)			PTSO (μg/μL)		
	5	10	25	5	10	25
G. candidum	28.0 ± 3.16	32.0 ± 3.16	39.0 ± 2.24	34.8 ± 3.49	41.3 ± 3.96	47.8 ± 2.49
A. alternata	27.0 ± 2.12	36.3 ± 2.59	42.5 ± 3.28	26.5 ± 1.50	34.3 ± 2.68	37.0 ± 1.22
Phyllosticta spp	38.8 ± 3.03	49.8 ± 1.92	57.0 ± 1.87	46.3 ± 2.17	64.8 ± 2.38	70.3 ± 1.09
P. cinnamomi	25.3 ± 2.17	33.3 ± 1.92	47.8 ± 1.48	39.0 ± 3.08	52.0 ± 1.22	57.3 ± 1.30
F. oxysporum	50.0 ± 1.58	59.8 ± 2.38	68.8 ± 2.86	37.5 ± 1.80	50.8 ± 3.49	57.0 ± 3.39
F. graminearum	49.5 ± 3.64	55.5 ± 2.50	65.8 ± 1.92	26.0 ± 1.58	31.5 ± 2.29	46.3 ± 1.92
P. expansum	34.3 ± 0.83	39.3 ± 0.83	47.5 ± 2.06	34.3 ± 3.49	45.3 ± 0.83	49.5 ± 1.80
P. digitatum	12.8 ± 1.92	16.5 ± 1.12	22.3 ± 3.34	10.8 ± 0.83	14.5 ± 1.12	21.8 ± 1.09

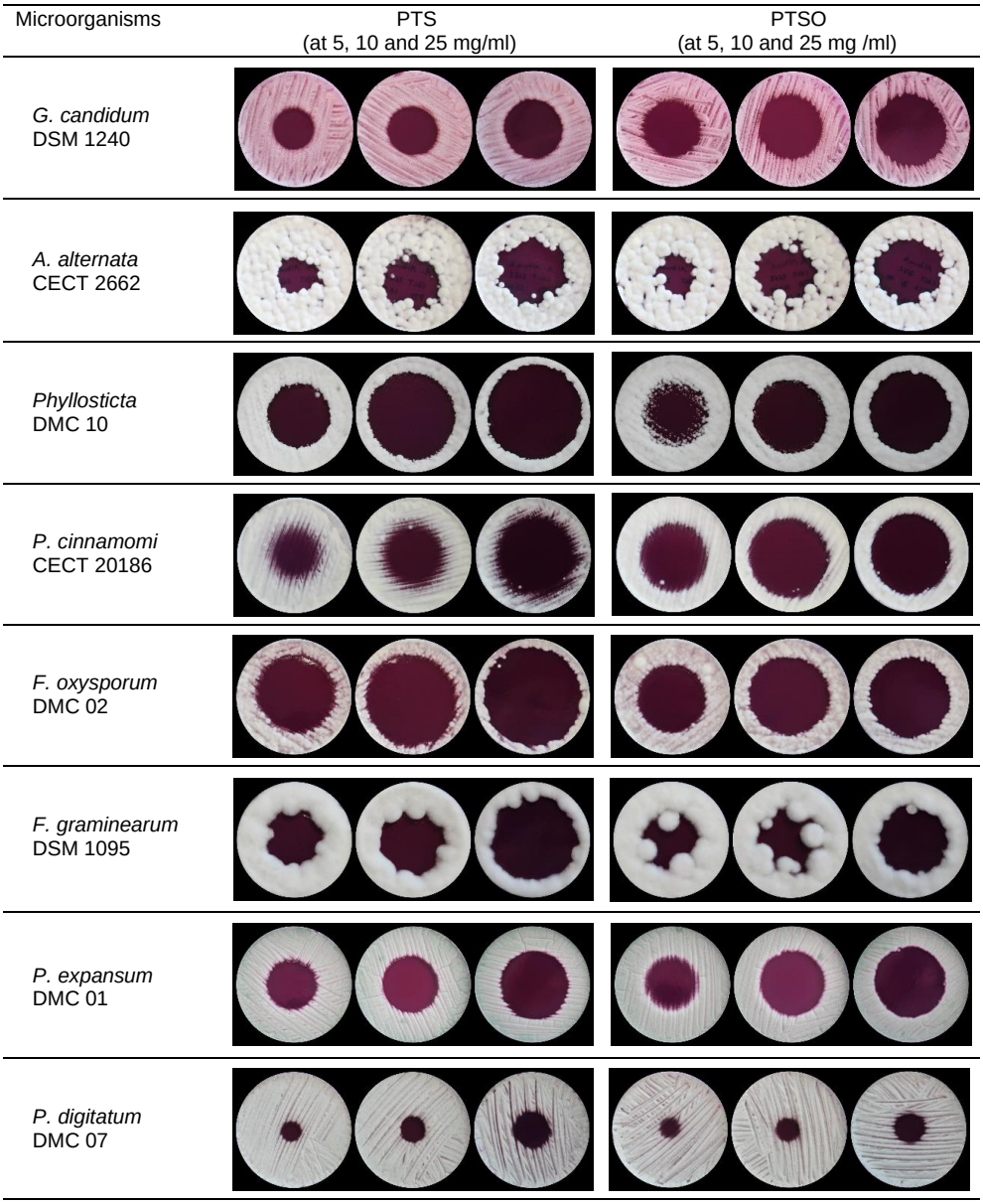

Figure 2. Antifungal activity of the gaseous phase of PTS and PTSO against phytopathogenic fungi.

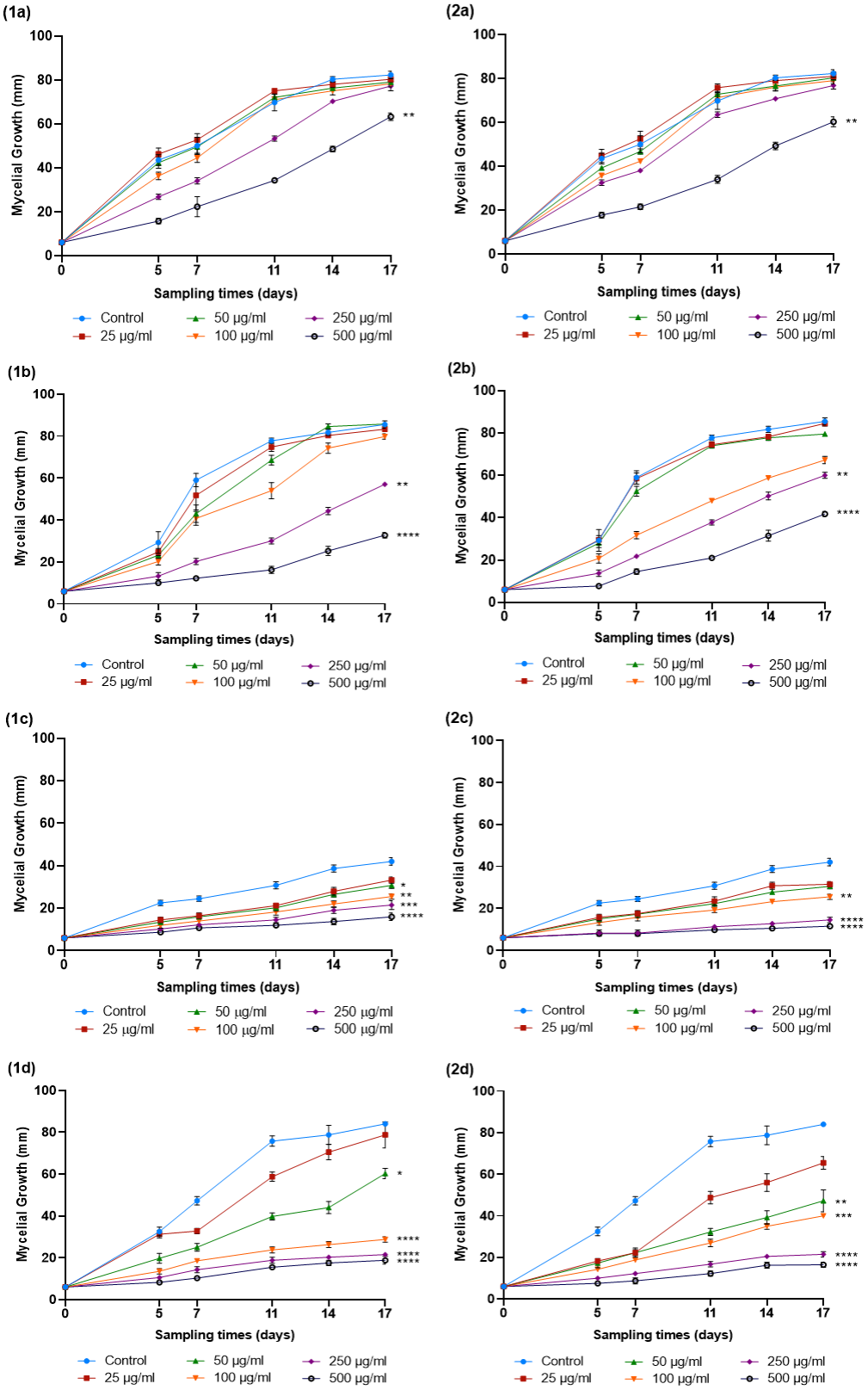

Figure 3. Mycelial growth of fungi on agar plates supplemented with PTS (left column) and PTSO (right column) at 25, 50, 100, 250 and 500 µg/mL over time compared to control, using the Dunnett test at a 95% confidence level. Values are means with SD in bars. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ respect to control. **1)** PTS **2)** PTSO **a)** *G. candidum* **b)** *A. alternata* **c)** *Phyllosticta* spp. **d)** *P. cinnamomi.*

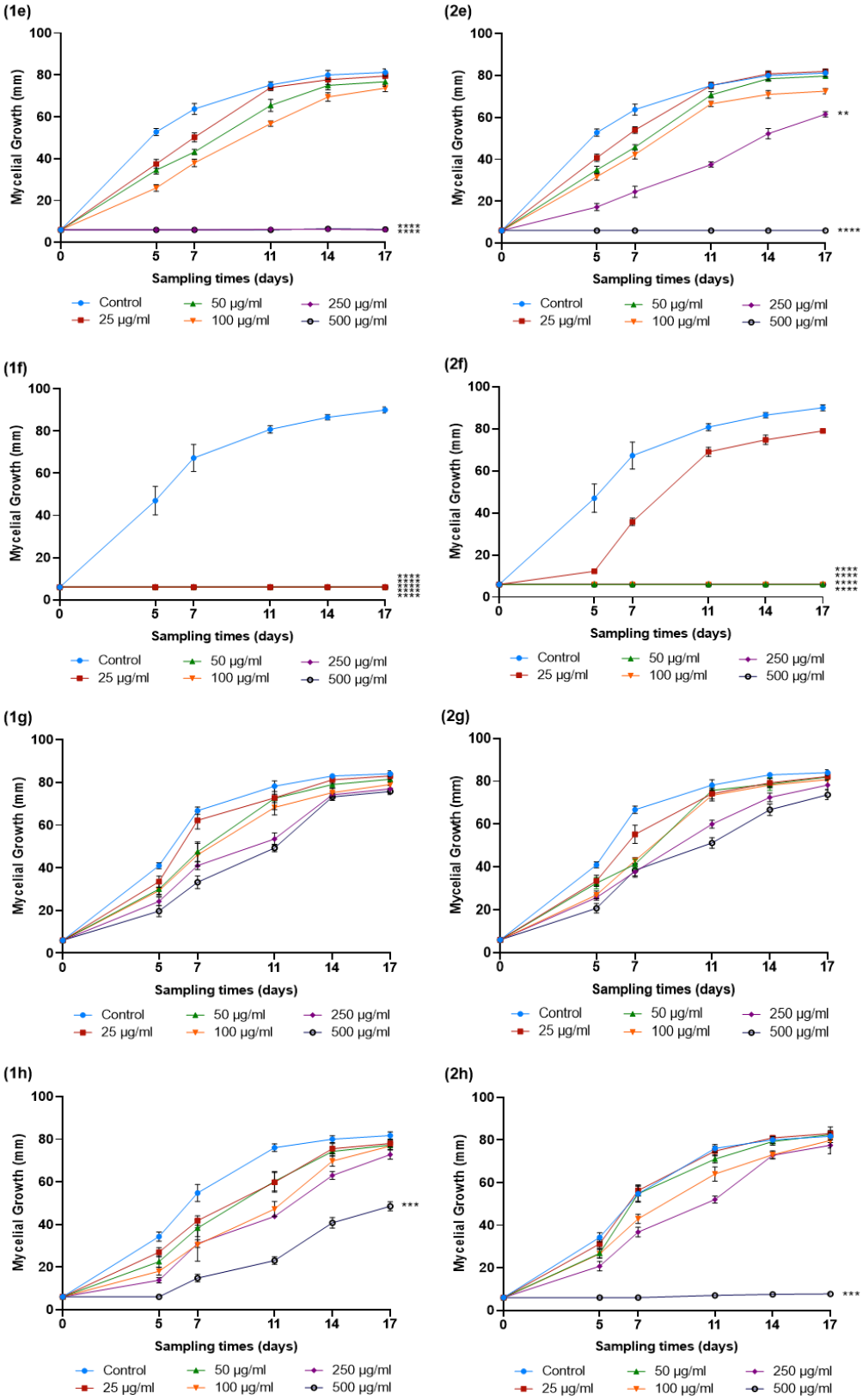

Figure 4. Mycelial growth of on agar plates supplemented with PTS (left column) and PTSO (right column) at 25, 50, 100, 250 and 500 µg/mL over time compared to control, using the Dunnett test at a 95% confidence level. Values are means with SD in bars. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ respect to control. **1)** PTS **2)** PTSO **e)** *F. oxysporum* **f)** *F. graminearum* **g)** *P. expansum*. **h)** *P. digitatum*.

3.3. *In vitro* activity against aphids

Insecticidal and repellent capacity of PTS and PTSO were assessed against adult aphids and presented in Figure 5. In the contact toxicity assay, the mortality rate of the aphid population after 24 hours of exposure to impregnated leaves was investigated. We found that the exposure of *A. gossypii* to both compounds significantly reduced the population at all concentrations evaluated with respect to the negative control ($p < 0.05$). The average mortality of aphids treated with 500 and 1000 µg/mL of PTS and 500 µg/mL of PTSO were 17, 19 and 19% respectively, which is lower than the mortality rate of the population treated with the insecticide used as positive control (28%). On the other hand, the death of individuals treated with PTS ≥ 2500 µg/mL and PTSO ≥ 1000 µg/mL equalled or exceeded the mortality rate of the positive control. However, none of the treatments showed significant differences compared to the positive control, except for the group of aphids treated with PTSO at 5000 µg/mL which registered a significant increase of mortality, reaching 42% after 24 hours of exposure ($p < 0.05$).

Moreover, whereas the insecticidal capacity of PTSO was found to be higher than that of PTS, the behaviour of both compounds was quite homogeneous in terms of repellent activity. At concentrations of 500 and 1000 µg/mL, PTS and PTSO demonstrated a repellent action of 53-56% and 39-47%, respectively. Even though these treatments showed no significant differences, neither between them nor with respect to the positive control (39% repellence), the responses were stronger with higher concentrations, displaying a significant increase of the repellent activity with respect to the control ($p < 0.05$).

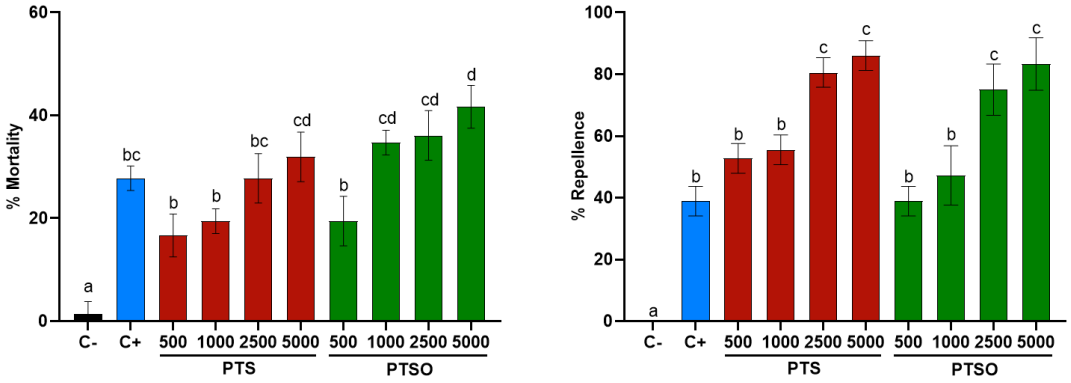

Figure 5. *In vitro* activity of different concentrations of PTS and PTSO against *A. gossypii*: A) Mortality rate due to contact toxicity expressed as percentage, B) Repellent activity expressed as percentage. Each panel includes a group in which the leaves cuttings were immersed in water (C-), in a commercial biocide/pesticide (C+) and in PTS or PTSO at 500; 1,000; 2,500 and 5,000 µg/mL. For both panels, bars with different letters indicate significant differences according to Tukey test ($p < 0.05$).

3.4. Antimicrobial effect and PTS/PTSO quantification in soil

When non-treated, *A. tumefaciens* and *F. oxysporum* generated growth curves according to what was expected, reaching the concentration of 10^8 CFU/g of soil at the

end of the study. Although Metam sodium and the two concentrations of PTS and PTSO tested significantly reduced the population of *A. tumefaciens* and *F. oxysporum* when compared to the untreated soil ($p < 0.05$), the behaviours of the bacterium and the fungus in the presence of the antimicrobials were very unlike. As shown in Figure 6, within 24 hours of exposure *A. tumefaciens* density were narrowed 3 logarithmic units (from 10^7 to 10^4 CFU/g) and, surprisingly, thereupon the population remained steady till the end of the assay. In contrast to the bacterial response, *F. oxysporum* was drastically affected by all the treatments during the first 11 days of sampling. As illustrated in Figure 7, from day 15, the progressive recovery of the fungus was observed in soil treated with Metam sodium and a single application of PTS and PTSO at 100 and 500 µg/g, being less pronounced with 500 µg/g. During the 45 days of the assay, *F. oxysporum* could not be isolated from the soil treated with 3 applications of PTS and PTSO at both 100 and 500 µg/g, which suggests a greater efficacy of the treatments based on repeated lower doses compared to those including a single initial dose at a higher concentration.

Figure 8 shows the concentrations of PTS and PTSO detected in soil by HPLC-UV. In each of the treatments, both compounds evolved in the same way over time. Due to their volatility, neither PTS nor PTSO remained at the established concentration in soil 1 hour after being applied, time at which the first determination was made ([PTS] = 16.04 ± 0.36 and 112.20 ± 0.1168 µg/g instead of 50 and 250 µg/g) ([PTSO] = 37.02 ± 0.54 and 159.20 ± 2.11 µg/g instead of 50 and 250 µg/g). The steady reduction in PTS and PTSO concentration explains why the treatment based on 3 applications of 100 µg/g of active compounds obtained better results than those based on 1 single dose of 500 µg/g for both, *A. tumefaciens* and *F. oxysporum.*

Additionally, although the doses administered of PTS and PTSO for each application were equal (50 µg/g or 250 µg/g of each, depending on the total concentration of active substance), PTSO was shown to be somewhat more stable since its concentration decreases less drastically.

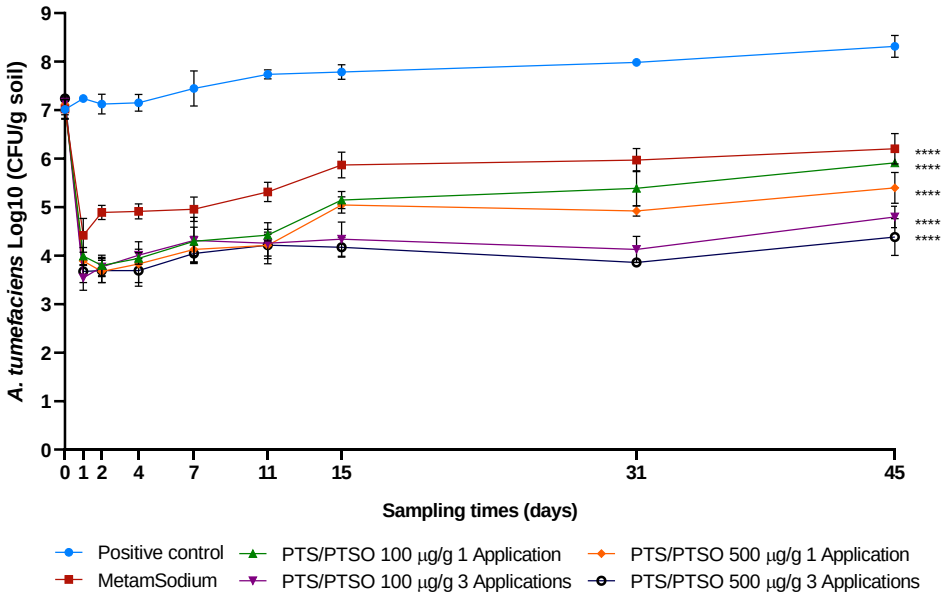

Figure 6. Density of *A. tumefaciens* in soil expressed as Log10 CFU/g of soil over time. Significant reductions in the population of each treated microcosm in comparison with the non-treated group (control) were established according to the Dunnett test at a 95% confidence level. Values are means with SD in bars. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ respect to control.

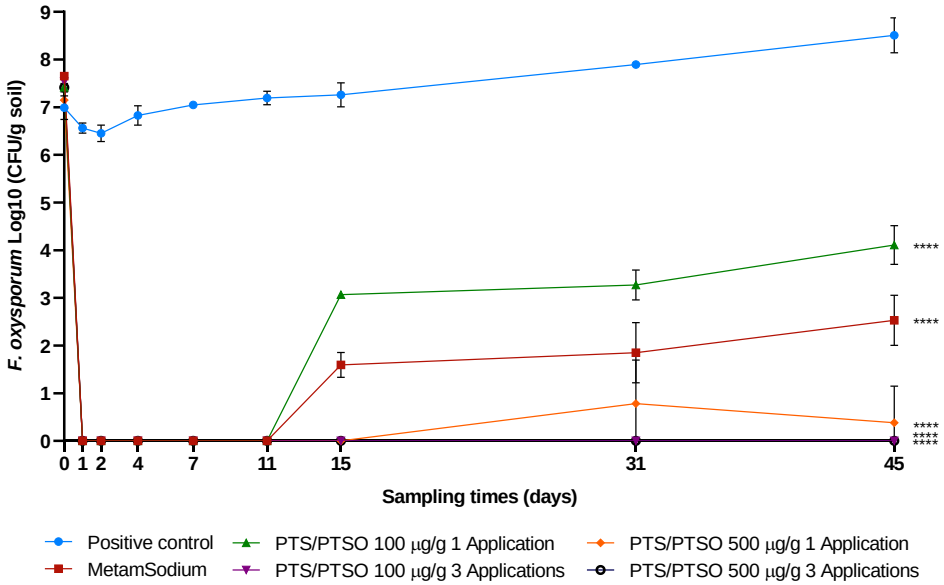

Figure 7. Density of *F. oxysporum* in soil expressed as Log10 CFU/g of soil over time. Significant reductions in the population of each treated microcosm in comparison with the non-treated group (control) were established according to the Dunnett test at a 95% confidence level. Values are means with SD in bars. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ respect to control.

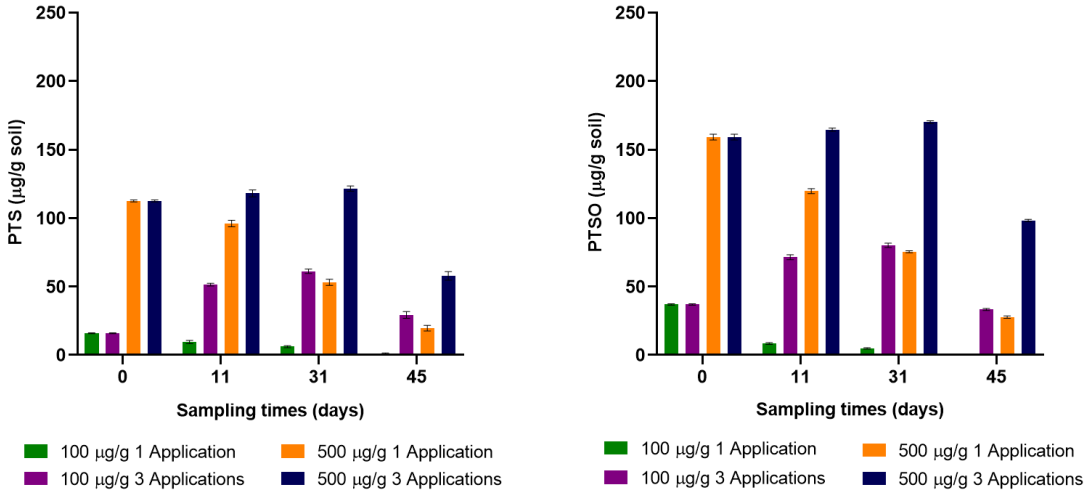

Figure 8. Concentration of PTS (left) and PTSO (right) reached in soil 1 hour (day 0), 11 days, 31 days and 45 days after the first application of the treatment.

4. Discussion

In this work, PTS and PTSO have demonstrated broad antimicrobial activity against phytopathogens that represent a significant threat to many economically important crops, including 6 bacterial and 8 fungal strains.

Organosulfur compounds PTS and PTSO antimicrobial activity had been previously studied against a wide range of human and animal pathogens, including those affecting the poultry industry and aquaculture (Aguinaga-Casañas et al., 2022; Cabello-Gómez et al., 2022; Sorlozano-Puerto et al., 2018). Despite of its instability, allicin significant microbicidal effect has been demonstrated on soil-borne plant pathogenic fungi (Arnault et al., 2013) and, to a lesser extent, on phytopathogenic bacteria (Wallock-Richards et al., 2014). However, there is very little information available that refers to the bioactive properties of PTS and PTSO against phytopathogens and plant pests, and that supports their application within the framework of plant health. Hayat et al., (2016) found that potent broad-spectrum biofungicidal effect of *Alliaceae* extracts might not only be attributed to allicin but also to other organosulfur compounds, stressing the importance of identify such compounds and their bioactivity. Previous reports have suggested that onion essential oils, and particularly PTS and PTSO, have potential applications in post-harvest preservation (Benkeblia, 2004; Mylona et al., 2019). The present investigation is so far the first study to characterize the activity of these compounds *in vitro* against a wide range of target organisms implicated in plant disease breakouts.

Our results indicate that PTSO is more active than PTS against bacteria, what was also reported by Sorlozano-Puerto et al. (2018). Similarly, the higher fungicidal activity of PTSO was also observed in comparison to PTS but, interestingly, no differences were appreciated between both compounds with regard to the fungistatic effect, which was assessed through the mycelial growth inhibition method. This has already been described by our group in previous research on the antifungal activity of PTS and PTSO against the soil-borne pathogenic fungus *V. dahliae* (Falcón-Piñeiro et al., 2021). Additionally, according to the results presented in this study, both compounds preserve the antimicrobial activity in their gas phase. However, when compared the MFC and MBC values of PTS and PTSO, it was found that their antifungal effect was higher than their antibacterial effect. These results are consistent with the data obtained in previous *in vitro* investigations in which the antimicrobial capacity of PTS and PTSO was examined against yeasts and bacteria for their potential use in human therapy (Sorlozano-Puerto et al., 2021).

On the basis of our findings, the persistence of a soil-borne pathogenic bacterium and fungus in soil when treated with PTS and PTSO was assessed. As acknowledged by Arnault et al. (2013), the potential of thiosulfinates contained in *Allium* species to be employed in pest management and, in particular, to serve as biofumigants, has yet to be investigated. From the results obtained in this study it can be concluded that PTS and PTSO have a significant effect on soilborne pathogens. Furthermore, we have established a correlation between the antimicrobial effect of both compounds and their persistence in soil. PTS and PTSO quantification in soil by HPLC-UV demonstrated that both rapidly volatilize in varying degrees, being PTSO more stable than PTS. Due to their high volatility, the concentration of PTS and PTSO in soil decreases immediately after the application, which allows the microorganism to recover. It can therefore be concluded

that their antimicrobial activity in soil is not only linked to the concentration applied initially but, more importantly, to their persistence in soil, which highlights the importance of establishing an optimal application protocol based on repeated doses over time. What is more, despite not being the objective of this study, the evaluation of the antifungal activity of PTS and PTSO in olive plantlets carried out in a previous work of our group evidenced the absence of phytotoxicity, not observing any effect on the appearance or development of the plants (Falcón-Piñeiro et al., 2021). The absence of phytotoxicity and the non-permanence of the compounds in soil supports the safety profile of PTS and PTSO as well as their use as a sustainable alternative for pre- and post-plant soil fumigation. However, further studies on how these compounds influence plant physiology and stress response mechanisms are needed.

Additionally, the results obtained in the soil sanitization assay indicate that PTS and PTSO antifungal activity is higher than their antibacterial activity, which had previously been suggested according to the *in vitro* antimicrobial activity tests performed in this investigation. While the high permeability of volatile organosulfur compounds through the phospholipid membranes and their ability to interact with thiols containing compounds may explain their antimicrobial activity (Hayat et al., 2016; Miron et al., 2000), the higher permeability of the fungal chitin cell wall compared to the peptidoglycan cell wall of bacteria support our findings that PTS and PTSO influence fungal growth to a greater extent (Khan & Zhihui, 2010; Lemar et al., 2002). Another explanation might be the interaction of PTS and PTSO with small secreted cysteine-rich proteins (SSCPs), specific of the secretomes of filamentous and dimorphic fungi (Feldman et al., 2020). These molecules present a broad functional versatility and, although most of them remain unclassified, it has been demonstrated that SSCPs have an essential role in fungal reproduction and dispersal (Cai et al., 2020; Z. Zhao et al., 2021). They are also involved in environmental interactions, fungi-plant interactions and substrate colonization (Frías et al., 2011; Viterbo & Chet, 2006). Lu & Edwards, (2016) functionally characterized SSCPs of *F. oxysporum* by their detection in the secretome by nano LC-MS/MS and the subsequent identification in infected wheat heads through gene expression profiling, thus demonstrating the role of SSCPs in the pathogenesis of the disease caused by *F. oxysporum* in wheat.

According to several studies, antimicrobial plant derived compounds might also possess insecticidal and repellent activity within their bioactive properties (Semerdjieva et al., 2021). Jiang et al., (2016) found that linalool is responsible for the insecticidal and repellent activities of *Cinnamomum camphora* essential oil against cotton aphids. Similarly, other authors showed that glucosinolates extracted from *Tropaeolum tuberosum* and capsaicinoids extracted from *Capsicum chinese* can successfully control *Aphis cytisorum* (Claros Cuadrado et al., 2019). Insecticidal and repellent activity of volatile compounds of onion and garlic had already been established against aphid *Myzus persicae* by contact toxicity test and choice test using commercial essential oils (Hori, 1996). In addition, a non-standardized blend of aqueous extracts of onion and garlic was effectively used as an alternative pesticide that was able to significantly reduce aphids'

population of different species infecting date palm trees, as reported by Ali Al-Shuraym (2022). In this study we have also demonstrated the effect of PTS and PTSO against the aphid species *A. gossypii*. Our *in vitro* results showed that the mortality rates achieved by both compounds equals that of commercial chemical insecticide from a concentration of 0.25% PTS and 0.1% PTSO. What is more, the repellent capacity exhibited by 0.1% PTS and PTSO significantly exceed the effect of DEET. While PTSO shows greater biocidal activity against *A. gossypii*, the greater volatility of PTS compared to PTSO, as demonstrated by HPLC-UV determination, explains why PTS has a greater repellent capacity.

Bioactive compounds from plant material with proven antibacterial, antifungal and insecticidal activity are an important natural source for the development of new environmentally safe plant protection products (Bakkali et al., 2008; Isman, 2020). Onion is one of the most cultivated and consumed vegetables worldwide, since 93 million tons are globally produced per year (Top Onion Producing Countries, 2022). Taking into account those that do not reach the consumer due to their low quality and the inedible parts, onion processing creates massive wastes that are a rich source of bioactive compounds (Benítez et al., 2011; Mourtzinos et al., 2018). Therefore, to orientate the valorisation of onion solid wastes, which reach 6 tons annually in European countries and have detrimental effects on the environment when not processed correctly, towards the formulation of novel and sustainable products suitable for agricultural production does not only reduce the environmental damage and provide low-cost raw material but also cover important needs for the agrifood sector (Katsampa et al., 2015; Kumar et al., 2022).

Even thought our results suggest that PTS and PTSO from *A. cepa* display strong activity against pathogenic microorganisms and aphids, and provide useful information that support their use for crop disease control, the present work has not evaluated how these organosulfur compounds influence soil microbial populations, natural enemies or pollinators. Therefore, further studies should focus on the analysis of the effect of PTS and PTSO on soil microbiome and non-target species.

5. Conclusions

PTS, and specially PTSO, showed antimicrobial effect against a wide range of bacteria and fungi infecting plants. This study revealed that the degree of efficacy of PTS and PTSO depend on the target species, being more effective against the fungal strains evaluated. Despite their rapid volatilization from soil, the combination of these compounds successfully controlled soilborne microorganism population. Both of them had similar, if not more, biocidal and repellent effect than commercial fumigants. Although PTS and PTSO will be further evaluated in field experiments for potential control of pathogen populations in crops, these results encourage their use for the development of sustainable biopesticides that contribute to environmental health. Moreover, both metabolites are found to be promising candidates for Integrated Pest

Management, whose bases include sustainable pest control, reduction of pesticide residues and the use of natural resources.

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1.2. *In vitro* screening of natural solutions for the control of *Xylella fastidiosa*

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Abstract: Effective preventive measures and therapies are lacking for the control of olive quick decline syndrome (OQDS), caused by *Xylella fastidiosa.* This xylem inhabiting bacterium represents a serious threat to agriculture in the Mediterranean region and has caused significant damage to olive trees in Italy. In this study we explored the potential bio-based approaches using organosulfur compounds (OSCs) from onion (*Allium cepa*) and the biocontrol bacterium *Pseudomonas lactis* PV8 to manage *X. fastidiosa* subsp. *pauca* strain ST53 in vitro. A standardized onion extract was successfully obtained, characterized, and quantified for its OSC content, with propyl propane thiosulfinates (PTS) and thiosulfonates (PTSO) identified as the main active compounds. Antibacterial assays conducted with the onion extract, as well as with PTS and PTSO individually, showed strong antibacterial activity against *X. fastidiosa*, with inhibition zones comparable to those of conventional antibiotics. Similarly, *P. lactis* PV8 demonstrated significant antagonistic activity through direct competition with the pathogen. While further research is required to assess the efficacy of these bio-based solutions in planta, this study lays the groundwork for sustainable strategies aimed at reducing the spread of OQDS and managing *X. fastidiosa* in olive trees.

Keywords: *Xylella fastidiosa; Pseudomonas lactis;* PTS, PTSO, onion extract

In progress.

1. Introduction

Xylella fastidiosa is a Gram-negative gamma proteobacterium that belongs to the family *Xanthomonodaceae* (Bansal et al. 2022). This xylem-dwelling bacterium has been detected in more than 600 plant species, according to recent surveys, highlighting its extensive host range. *X. fastidiosa* can remain asymptomatic in several host plants, yet it causes devastating diseases in others, leading to substantial economic losses (Delbianco et al. 2023). Some of the most destructive and economically significant diseases caused by this bacterium include Pierce's disease in grapevine (*Vitis vinifera*), citrus variegated chlorosis (CVC) in sweet orange (*Citrus sinensis*), phony peach disease affecting *Prunus persica*, almond and coffee leaf scorch affecting *Prunus dulcis* and *Coffea arabica*, and plum leaf scald affecting *Prunus domestica* (Surano et al. 2022). The genetic variability of *X. fastidiosa* plays a crucial role in its ability to infect a wide range of plant species. This variability arises mainly through homologous recombination, which lead to rapid evolutionary changes that allow the bacterium to adapt to and colonize new host plants (Nunney et al. 2014). *X. fastidiosa* is divided into 5 subspecies, largely separated due to geographical constraints, being subsp. *fastidiosa*, *multiplex* and *pauca* the most commonly reported. *X. fastidiosa* was originally confined to the Americas, with *X. fastidiosa* subsp. *fastidiosa* and *X. fastidiosa* subsp. *multiplex* being widespread in the United States, while *X. fastidiosa* subsp. *pauca* is found predominantly in South America (Vanhove et al. 2019). In 2013, this bacterium was detected for the first time in Europe, in the region of Apulia in southern Italy, where *X. fastidiosa* subsp. *pauca* strain ST53 was identified as the causal agent of olive quick decline syndrome (OQDS) (Surano et al. 2023). The strain ST53 is linked to Central American isolates according to genomic studies, with the genotype also reported in Costa Rica, and was likely introduced through infected plant material, as asymptomatic plants for planting can serve as an efficient source of inoculum for new outbreaks (Giampetruzzi et al. 2017).

X. fastidiosa is a vector-borne bacterium primarily transmitted by common xylem sap-feeding insects. The rapid spread of OQDS in olive groves has been linked to efficient transmission of the bacterium by the spittlebug, *Philaenus spumarius* (White et al. 2020). OQDS is characterized by leaf scorch, marginal necrosis, and the desiccation of branches and twigs, symptoms that typically begin at the top of the tree canopy and gradually spread to the entire crown, ultimately leading to tree death (Saponari et al. 2019). Bacterial cells attach to the plant xylem vessels, which transport water and mineral elements from the soil to the aerial parts. As the cells proliferate, they aggregate and form a biofilm matrix composed of secreted molecules, primarily exopolysaccharides. These aggregates block the xylem vessels, disrupting water transport and contributing to the decline of the tree (D'attoma et al. 2019).

The introduction of *X. fastidiosa* in Apulia triggered an epidemic spread of OQDS, decimating olive trees across the region. Favourable epidemiological conditions, including an abundant insect vector population and the absence of effective treatments for infected plants, have made controlling the spread of the bacterium particularly challenging (Giampetruzzi et al. 2020; Surano et al. 2023). This poses a significant threat

to Mediterranean countries, where olive cultivation holds great economic, cultural, and environmental significance (Bizos et al. 2020; Bajocco, Raparelli, and Bregaglio 2023). Currently, most strategies to manage *X. fastidiosa* aim to limit its spread through insecticide use and plant removal. As Europe transitions to a more sustainable agricultural model, alternative approaches, including plant secondary metabolites and biological control strategies, are being explored (Moll et al. 2021).

Organosulfur compounds (OSCs) from onion (*Allium cepa*), such as thiosulfinates and thiosulfonates, have demonstrated significant antimicrobial activity against various plant pathogens, including bacteria and fungi (Lanzotti, Bonanomi, and Scala 2013). Additionally, in olives, these compounds demonstrated the ability to significantly reduced vascular colonization by *Verticillium dahliae*, which, like *X. fastidiosa*, is a xylem-inhabiting pathogen (Falcón-Piñeiro et al. 2021).

On the other hand, *Pseudomonas* species are recognized for their potential in managing plant diseases. These bacteria can suppress various pathogens through competition for resources and the production of antimicrobial compounds (Dimkić et al. 2022). Studies have shown that *Pseudomonas* spp. effectively reduce disease severity caused by *X. fastidiosa* and other xylem inhabiting pathogens, making them promising candidates for biological control strategies. Their ability to form protective biofilms on plant tissues enhances plant resilience against pathogen colonization (Niem et al. 2020; Achari and Ramesh 2014).

The aim of this study was to explore in vitro bio-based solutions for controlling *Xylella fastidiosa* subsp. *pauca* strain ST53, by utilizing a standardized onion extract rich in organosulfur compounds (OSCs) and *Pseudomonas lactis* PV8, with the aim of laying the groundwork for their potential use in the control of olive quick decline syndrome (OQDS).

2. Material and Methods

2.1. Microorganisms

The biocontrol bacterium *Pseudomonas lactis* strain PV8 was isolated from plant tissues in a family-owned orchard located in the Sierra Nevada Natural Park, Granada, Spain, as part of a biodiversity study under the VEGELAB research project, in collaboration between the University of Granada and DOMCA (Granada, Spain). This psychrophilic bacterium was selected for further investigation due to its antagonistic activity against *Pseudomonas* spp and *Fusarium* spp., and was identified by 16S rRNA sequencing, with 99.8% similarity. PV8 was grown on tryptone soy agar (TSA) (Bioser, Barcelona, Spain) at 25°C for 48 h.

Xylella fastidiosa subsp. *pauca* strain ST53 was used in this study. It was grown on PD3 agar at 25°C for one week (Davis, Purcell, and Thomson 1980).

2.2. Organosulfur compounds

PTS and PTSO at 20% were provided by DOMCA SA (Granada, Spain). All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated.

2.3. Onion extract

A standard process was followed to obtain an onion extract rich in OSCs from low-quality onion bulbs, unsuitable for human consumption. The onions were provided by a local producer (Fuente Vaqueros, Granada, Spain), peeled and immediately washed twice in a 1% acetic acid aqueous solution for 30 min, then rinsed with water. The cleaned onions were chopped and crushed using a cutter (CUT-MIX-200, CASTELL VALL Barcelona, Spain). The resulting onion paste was macerated in ethanol aqueous solution at 30% at room temperature for 24 h to extract the active compounds. A mixture of oxidants, including peracetic acid, was added during maceration to increase the concentration of thiosulfonates and thiosulfinates in the final extract. After maceration, the mixture was filtered to remove solids. The filtered solution was then subjected to hydro-distillation to concentrate the OSCs. The distillate was subsequently extracted using ethyl acetate in proportion (1:1), which allowed for GC/MS analysis of the compounds present. The organic phase, containing the OSCs of interest, was dried with anhydrous sulphate salt and then filtered. The solvent was removed under reduced pressure at 40°C, yielding the final onion extract, which was analysed by gas chromatography-mass spectrometry (GC/MS) to confirm its OSCs composition.

2.3.1. Extract GC/MS characterization

To identify the OSCs in the onion extract after removing water from the distillate, a gas chromatographic method coupled with a mass spectrometer detector was utilized. This analysis was performed using a Gas Chromatographer 7820A coupled to a Mass Spectrometer 5977B (Agilent Technologies, Santa Clara, CA, USA), equipped with an electron impact ionization source operating at 70 eV and a quadrupole analyser. The method was based on a procedure recently published (Pastor-Belda et al. 2020). The selected column was an Agilent ultra-inert DB-5MS UI (30 m x 0.25 mm x 0.25 μm; 5% diphenyl-95% dimethylpolysiloxane) with a 4mm ID liner trap. The method parameters were set as follows: helium (99.99%) was used as the carrier gas at a flow rate of 1.0 mL/min; the injection volume was 1 μL in split-less mode; the injector temperature was 280 °C. The oven temperature program consisted of an initial isothermal stage at 50 °C for 1 minute, followed by an increase to 160 °C at 25 °C/min, and then to 250 °C at 30 °C/min, where the temperature was held for an additional 1.3 minutes. The mass spectrometer parameters were as follows: ionizing voltage of 70 eV, temperature of 230 °C, quadrupole temperature of 150 °C, transfer line temperature of 300 ºC, scanning range of 50–550 amu (atomic mass units), and a solvent delay of 4 minutes. Most of the compounds in the total ion chromatogram were identified by comparison with the NIST mass spectral library.

2.3.2. OSCs identification and quantification by HPLC-UV

To monitor the total amount of OSCs obtained after the hydro-distillation of onion macerates, a liquid chromatographic method was performed using an HPLC Agilent Infinity 1260 (Agilent Technologies, Santa Clara, CA, USA) equipped with a DAD (UV-detector), a quaternary pump, an on-line degasser, an autosampler, and a column thermostat. The chromatographic method was based on a procedure previously published (Abad et al. 2015). The selected column was an Agilent Zorbax Eclipse Plus C18 (4.6 x 50 mm, 1.8 μm), and all analyses were conducted at 25 °C. The mobile phases used were MeCN (A) and an aqueous solution of perchloric acid 30 mM (B). The injection volume was set at 10 μL, and the flow rate at 0.85 mL/min. The detector wavelength was fixed at 200 nm. The gradient selected is described in Table 1.

Table 1. Ratio of solvents for the HPLC-UV analytical method.

Time (min)	% Mobile phase A	% Mobile phase B
0	50	50
1	65	35
2	75	25
3	85	15
4	100	0
6	100	0
10	0	0
12	0	0

The analytical method was validated for the quantification of OSCs present in the extract after hydro-distillation. The samples were directly injected without dilution, after filtration through a 0.22 μm nylon filter. A commercial standard was used for the calibration curve with five different concentration levels ranging from 80 to 500 mg/L, each prepared in duplicate and injected in triplicate. The limit of detection (LOD) and limit of quantification (LOQ) were established at 2 and 6 ppm, respectively. The method demonstrated linearity with r^2 values greater than 0.99 and was considered precise in terms of repeatability based on the relative standard deviation values obtained.

2.4. In vitro antimicrobial activity of OSCs against *X. fastidiosa*

The antimicrobial activity of PTS, PTSO and Onion extract was evaluated by the agar well diffusion method following the methodology previously described with some modifications (Zicca et al. 2020). Three drops of 20 μL of *X. fastidiosa* suspension at 10^8 CFU/mL in saline solution (NaCl 0.9%) were placed at the top of PD3 agar plates and allowed to flow towards the opposite side of the plate to create three parallel rows of culture. After 24 hours of incubation, an 8 mm well was made at the top of the *X. fastidiosa* rows, where 200 μL of the compound was deposited. The tested concentrations were 50, 25, 10, 5, and 2.5 μg/μL. A negative control using double-distilled water and a positive control consisting of a mixture of ampicillin at 1 μg/μL and streptomycin at 0.025 μg/μL were included. The inhibition area was measured after 7 days of incubation. Tests were performed in triplicate.

2.5. In vitro antagonistic activity of *P. lactis* PV8 against *X. fastidiosa*

The antagonistic activity of *P. lactis* PV8 against *Xylella fastidiosa* was assessed on PD3 agar by the method described in Section 2.4 *In vitro antimicrobial activity of OSCs against X. fastidiosa*. Both PV8 and *X. fastidiosa* suspensions were prepared in saline solution (NaCl 0.9%). Additionally, the strain PV8 was evaluated for the production of antimicrobial compounds in liquid culture (Zicca et al. 2020). PV8 was inoculated at 2% into 20 mL of LB broth (Shahid et al. 2021), and incubated at 25°C under shaking (140 rpm) for 48 h. The culture filtrate was then obtained by centrifugation at 13,000 rpm for 10 min, followed by filtration of the supernatant through 0.22 µm filter. 200 µL of either PV8 suspension at 10^8 CFU/mL or culture filtrate was deposited into 8 mm well made at the top of the *X. fastidiosa* rows. The inhibition zone was measured after 7 days of incubation. Tests were performed in triplicate.

3. Results

3.1. Onion extract characterization

The onion extract was analysed by GC/MS to identify the main OSCs. The compounds detected in the onion extracts by GC/MS are shown in Figure 1, along with their retention times: Methyl propyl disulphide (rt = 7.87 min), Dipropyl disulphide (rt = 12.67 min), Dipropyl trisulphide (rt = 13.74 min), Propyl propane thiosulfonate (rt = 19.48 min), and Dipropyl tetra sulphide (rt = 23.08 min). Figures 2, 3, 4, 5, and 6 display the GC/MS spectra for each identified compound.

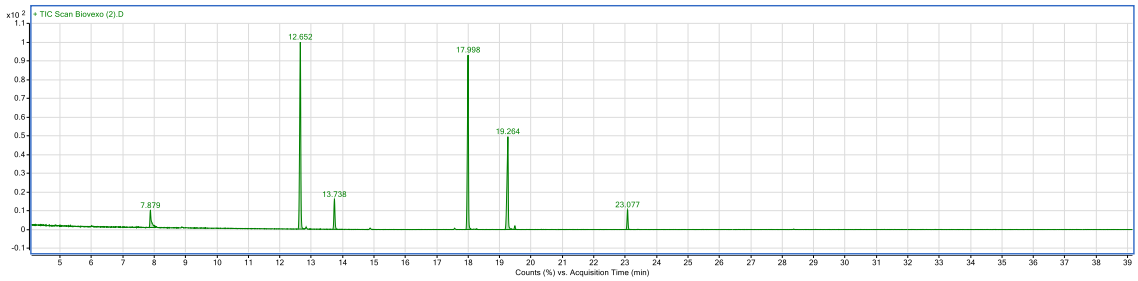

Figure 1. GC-MS spectrum of onion extract, with retention times indicated for key compounds.

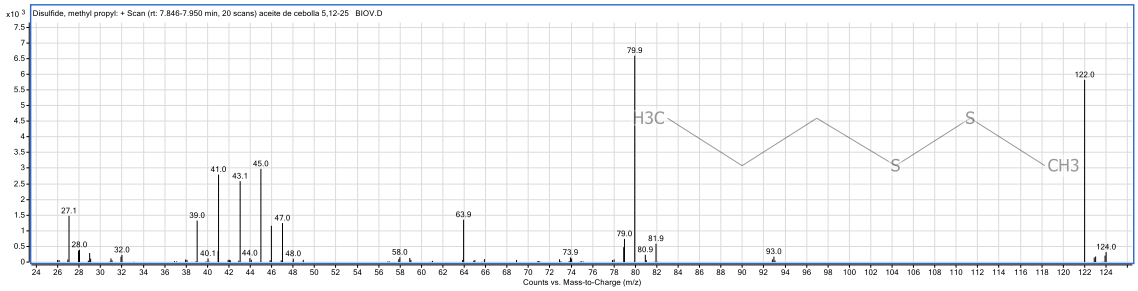

Figure 2. GC-MS spectrum of Methyl propyl disulphide.

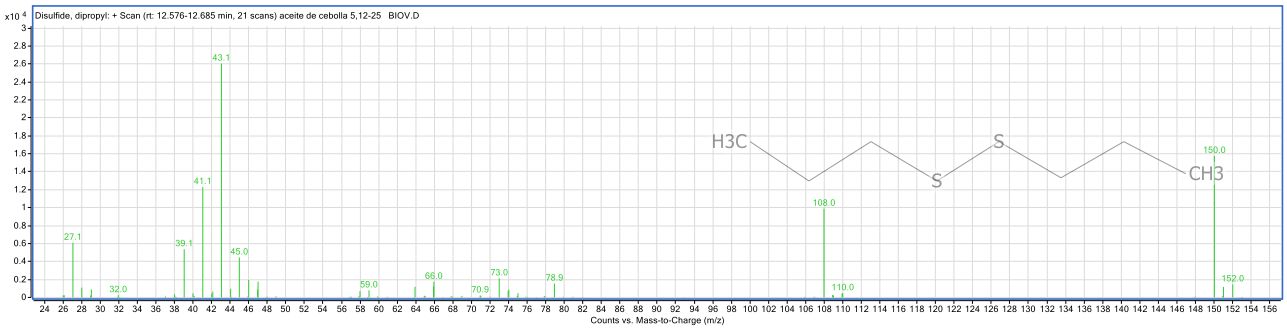

Figure 3. GC-MS spectrum of Dipropyl disulphide.

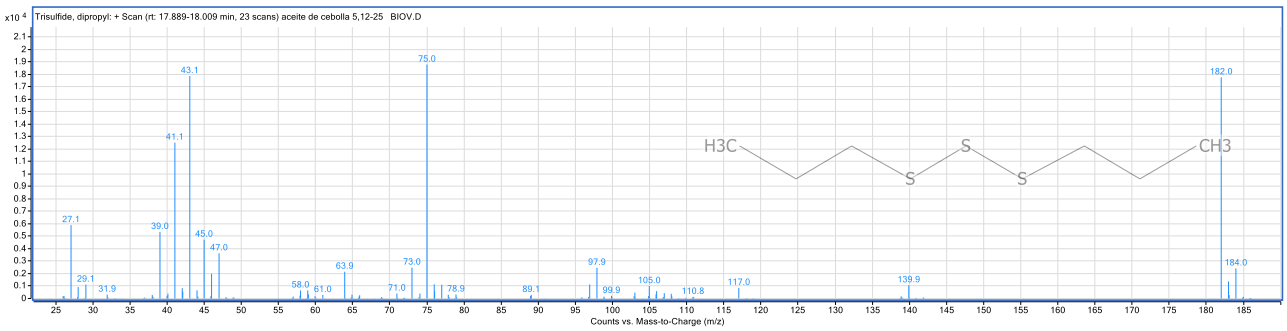

Figure 4. GC-MS spectrum of Dipropyl trisulphide.

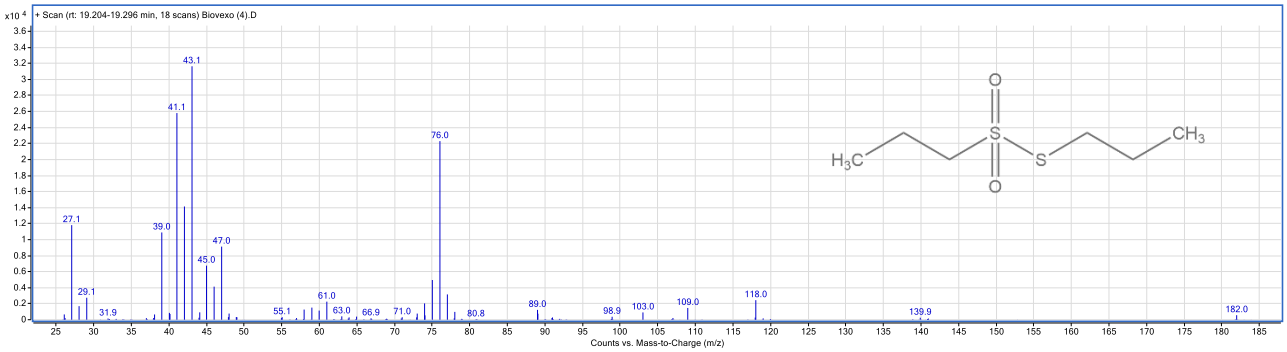

Figure 5. GC-MS spectrum of Propyl propane thiosulfonate.

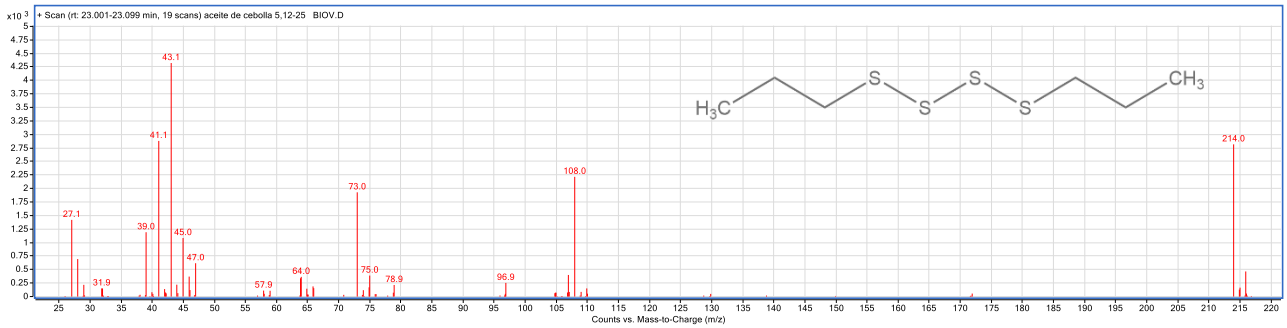

Figure 6. GC-MS spectrum of Dipropyl tetrasulphide.

The onion extract was then analysed by HPLC-UV to quantify the total amount of OSCs. Interestingly, propyl propane thiosulfinate (PTS), which was not detected by GC/MS, was detected in the HPLC chromatogram (Figure 7). This discrepancy may arise from the inability of this compound to be detected by GC/MS, possibly due to problems with vaporization and ionization. The total concentration of OSCs in the onion extract

obtained was 3300 mg/L. The predominant compounds were thiosulfinates (PTS) and thiosulfonates (PTSO), with retention times of 1.679 s and 2.285 s, respectively. Together, they accounted for 47% of the total OSC content. This information is summarized in Table 2.

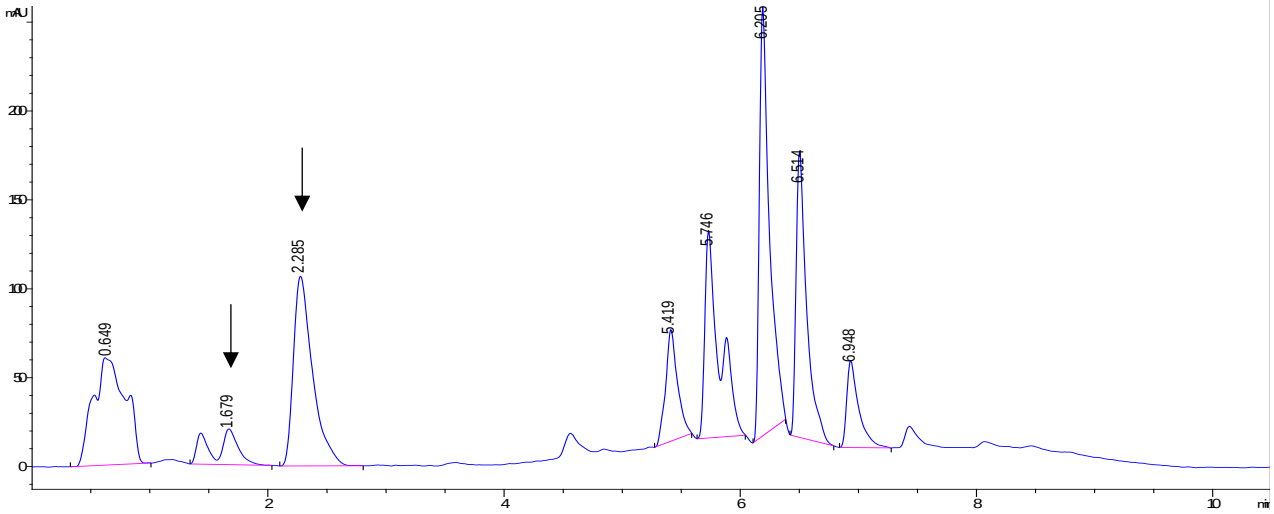

Figure 7. HPLC-UV chromatograms of onion extract, with retention times indicated for key compounds.

Table 2. Extraction yield.

OSCs total amount (mg/L)	PTS + PTSO (%)
3300	47

3.2. Effect of organosulfur compounds and *P. lactis* PV8 on the growth of *X. fastidiosa*

The antibacterial activity of PTS and PTSO, as well as onion extract rich in these organosulfur compounds, was tested *in vitro* against *X. fastidiosa*. As shown in Table 3, all three treatments exhibited inhibitory effects on the growth of *X. fastidiosa*. Both PTS and PTSO showed very similar antibacterial effects across all concentrations tested. At 50, 25, 10 and 5 µg/µL, both compounds completely inhibited bacterial growth, with values greater than 75 mm, maintaining a strong inhibitory effect as their concentration decreases. Only at the lowest concentration tested, 2.5 µg/µL, bacterial growth was observed (Figure 8 and 9), with inhibition values of 69.5 ± 2.24 mm for PTS and 64.6 ± 1.06 mm for PTSO. At 50 µg/µL and 25 µg/µL, the onion extract showed similar antibacterial activity to PTS and PTSO, with inhibition values > 75 mm. As the concentration decreased, the inhibition data revealed a clear dose-dependent response, while the onion extract still maintained a strong antibacterial effect (Figure 10). At 10 µg/µL, it produced significant inhibition, with a value of 59.2 ± 1.25 mm. This inhibitory effect was consistent at lower concentrations, with inhibition values of 51.5 ± 1.07 mm at 5 µg/µL and 51.5 ± 1.14 mm at 2.5 µg/µL. The positive control, consisting of ampicillin and streptomycin, produced an inhibition zone of 33.0 ± 3.51 mm (Figure 11), demonstrating a greater efficacy with PTS, PTSO, and the onion extract that with antibiotics at all tested concentrations.

Table 3. In vitro antimicrobial activity of PTS, PTSO and onion extract against *X. fastidiosa*, expressed as the average radius ± standard deviation of inhibition zone (mm).

Concentration (μg/μL)	PTS	PTSO	Onion extract
50	> 75	> 75	> 75
25	> 75	> 75	> 75
10	> 75	> 75	59.2 ± 1.25
5	> 75	> 75	51.5 ± 1.07
2.5	69.5 ± 2.24	64.6 ± 1.06	51.5 ± 1.14

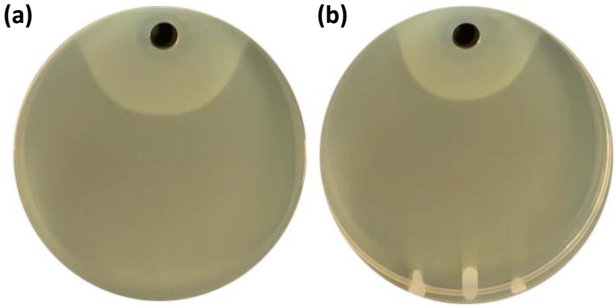

Figure 8. Antimicrobial activity of PTS against *X. fastidiosa*. PTS was applied in the well located at the top of the plates at **(a)** 5 μg/μL and **(b)** 2.5 μg/μL.

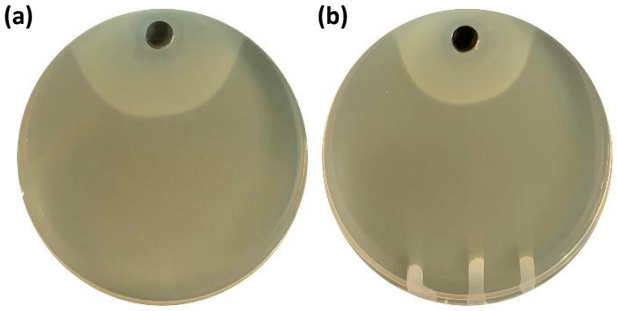

Figure 9. Antimicrobial activity of PTSO against *X. fastidiosa*. PTSO was applied in the well located at the top of the plates at **(a)** 5 μg/μL and **(b)** 2.5 μg/μL.

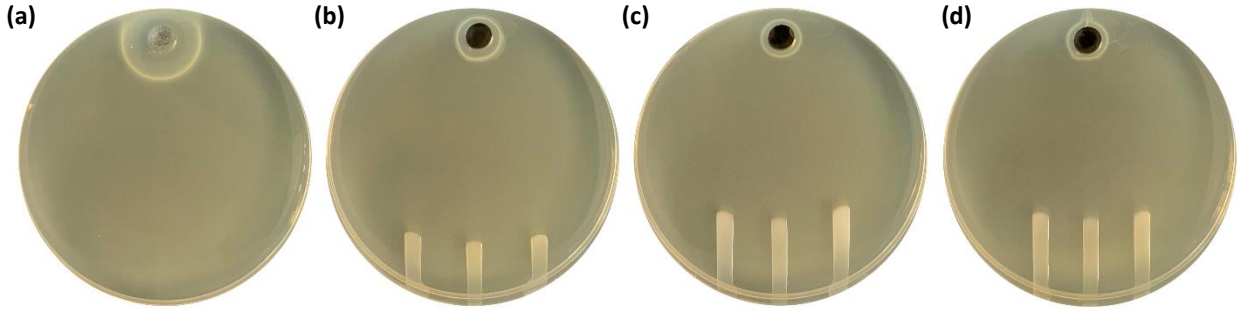

Figure 10. Antimicrobial activity of onion extract against *X. fastidiosa*. The onion extract was applied in the well located at the top of the plates at **(a)** 25 μg/μL **(b)** 10 μg/μL **(c)** 5 μg/μL and **(d)** 2.5 μg/μL.

Figure 11. Antimicrobial activity of the mixture of ampicillin at 1 µg/µL and streptomycin at 0.025 µg/µL against *X. fastidiosa.* Antibiotics were applied in the well located at the top of the plates.

The ability of *P. lactis* PV8 to inhibit the growth of *X. fastidiosa* was evaluated. It demonstrated strong antibacterial activity against *X. fastidiosa*, with an inhibition zone of 35.3 ± 0.65 mm, as shown in Table 4. The inhibition zone produced by *P. lactis* PV8 (Figure 12a) was similar to that of ampicillin and streptomycin, used as positive control, that measured 33.0 ± 3.51 mm (Figure 11). In contrast, the culture filtrate did not show any inhibitory activity, indicating that *P. lactis* PV8 did not produce active antimicrobial metabolites in LB broth under the conditions tested (Table 4, Figure 12b).

Table 4. In vitro antagonistic activity of *P. lactis* PV8 and culture filtrate against *X. fastidiosa*, expressed as the average radius ± standard deviation of inhibition zone (mm).

	***P. lactis* PV8**
Bacterial suspension	35.3 ± 0.65
Culture filtrate	0

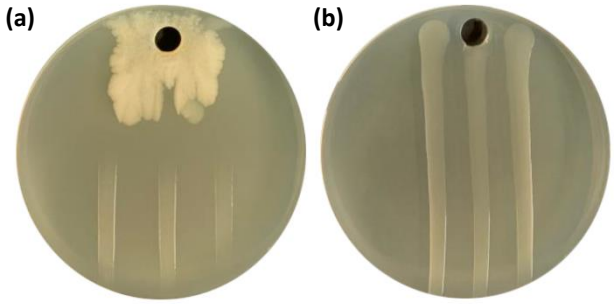

Figure 12. Antagonistic activity of *P. lactis* PV8 against *X. fastidiosa.* **(a)** PV8 suspension and **(b)** culture filtrate were applied in the well located at the top of the plates.

4. Discussion

The results of this study provide important insights into potential bio-based solutions for managing *X. fastidiosa* subsp. *pauca* strain ST53. The antimicrobial activity of OSCs from onion extract showed significant inhibitory effects against X. *fastidiosa*, with both individual compounds PTS and PTSO, as well as the onion extract, demonstrating strong bactericidal properties in a dose dependent manner. The strong inhibition zones generated in comparison to antibiotics, which are banned in the European Union as a treatment against bacterial plant diseases (Quetglas et al. 2022), suggest that these compounds could serve as effective alternatives to chemical

treatments, which is crucial in the context of sustainable agriculture. These findings are consistent with previous studies showing the effectiveness of these compounds against a wide range of phytopathogens (Falcón-Piñeiro et al. 2023). Nevertheless, while OSCs from onion have shown antimicrobial properties in other contexts, their potential as a control measure for *X. fastidiosa* had not yet been explored. To the best of our knowledge, this is the first study to evaluate the antimicrobial activity of OSCs from onion specifically against *X. fastidiosa*. In contrast, there has been limited research on the use of garlic extract against this pathogen. A recent investigation reported that a garlic-based solution effectively inhibited the planktonic growth of *X. fastidiosa in vitro* (Vizzarri et al. 2023).

Studies on the effect of OSCs from onion on *Xanthomonas* species, which share a taxonomic relationship with *X. fastidiosa* as both belong to the family *Xanthomonadaceae*, may offer valuable insights into the management of *X. fastidiosa* with this solution (Bansal et al. 2023). *In vitro* studies have demonstrated the efficacy of OSCs from onion and garlic against *Xan. campestris* and *Xan. oryzae* pv. *Oryzae* (Hussain et al. 2021; Srinivas et al. 2024). Additionally, onion essential oil vapours demonstrated strong antimicrobial effects against *Xan. campestris* on infected Cucurbitaceae, Brassicaceae, and Solanaceae seeds without affecting germination rates (Chung et al. 2022).

Alongside OSCs, the use of biological control bacteria, such as *Pseudomonas* species, presents an additional bio-based strategy for managing *X. fastidiosa*. In this study, *P. lactis* PV8 exhibited strong antagonistic activity against *X. fastidiosa* in vitro, producing inhibition zones comparable to those generated by conventional antibiotics. This aligns with previous researches, where *Pseudomonas* sp. have shown significant *in vitro* activity against *X. fastidiosa*, as well as against the related pathogen *Xanthomonas citri* (Mourou et al. 2022; Poveda et al. 2021). The ability of *Pseudomonas* species to colonize plant tissues and form biofilms may contribute to its effectiveness *in planta*, as biofilms can enhance the resilience of plants by occupying ecological niches that would otherwise be exploited by pathogenic bacteria (Heredia-Ponce et al. 2021). According to previous studies, biofilm formation allows *Pseudomonas* species to establish stable populations within the xylem. In grapevines (*V. vinifera*) affected by Pierce's disease, endophytic inoculation with *P. fluorescens* promoted xylem colonization, allowing it to compete with *X. fastidiosa* and reduce both disease severity and bacterial titre. Nevertheless, *X. fastidiosa* was still present in the treated plants at the end of the experiment (Bragard et al. 2019).

However, it is noteworthy that the culture filtrate of *P. lactis* PV8 did not show any inhibitory activity, suggesting that the bacterium does not produce diffusible antimicrobial compounds under the tested conditions. This implies that the inhibitory effect of *P. lactis* PV8 may primarily result from direct interaction or competition with *X. fastidiosa*, rather than the secretion of antimicrobial metabolites. This contrasts with findings from other studies, where biocontrol *Pseudomonas* strains have been shown to produce a variety of bioactive compounds, including siderophores, antibiotics, and lytic enzymes, which are critical for their antagonistic activity against plant pathogens (Biessy

and Filion 2018; Höfte and Altier 2010). Other authors have reported that metabolites from *Pseudomonas* sp. isolated from citrus trees disrupt biofilm formation by *Xan. citri*, reducing disease severity in *Citrus sinensis* (orange) and *Citrus limon* (lemon) plants (Pistori et al. 2018; Poveda et al. 2021). Further studies are needed to explore the potential of *P. lactis* PV8 to produce bioactive compounds under different conditions, as well as its ability to colonize olive trees and suppress *X. fastidiosa* in planta.

5. Conclusion

This study highlights the potential of bio-based approaches, such as organosulfur compounds (OSCs) from onion and *Pseudomonas lactis* PV8, as alternative strategies for the management of *X. fastidiosa* subsp. *pauca*. An onion extract rich in OSCs was successfully obtained, demonstrating promising antimicrobial properties. The enhanced concentration of thiosulfinates and thiosulfonates in the optimized extract is directly linked to its increased antimicrobial efficacy, making it a viable candidate for future applications in controlling *X. fastidiosa*. Furthermore, the ability of *P. lactis* PV8 to inhibit *X. fastidiosa* growth in vitro underscores the relevance of beneficial microorganisms in integrated pest management strategies. Although further research is needed to fully understand the potential of *P. lactis* PV8 to produce bioactive compounds, as well as both PV8 and onion-derived OSCs behaviour in planta, this study provides a foundation for exploring these sustainable solutions in the field. As Europe transitions towards more sustainable agricultural practices, the development and application of natural, eco-friendly control methods, such as OSCs and biocontrol agents like *Pseudomonas* spp., will be essential in managing plant diseases and reducing reliance on chemical treatments.

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CAPÍTULO 2. APLICACIÓN DE COMPUESTOS ORGANOSULFURADOS DERIVADOS DE CEBOLLA EN OLIVO: CONTROL DE LA VERTICILOSIS Y EVALUACIÓN DE LA CAPACIDAD BIOESTIMULANTE

2.1. Antifungal Activity of Propyl-Propane-Thiosulfinate (PTS) and Propyl-Propane-Thiosulfonate (PTSO) from *Allium cepa* against *Verticillium dahliae*: *In Vitro* and *In Planta* Assays

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Abstract: *Verticillium* wilt, caused by *Verticillium dahliae*, is the most devastating soil-borne fungal disease of olive trees worldwide. Currently there is no effective measure available to control the pathogen in diseased plants in open field conditions. Searching more effective and sustainable solutions are a priority for the olive sector. Existing alternatives for disease control include the use of biological control microorganisms and compounds of natural origin from plants, such as Alliaceae. Propyl propane thiosulfinate (PTS) and propyl propane thiosulfonate (PTSO) are two organosulfur compounds derived from *Allium cepa* with a widely documented antimicrobial activity. The aim of this study was to evaluate the antifungal activity of PTS and PTSO against the defoliating and non-defoliating *V. dahliae* pathotypes. Firstly, several *in vitro* tests were performed (Minimum Antifungal Concentration, susceptibility studies according to Kirby-Bauer disk-diffusion method, antifungal activity through aerial diffusion and effect on mycelial growth). The ability of both compounds to sanitize soil was evaluated using sterile substrate inoculated with *V. dahliae*. Finally, challenges in growth chambers were carried out. PTS and PTSO generated growth inhibition zones in agar diffusion and gas phase, and mycelial growth of all *V. dahliae* strains was significantly altered. *V. dahliae* population in soil was considerably reduced after the sanitization. Finally, *in planta* assays demonstrated the ability of these compounds to reduce disease related parameters and their contribution to control the phytopathogen. In conclusion, results showed that PTS and PTSO from *Allium cepa* display *in vitro* and *in vivo* antifungal activity against *V. dahliae* and suggested that both compounds could be used as natural and environmentally friendly tools for *Verticillium* wilt management.

Keywords: Verticillium wilt; onion; organosulfur compounds; olive trees; pest management

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1. Introduction

Verticillium wilt caused by fungus *Verticillium dahliae* is currently considered the main and most devastating soilborne fungal disease of olive trees (*Olea europaea* L.) (Landa et al. 2019). Although it affects more than 400 plant species, such as cotton, tomato, almond and peach, the high incidence of this disease in all Mediterranean olive-growing regions is threating olive trees and olive oil production, causing important economic losses (Castro et al. 2020). The severity of the attacks by *V. dahliae* on olive and cotton highly depends on the virulence of the pathotype infecting the olive trees (Gramaje et al. 2013). *V. dahliae* isolates can be classified into defoliating (D) pathotype, that is highly virulent, and nondefoliating (ND) pathotype, based on their ability to cause defoliation of green leaves from shoots and twigs (Navas-Cortés et al. 2008). The D pathotype can be lethal to the plant and causes extensive and early drop of infected green leaves and, eventually the complete defoliation and necrosis. The ND pathotype causes dieback of olive twigs and branched without leaf shedding, besides necrosis of inflorescences and leaf chlorosis (Keykhasaber, Thomma, and Hiemstra 2018; Jiménez-Fernández et al. 2016). However, trees infected by the ND pathotype can show complete remission from symptoms (Gramaje et al. 2013).

V. dahliae is a strictly asexually reproducing fungus, characterized by the production of microsclerotia as resistant structures (Jimenez-Diaz et al. 2012). The formation of these structures is a critical factor in the survival, dissemination and epidemiology of the pathogen (Varo, Raya-Ortega, and Trapero 2016), since they can survive in soil without a host for more than 10 years (Castro et al. 2020). Microsclerotia germinate in soil in response to root exudates, giving rise to hyphae that can penetrate the olive root and grow across the root cortex. This way, hyphae invade the xylem vessels and forms conidia that is spread by olive tree stem, giving rise to an extensive xylem colonization and functional impairment (Jiménez-Fernández et al. 2016).

Verticillium wilt is a hard to control disease, since *V. dahliae* can survive in soil for years and to grow confined in the host's xylem during the parasitic phase, preventing treatments by topically application fungicides (Jiménez Díaz et al. 2009). To date the only method that has proven to be effective against this pathogen is soil disinfection with fumigants such as methyl bromide or chloropicrin. However, the potential risk involved in applying these chemicals, for both human health and environment has led to their banning in many countries (Sarfraz et al. 2020; Goicoechea 2009; Short et al. 2015). The lack of effective fungicides, and the increasing restrictions on the use of chemical compounds in managing plant diseases, is pushing the search for new alternative methods to control *V. dahliae* (Varo, Raya-Ortega, and Trapero 2016). An interesting and sustainable option is the use of plant-derived natural products.

In recent years, the antimicrobial activity of several organosulfur compounds like thiosulfinates and thiosulfonates, obtained from *Allium* genus plants such as onion (*Allium cepa* L.), Welsh onion (*Allium fistulosum* L.) or garlic (*Allium sativum* L.), has been extensively studied (Deberdt et al. 2012; Sorlozano-Puerto et al. 2021; 2018; Borlinghaus

et al. 2014; Sarfraz et al. 2020). In onion, the most common organosulfur compounds are isoalliin (S-propenyl-L-cysteine sulfoxide) and propiin (S-propyl-L-cysteine sulfoxide). When an onion is crushed or cut, propiin changes into propyl-propane thiosulfinate (PTS) due to the action of the enzyme (Keusgen et al. 2002). Despite PTS is more stable than other thiosulfinates like allicin (Sarfraz et al. 2020), it is also a labile compound that changes into di-propyl disulfide and propyl-propane thiosulfonate (PTSO) (Guillamón 2018).

Some potential uses of PTS and PTSO in animal nutrition have been proposed because of their anti-inflammatory (Vezza et al. 2019), anti-bacterial (Peinado et al. 2012) and anticoccidial properties (Kim et al. 2013). Furthermore, their ability to modulate the gut microbiota and improve productivity in piglets and laying hens has been recently demonstrated (Rabelo-Ruiz, Teso-Pérez, et al. 2021; Rabelo-Ruiz, Ariza-Romero, et al. 2021). In addition, both PTS and PTSO have been shown to be toxicologically safe in different studies carried out in cell models and in experimental animals (Cascajosa-Lira et al. 2021; Lira et al. 2020; Llana-Ruiz-Cabello et al. 2015).

While their precise mechanism of action is not yet completely understood, these onion-derived products have three characteristics that could explain their antimicrobial effect: (i) they react with thiol groups of the microbial metabolism altering integrity of membranes (Focke, Feld, and Lichtenthaler 1990); (ii) they can react with glutathione, decreasing its intracellular levels, which entails oxidative stress and cellular apoptosis (Gruhlke et al. 2010; Roth, P. J. and Theato 2013); and(iii) they can inhibit RNA synthesis through the inhibition of RNA polymerase (Feldberg et al. 1988; Borlinghaus et al. 2014).

The use of organosulfur compounds from *Allium* in agriculture has been mainly focused on antifungal activity of garlic derivatives against plant pathogens such as *Botrytis cinerea, Penicillium expansum, Bipolaris sorokiniana, Phytophthora infestans, Fusarium* and *Rhizopus* spp., among others (K. Abdulaziz, Musa, and Aisha 2018; Daniel, Lennox, and Vries 2015; Perelló, Gruhlke, and Slusarenko 2013; Curtis et al. 2004). Nevertheless, studies that have evaluated the antifungal activity of similar derivates from onion are limited and they are mainly focused on the effectiveness of onion essential oil or aqueous extracts (Kocić-Tanackov et al. 2012; Shams-Ghahfarokhi et al. 2006; Genatrika E, Sundhani E 2020).

The aim of the present study was to evaluate the antifungal activity of volatile organosulfur compounds PTS and PTSO from *A. cepa* against the plant pathogenic fungi *V. dahliae*, both in vitro and in planta, and the determination of their potential use as soil sanitizer providing a new method to control Verticillium wilt in olive trees.

2. Materials and Methods

2.1. Antifungals

PTS and PTSO (98% purity) from *A. cepa* were supplied by DOMCA SAU (Granada, Spain). The pure compounds were used for *in vitro* assays. A blend of PTS and

PTSO in proportion 1:1 (w/w) at different concentrations was used for soil sanitization tests and in planta assays.

2.2. *Verticillium dahliae* Isolates

Three *V. dahliae* isolates were used in this study (Table 1). These isolates belong to the culture collection of the Department of Crop Protection, Institute for Sustainable Agriculture, Spanish National Research Council, Córdoba, Spain (Gramaje et al. 2013). The *V. dahliae* isolates were selected as representative of the pathotypes and mycelial compatibility groups most widely distributed in southern Spain (Jiménez-Díaz et al. 2011). The liquid culture media used were RPMI-1640 medium with L-glutamine, supplied by Labclinics (Barcelona, Spain). Glucose was added to a final concentration of 0.2% and pH was adjusted to 7.0 with acid morpholine propane sulfonic (0.165 M) buffer. The solid culture medium used was Rose-Bengal agar supplied by Scharlau (Barcelona, Spain). Inoculum of *V. dahliae* isolates were prepared in saline solution.

Table 1. *Verticillium dahliae* isolates used in this study, along with reference code, vegetative compatibility grouping (VCG) and pathotype.

Reference Code	VCG[1]	Pathotype[2]
V136I	1A	D
V1235I	2B	ND
V1266I	4B	ND

[1]VCG = Vegetative compatibility group.
[2] D = defoliating pathotype, ND = nondefoliating pathotype

2.3. Antifungal *In Vitro* Assays

2.3.1. Agar Diffusion Test

The chosen method to study antifungal activity in solid medium was the disk-diffusion method proposed by Kirby–Bauer (A. W. Bauer et al. 1966) and modified by Calvo and Asensio (Calvo and Asensio 1999). Sterilized cellulose discs of 6 mm of diameter (Whatman® antibiotic test discs, Buckinghamshire, UK) impregnated with 20 µL PTS or PTSO at different concentrations (2.5, 5, 10, 25 and 50 µg/µL) were placed in the centre of Rose-Bengal agar plates. These plates were already inoculated with the appropriate *V. dahliae* isolate using a conidial suspension adjusted to 10^6 CFU/mL in saline solution, so that the growth after 4 days incubation at 25°C was confluent. The inhibition zone of fungal growth was proportional to the degree of inhibition produced. Two independent tests were carried out, and each sample was tested in duplicate. EC50 values were also calculated.

2.3.2. Minimum Fungicidal Concentration (MFC)

Standard broth microdilution method was carried out according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (CLSI 2017). Decreasing concentrations of PTS and PTSO were prepared by making 1:2 dilutions from a starting concentration of 10000 µg/mL (10000; 5000; 2500; 1250; 625; 312.5; 156.25; 78.125; 39.06; 19.53 and 9.76 µg/mL) in RPMI-1640 medium. These dilutions were subsequently

inoculated with the corresponding *V. dahliae* isolate and incubated for 24 h at 25 °C in the dark. As a negative control, same broth volume was inoculated with *V. dahliae* and incubated in the same conditions. Natamycin, a polyene macrolide with antifungal properties, was used as a positive control. All assays were performed in duplicate and each sample was tested twice.

2.3.3. Antifungal Activity of Gaseous PTS and PTSO

To study the antifungal activity of the compounds associated with their gas phase, the methodology described in section 2.3.1 was carried out with some modifications (Leontiev et al. 2018; Sorlozano-Puerto et al. 2021). *V. dahliae* colonies were suspended in saline solution to obtain a conidial suspension adjusted to 10^6 CFU/mL that was used to inoculate the Rose-Bengal agar plates. As previously described, sterilized cellulose discs were impregnated with 20 µL of PTS or PTSO at different concentrations (2.5; 5; 10; 25 and 50 µg/µL) and placed, not in the agar, but in the centre of the petri dish lids, so that the agar plates with *V. dahliae* were placed inverted over the lid. Therefore, the tested compounds and the inoculated agar did not come into direct contact, except by diffusion through the air. After incubation at 25 °C for 4 days in the dark, the diameter of the inhibition zones was measured (mm). Assays were performed in duplicate and each sample was tested twice. EC_{50} values were also calculated.

2.4. Effect of PTS and PTSO on Mycelial Growth

The protocol chosen to carry out this assay was based on the method proposed by Liu–Yan (Liu et al. 2017). First, agar plates containing Rose-Bengal medium supplemented with PTS and PTSO at different concentrations (25, 50, 75 and 100 µg/mL) were prepared. Non-supplemented Rose-Bengal agar plates were used as positive control of *V. dahliae* growth. Next, 10 µL drops of a previously titrated *V. dahliae* inoculum at 106 CFU/mL were placed in the centre of the agar plates, and these were incubated at 25°C for 16 days in the dark. Mycelial growth was determined by measuring the diameter (mm) of the mycelium over time and EC_{50} was calculated by using data from the last day of the assay. Percentage of mycelial growth inhibition was expressed according to the following formula:

$$\% \text{ inhibition} = [(C-T)/C] \times 100$$

Where C is the diameter of the fungal colony in the control plate, and T is the diameter of the fungal colony in each treatment plate. Two independent tests were carried out and each sample was tested in duplicate.

2.5. Soil Sanitization

The soil used for this study was a universal substrate (Composana, Münster, Germany) that was autoclaved in a steam sterilizer (Raypa, Terrasa, Spain) at 121°C for 20 minutes. Sterility of soil was confirmed by incubating on Rose-Bengal agar at 25°C for 4 days (Falade et al. 2016). Plastic bags were filled with 400 g of sterilized soil. Bags were inoculated by adding 10 mL of a conidial suspension of *V. dahliae* isolate V136I adjusted

to 10^7 CFU/mL, so that the final population in the inoculated soil was approximately 10^5 CFU/g (Deberdt et al. 2012). A bag without inoculum was used as negative control and another bag only with inoculated soil was used as positive control. In the remaining bags, the blend of PTS and PTSO in proportion 1:1 was applied 72 h after soil infestation at different concentrations (100; 500 and 1,000 μg/mL). Bags were shaken to homogenize their content and sealed to avoid leaks and contamination. *V. dahliae* population was quantified at 1, 2, 3 and 4 days after treatment. At each time, 25 g of soil from each bag was suspended in 225 mL of buffered peptone water (Scharlau, Barcelona, Spain).). A lab paddle blender (MASTICATOR, IUL, Barcelona, Spain) was used to obtain a better homogenised sample for analysis. Serial dilutions were placed in Rose-Bengal agar and incubated at 25°C for 4 days in the dark (Deberdt et al. 2012). Then, colonies were counted and the concentration *V. dahliae* in soil was expressed as Log10 CFU/g. Each test was carried out twice and each sample was tested in duplicate.

2.6. Effect of PTS and PTSO on Verticillium Wilt Suppression

In planta experiments were carried out in growth chambers using 7-month-old olive plantlets of cv. Picual, which grew in soil artificially infested with the highly virulent defoliating pathotype of *V. dahliae* V136I 1A, and incubated under optimal environmental conditions for Verticillium wilt development (Jiménez-Díaz et al. 2011). Inoculum consisted of an infested cornmeal-sand mixture (CMS; sand: cornmeal: deionized water, 9:1:2, w/w) produced as described by Jiménez-Fernández et al (Jiménez-Fernández et al. 2016). The infested CMS was homogenized, dried in an incubator adjusted to 33°C for 3 days, and thoroughly mixed with a pasteurized soil mixture (clay loam: peat, 2:1, v/v) at a rate of 1:20 (w/w) to reach an inoculum density of 105 CFU/g soil of *V. dahliae* as determined by dilution-plating on chlortetracycline-amended water agar (CWA; 1 L distilled water, 20 g agar, 30 mg chlortetracycline) (Jiménez-Fernández et al. 2016). The *V. dahliae* infested soil was treated with two doses of the blend of PTS/PTSO (250 and 500 μg/mL) and kept in sealed plastic bags for 2 days. Then, plants were uprooted from the potting substrate, gently shaken to retain only the rhizosphere soil, and placed in 1500 mL pots filled with this *V. dahliae*-infested soil mixture. Non-inoculated plants were treated similarly and transplanted to the pasteurized soil mixture with non-infested CMS at the same rate as infested CMS. Once planted, a group of plantlets was not treated, and other groups received via irrigation 100 mL of the blend of PTS/PTSO (250 and 500 μg/mL) a week after being planted.

Both inoculated and control plants were incubated in a growth chamber adjusted to 22 ± 2 °C, 60–80% relative humidity and a 14-h photoperiod of fluorescent light of 360 μmol m-2 s-1 for 3 months. There were 10 replicated pots (one plant per pot) for the different treatments and for the inoculated and non-inoculated plants in a completely randomized design. Disease reaction was assessed by the incidence (percentage of plants showing disease symptoms) and severity of foliar symptoms. Symptoms were assessed on individual plants on a 0 to 4 rating scale according to the percentage of affected leaves and twigs at 2- to 3-day intervals throughout the duration of the trial (Jiménez-Fernández et al. 2016). Upon termination of the experiment, the extent of colonization

by *V. dahliae* was determined by isolation of the fungus in CWA (Jiménez-Fernández et al. 2016). From 6-cm-long stem pieces sampled from the main stem at the same time than similar samples were processed for extraction of xylem microbiome. Data of pathogen isolation from the stem were used to calculate the intensity of stem vascular colonization for each individual plant, according to a stem colonization index (SCI) as described before (Jiménez-Fernández et al. 2016).

2.7. Statistical Analyses

The average data standard deviations were determined with Excel software (Microsoft Corp., Redmond, WA, USA). Statistical analyses were performed using the SPSS-PC 15.0 software (SPSS, Chicago, IL, USA). Data on microbiological counts were subjected to ANOVA. Data of in soil and in planta assays were analysed according to Tukey HSD test. Error probability values less than 0.05 were considered not significant.

3. Results

3.1. *In Vitro* Antifungal Assays

3.1.1. Antifungal Activity

The antifungal activity of organosulfur compounds PTS and PTSO has been evaluated against 3 different isolates of *V. dahliae* (Table 1). A summary of the antifungal activity data obtained by the disk-diffusion method in agar is shown in Table 2, expressed as the average diameter ± standard deviation (in mm). All the *V. dahliae* isolates were sensitive to both compounds in a dose-dependent manner, becoming more evident from a concentration of 10 μg/μL, with inhibition zones between 50 and 87 mm. The antifungal activity of PTS and PTSO was similar in this trial, without any remarkable variations between the inhibition zones as shown in Figure 1.

The results obtained in the agar diffusion test, which are purely qualitative, were supported by the determination of MFC. The MFC values obtained (Table 3) confirm the potential of PTS and PTSO against *V. dahliae* isolates. Unlike in the agar diffusion test in which no significant differences were observed between both compounds, the MFC data indicated that antifungal susceptibility of PTSO was significantly higher than that of PTS ($p < 0.01$). This antifungal activity was especially relevant against *V. dahliae* V136I 1A, with a MFC value of 7.91 μg/mL of PTSO.

Table 2. Antifungal activity of PTS and PTSO against different *Verticillium dahliae* isolates (V136I 1A, V1235I 2B and V1266I 4B) by disk-diffusion method, expressed as the average diameter ± standard deviation of inhibition zone (mm), and EC_{50} values.

Compound	Concentration (μg/μL)	V136I 1A	V1235I 2B	V1266I 4B
PTS	**2.5**	32.0 ± 3.16	47.3 ± 2.86	37.8 ± 3.49
	5	44.0 ± 3.16	56.0 ± 1.87	48.8 ± 2.38
	10	61.3 ± 2.38	64.5 ± 2.69	68.3 ± 3.34
	25	69.0 ± 1.41	72.3 ± 2.59	80.8 ± 1.92
	50	75.3 ± 1.92	80.5 ± 2.18	86.0 ± 3.16
	EC50 (μg/μL)	7,7 ± 0.03	10,0 ± 0.56	8,3 ± 0.17
PTSO	**2.5**	28.3 ± 2.38	40.0 ± 3.16	28.8 ± 3.34
	5	38.8 ± 2.38	48.8 ± 0.83	42.3 ± 2.59
	10	50.8 ± 2.38	56.5 ± 1.12	65.0 ± 3.24
	25	58.5 ± 1.12	65.0 ± 3.39	76.3 ± 1.64
	50	71.5 ± 3.04	75.8 ± 3.03	87.3 ± 0.83
	EC_{50} (μg/μL)	10,8 ± 0.35	11.6 ± 0.42	8.8 ± 0.24

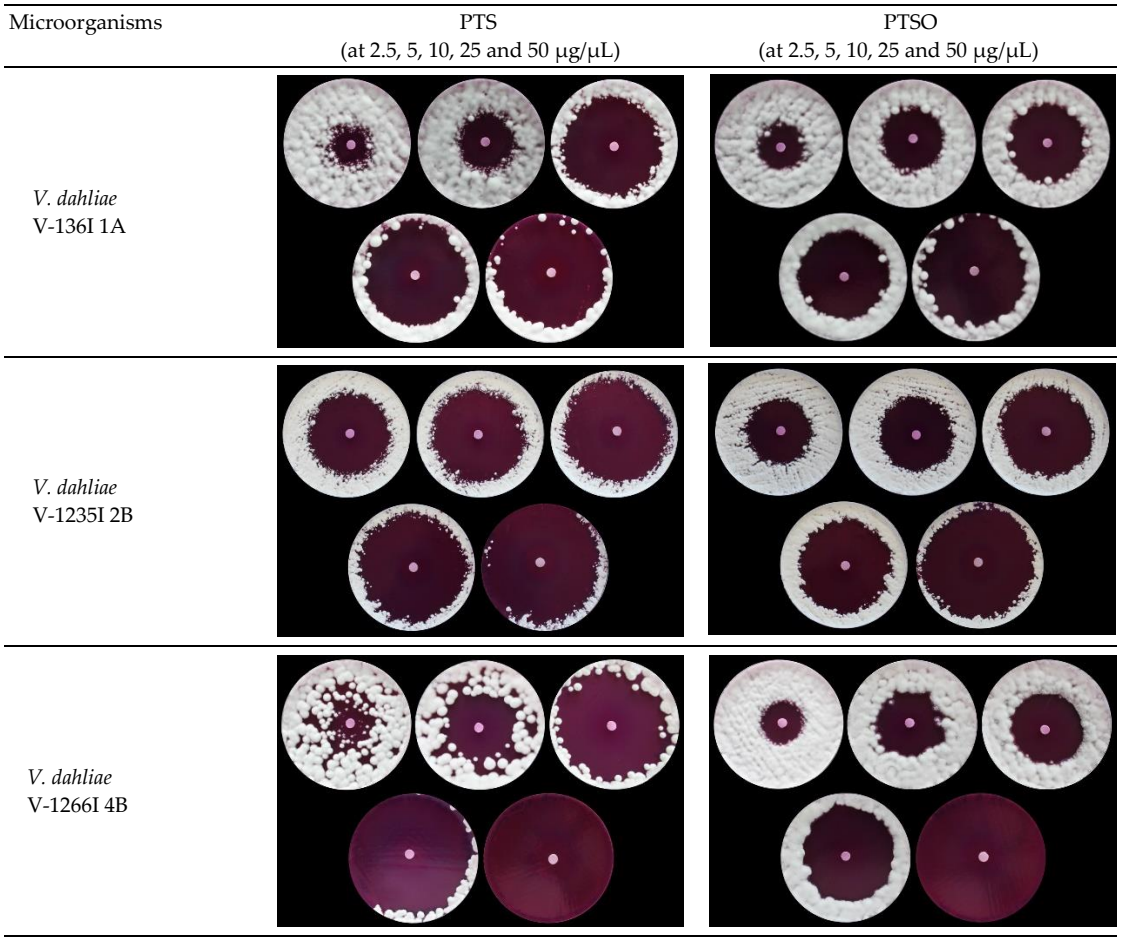

Figure 1. Antifungal activity of PTS and PTSO against *Verticillium dahliae* V136I 1A, V1235I 2B and V1266I 4B isolates by Disk-Diffusion method. The image shows inhibition zones at doses of 2.5 μg/μL, 5 μg/μL, 10 μg/μL, 25 μg/μL and 50 μg/μL from left to right and from top to bottom.

Table 3. Minimum Fungicidal Concentration (MFC) against different isolates of *Verticillium dahliae*.

	MFC (μg/mL)	
Isolates	**PTS**	**PTSO**
V. dahliae V136I 1A	78.13	19.53
V. dahliae V1235I 2B	78.13	39.06
V. dahliae V1266I 4B	78.13	39.06

3.1.2. Antifungal Activity of the Gas Phase

Table 4 shows the results of the volatility-linked activity assay, expressed as the diameter of the inhibition zone ± standard deviation (in mm). PTS and PTSO inhibited growth in all *V. dahliae* isolates tested in the present study through their gaseous phase without coming into contact with the medium and thus, with the fungus, except for its aerial diffusion. The vapor produced by both compounds reached the agar medium, inhibiting fungal growth in a circular area above the drop placed in the lid of the Petri dish (Figure 2). The absence of fungal growth suggests a predominant biocidal effect, being particularly remarkable for PTS at doses of 25 and 50 μg/μL against ND pathotypes V1235I 2B and V1266I 4B.

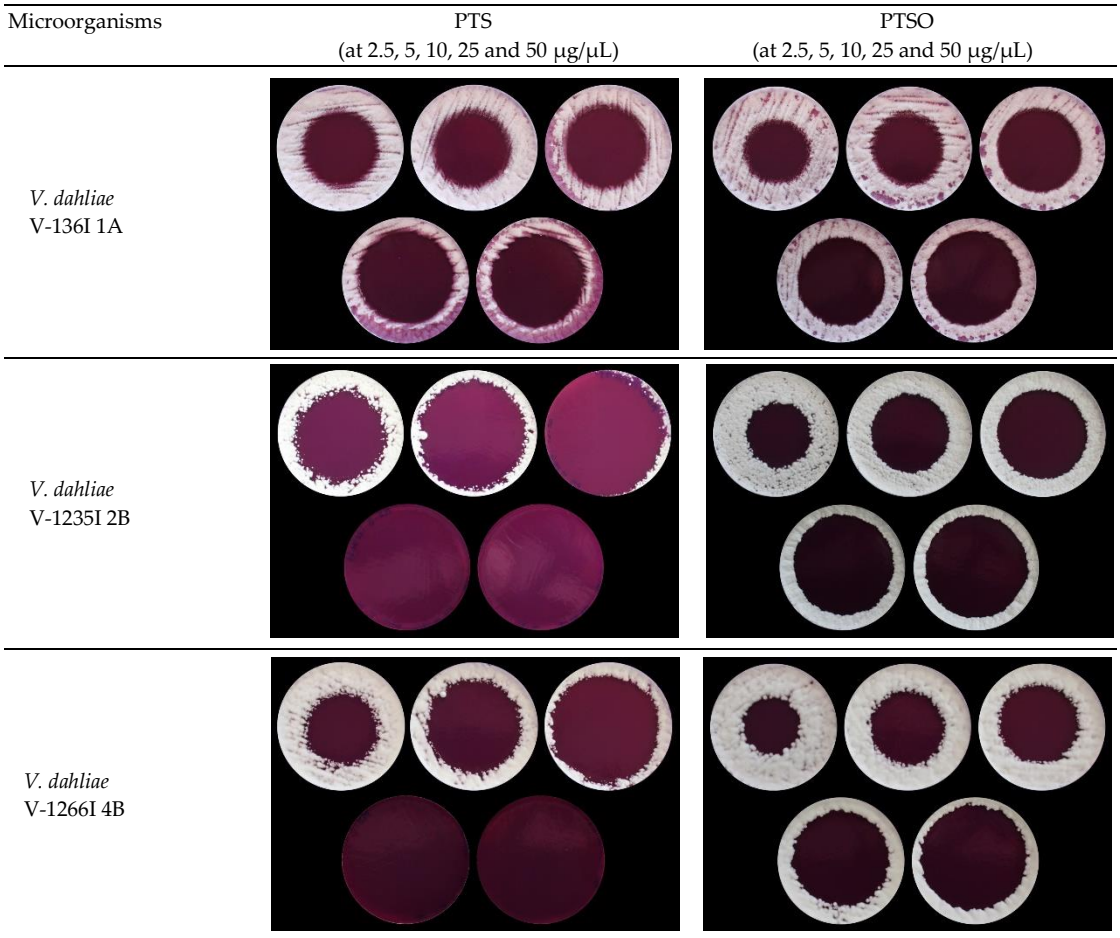

Figure 2. Antifungal activity of PTS and PTSO against *Verticillium dahliae* V136I 1A, V1235I 2B and V1266I 4B strains via the gas phase. The image shows inhibition zones at doses of 2.5 μg/μL, 5 μg/μL, 10 μg/μL, 25 μg/μL and 50 μg/μL from left to right and from top to bottom.

Table 4. *In vitro* antifungal activity of PTS and PTSO against different isolates of *Verticillium dahliae* (V136I 1A, V1235I 2B and V1266I 4B) via the gas phase, expressed as the average diameter ± standard deviation of inhibition zone (mm), and EC_{50} values.

Compound	Concentration (μg/μL)	V136I 1A	V1235I 2B	V1266I 4B
PTS	**2.5**	48.5 ± 1.12	55.0 ± 1.41	45.0 ± 1.41
	5	53.8 ± 3.49	71.5 ± 2.06	58.0 ± 2.24
	10	63.8 ± 3.49	79.3 ± 1.30	72.0 ± 1.41
	25	69.0 ± 2.12	87.0 ± 1.58	84.3 ± 1.92
	50	75.3 ± 2.86	92.5 ± 2.06	97.3 ± 1.92
	EC_{50} (μg/μL)	9,9 ± 0.29	7.0 ± 0.15	10,3 ± 0.09
PTSO	**2.5**	38.5 ± 1.12	40.5 ± 2.06	39.0 ± 1.58
	5	46.0 ± 1.41	52.3 ± 1.48	48.5 ± 1.12
	10	54.5 ± 2.96	62.3 ± 1.92	54.3 ± 1.30
	25	62.3 ± 1.79	69.0 ± 1.58	66.0 ± 1.87
	50	66.3 ± 2.28	73.0 ± 2.24	71.5 ± 1.66
	EC_{50} (μg/μL)	8,8 ± 0.22	7,2 ± 0.08	9,9 ± 0.11

3.2. Effect of PTS and PTSO on Mycelial Growth

To complete the *in vitro* characterization of antifungal activity studies, the fungistatic capacity was evaluated by the mycelial growth progression test in agar plates. The results obtained are presented in Figure 3. Mycelium Growth Curves provide additional information on the interaction between the compounds tested and the fungal growth. As in the previous assay, both PTS and PTSO showed a remarkable inhibitory effect on the *V. dahliae* mycelial growth.

These results demonstrate the correlation between the inhibition produced and the concentration of both compounds. It should be noted that the increase over time was more pronounced at the lowest concentrations tested, whereas at the concentration of 100 μg/mL the fungal growth was practically imperceptible. Treatments of 50 μg/mL, 75 μg/mL and 100 μg/mL of PTS or PTSO showed significant differences ($p< 0.05$) as compared to the control group. The effectiveness of PTSO at 25 μg/mL was lower against V136I isolate. Likewise, PTS at 25 μg/mL was less effective against V136I 1A and V1266I 4B. These results are further reflected in Table 5, which shows the inhibition of mycelium growth caused by each treatment compared to the normal growth of the control group, expressed as percentage.

Table 5. Percentage of mycelial growth inhibition of *Verticillium dahliae* isolates at different concentration of PTS and PTSO: control group; 25 µg/mL; 50 µg/mL; 75 µg/mL and 100 µg/mL.

Compound	Concentration (µg/µL)	V136I 1A	V1235I 2B	V1266I 4B
PTS	**25**	28.39	64.87	37.16
	50	38.85	74.74	62.40
	75	60.86	82.26	66.43
	100	70.09	86.71	71.27
PTSO	**25**	29.22	58.13	48.93
	50	36.32	71.48	64.62
	75	64.84	84.09	81.77
	100	69.17	87.38	85.17

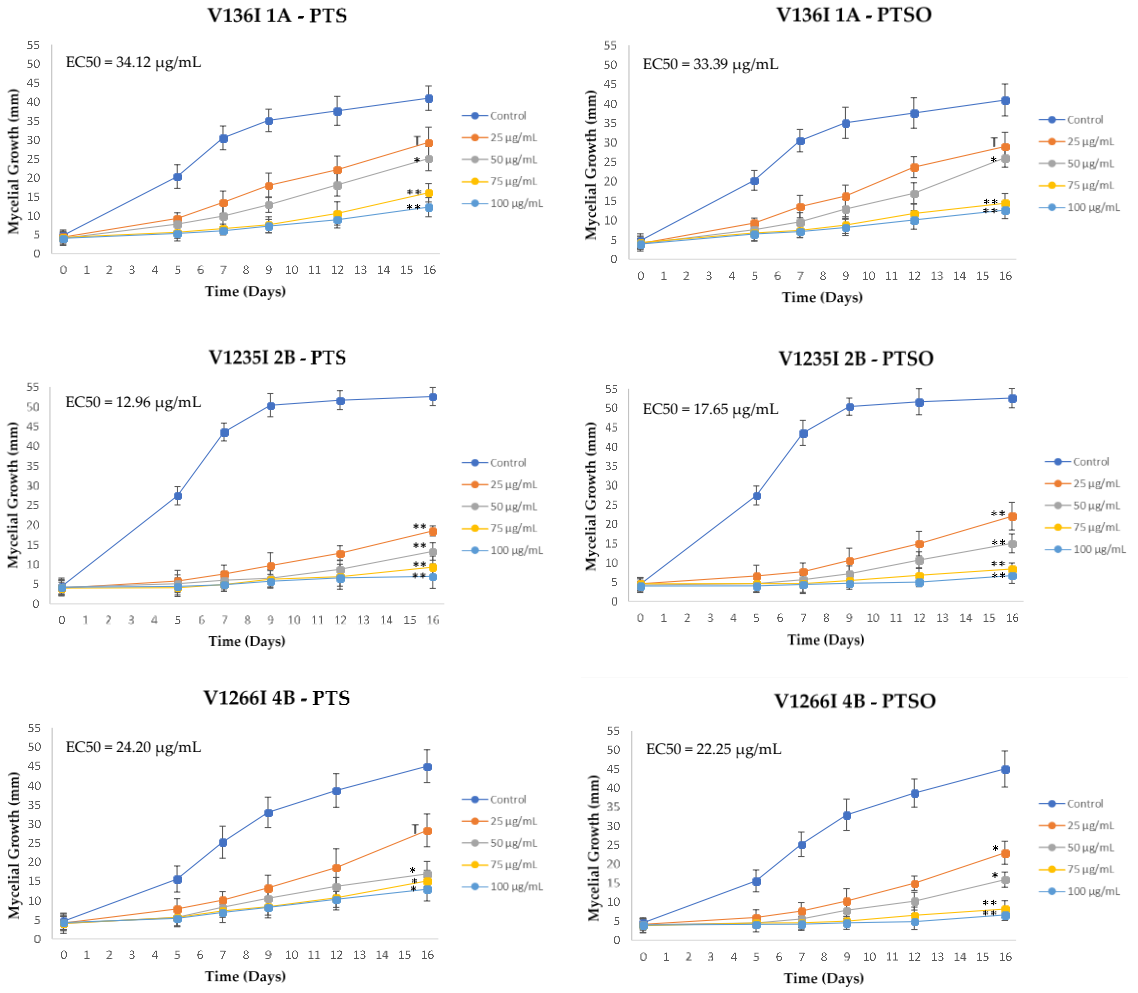

Figure 3. Mycelium Growth Curves of *Verticillium dahliae* isolates at different concentration of PTS and PTSO: control group; 25 µg/mL; 50 µg/mL; 75 µg/mL and 100 µg/mL. Values are means with SD in bars. T $p < 0.1$; * $p < 0.05$; ** $p < 0.01$ respect to control. EC50 values have been calculated for each *V. dahliae* isolate and each organosulfur compound.

3.3. Application of PTS and PTSO in Soil Sanitization

V. dahliae population in the untreated group (C +) evolved as expected, achieving 5 Log10 CFU/g on day 4. In addition, the absence of growth in the negative control

(sterilized soil that was not inoculated with *V. dahliae*), confirmed that sterility conditions were maintained during all the assay.

The different doses of the blend of PTS/PTSO achieved a significant reduction ($p<0.05$) on the population of *V. dahliae* in soil, as shown in Figure 4. Soil treated with a dose of 100 µg/mL reduced the fungal concentration by approx. 2 Log10 CFU/g during the assay. The effects observed in soil treated with a dose of 500 µg/mL were more remarkable, showing absence of growth at days 1 and 2 after treatment. Nevertheless, fungal growth was observed in days 3 and 4 reaching 1.5 Log10 CFU/g. Finally, the highest inhibition occurred for the dose of 1000 µg/mL with reductions of the counts up to 4-5 logarithmic units as compared to the control.

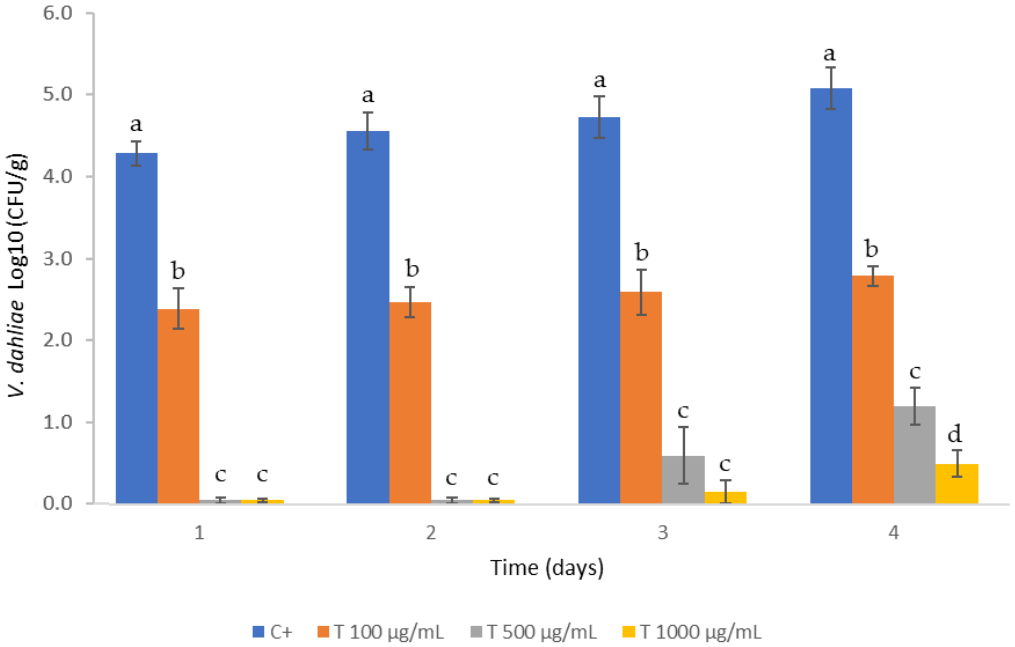

Figure 4. Concentration of *Verticillium dahliae* in soil after treatment with the blend of PTS and PTSO in proportion 1:1 at different concentrations (100; 500 and 1000 µg/mL) compared to a positive control group (C +) which consists of sterilized soil inoculated with *V. dahliae* but without treatment. Values are means with standard deviation in bars. For each sampling day, bars with a different letter indicate significant differences according to Tukey HSD test at $p<0.05$.

3.4. Effect of PTS and PTSO on Verticillium Wilt Suppression

All soil treatments significantly reduced all disease parameters in the study compared to that reached on non-treated *V. dahliae* infested soil ($p<0.05$). After 3 months of incubation under optimal environmental conditions for Verticillium wilt development, disease incidence was 100% and severity of Verticillium wilt symptoms reached 3.3 (on a 0-4 rating scale) in *V. dahliae* infested but non-treated soil (Figures 5.a.b and 6.a.b).

Interestingly, plants growing in treated infested soil showed a disease incidence of 60 and 20% (Figure 6.a), a severity of symptoms of 1.9 and 0.6 (Figure 6.b), and an intensity of vascular colonization of 60 and 43% (Figure 6.c), on soil treated with blend of PTS/PTSO at doses of 250 and 500 µg/mL, respectively (Figure 5.a).

Moreover, no Verticillium wilt symptoms were observed on plants growing in treated soil and further irrigated with 100 mL of the blend of PTS/PTSO one week after transplanting at any of the two doses tested (Figure 5.b and 6.a.b). At the end of the experiment, *V. dahliae* inoculum density was clearly reduced (Figure 6.d).

Significantly reductions were observed in soil treated with 250 μg/mL of the blend of PTS/PTSO according to Tukey test. The highest reduction was observed in case of *V. dahliae* infested soil treated with 500 μg/mL of the blend of PTS/PTSO and subsequently irrigated with the same product one week after planting. In this sample, inoculum density of *V. dahliae* was reduced with differences up to 2 logarithmic units as compared to the infested non-treated control group (Figure 6.d). Although a dose-dependent trend was observed, there were no significant differences between the four treatments in relation to the fungal concentration (Figure 6.d).

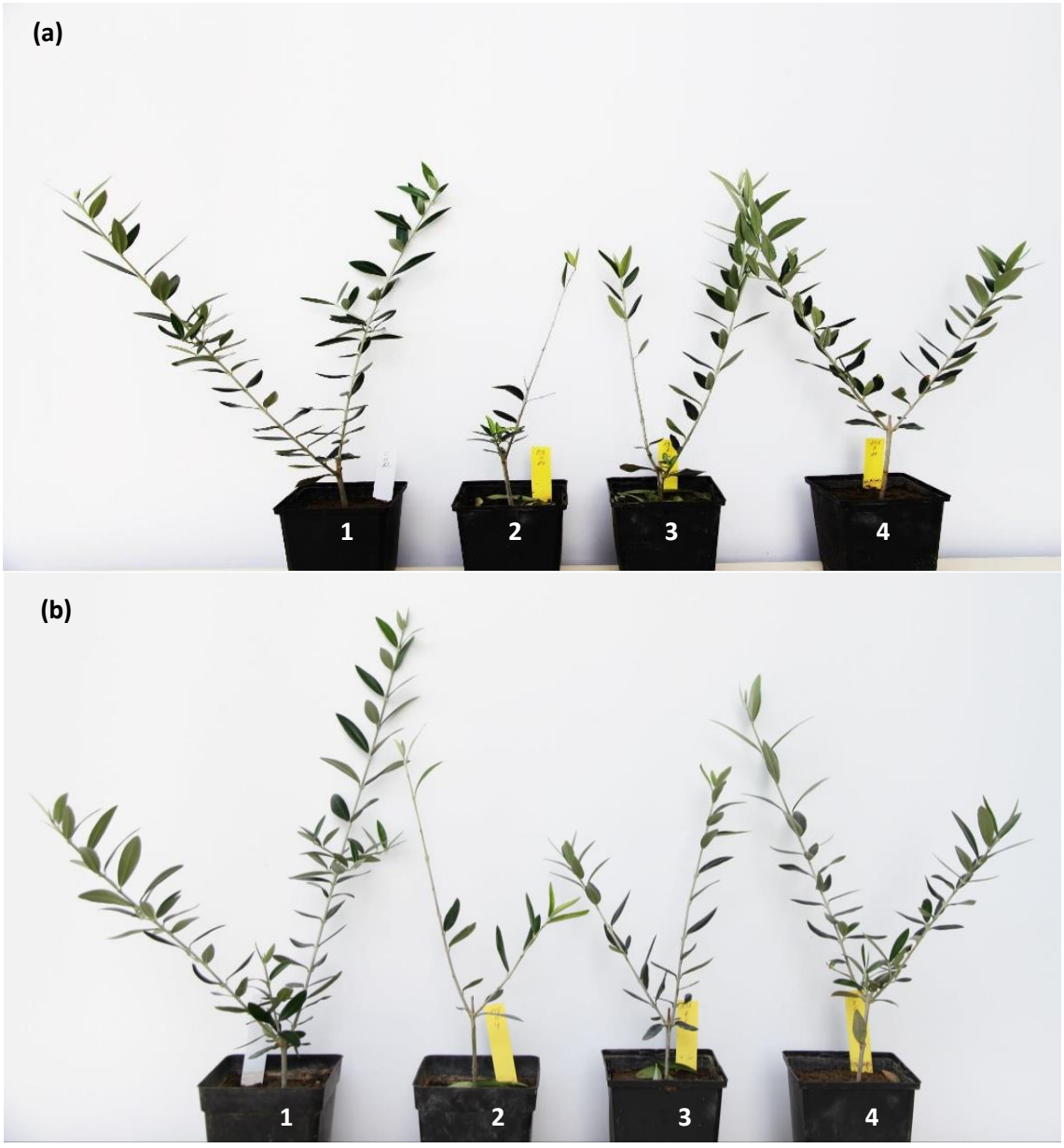

Figure 5. Symptoms of Verticillium wilt in olive plantlets of cv. Picual that grew for 2 months in soil infested with the defoliating pathotype V136I 1A of *Verticillium dahliae* treated with the blend of PTS and PTSO in proportion 1:1 at different concentrations: (a) Plants that were not further treated with AP; (b) Plants that were treated once with 100 mL one week later via irrigation. In both panels (a) and (b), it can be observed: 1) Negative control group without fungal inoculation or treatment, 2) Positive control group that grew in soil infested with *V. dahliae* and not treated 3) Plants that grew in *V. dahliae* infected soil treated with the blend of PTS/PTSO at 250 µg/mL, and 4) Infected test group treated with the blend of PTS/PTSO at 500 µg/mL.

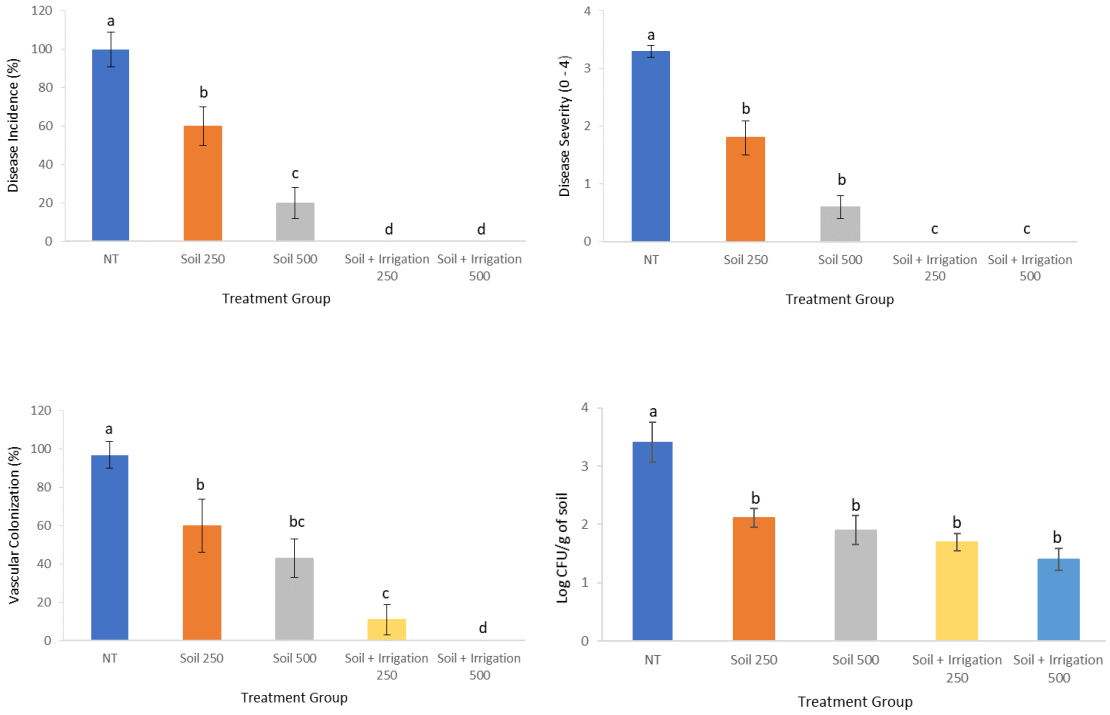

Figure 6. Disease reaction of olive trees of cv. Picual that grew in soil infested with the defoliating *Verticillium dahliae* (NT) pathotype treated with the blend of PTS and PTSO in proportion 1:1 at 250 and 500 µg/mL, 92 days after transplanting to the infested soil: (a) Disease incidence expressed as percentage (b) Disease severity expressed in scale from 0 to 4 (c) Intensity of vascular colonization expressed as percentage (d) Density of *V. dahliae* in soil expressed as Log10 CFU/g of soil. All panels include results from plants previously treated with the blend of PTS/PTSO or treated once with 100 mL of their respective dose one week later (Soil + Irrigation). For each panel, bars with a different letter indicate significant differences according to Tukey HSD test at $p<0.05$.

4. Discussion

The increasing trend in the number of soilborne fungal diseases outbreaks that has been observed over the last decade, with the consequent loss in crop production and increased costs, have become a major problem for plant's health (Panth, Hassler, and Baysal-Gurel 2020). Moreover, it has been widely reported that the proportion of soilborne pathogens and global warming are directly related, considering that the temperature determines the distribution of soil microbial communities and influences the distribution of fast-growing opportunistic fungal infections (Delgado-Baquerizo et

al. 2020). Therefore, the search for new integrated soilborne disease management strategies has become a priority.

In this context, the broad antimicrobial activity of organosulfur compounds derived from *Allium* spp. against a variety of bacteria and fungi has already been demonstrated in various *in vitro* studies (Sarfraz et al. 2020; K. Abdulaziz, Musa, and Aisha 2018; Leontiev et al. 2018; Arnault et al. 2013; Sorlozano-Puerto et al. 2021). Although the mechanism of action of organosulfur compounds is not yet deeply studied, the antimicrobial activity of these compounds could be associated with their capacity to react with thiol groups of microbial enzymes, RNA synthesis blocking mechanisms, induction of oxidative stress and cellular apoptosis (Roth, P. J. and Theato 2013; Feldberg et al. 1988; Sorlozano-Puerto et al. 2018).

Some plant extracts from other botanical species such as *Vaccinium myrtillus L., Laurus nobilis L., Eucalyptus camaldulensis Dehnh, Mentha piperita L., Thymus vulgaris L.*, and *Lavandula angustufolia Mill*. (Bayar et al., 2018; Üstüner et al., 2018; Erdogan et al., 2016) have shown to be able to inhibit *in vitro* mycelial growth of different phytopathogenic fungi, including *V. dahliae*. Moreover, in inoculated olive plants, the essential oil of *Thymus* sp. completely inhibited mycelial growth, and reduced microsclerotia viability and Verticillium wilt disease (Varo et al. 2017).

In the present study, the antifungal efficacy of PTS and PTSO from *A. cepa* has been evaluated against three isolates of soilborne fungus *V. dahliae*, being two of them of the non-defoliating (ND) pathotype and the third one a representative of defoliating pathotype (D) and VCG infecting olive trees in southern Spain. Even though at the plant level, the D pathotype shows a greater virulence and lethality compared to the ND (Gramaje et al. 2013), our results showed no differences in the antifungal activity of PTS and PTSO between D and ND isolates neither in the disk-diffusion tests nor the MFC values obtained. However, the D pathotype appeared to be somewhat less affected to the gas phase of PTS and PTSO during the volatility-linked activity test, while the antifungal effect was quite more noticeable against the isolates of the ND pathotype. In line with our results, other authors have recently reported the antimicrobial activity of PTS and PTSO against bacteria and yeast through their gas phase (Sorlozano-Puerto et al. 2021).

Once verified the antifungal activity of PTS and PTSO *in vitro* tests, a soil sanitization assay was performed using a preparation based on both organosulfur compounds in 1:1 proportion. As reported by Panth et al. (Panth, Hassler, and Baysal-Gurel 2020), plants belonging to *Allium* spp. such as onion have been used in biofumigation, a soilborne pathogen management approach that consists of grinding a plant cover set up during the fallow period and incorporate the biomass obtained into the soil, so that it can release active organic compounds. By using crushed onion, it has been possible to verify that the pesticidal activity of the organosulfur compounds released into the soil is comparable to that of methyl bromide. These results, described

by Arnault et al. (Arnault et al. 2013), not only confirmed the broad spectrum of action of onion active compounds, but also showed that they stimulate vegetative growth.

The assay carried out in sterilized soil infested with *V. dahliae* displayed very positive results in terms of disease control and reduction of fungal concentration. In fact, PTS and PTSO reduced the microbial density of *V. dahliae* in the infested soil up to 4 logarithmic units. Despite the fact that the treatment did not completely eradicate the presence of the fungus in the soil, its great effect on the fungal population was highly remarkable as a potential control measure for this disease. Deberdt et al. (Deberdt et al. 2012) treated soil infested with the plant pathogenic bacteria *Ralstonia solanacearum* with *Allium fistulosum* extract (Welsh onion), and found out that, although the pathogen may have survived at low levels in the soil and was even able to infect tomato roots, it was unable to fully colonize or cause observable symptoms. In case, of Verticillium wilt in olive trees, it has been reported the progressive disease remission of symptoms, in particular when olive trees are infected by the ND pathotype. This phenomenon is called symptomatic recovery of the disease plant, and it was first described in 1965 by Wilhelm and Taylor (Keykhasaber, Thomma, and Hiemstra 2018a). The recovery in conditions of moderate infection is related to the inactivation of *V. dahliae* in the xylem of the plant, being a new infection necessary to develop the disease again (Jiménez Díaz et al. 2009). Thus, reducing the severity of the infection, as well as protecting the root system of the recovered olive trees are essential for the Verticillium wilt integrated control approach. This could be applied to PTS and PTSO, that even though they are not able to fully eradicate the fungus in soil, the reduction produced is enough to minimize the impact of the disease in the plant.

From the results obtained in planta assays it could be deduced that organosulfur compounds from onion may improve the microbiological quality of soil, thus further studies are needed to stablish their influence on the soil microbiome and whether they can be used as pre-plant treatment.

As previously mentioned, scientific reports on the activity of organosulfur compounds from onion against plant pathogens are limited. On the contrary, more evidences on the antifungal activity of similar compounds from garlic, such as diallyl thiosulfinate or allicin (Leontiev et al. 2018; Sarfraz et al. 2020) are found. Some authors have reported the antifungal activity of an allicin against *V. dahliae*, both *in vitro* and in leaf disk bioassay (Hayat et al. 2016). Recently, Ali et al., (Ali et al. 2021) demonstrated the ability of an aqueous garlic extract containing allicin to reduce disease severity index in eggplant seedlings against *V. dahliae*.

Even though further studies are needed, both in greenhouse and in field trials evaluating the effects of the compounds over time in a larger number of plants, in which different doses and methods of application of the compounds are assessed, the antifungal activity of PTS and PTSO against one of the most aggressive soilborne pathogens has been proved in this study, providing new knowledge on the activity of *Allium* organosulfur compounds and their potential use for plant disease control.

5. Conclusions

Both PTS and PTSO showed a remarkable ability to reduce the growth of *Verticillium dahliae*, highlighting their antifungal activity in gas phase, which is related to the volatility of these metabolites. Moreover, these organosulfur compounds from *A. cepa* can be an alternative to chemical soil disinfectants. Although additional studies in real field conditions are necessary to confirm their potential in integrated pest management, results obtained after *in vitro* and in planta assays support that both compounds could be a useful and environmentally friendly tool in Verticillium wilt management.

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2.2. Evaluation of the Biostimulant Activity and Verticillium Wilt Protection of an Onion Extract in Olive Crops (*Olea europaea*)

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Abstract: The olive tree is crucial to the Mediterranean agricultural economy but faces significant threats from climate change and soil-borne pathogens like *Verticillium dahliae*. This study assesses the dual role of an onion extract formulation, rich in organosulfur compounds, as both biostimulant and antifungal agent. Research was conducted across three settings: a controlled climatic chamber with non-stressed olive trees, an experimental farm with olive trees under abiotic stress; and two commercial olive orchards affected by *V. dahliae*. Results showed that in climatic chamber, onion extract significantly reduced MDA levels in olive leaves, with a more pronounced reduction observed when the extract was applied by irrigation compared to foliar spray. The treatment also increased root length by up to 37.1% compared to controls. In field trials, irrigation with onion extract increased the number of new shoots by 148% and shoots length by 53.5%. In commercial orchards, treated trees exhibited reduced MDA levels, lower *V. dahliae* density, and a 26.7% increase in fruit fat content. These findings suggest that the onion extract effectively reduces oxidative stress and pathogen colonization, while enhancing plant development and fruit fat content. This supports the use of the onion extract formulation as a promising, sustainable alternative to chemical treatments for improving olive crop resilience.

Keywords: olive tree; onion extract; *Allium cepa*; biostimulant; antifungal; *Verticillium dahliae*

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1. Introduction

The olive tree (*Olea europaea*) is one of the most economically important crops in the Mediterranean region (Palomo-Ríos et al. 2021). In 2017, the total area devoted to olive cultivation worldwide was approximately 10.8 million ha and olive production was 2,087,278 tons (Brito, Dinis, Moutinho-Pereira, et al. 2019). While countries of the Mediterranean basin are responsible for more than 90% of the world's olive production (Manetsberger et al. 2023), olive oil and table olives consumption extend to 179 countries (Cardoni and Mercado-Blanco 2023). The localized production together with the high global demand for olive products underlines not only the economic, but also the social and environmental importance of this crop in producing countries. Despite being one of the crops best adapted to the climate of the Mediterranean region (Kaniewski et al. 2023), characterized by warm and dry summers, with high levels of solar radiation (Arenas-Castro et al. 2020), climate change has a detrimental effect on olive performance (Dias et al. 2021). This area is highly vulnerable to adverse environmental conditions caused by climate change (De Ollas et al. 2019). According to the European Commission's Joint Research Centre (JRC), the major effects are extremely high temperatures and changes in precipitation patterns, which are becoming ever scarcer, leading to severe drought events (Toreti et al. 2024). Temperature and water availability have been reported to be the main factors that compromise for olive growth and yield, respectively (Honorio et al. 2024). Additionally, climate change may be related to an increased incidence of microbial diseases, such as Verticillium wilt caused by *Verticillium dahliae* (Goicoechea 2020).

V. dahliae is an ascomycete fungus responsible for the most relevant soil-borne disease affecting Mediterranean olive crops (Calderón et al. 2014). The incidence of Verticillium wilt is highly influenced by the population of the fungus in the soil, which is significantly affected by soil temperature and humidity (Requena-Mullor et al. 2020). Moreover, *V. dahliae* forms resistant structures, called microsclerotia, that can survive up to 10 years in the soil in the absence of a host and during adverse conditions (Harting et al. 2020). Microsclerotia germination depends on water availability and is induced in response to root exudates (Hu et al. 2014). They form hyphae, that penetrate the roots, reach the xylem vessels and give rise to conidia. They extensively colonize the xylem, which leads to water stress and causes yield losses and tree mortality (Jiménez-Fernández et al. 2016). Specific symptoms rely on the infective pathotype: defoliating (D) and non-defoliating (ND) (Mercado-Blanco et al. 2003). The D pathotype can be lethal and causes the drop of green leaves and complete defoliation, whereas the ND pathotype is less aggressive and is associated with a slow decline syndrome (Markakis et al. 2010).

Because of the general awareness regarding the abiotic and biotic stress threatening olive crops, current efforts have been directed towards the development of natural, eco-friendly solutions, such as plant-based extracts, which can enhance the resilience of crops to these stresses while reducing the reliance on synthetic chemicals (Asif et al. 2023; Hegedűs et al. 2022). Plant extracts are regarded as safe, low in toxicity, and highly biodegradable (Li Destri Nicosia et al. 2016). Their use as biostimulants and

natural antimicrobials has gained significant attention in recent years, showing promising results in enhancing crop performance and disease resistance (Kisiriko et al. 2021). Many authors have described the application of seaweed (*Ascophyllum nodosum*), moringa (*Moringa oleifera*) and liquorice (*Glycyrrhiza glabra*) extract, among others, as biostimulants in a variety of woody fruit crops, including citrus plants (*Citrus sinensis* and *Citrus clementina*), grapes (*Vitis vinifera*), and trees from the *Prunus* genus, such as plum, peach and almond trees (Thanaa et al. 2016; Godlewska, Ronga, and Michalak 2021; Fornes, Sánchez-Perales, and Guardiola 2002; Salvi et al. 2019; Kumari et al. 2023; Bakhsh et al. 2020). In addition, the antimicrobial activity of plant extracts such as tea (*Camellia sinensis*), thyme (*Thymus vulgaris*), *Brassica* vegetables, cork oak (*Quercus suber*) and eucalyptus (*Eucalyptus globulus*) against a wide range of phytopathogens has been reported (Lagrouh, Dakka, and Bakri 2017; Mohd Israfi et al. 2022; He et al. 2024; Yang and Zhang 2019).

Allium plants include over 900 species, with the most studied being garlic, onion, leek, and shallot. The primary bioactive compounds in *Allium* species are organosulfur compounds (OSCs), which are produced during tissue damage as a defence mechanism. Key OSCs include S-alk(en)yl-L-cysteine sulfoxides (ACSO), precursors to thiosulfinates, thiosulfonates, and sulfides. In onion (*Allium cepa*), the main sulfur compounds are isoalliin, which converts to lachrymatory factor, methiin, and propiin, which, through the action of alliinase, lead to dipropyl thiosulfinate (PTS) and propyl-propane thiosulfonate (PTSO) (Guillamón et al. 2021). Extracts from the *Allium* genus have shown promising bioactive properties including antimicrobial, repellent and antioxidant, among others (Falcón-Piñeiro et al. 2023; Guillamón et al. 2023). The literature suggests that *Allium* extracts may have a dual function in agriculture as both biostimulants and antimicrobial agents. Various studies have demonstrated the capacity of garlic (*Allium sativum*) extracts to improve the growth, yield, fruit quality and stress tolerance of legumes (*Vicia faba* and *Phaseolus vulgaris*), eggplant (*Solanum melongena*), and pepper (*Capsicum annuum*) (Zulfiqar et al. 2020; Godlewska, Ronga, and Michalak 2021). Other authors have revealed that garlic extract exhibits similar antifungal activity as the chemical fungicides carbendazim and kanamycin B against *Colletotrichum musae* or *Lasiodiplodia theobromae* (Mohd Israfi et al. 2022). Additionally, we demonstrated that organosulfur compounds from onion reduce the incidence and severity of Verticillium wilt, as well as the density of *V. dahliae* in the soil, in trials conducted in a climatic chamber with seven-year-old olive plantlets var Picual (Falcón-Piñeiro et al. 2021).

The aim of this study was to assess the dual biostimulant and antifungal activity of an onion extract formulation rich in organosulfur compounds. This investigation was conducted using three experimental approaches: (1) a controlled climatic chamber trial to assess the biostimulant effect on non-stressed, 1-year-old olive trees, focusing on oxidative stress and plant development parameters; (2) a field trial on an experimental farm to investigate the biostimulant effect on budbreak and shoot length in 4-year-old olive trees experiencing abiotic stress; and (3) two field trials in commercial olive orchards to evaluate both the biostimulant and antifungal effects on mature olive trees

naturally infected with *V. dahliae*, by measuring oxidative stress and fruit quality parameters, as well as pathogen density. This multi-tiered approach was designed to provide comprehensive insights into the potential of the onion extract as a sustainable solution for enhancing olive tree resilience and controlling fungal pathogens across different environmental settings.

2. Results

2.1. Biostimulant activity under controlled conditions

To determine ROS accumulation and membrane damage in control and treated 1-year-old olive plantlets in climatic chamber, malondialdehyde (MDA) was measured. The results are shown in Figure 1. Figure 1a illustrates the changes in MDA levels in olive leaves in response to 3 treatment applications, administered 24 hours before the first sampling, on day 15, and day 30 post-sampling. No significant differences were observed in MDA levels across all groups 24 h after the first application, indicating minimal oxidative stress and membrane disruption. Fifteen days after the first application a significant reduction of MDA was observed in olive plantlets treated by foliar spray and irrigation compared to the control ($p < 0.05$). This difference was maintained in olive trees treated by irrigation on day 30 (30 days after the first application and 15 days after the second). In the case of olives plantlets treated by foliar spray, a statistical trend towards a reduction in MDA levels compared to control plants was observed ($p < 0.1$). Finally, on day 60 of the experiment (30 days after the third application), the MDA levels equalized.

The MDA levels in roots, showed in Figure 1b, did not show any significant variation in the treated plant with respect to control plants at any sampling time. In the case of olive plantlets treated by irrigation, a statistical trend towards a reduction in MDA was observed on days 15 and 30 of sampling ($p < 0.1$).

The antioxidant capacity was evaluated by measuring the reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) using the FRAP (ferric-reducing antioxidant potential) method (Figure 2). Figure 2a depicts the concentration of Fe^{2+} in leaves over time. There were no significant differences across groups on the first and second days of sampling, even if the foliar spray treatment shows a slight reduction on day 15 compared to the control. A more pronounced effect is observed on day 30, since Fe^{2+} is significantly reduced in the foliar samples of plants treated by both foliar spray and irrigation ($p < 0.05$). This difference compared to the control disappeared 30 days after the last application of the treatment (day 60).

Concentration of Fe^{2+} in roots is shown in Figure 2b. As in the leaf samples, no differences were observed between the different groups on the first day of sampling. A significant reduction in Fe^{2+} levels was observed in leaves from plants treated by irrigation compared to both the control and foliar spray groups 15 days after the first treatment application ($p < 0.05$). This trend persisted on day 30. By day 60, the Fe^{2+} levels

in the irrigation group remained low ($p < 0.05$), with the control and foliar spray groups showing more variability, but without significant differences between them.

The average leaf weight and root length of the olive plantlets were assessed on the final day of sampling. As shown in Table 1, the leaf weight of olive plantlets treated by foliar spray and irrigation did not significantly differ from each other or from the control. In contrast to leaf weight, root length results showed significant differences among groups. Root length significantly increased in plants treated by both foliar spray and irrigation compared to the control ($p < 0.01$). No significant differences were observed between the two application methods.

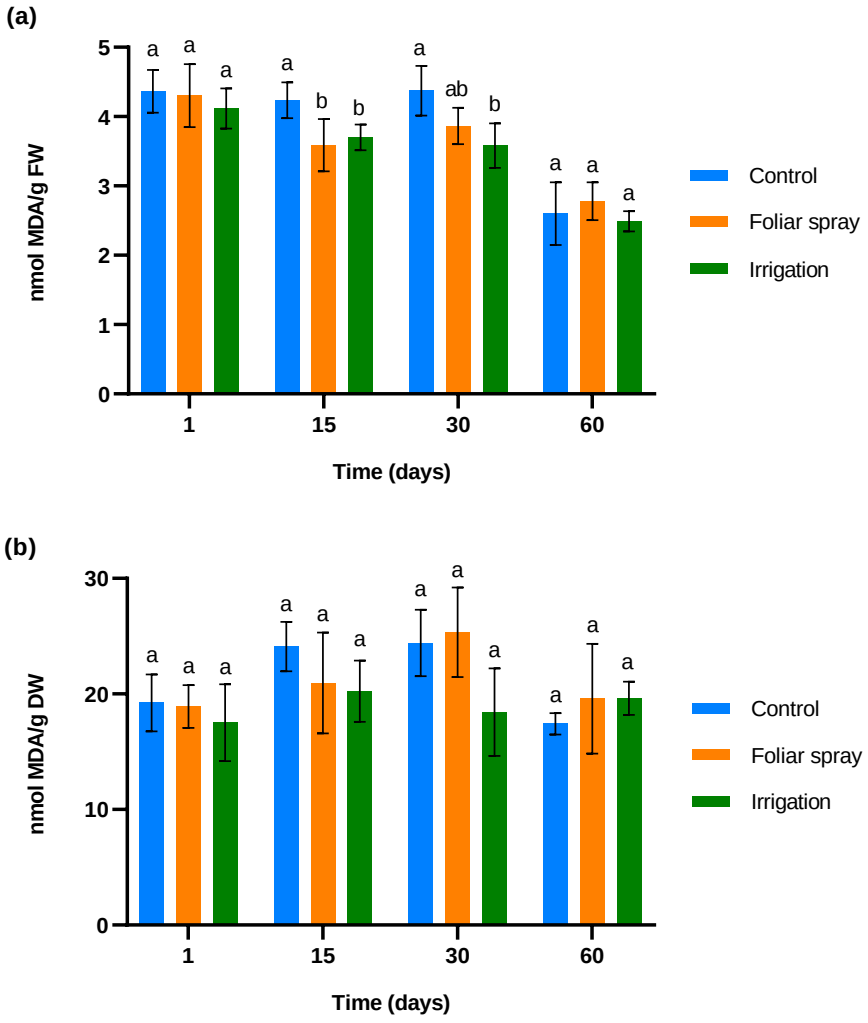

Figure 1. Effect of onion extract formulation applied by foliar spray and irrigation on MDA content of 1-year-old olive plantlets compared to the control: (**a**) leaves (**b**) lyophilized roots. Data are mean ± SD. Bars with different letters indicate significant differences according to Tukey test ($p < 0.05$).

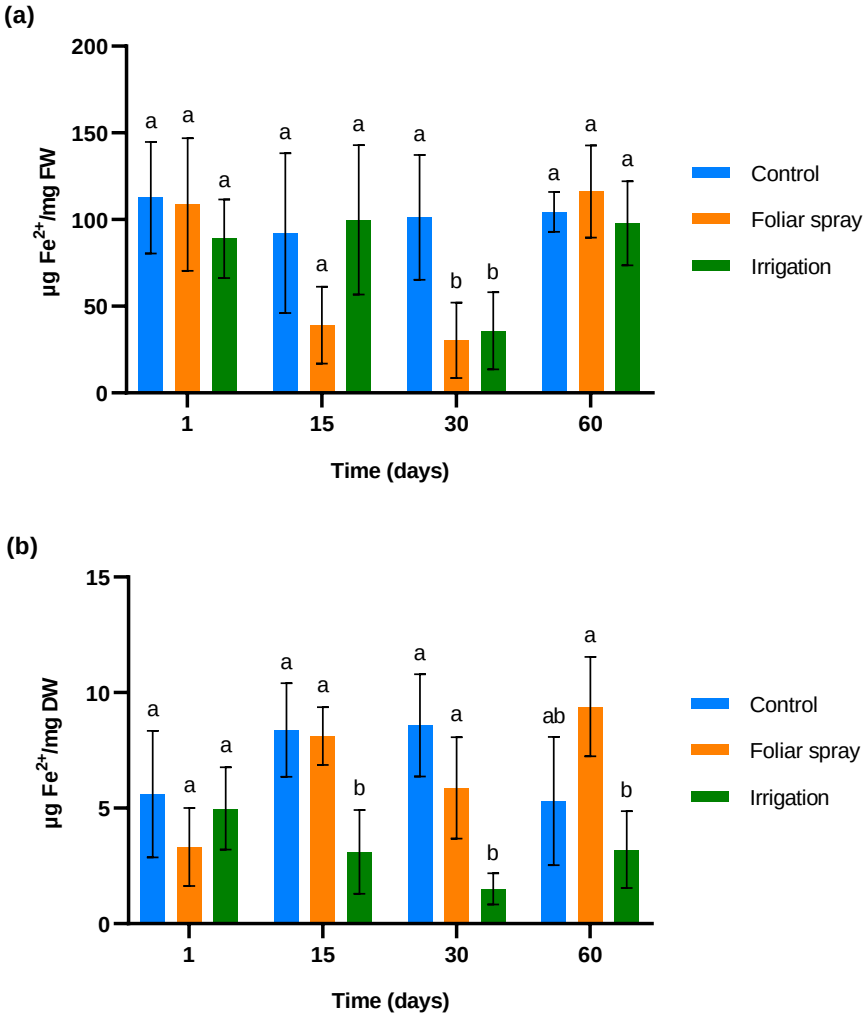

Figure 2. Effect of onion extract formulation applied by foliar spray and irrigation on ferric reducing power in 1-year-old olive plantlets compared to the control: (a) leaves (b) lyophilized roots. Data are mean ± SD. Bars with different letters indicate significant differences according to Tukey test ($p < 0.05$).

Table 1. Average leaf weight and root length of olive plantlets treated with onion extract by foliar spray and irrigation under controlled conditions. Different letters indicate significant differences according to Tukey test ($p < 0.05$).

	Leaf weight (mg)	Root length (cm)
Control	131.6 ± 36.1 a	12.2 ± 2.6 a
Foliar spray	139.0 ± 1.4 a	18.6 ± 1.0 b
Irrigation	129.4 ± 12.9 a	19.5 ± 1.6 b

2.2. Biostimulant activity under field conditions

2.2.1. Budbreak induction in 4 years old olive trees

The biostimulant activity of the onion extract has been assessed through a budbreak study in 4-year-old olive trees under conditions of severe defoliation due to abiotic stress in field trial. The results are shown in Figure 3. Significant differences were observed between the number of shoots in control olive trees and those treated by foliar

spray ($p < 0.01$) and irrigation ($p < 0.001$) at both evaluation times (Figure 4). While the number of shoots on the olive trees treated by foliar spray and irrigation was similar after 2 applications of onion extract, after 3 applications the number of shoots significantly increased in the trees treated by irrigation compared to those treated by foliar spray ($p < 0.01$) (Figure 3a). The length of shoots did not differ significantly between control olive trees and those treated with foliar spray at any of the evaluation times. However, significant variations were found in olive trees irrigated with onion extract compared to the control after 2 ($p < 0.05$) and 3 applications ($p < 0.001$), and foliar spray-treated plants after 3 applications ($p < 0.01$) (Figure 3b).

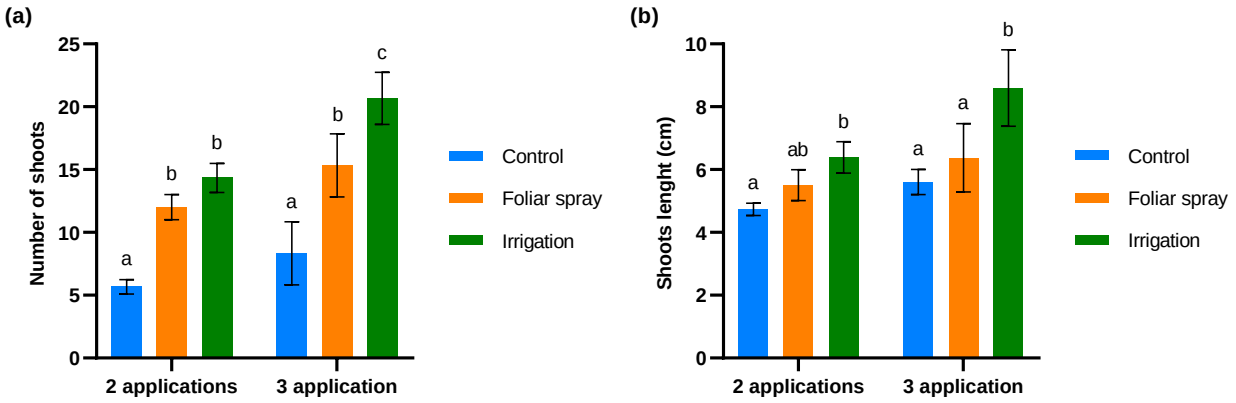

Figure 3. Evaluation of budbreak in 4-year-old olive trees treated with onion extract by foliar spray and irrigation compared to the control: (**a**) number of new shoots (**b**) shoots length (cm). For both panels, bars with different letters indicate significant differences according to Tukey test ($p < 0.05$).

(**a**) (**b**)

Figure 4. Cont.

(**c**) (**d**)

Figure 4. Status of olive trees (**a**) before the first application (**b**) control plants 20 days after the third application (**c**) plants treated by foliar spray 20 days after the third application (**d**) plants treated by irrigation 20 days after the third application.

2.2.2. Biostimulant activity in mature olive trees

MDA content and the reduction of Fe^{3+} to Fe^{2+} were also determined in leaf samples from mature olive trees naturally infected with *V. dahliae* in commercial olive orchards located in Linares and Santaella. Results are shown in Figures 5 and 6, respectively. In both locations, MDA levels in leaf samples from the control area were higher than in those from the treatment area. In Linares, although the difference was not statistically significant, there was a trend towards the reduction of MDA in the treated group ($p < 0.1$). In Santaella, leaves from treated trees showed a significant reduction in MDA compared to the control ($p < 0.05$). While the MDA content in the control groups at both locations was similar, Fe^{2+} levels differed significantly. At the Linares orchard, a significant reduction in Fe^{2+} was observed in the treated group with respect to the control ($p < 0.01$), whereas at Santaella they were similar. Additionally, proline content was measured. As shown in Figure 7, no significant differences were observed between control and treatment groups within the same orchard. Interestingly, proline content was significantly lower at the Santaella orchard.

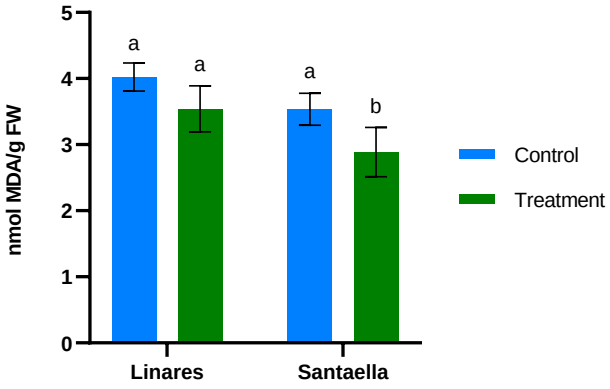

Figure 5. Effect of onion extract formulation applied by irrigation on MDA content of adult olive trees in orchards located in Linares and Santaella. Data are mean ± SD. Bars with different letters indicate significant differences according to Tukey test ($p < 0.05$).

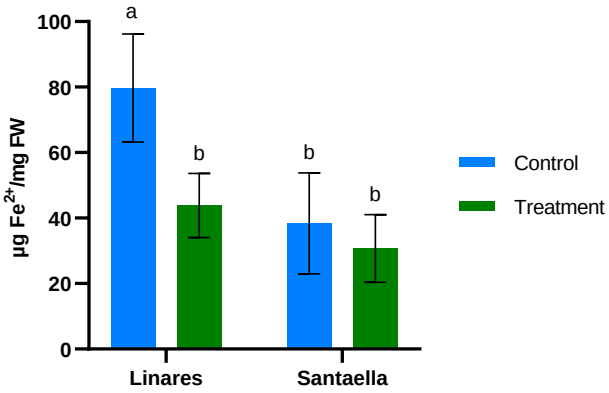

Figure 6. Effect of onion extract formulation applied by irrigation on ferric reducing power in adult olive trees in orchards located in Linares and Santaella. Data are mean ± SD. Bars with different letters indicate significant differences according to Tukey test ($p < 0.05$).

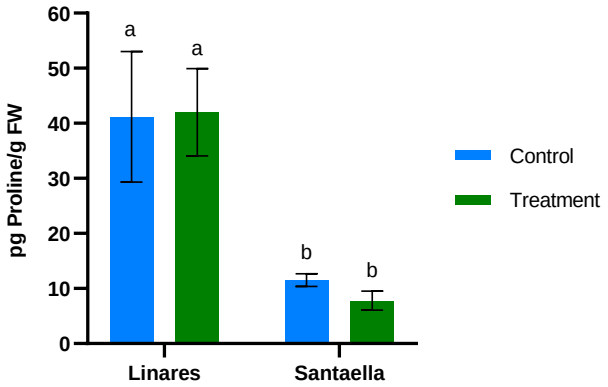

Figure 7. Effect of onion extract formulation applied by irrigation on proline content power in adult olive trees in orchards located in Linares and Santaella. Data are mean ± SD. Bars with different letters indicate significant differences according to Tukey test ($p < 0.05$).

The weight, volume, moisture and fat content per 100 fruits was measured for both treated and untreated olive trees at farms located in Linares and Santaella. As shown in Figure 8a, b, the analyses of weight and volume revealed no significant differences between fruits from the control and treatment zones within the same farm. The results for weight and volume were correlated. Additionally, these data revealed significant differences between the two farms. Fruits from the farm in Linares were significantly smaller and had lower volumes compared to those from the farm in Santaella ($p < 0.05$). The analysis of humidity content (Figure 8c) revealed that there were no statistically significant differences between the moisture levels of fruits from treated and control trees in the Linares orchard. However, a significant reduction in moisture content was observed in the fruits from treated trees compared to control trees in the Santaella orchard ($p < 0.001$). Regarding fat content (Figure 8d), the fruits from treated olive trees in Santaella showed a statistically significant increase compared to the fruits from control trees ($p < 0.001$). While no statistically significant differences were observed in the fat content of fruits from Linares, there was a statistical trend towards an increase in fruit fat content in the treated trees compared to the control ($p < 0.1$).

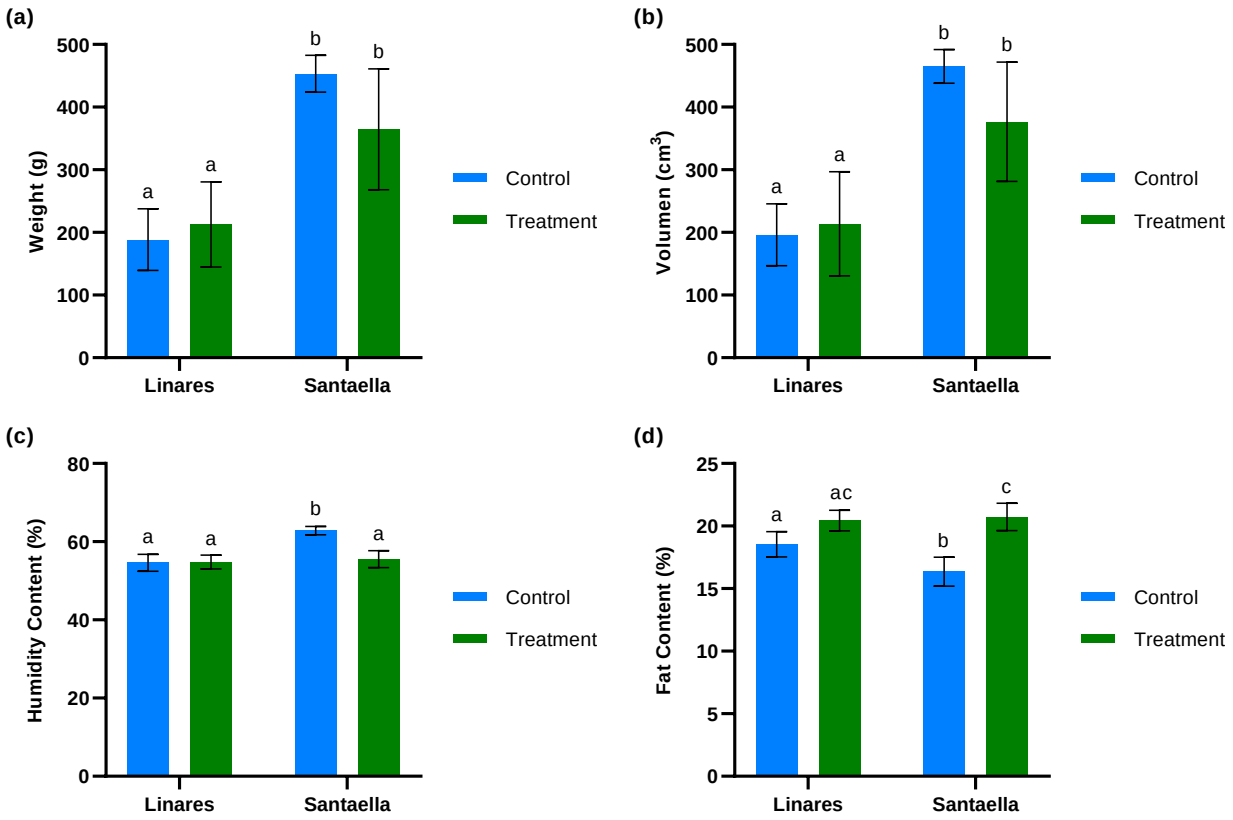

Figure 8. Analysis of fruits from treated and control olive trees in orchards located in Linares and Santaella: (**a**) Weight/100 fruits (g) (**b**) Volume/100 fruits (cm^3) (**c**) Humidity content/100 fruits (%) (**d**) Fat content/100 fruits (%). For both panels, bars with different letters indicate significant differences according to Tukey test ($p < 0.05$).

2.3. Antifungal activity against *V. dahliae* under field conditions

The impact of the onion extract formulation on the infection rates and gene copy numbers of *V. dahliae* in olive trees was assessed in both locations (Table 2). In Linares orchard, the percentage of infected trees was slightly higher in the treated group compared to the control, though these differences were not statistically significant. However, the log_{10} gene copies were significantly lower in the treated trees compared to the control group ($p < 0.0001$). A similar pattern was observed in Santaella orchard. No significant differences in infection rate were observed between control and treated group, but a significant reduction of the log_{10} gene copies was observed in the treatment group compared to the control ($p < 0.0001$).

Table 2. Percentage of infected olive trees and number of genes copies expressed as Log_{10} of *V. dahliae* in control and treated areas in Linares and Santaella orchards. Different letters indicate significant differences according to Tukey test ($p < 0.05$).

	Infection rate (%)	**Log_{10} copies**
Linares Control	63 ^{ab}	4.0 ± 0.3 ^{a}
Linares Treatment	70 ^{a}	1.4 ± 0.1 ^{b}
Santaella Control	60 ^{ab}	4.3 ± 0.1 ^{a}
Santaella Treatment	53 ^{b}	2.0 ± 0.1 ^{b}

3. Discussion

The olive tree is increasingly susceptible to climate change effects (Orlandi et al. 2020). These climatic shifts exacerbate existing challenges, notably the prevalence of *V. dahliae*. Climate change and disease pressure not only undermines the resilience of olive trees but also jeopardize the long-term viability of the olive oil industry (Sousa et al. 2020). A major concern in managing the effects of abiotic and biotic stress is the need to improve the sustainability of the agricultural industry through novel, eco-friendly strategies to control plant diseases and stimulate plant resilience (Sabetta et al. 2021; Carvalho et al. 2022). In our study we demonstrated the dual biostimulant and antifungal activity of an onion extract formulation rich in organosulfur compounds on olive trees under non-stressful conditions in climatic chamber, as well as under abiotic and biotic stress in field trials.

In the experiment conducted in climatic chamber, treatments were applied to olive trees in good condition, that were not under abiotic or biotic stress. Our findings did not reveal any significant variations in the MDA content in roots. However, a reduction was observed in leaves on days 15 and 30 of sampling. MDA is a product of membrane lipid peroxidation, which occurs when lipids in cellular membranes are damaged by free radicals (Sofo et al. 2004). Multiple researchers have used MDA as an indicator of oxidative stress in olive trees (Ali et al. 2019). Our results demonstrate not only that the onion extract formulation has no phytotoxic effects on 1-year-old olive trees but also suggest that it could reduce lipid peroxidation under stress conditions. Onion extract reduced the ferric reducing capacity on days 15 and 30 of sampling. The ferric reducing capacity in roots was only influenced by application through irrigation. FRAP is an indicator of a tissue's ability to neutralize free radicals and reduce oxidative stress, providing an indirect measure of total antioxidant activity. The reduction in ferric reducing ability, indicated by a lower Fe^{2+} content, is generally as-sociated with increased oxidative stress (Kaur et al. 2017). However, in plants experiencing moderate or low oxidative stress, a high reducing capacity may not be necessary, as the production of reactive oxygen species (ROS) is also minimal. In this context, iron regulation could be modulated to prevent the accumulation of free Fe^{2+}, which could generate ROS through the Fenton reaction (Schweikert, Liszkay, and Schopfer 2002). Consequently, lower Fe^{2+} levels would indicate a more balanced re-dox state.

Although the onion extract had a significant effect on MDA and Fe^{2+} levels 15 days after the first and second applications, 30 days after the third application the levels were comparable to those of the control, which may be attributed to various reasons. On one hand, this could be due to the acclimatization of the olive plantlets in the climatic chamber. On the other hand, the volatility of the organosulfur compounds present in the onion extract could explain the absence of differences 30 days after the last application. In a previous study, we assessed the persistence of organosulfur compounds from onion extract in soil by HPLC–UV (Falcón-Piñeiro et al. 2023). This study demonstrated that the compounds rapidly volatilize to varying degrees, with concentrations reducing by up to 50% 15 days after application.

Additionally, results from climatic chamber trial demonstrated that both foliar spray and irrigation treatments positively influenced root growth. Treated 1-year-old olive plantlets exhibited more robust root structures. Ali et al. (Ali et al. 2021) described a similar effect in eggplant seedlings treated with allelochemicals from *Allium* species applied via foliar spray. As stated by Pedranzani et al. (Pedranzani et al. 2016), a more developed root structure enhances plant stress resilience by improving access to water and nutrients as well as hydraulic conductivity. *V. dahliae* colonizes the xylem of the plant and causes vessel occlusion, reducing water transport and leading to stomatal closure (Fradin and Thomma 2006). Therefore, stimulation of root development not only positively influences conditions under water or saline stress, but also enhances resistance to the symptoms of Verticillium wilt. This growth biostimulation effect is consistent with the results obtained at the Neval experimental farm. In the field trial involving 4-year-old trees, the application of onion extract provided a biostimulant effect, as evidenced by the increased budbreak rate. Our results are in accordance with those reported by other authors that obtained higher budbreak rate on 10-year-old apple trees exposed to sulfur natural compounds (Rady and Seif El-Yazal 2014). These results are of great relevance in the current context of a climate emergency. Temperature exerts a significant influence on the phenological phases of bud break and flowering of olive trees. It also controls the winter dormancy and the onset of vegetative growth that begins in spring, stages that condition the beginning of new structures development. The increase in global temperatures as a result of climate change may substantially reduce the number of chill hours in winter and raise the average temperature in spring, which, according to several studies, adversely affect budbreak and flowering (Benlloch-González et al. 2019; Alcalá and Barranco 2019; Paola, Vincenza, and Paola 2021).

Both the determinations conducted in the climatic chamber and the budbreak study carried out on the experimental farm indicate that the application of the formulation by irrigation was more effective. Furthermore, the budbreak study highlights the importance of performing 3 successive applications by irrigation. The results show that the budbreak rate and shoot length significantly increased after the third irrigation application compared to the results obtained after the second.

The Linares and Santaella orchards were naturally infected with *V. dahliae*. Onion extract reduced MDA content and, consequently, membrane lipid peroxidation in treated olive trees at both orchards. These results are consistent with those described in the climate chamber trial, and support the ability of the onion extract to reduce oxidative stress and membrane damage under stress conditions. Similarly, allelochemicals from *Allium* species have been shown to reduce MDA content in plants under biotic stress. Reduced MDA content has been documented in eggplants infected with *V. dahliae* when treated with an extract rich in these compounds from garlic (Ali et al. 2021). Such effects could be explained by the antioxidant properties of organosulfur compounds from *Allium* species (Mellado-García et al. 2016). The mechanisms by which organosulfur compounds exert their antioxidant capacity have been de-scribed in previous research. According to Cascajosa-Lira et al., they scavenge free radicals and induce the activity of

cellular antioxidant enzymes (Cascajosa-Lira et al. 2024). In addition, Llana-Ruiz-Cabello et al. reported that organosulfur compounds provide nonenzymatic antioxidant protection, demonstrating their ability to enhance lipid stability (Llana-Ruiz-Cabello et al. 2015). On the other hand, the results of FRAP analyses performed on olive plant tissue in both climate chambers and field trials show that onion extract does not induce the reduction of Fe3+ to Fe2+. Similarly, it does not promote the accumulation of proline, which is known to confer a protective effect by neutralizing free radicals (Naliwajsju and Maria 2021).

Water deficit, imposed by climate change, increased soil salinity due to irrigation with low-quality water, and high incidence of pathogens causing vessel occlusion, is a limiting factor in olive cultivation (Tadić et al. 2021) and affect fruit setting and quality (Bompadre et al. 2013). Drought stress during the phases of fruit setting results in small fruits with low fat content (Greven et al. 2009). Additionally, the oils obtained from these fruits have occasionally been found to be excessively bitter (Brito, Dinis, Moutinho-Pereira, et al. 2019). The application of onion extract was associated with an increase in the fat content of the fruits from *V. dahliae*-infected olive trees at both the Linares and Santaella orchards. As mentioned above, *V. dahliae* reduces hydraulic conductivity by colonizing the xylem (Fradin and Thomma 2006). The increase in fat content of the fruits from trees treated with onion extract could be explained by several mechanisms. In this study, onion extract has been shown to promote root development, which could improve the plant's ability to absorb water and mitigate the effect of *V. dahliae* on hydraulic conductivity. Additionally, the antioxidant properties of the extract, as reported in this study, may protect tissues from oxidative stress caused by the infection, helping to stabilize metabolic processes. Lastly, the onion extract could have a direct effect on *V. dahliae* population, thereby limiting the severity of the damage it causes. This hypothesis is further reinforced by the RT-qPCR results. Quantification of *V. dahliae* by RT-qPCR in leaf samples from treated and control olive trees indicated that, while the treatment did not substantially reduce the percentage of infected trees, it consistently resulted in a significant reduction in the pathogen density in leaves, as reflected by the lower number of gene copies in both locations. These results are in accordance with those reported by our group in a previous study, where the effect of organosulfur compounds from onion on Verticillium wilt suppression was evaluated in 7-month-old olive plantlets var Picual (Falcón-Piñeiro et al. 2021). The plantlets were grown in soil artificially infested with *V. dahliae* in climatic chamber under optimal conditions for Verticillium wilt development. The application of the organosulfur compounds by irrigation reduced *V. dahliae* density in the soil, vascular colonization, and symptom severity.

To our knowledge, the use of *Allium* extracts for the control of Verticillium wilt in olive trees has not been extensively studied, despite their strong antifungal activity against pathogenic fungi. Pomegranate (*Punica granatum*) and carob (*Ceratonia siliqua*) extracts have been shown to be effective in reducing disease severity when applied by irrigation (Antón-Dominguez et al. 2024). The results of this study support the

application of plant extracts by irrigation, as foliar application proved ineffective in controlling the disease.

The biostimulant and antifungal activity observed in our study are likely due to the dual effect of multiple bioactive compounds present in the onion extract, particularly organosulfur compounds such as PTS and PTSO. These compounds are known for their potent antifungal properties, which they exert by disrupting the cellular integrity and metabolic functions of pathogenic microorganisms, such as *V. dahliae* (Sorlozano-Puerto et al. 2021). This disruption may occur through the inhibition of essential enzymatic activities required for fungal growth and the destabilization of fungal cell membranes, ultimately reducing pathogen viability and colonization (Focke, Feld, and Lichtenthaler 1990). Moreover, these organosulfur compounds may also enhance the plant's innate defence mechanisms by upregulating genes involved in antioxidant responses, thereby mitigating oxidative damage under both biotic and abiotic stress conditions (Zhu et al. 2022; Miekus et al. 2020). The interplay between these compounds is particularly compelling, as they may work synergistically to protect against biotic stressors while supporting overall plant health by boosting antioxidant capacity and promoting growth under varying environmental conditions. This dual action could be the key to the onion extract's effectiveness as both an antifungal and a biostimulant agent.

The dual functionality of the onion extract formulation could not only effectively aid in stress mitigation but also aligns with the growing demand for sustainable agricultural practices. Nevertheless, since Picual is a cultivar primarily used for olive oil production (Barranco and Rallo 2000), studying the potential impact of onion extract on olive oil is essential. The interaction between onion-derived compounds and olive oil constituents may influence key quality parameters such as oxidative stability and sensory attributes, which are pivotal to its desirability in the marketplace. A previous trial demonstrated that while the application of a biostimulant based on tropical fruit extract altered the organoleptic properties of olive oil from cultivar Racioppella, treatment with glycine-betaine had no effect (Cirillo et al. 2022). Additionally, research with the Empeltre variety has shown that the incorporation of garlic into the olive paste during the malaxation process significantly increased the total phenolic content and antioxidant capacity of the resulting olive oil (Abenoza and Sánchez-Gimeno 2021). Further studies are needed to evaluate the potential effects of onion extract on oxidative stability, fatty acid and phenolic compound content in olive oil.

Finally, given that the application of various active compounds with specific functions is a common practice in the agronomic management, future studies should investigate the synergy between onion extract and other sustainable alternatives that have been shown to protect olives from abiotic and biotic stress and enhance productivity, such as seaweed extracts (Graziani et al. 2022), salicylic acid (Brito, Dinis, Ferreira, et al. 2019) and sodium nitroprusside (SNP) (Ullah et al. 2024; Biosci and Elhami 2015).

4. Materials and Methods

4.1. Onion extract

A formulation based on an onion extract (*Allium cepa* L. bulb extract) obtained from discarded onions was tested. The product (TROFIC®) was provided by DOMCA S.A.U. (Granada, Spain), and was composed by 85% of a standardized bulb onion extract containing 50% of organosulfur compounds derived from propiin, such as propyl thiosulfinates and thiosulfonates. It also included other components essential for stability, such as soy lecithin and ascorbic acid.

4.2. Evaluation of biostimulant activity in climatic chamber

Sixty 1-year-old Picual olive plantlets, approximately 50 cm in height, were randomly selected and transplanted into new pots. They were divided into 3 experimental groups: control plants that received no treatment, plants treated by foliar spray, and plants treated by irrigation. The olive plantlets were numbered and kept in a climate chamber under non-stressful conditions, with a photoperiod of 8 hours of light and 16 hours of darkness, and a maximum/minimum temperature of 25/12°C. The product was diluted to 500 mg/L. A volume of 150 mL per plantlet was applied by irrigation. For the foliar spray treatment, the necessary volume to cover the entire aerial part was applied, approximately 3 mL. Three applications were performed: one at the beginning of the trial, another after 15 days, and a final application 30 days after the beginning of the trial. Destructive sampling of leaves and roots from 5 olive plantlets was performed at 4 different times: 24 hours after the first application of the treatment (T1), after 15 days (T2), after 30 days (T3), and after 60 days (T4). On the days when sampling and treatment application coincided (T2 and T3), sampling was performed first and then the treatment was applied to the remaining plantlets. Leaves and roots were frozen in liquid nitrogen, pulverized using a grinder, and immediately stored at -80ºC until analysis. For each sampling time, MDA was determined, and FRAP analysis was performed. Additionally, after the final sampling, leaf biomass and root length were evaluated.

4.2.1. Measurement of MDA

The MDA content was determined following the TBARS procedure proposed by Heath and Packer with some modifications (Heath and Packer 1968). For each leaf sample, 300 mg were macerated with 20% (w/v) TCA (trichloroacetic acid) and 4% (w/v) BTH (butylated hydroxytoluene) and centrifuged at 12,000 rpm for 10 min. Supernatant was mixed with 0.5% (w/v) TBA (thiobarbituric acid), and the mixture was incubated at 95 °C for 30 min in a water bath. The samples were cooled on ice to stop the reaction, and then centrifuged at 12,000 rpm for 10 minutes. The absorbance of the supernatant was measured at 532 nm and 600 nm. A standard curve was generated using known concentrations of MDA, and results were expressed as nmol MDA/g sample. Each sample was analysed in triplicate, and the absorbance of each replicate was measured twice.

The determination of MDA in roots was conducted following the same method using 30 mg of lyophilized samples. The lyophilization process was essential as the residual water and soil interfered with the accurate quantification of the metabolite.

4.2.2. FRAP Assay

The ferric reducing antioxidant power assay (FRAP) was conducted according to Benzie and Strain (Benzie and Strain 1996). Some modifications were applied to the protocol. For each leaf sample, 300 mg were macerated with 80% (v/v) acetone and shaken for 15 minutes at 4°C in darkness. After centrifugation at 5000 rpm at 4°C for 15 minutes, FRAP reagent was added to the supernatant. Samples were incubated for 30 minutes at 37°C in darkness, and absorbance was measured at 595 nm. The amount of Fe^{2+} in the samples was calculated from a standard curve of known concentrations. Results were expressed as μg Fe^{2+}/mg sample. Each sample was analysed in duplicate, and absorbance was measured twice for each replicate.

The same protocol was conducted for the FRAP assay in root tissue, using 20 mg lyophilized samples.

4.3. Evaluation of biostimulant activity in young olive trees in experimental farm

The field trial was conducted from March to June 2021, in collaboration with Neval, an agricultural R&D laboratory accredited by the Spanish Ministry of Agriculture for the execution of officially recognized tests, in an experimental farm located in Xilxes, Valencia, Spain (30N 740759.99 4406967.05 UTM WGS84). The soil was classified as Loam according to the USDA (United States Department of Agriculture). It contained 45% sand, 34% silt and 21% clay (analysed by Bouyoucos hydrometer method (van Reeuwijk 2002)), and 1.21% organic matter (measured according to Walkley-Black procedure (van Reeuwijk 2002)).

The biostimulant activity was evaluated in 4-year-old, completed defoliated olive trees var Picual. They were transplanted from pots to soil with a planting frame of 3.5 × 2 m. The soil had not received any previous treatment. The state of severe defoliation of the olive trees was due to abiotic stress caused by the transplant. Treatment application started after a one-month acclimation period. The number of shoots was quantified before the first application. The efficacy of onion extract was evaluated by foliar spray and irrigation in comparison with the control (water). The product was diluted to 500 mg/L water. Foliar spraying was carried out using a backpack sprayer and a spray volume equivalent to 700 L/ha, while for irrigation 10 L/tree were applied. Three applications were made at 21 days intervals. Three replicates were carried out, with 3 olive trees each, in a completely randomized manner. From transplantation until the end of the study, the weekly irrigation regime established by the farmer was maintained to avoid drought stress. To evaluate the efficacy of the treatment, the length of the shoots and the number of new ones were assessed 20 days after the second and third application.

4.4. Evaluation of biostimulant and antifungal activity in olive orchards

The trial was conducted in two Picual olive orchards situated in Linares, Jaen (30N 444908.38 4209274.77 UTM WGS84), and Santaella, Córdoba, Spain (30N 335550.44 4153847.83 UTM WGS84), both of which are owned by DCOOP, a cooperative headquartered in Antequera (Málaga Spain) and recognized as the world's largest producer of olive oil. The trial lasted 14 months, from April 2021 till June 2022. According to the USDA, the soil of Linares orchard was classified as sandy loam (68% sand, 18% silt and 14% clay) and had 1.13% organic matter, while the soil of Santaella was classified as loamy sand (78% sand, 13% silt and 9% clay) and had 0.8% organic matter. These analyses were carried out following the same methods as those specified in *4.3. Evaluation of biostimulant activity in young olive trees under field conditions*. Both are intensive irrigated orchards. The olive trees in the Linares orchard (10 × 10 m planting frame) were bicentennial and were irrigated 12 h every 4 days. In contrast, the 25-year-old olive trees in Santaella orchard (7 × 7 m planting frame) received 3 hours of daily irrigation from April to October. Both had a high incidence of Verticillium wilt, and symptoms of decline syndrome were observed.

The orchards were divided in 2 areas: treatment and control, outlined in red and yellow in Figures 9 and 10. The treatment was applied in spring, as established by the farmer, through the irrigation system at 5 L/ha. Application and sampling dates are described in Table 3. Three applications were made at 1-month intervals during springs of 2021 and 2022, starting in April. The following samplings were carried out on the following times: fruit sampling was carried out in November 2021; and leaf sampling in June 2022, 2 weeks after the last application.

Twenty olive trees were sampled in each area. The sampled olive trees, randomly selected, were located in the central area, avoiding olive trees on the edges. From each olive tree, 20 olives were taken by hand, 5 olives for each orientation (South, North, East and West). The fruits were collected at eye level, both from the most superficial branches and from the inner area of the olive tree. Sampling was performed looking the other way to avoid subjectivity, with the aim of preventing preferences for a specific olive ripeness or size. In total, 400 olives were obtained from each area and orchard.

Leaf samples were taken from 30 olive trees in each of the delimited areas, avoiding those on the edges. At least 100 leaves were collected from each tree, ensuring samples were taken from all orientations. Leaves were sampled randomly, selecting those at eye level from both the outer branches and those in the inner area of the olive tree.

Figure 9. Farm located in Linares, Jaen. The treatment area is outlined in red, and the control area in yellow. Image obtained through the Geographic Information System for Agricultural Plots (SIGPAC).

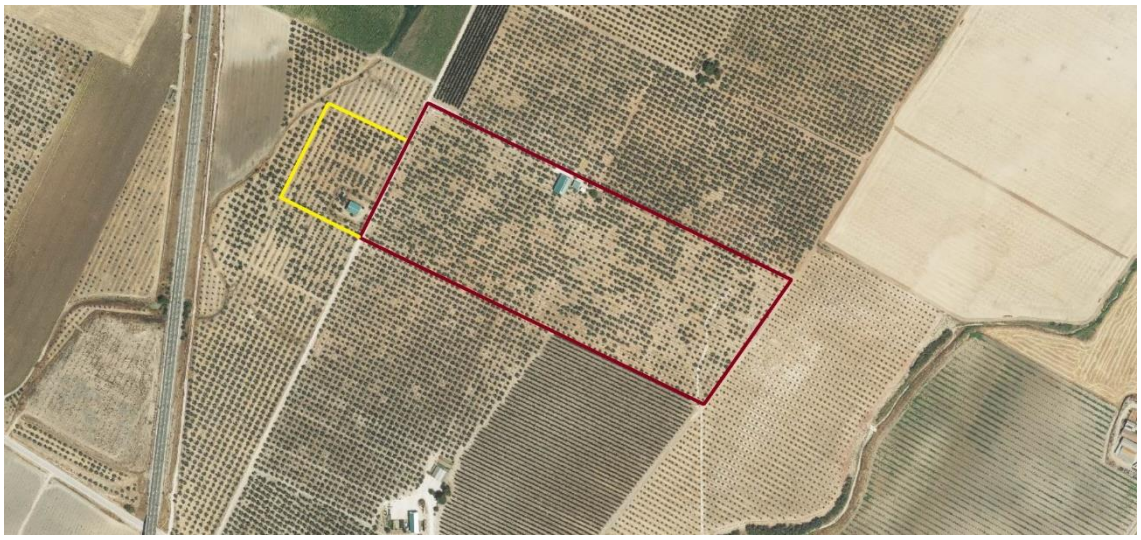

Figure 10. Farm located in Santaella, Córdoba. The treatment area is outlined in red, and the control area in yellow. Image obtained through SIGPAC.

Table 3. Treatment applications, evaluations and sampling dates.

Date	Application	Sampling
April 2021	1st	
May 2021	2nd	
June 2021	3rd	
November 2021		Fruits
April 2022	4th	
May 2022	5th	
June 2022	6th	Leaves

4.4.1. Fruit analysis

Fruits from 5 olive trees were analysed together, grouped according to plant proximity. Weight and volume of each group of 100 fruits were established and the

average was calculated. Volume was measured using the Archimedes principle. Olives were placed in 500 mL of water and the displacement was measured. Water content was determined gravimetrically from 30 g of grounded olives after drying in an air oven at 105°C for 24 h, according to UNE (Spanish Standard) 55031:1973 (Asociación Española de Normalización y Racionalización (AENOR) 1973). Fat content was measured from the dried sampled used in the moisture determination by Soxhlet method following UNE (Spanish Standard) 55030:1961 guidelines (Asociación Española de Normalización y Racionalización (AENOR) 1961). The fat was separated with hexane in a Soxhlet extractor for 6 h. The solvent was removed in a rotary evaporator at 40 °C, and the remaining oil was dried in an oven at 60°C until constant weight.

4.4.2. Leaf analysis

Leaf samples from each tree were numbered and analysed separately. Leaves were frozen in liquid nitrogen, pulverized using a grinder, and subsequently stored at -80 °C. The determination of MDA and the study of antioxidant capacity (FRAP method) were carried out following the protocols described in *4.2 Evaluation of biostimulant activity in climatic chamber*.

Proline was analysed from an amino acid extract. The amino acids were extracted from N2 pulverized material with a mix of ethanol/chloroform/water (12/5/1; v/v/v). Norvaline and sarcosine were used as internal standards. The extract was centrifuged at 3,500 x g during 10 min at 4°C and the supernatant was separated into chloroform and aqueous phases by the addition of HCl 0.1 N and chloroform. The mixture was centrifuged at 3500 x g during 5 min to separate phases; then, the aqueous phase was dried under nitrogen flow and stored at 20°C under an inert atmosphere. The dried samples were resuspended in 0.9 mL of HCl 0.1 N, sonicated and filtered with nylon filter (0.22 μm) and suitably diluted. Combining o-phthalaldehyde (OPA) and fluorenylmethyl chloroformate (FMOC) chemistries was used for pre-column derivatization of amino acids. Chromatographic analysis was done by following the method proposed by Palma et al. with some modifications (Palma et al. 2019). The amino acids were quantified by HPLC (Agilent 1,260 Infinity) with an ACE 5 C18-PFP 4.6mm 250 mm column and a fluorometer using excitation and emission wave-lengths of 340 and 450 nm (0–15 min) and 260 and 325 nm (16–33 min). Amino acids were eluted at a flow rate of 1 mL/min using an elution gradient with sodium acetate buffer 25 mM pH 6.8 (A) and acetonitrile/methanol/water mix (45/45/10, v/v/v) (B). The gradient profile, expressed as (t [min]; %A), was: (0; 80%), (20; 40%), (24; 40%), (26; 0%), (31; 0%) and (33; 80%). Results were expressed as pg Proline/g sample.

V. dahliae was detected and quantified in leaf samples by RT-qPCR. DNA extraction from leaves was carried out using the Plant/Fungi DNA Isolation Kit (Norgen Biotek, Thorold, Canada) following the instructions provided by the manufacturer. The same kit was used to extract DNA from a *V. dahliae* pure culture (isolate V136I; provided by the Department of Crop Protection, Institute for Sustainable Agriculture, Spanish National Research Council, Córdoba, Spain). DNA was stored at -20 °C for further use.

To generate a standard curve, *V. dahliae* V136I DNA was amplified by conventional PCR in a 2720 Thermal Cycler (Applied Biosystems, Singapore) using primers targeting the internal transcribed spacer (ITS) (Keykhasaber et al. 2017): VerDITS-F 5'-CCGGTCCATCAGTCTCTCTG -3' and VerDITS-R 5'-CACACTACATATCGCGTTTCG -3'. These primers amplify a 132-bp fragment. The amplification was caried out in a final reaction volume of 25 µL that contained 5 µL DNA, 2 µL primers 10 µM (5µM-Fw+5µM-Rev), 12.5 µL AmpliTaq Gold 360 Master Mix (Thermo Fischer Scientific, Vilnius, Lithuania) and ultrapure nuclease-free water up to the reaction volume (Thermo Fischer Scientific, Bremen, Germany). Cycling conditions consisted of 5 min at 95 °C, 40 cycles of 5 s at 95 °C, 15 s at 55 °C and 30 s at 72 °C, and a final extension step of 7 min at 72 °C. The PCR product was run on a 1.5% agarose gel to verify the absence of other DNA fragments. DNA concentration was measured using Qubit 4 Fluorometer (Invitrogen, Darmstadt, Germany), and the number of copies was calculated. For absolute quantification, ten-fold serial dilutions of DNA were prepared in nuclease-free water, yielding concentrations ranging from 10^8 to 10^1 copies/µL.

For the identification of infected trees and the quantification of *V. dahliae*, RT-qPCR was conducted on Bio-Rad CFX Connect Real-Time PCR Detection System (Bio-Rad, Feldkirchen, Germany). The reaction volume (20 µL) contained 5 µL DNA, 2 µL primers 10 µM (5µM-Fw+5µM-Rev), 10 µL iTaq Universal SYBR Green Supermix (Bio-Rad, Feldkirchen, Germany) and 3 µL ultrapure nuclease-free water. Each run included a positive control of *V. dahliae* V136I DNA and a no template control (NTC) in which the DNA was replaced with nuclease-free water. Two simultaneous replicates were carried out for each sample. The RT-qPCR programme consisted of an initial step of denaturation for 5 min at 95°C, 40 cycles of 5 s at 95°C, 45 s at 63°C and 3 s at 77°C. A melting curve was performed from 65 °C to 95°C with a heating rate of 0.5 °C/s (Serrano et al. 2023). The initial copy number of each sample was calculated based on the slope and intercept generated by the corresponding standard curve using the RT-qPCR CFX Manager software v.3.1 (Bio-Rad). Results were expressed as Log10 copies to normalize the data.

4.4. Statistical treatment

GraphPad prism 8.0 software (GraphPad Software Inc., San Diego, California) was used for statistical analysis. Shapiro–Wilk normality tests were used to determine normal distribution of data subjected to ANOVA. A one-way ANOVA test supplemented with Tukey's post hoc was used to compare leaves weight and roots length. A two-way ANOVA test supplemented with Tukey's post-hoc test was used for the evaluation of statistically significant differences in the MDA and Fe^{2+} levels obtained from the climatic chamber trial, as well as in the number and length of shoots. This analysis considered two independent variables: the application method and the number of treatment applications. Similarly, for the statistical analysis of results from the olive orchard trial, which included MDA, Fe^{2+} and Proline content, fruit parameters and RT-qPCR results, a two-way ANOVA test supplemented with Tukey's post-hoc test was also employed,

considering treatment and orchard location as independent variables. Differences were considered statistically significant when $p < 0.05$.

5. Conclusions

This study evaluated the biostimulant activity and protection against Verticillium wilt provided by an onion extract in olive crops, under both controlled conditions and field trials. In climatic chamber experiments, the treatment led to a significant reduction in malondialdehyde (MDA) levels. Additionally, the treatment positively affected root development. Field trials with 4-year-old olive trees under abiotic stress demonstrated that the onion extract promoted a significant increase in budbreak and shoot length, particularly in plants treated by irrigation. Finally, in two commercial orchards naturally infected with *V. dahliae* (Linares and Santaella), the onion extract formulation reduced MDA content in both locations, indicating a potential protection against pathogen-induced oxidative stress. Although the infection rate did not differ significantly between treated and control groups, the log10 gene copies of *V. dahliae* were significantly lower in treated trees in both locations, suggesting a reduction in pathogen density. Furthermore, fruit analysis revealed an increase in fat content in the fruits from treated trees.

For field practitioners, these findings suggest several practical applications. The onion extract should be applied to olive trees through irrigation in consecutive applications to maximize its effectiveness. This method has proven to be more effective in promoting olive tree development and overall health. By incorporating onion extract into integrated pest management strategies, oxidative stress and pathogen density, particularly in orchards affected by *V. dahliae*, could be reduced, while simultaneously increasing the fat content in the fruit.

In conclusion, onion extract standardized in organosulfur compounds demonstrate promising potential as both a biostimulant and a disease management tool for olive crops. Its ability to reduce oxidative stress and improve budbreak and root growth suggests that this extract could help mitigate stress, enhance recovery in affected plants, and control *V. dahliae* infection. These findings highlight the value of onion extract as a sustainable alternative to conventional chemical products and pave the way for its use in producing more resilient crops. Future research should delve deeper into the underlying mechanisms driving these effects and work towards optimizing application methods to enhance benefits across diverse agricultural contexts. Additionally, it is crucial to conduct a comprehensive physicochemical characterization of the resulting olive oil to fully understand the impact of these treatments on oil quality.

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2.3. Assessing the Safety of Two Organosulfur Compounds Derived from Onion in Western Honey bee (*Apis mellifera*)

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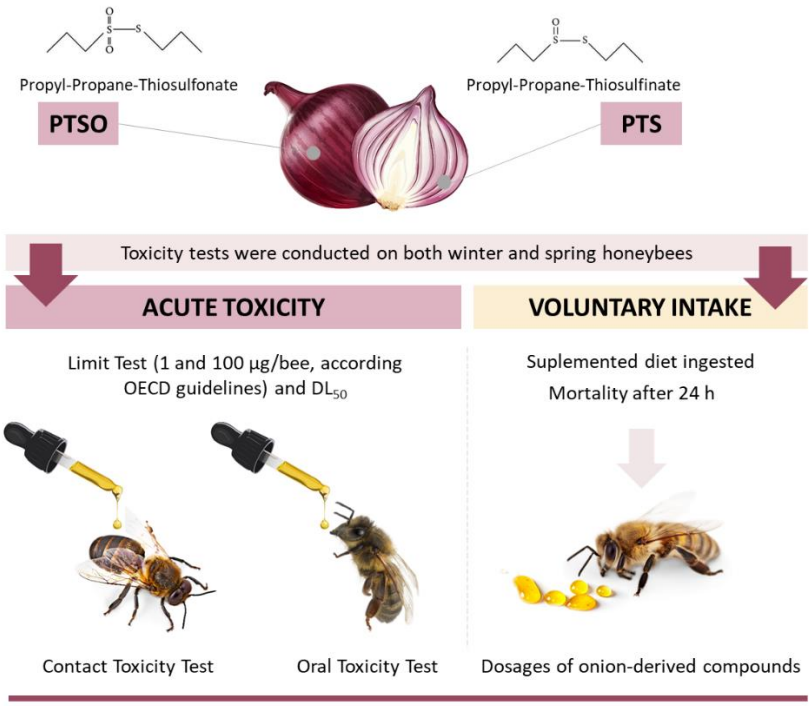
Abstract: The western honey bee is the main species responsible for wild and cultivated plants pollination worldwide. Honey bee population decline seriously threatens biodiversity conservation and agricultural production. Even though many factors have contributed to the weakening of bee numbers and colony losses, including the loss of natural habitat and landscape simplification, the use of synthetic agrochemicals play a major role. This work aims to analyse the toxicity of propyl propane thiosulfinate (PTS) and propyl propane thiosulfonate (PTSO), two organosulfur compounds derived from *Allium cepa* with antimicrobial and pesticide activity that support their use for the development of sustainable biopesticides, in winter and spring honey bees. Acute toxicity assays were performed by contact and oral exposure, using the insecticide dimethoate as toxic standard. Additionally, a study was carried out in order to assess the dose at which honey bees do not voluntarily ingest each compound. In both winter and spring honey bees, the LD_{50} of PTS and PTSO are higher than the maximum field application dose (1 μg/bee). Winter honey bees were found to be more resistant to PTS and PTSO than spring honey bees. Assessment of the effects of exposure to both compounds combined showed no synergism. Lastly, neither winter honey bees nor spring honey bees voluntarily ingested PTS or PTSO at higher concentrations at which mortality was recorded. Therefore, both organosulfur compounds offer a sustainable alternative for pest control while potentially safeguarding pollinators.

Keywords: *Apis mellifera*; propyl-propane-thiosulfinate; propyl-propane-thiosulfonate; *Allium cepa*.



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1. Introduction

Pollinators are directly related to the reproduction of plant species and the conservation of ecosystems (Carvalheiro et al. 2021). Pollination, that was identify by the European Food Safety Authority (EFSA) as relevant ecosystem service (EFSA 2012), takes part in the preservation of wild plant biodiversity (Papa et al. 2022). On the other hand, it enhances the quality and yield of global crops production (Jones and Rader 2022). According to the Food and Agriculture Organisation of the United Nations (FAO), pollinators contribute to 35% of crop production worldwide and influence the yield of 87 of 117 main edible crops (Gallai and Vaissière 2009). The European Commission stated that, in the European Union alone, almost 5 billion euros of annual agricultural production is attributed exclusively to insect pollinators (European Commission 2018).

The western honey bee (*Apis mellifera* Linnaeus; Hymenoptera: Apidae) is, among a diverse group of vertebrates and invertebrates, the most important pollinator from both an ecological and economic point of view (P. Liu et al. 2022). Honey bees play a key role in agricultural ecosystems since, as estimated by FAO, they are primary responsible for the pollination of 71 of the 100 species that provide 90% of food worldwide (Y. Liu et al. 2020; Paudel et al. 2015). There is evidence of a significant decline in bees and other insect pollinators population (Brodschneider et al. 2016; Tlak Gajger et al. 2020; Gray et al. 2023), which leads to the disappearance of species that rely on them directly or indirectly and threatens food security (Vaidya et al. 2023). Even though the decline is caused by a wide range of reasons, the use of plant protection products (PPPs), pathogens, climate change, loss of natural habitat heterogeneity and simplification of agricultural landscapes are among the main threats (Halvorson et al. 2021; Fernandes et al. 2022).

Whereas PPPs protect crops from microbial pathogens and other harmful organisms, such as insects, and contribute to increase food production, their use can severely damage non-target species, including honey bees, due to their bioaccumulation and non-selective action (Murawska, Migdał, and Roman 2021). Honey bees come into contact with agrochemical through different exposure routes during foraging flights, including air particles, soil, water and plant matrices, such as nectar, pollen and propolis (Simon-Delso et al. 2018; Zioga et al. 2020). Direct contact or ingestion of PPPs can cause the death of honey bees as well as sublethal damage. Exposure to sublethal doses allows foragers honey bees take water, nectar, pollen and resins, where PPPs residues can accumulate, into the hive, thus exposing the entire colony (Stanley et al. 2016). Over 150 PPPs residues have been found in hive products, such as wax, pollen and bee bread (Cascajosa-Lira et al. 2023; Murcia-Morales et al. 2022).

During recent years, scientific organizations and government agencies have developed strategies to evaluate the ecological risks that PPPs pose for pollinators with the aim of reversing the decline in their population and diversity (Como et al. 2017). The European Commission presented in 2018 the EU Pollinators Initiative, the first EU framework to address and raise awareness of this issue (European Commission 2018).

This initiative, which established short and medium-term actions as well as long-term objectives, has been revised and updated. The revision includes, among other measures, the reduction of the use of more hazardous chemical pesticides by 50% by 2030 and their progressive replacement by more sustainable methods for pest management that help preserve honey bees (European Commission 2023). Over the last decades, methods of risk assessment specific to honey bees were developed by the Organization for Economic Cooperation and Development (OECD) and the European and Mediterranean Plant Protection Organization (EPPO), among others (Barascou et al. 2021). These guidelines for toxicity testing of PPPs on *Apis* species have been validated by EFSA according to regulation (EU) 1107/2009 and accepted internationally (EFSA et al. 2023). As a result of the standardization of toxicity evaluation methods, extensive empirical data regarding the potential impact of chemical compounds that can be found in products available on the market has been obtained and compiled in the literature as well as in databases (H. Thompson 2016; Cullen et al. 2019). Previous studies suggest that plant extracts and their active compounds are safer for non-target species, due to their lower persistence in the environment. This makes them potential candidates to substitute synthetic agrochemicals (Zhu et al. 2019; Cunha Pereira et al. 2020).

In this context, organosulfur compounds propyl-propane thiosulfinate (PTS) and propyl-propane thiosulfonate (PTSO) derived from onion (*Allium cepa*) have garnered increasing attention due to their functional properties, including flavouring, antimicrobial, antiparasitic, antitumoral and immunomodulatory activities, among others (Aguinaga-Casañas et al. 2022; Vezza et al. 2019; 2021; Guillamón et al. 2023; García-García et al. 2023; Sorlozano-Puerto et al. 2021). Both are volatile compounds that are formed through dismutation or disproportionation reactions from propiin (Guillamón et al. 2021), which is a natural constituent of onion fresh bulb tissue (Rose et al. 2005).

This has led to an expansion of their potential applications within the agri-food sector, where their diverse properties and natural origin could make them valuable as active agents for pest management or as promising ingredients for both human and animal nutrition (Cabello-Gómez et al. 2022; Cascajosa-Lira et al. 2021). The study of the possible applications of these organosulfur compounds in agriculture has recently started. Previous research on the inhibitory effect of PTS and PTSO on growth and mycotoxin production of *Fusarium* species *in vitro* and *in situ* on artificially inoculated wheat, oats and maize demonstrated that these compounds significantly reduced both parameters *in vitro*. Efficacy *in situ* depended on the specific *Fusarium* species-toxin pathosystem and water availability (Mylona et al. 2019). PTS and PTSO have also been shown to *have in vitro* and *in vivo* antifungal activity against *Verticillium dahliae*, the causal agent of Verticillium wilt, which is consider the most devastating soilborne fungal disease affecting olive trees (*Olea europaea* L. var. europaea). In addition, the same study showed that both compounds reduce disease related parameters and contribute to the control of the phytopathogen (Falcón-Piñeiro et al. 2021). A recent study has demonstrated the antimicrobial activity of PTS and PTSO against a wide range of

phytopathogenic bacteria and fungi *in vitro*. This study has also showed their repellent and biocidal properties against the cotton aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae), a major agricultural pest that acts as a vector for many serious plant viruses, such as the cucumber mosaic virus (Im et al. 2022). These results suggest that the properties of both organosulfur compounds are of great interest for the agricultural sector, which has recently experienced an increase in the demand for new environmentally-friendly non-synthetic PPPs (Tudi et al. 2021).

With the aim of further exploring the agri-food applications of these compounds, numerous toxicity assays, both in cell lines and in studies on murine models of chronic and acute toxicity, have been conducted, demonstrating their safe use (Cascajosa-Lira et al. 2023; 2020; Cascajosa-Lira, Andreo-Martínez, et al. 2022; Cascajosa-Lira, Pichardo, et al. 2022; Pilar Mellado-García et al. 2016)(Pilar Mellado-García et al. 2016; Cascajosa-Lira et al. 2023; Cascajosa-Lira, Pichardo, et al. 2022; Cascajosa-Lira, Andreo-Martínez, et al. 2022; Cascajosa-Lira et al. 2020). While a limited number of studies assessing the toxicity of garlic extracts in honey bees have been found (Xavier et al. 2015; Palareti et al. 2016), no data was found in the literature regarding the toxic potential of PTS and PTSO on pollinating organisms.

Taking these facts into account, the aim of the present study was to investigate for the first time the in vivo toxicity of PTS and PTSO in the western honey bee. To achieve this, toxicity tests were conducted on both winter honey bees and spring honey bees to assess whether their physical differences result in varying resistance to these compounds. Additionally, the voluntary intake of PTS and PTSO by honey bees was evaluated.

2. Results

2.1. Acute contact toxicity

The contact toxicity of the organosulfur compounds PTS and PTSO on winter and spring honey bees was evaluated through limit test. Results are shown in Figures 1 and 2. The mortality in the control groups, that include solvents (acetone and polysorbate) and water, was 0% in winter and spring trials. Moreover, the mortality of the toxic standard (dimethoate) was 100% in both cases. Regarding the tested products, no mortality was observed in either autumn or spring honey bees when applying 1 μg/bee of PTS or PTSO. It represents the maximum concentration at which these compounds would be applied under real field conditions. Winter honey bees were found to be resistant to PTS and PTSO at higher concentrations when applied topically. No significant differences were observed between the mortality percentage of winter honey bees treated with 100 μg of PTS or PTSO (6.67 and 4.44, respectively) compared to the control ($p > 0.05$). The average mortality of winter honey bees treated with 50 μg of each compound (i.e., with 100 μg of active ingredients) was only 4.44%. This was indicative of the absence of synergy between both *Allium* compounds. Contact with 200 μg a.i./bee (100 μg of PTS and 100 μg of PTSO) doubled the mortality rate of the group treated with 50 μg/bee of each compound, which shows the dose dependency of the contact toxicity

of PTS and PTSO. On the other hand, PTS and PTSO significatively affected spring honey bees' survival when applied topically at 100 µg/bee ($p < 0.05$), reaching 100 and 95.55 % mortality after 24 h of exposure respectively. Although this dosage represents a 100-fold increase over the actual dosage of the compounds under real field conditions, given their potential contact toxicity in spring honey bees, a test was conducted to determine the LD_{50}. After 24 h, the LD_{50} values for PTS and PTSO were 29.1 ± 0.91 and 33.3 ± 0.90 µg/bee respectively, with 95% confident limit. These values, presented in Table 1, are above the actual concentrations of use, estimated as a maximum of 1000 µg/mL. The mortality rate did not increase after 48 h of exposure in any contact toxicity test performed. Furthermore, sublethal damage was not observed in living individuals at the different study times or concentrations tested.

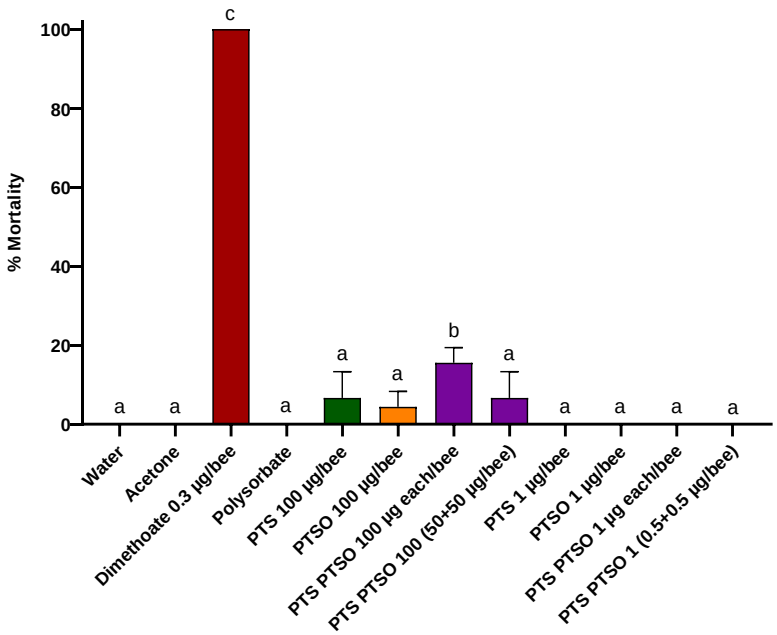

Figure 1. Determination of acute contact toxicity in winter honey bees by Limit Test. Mortality results are expressed as percentage. Bars with different letters indicate significant differences according to Tukey test ($p < 0.05$).

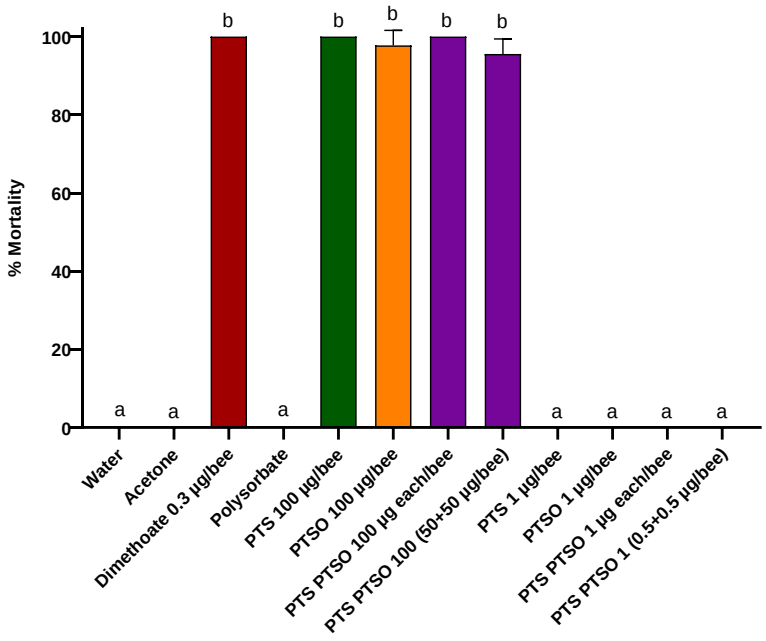

Figure 2. Determination of acute contact toxicity in spring honey bees by Limit Test. Mortality results are expressed as percentage. Bars with different letters indicate significant differences according to Tukey test ($p < 0.05$).

2.2. Acute oral toxicity

Figures 3 and 4 summarise the effects of exposure to oral doses of PTS and PTSO separately and combined. The average mortality for water, acetone and polysorbate controls did not exceed 10% at the end of the test, whereas oral administration of dimethoate caused 100% honey bees mortality after 24h of exposure. Neither PTS nor PTSO resulted in any toxic effect in winter and spring honey bees when they were tested at 1 µg/bee. Furthermore, no increase in toxicity was observed when the combinations of 0.5 µg PTS and 0.5 µg PTSO, and 1 µg PTS and 1 µg PTSO were ingested. As in the acute contact toxicity test, differences were observed in the tolerance of winter and spring honey bees to oral exposure to PTS and PTSO at 100 µg/bee (100 times the normal usage dose). No significant differences were observed in the 100 µg/bee PTS, 100 µg/bee PTSO and 50 µg PTS + 50 µg PTSO treatment groups compared to control groups ($p > 0.05$). The oral exposure to 100 µg PTS + 100 µg PTSO caused 8.89% mortality ($p < 0.05$) due to the doubling of the total amount of compounds administered. In contrast, no spring honey bees survived after ingestion of 100 µg PTS, PTSO or their 2 combinations (50 µg PTS + 50 µg PTSO, and 100 µg PTS + 100 µg PTSO). According to the OECD guideline for acute oral toxicity test, a study for the determination of LD_{50} values of PTS and PTSO was conducted. After 24 h, the LD_{50} values, showed in Table 1, were 19.0 ± 0.38 for PTS and 21.8 ± 0.34 µg/bee for PTSO, with 95% confident limit. As with LD_{50} contact, the maximum field application dose (1,000 µg/mL) is well below the oral LD_{50}. Mortality rate after the ingestion of 20 µg of PTS was 53.33%, and in all living honey bees sublethal effects were observed. In the case of PTSO, sublethal effects were observed in the study group exposed to 25 µg, in which a mortality rate of 66.67% was recorded. In both cases,

sublethal damages observed were difficulty flying and atypical muscle movements. Results didn't change after 48 h in any oral toxicity test performed.

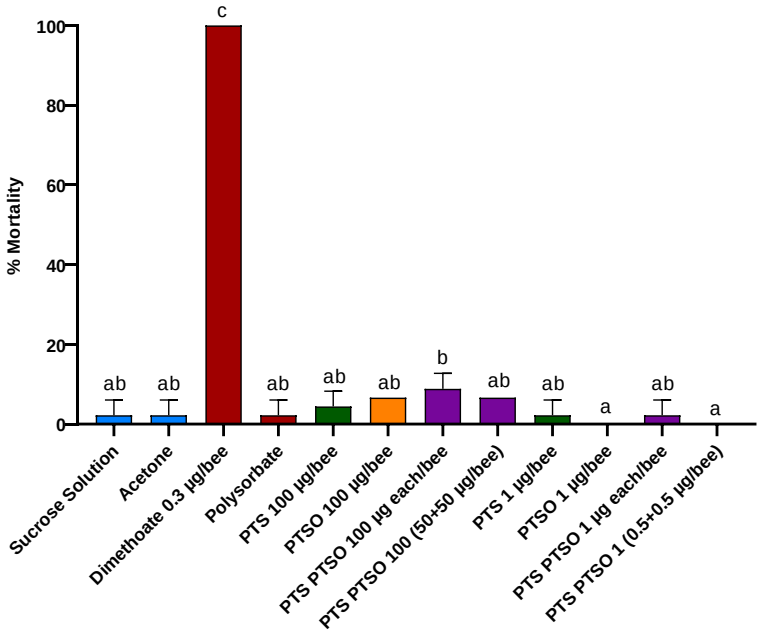

Figure 3. Determination of acute oral toxicity in winter honey bees by Limit Test. Mortality results are expressed as percentage. Bars with different letters indicate significant differences according to Tukey test ($p < 0.05$).

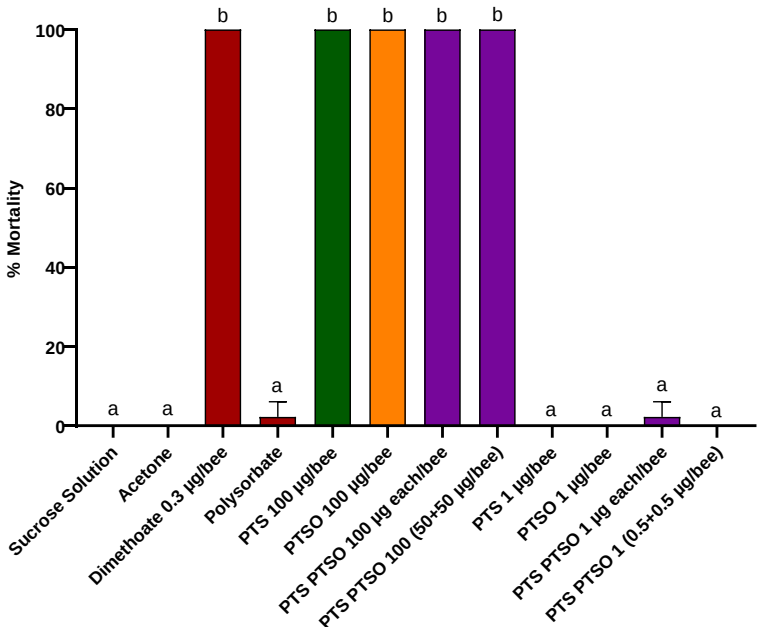

Figure 4. Determination of acute oral toxicity in spring honey bees by Limit Test. Mortality results are expressed as percentage. Bars with different letters indicate significant differences according to Tukey test ($p < 0.05$).

Table 1. LD_{50} contact and oral of PTS and PTSO for adult spring honeybees, expressed in μg of test substance per bee.

	PTS (μg/bee)	PTSO (μg/bee)
LD_{50} contact	29.1 ± 0.91	33.3 ± 0.90
LD_{50} oral	17.2 ± 0.41	19.7 ± 0.35

2.3. Voluntary intake of PTS and PTSO

Figure 5 shows the amount of food ingested by each group of winter honey bees after 6 h, and the mortality observed after 24 h. After 48 h the mortality rate did not change. Each winter honey bee ate an average of 14.66 mg of sucrose solution (negative control) after 6 hours, which represents 97.30% of the content of the feeder (200 μL). The average mortality of the negative control (sucrose solution) did not reach 10%. The mortality of the diluents acetone and polysorbate was less than 10%, which reaffirmed the suitability of using these compounds to dissolve both PTS and PTSO and dimethoate (toxic standard), respectively. Mortality of bees fed on dimethoate was 100%. The mortality of winter honey bees whose diet contained 10 μg a.i./μL showed significant differences with the negative control ($p < 0.05$). However, each individual ingested 0.29 and 1.11 mg of diet supplemented with PTS and PTSO, respectively (1.88% and 7.51%). This suggests that they died of starvation. From the concentration of 4 μg a.i./μL, no significant differences were observed between the mortality of the groups that were fed with supplemented diet and the negative control. Although the intake of the groups fed on supplemented diet with 4 and 2 μg a.i./μL differed significantly from the intake of the negative control ($p < 0.05$), individuals of these 4 study groups ingested a minimum of 50% of the food. There were no significant differences between the negative control and the groups fed on supplemented diet with 1; 0.1; 0.05 and 0.01 μg a.i./μL, which ate more than 13 mg/bee (> 94 % of the content of the feeder).

Voluntary intake and mortality data of spring honey bees are shown in Figure 6. Each spring honey bee ate an average of 10.20 mg sucrose solution after 24 h. Intake and mortality results from bees that were fed with polysorbate and acetone, as well as dimethoate and sucrose solution supplemented with 10 μg a.i./μL, followed the same pattern as winter honey bees' data. Furthermore, in this case a significant mortality was also observed in the groups in which the sucrose solution was supplemented with 4 μg a.i./μL ($p < 0.05$), in which individuals ate 5.20 mg PTS and 7.50 mg PTSO (47.33 and 66.18%). These intake percentages are similar to those of winter honey bees. However, in the case of winter honey bees, no significant differences were observed compared to the mortality of the negative control. This supports the results obtained in acute oral and contact toxicity tests, which indicate that spring honey bees are more sensitive to both active ingredients. Whereas from a concentration of 2 μg a.i./μL there is no mortality, no significant differences were observed in the intake of bees whose diet was supplemented with 1 μg a.i./μL or less compared to the negative control.

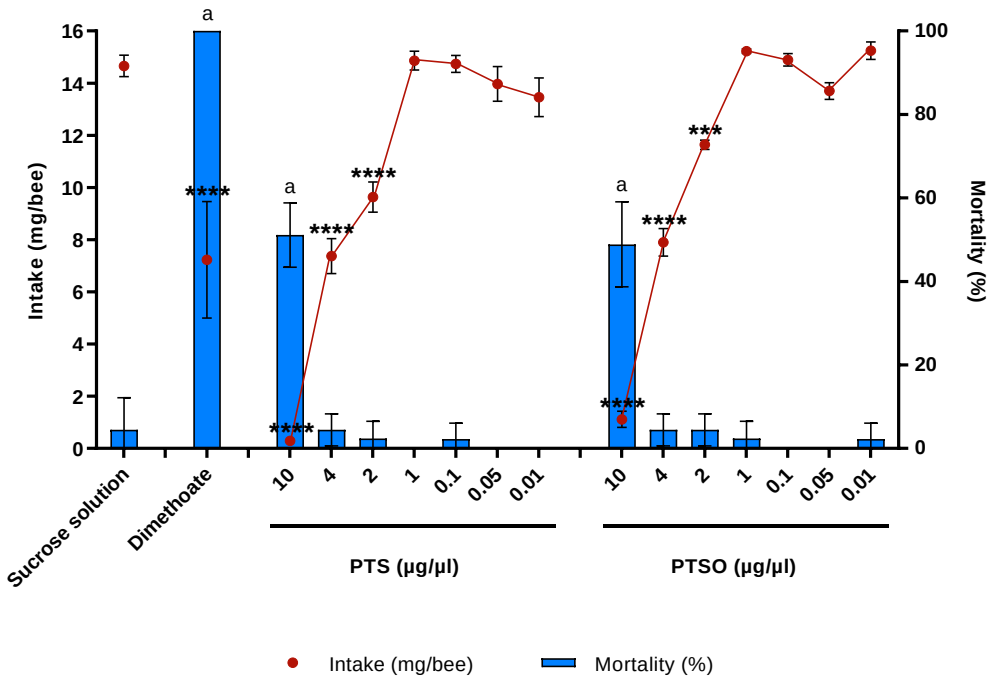

Figure 5. Correlation of the voluntary intake of food, consisting of PTS and PTSO in sucrose solution at different concentrations, and mortality in winter honey bees, in comparison with the negative control (sucrose solution) and using dimethoate as toxicity standard. Intake and mortality results are expressed in mg/bee and as percentage, respectively. Values are means with SD in bars. Bars with asterisks indicate significant differences in intake compared to the negative control according to Dunnett's test using a 95% confidence level (***$p < 0.001$; ****$p < 0.0001$). Bars with letters indicate significant differences in mortality rate compared to the negative control according to Dunnett's test ($p < 0.05$).

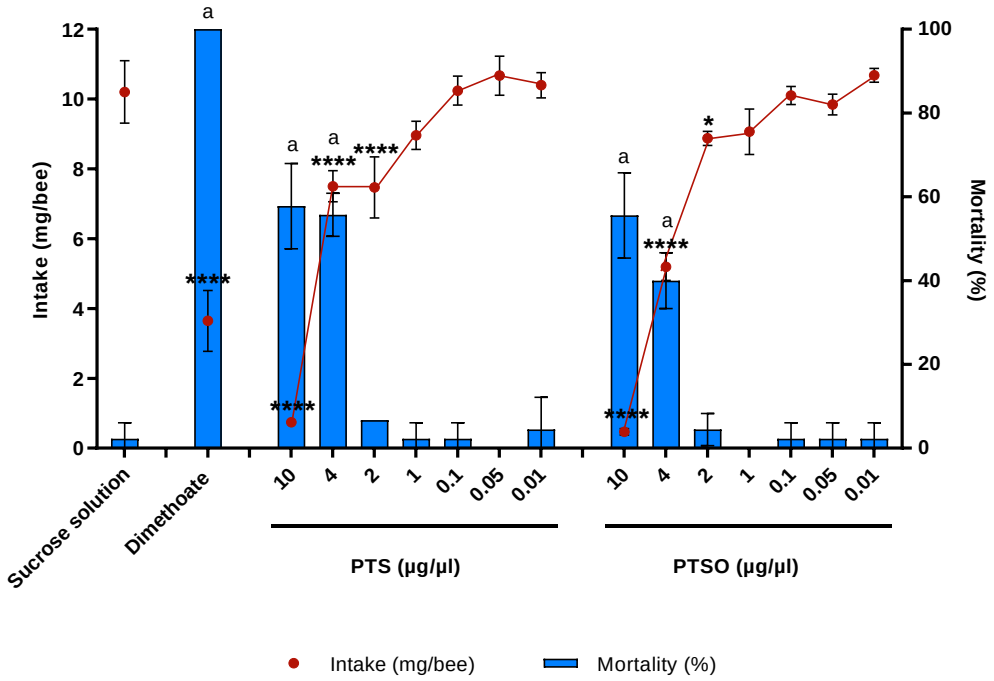

Figure 6. Correlation of the voluntary intake of food, consisting of PTS and PTSO in sucrose solution at different concentrations, and mortality in spring honey bees, in comparison with the

negative control (sucrose solution) and using dimethoate as toxicity standard. Intake and mortality results are expressed in mg/bee and as percentage, respectively. Values are means with SD in bars. Bars with asterisks indicate significant differences in intake compared to the negative control according to Dunnett's test using a 95% confidence level (***$p < 0.001$; ****$p < 0.0001$). Bars with letters indicate significant differences in mortality rate compared to the negative control according to Dunnett's test ($p < 0.05$).

3. Discussion

In recent years, due to the need for agricultural systems to be more sustainable, the use of plant-derived secondary metabolites as an alternative pest control strategy in crops has been promoted (Catani et al. 2023). These plant active compounds are considered environmentally safer than synthetic PPPs because of their lower persistence in the environment (Duke et al. 2010). Previous researches have demonstrated that PTS and PTSO derived from onion reduce the incidence and severity of verticillium wilt caused by the soil-borne fungus *V. dahliae* in olive seedlings (Falcón-Piñeiro et al. 2021), as well as the broad antimicrobial activity of both compounds against a wide range of phytopathogenic bacteria and fungi *in vitro* (Falcón-Piñeiro et al. 2023). This study also demonstrated through HPLC-UV quantification the low persistence of both compounds in soil, that rapidly volatilize after application. Despite generally being considered safer, these agents have sometimes been found to negatively affect non-target organisms, such as pollinators, and arthropods and microorganisms used for biological control (Tome et al. 2015). In a recent review, Giunti et al. (Giunti et al. 2022) emphasized the need to delve deeper into the detrimental effects of botanical control agents, which are typically rich in various active compounds. A previous report demonstrated the toxicity of a neem oil and a garlic extract that are placed on the market as insecticides on both larvae and adult honey bees at the use concentrations recommended by the manufacturer (Xavier et al. 2015). The same study stablished the lethal effect of citronella and eucalyptus commercial oils on adult honey bees. Other studies have reported that crude extracts of garlic and lemon grass (*Cymbopogon citratus*) do not cause toxic effect on adult honey bees at 10000 μg/bee, whereas extracts of Piper species were highly toxic at the same concentration (Palareti et al. 2016). To the best of our knowledge, this is the first report assessing the safety of PTS and PTSO from onion in the western honey bee, covering acute toxicity and repellent effect through voluntary intake, and comparing individuals' response from 2 different seasons.

In this study, we showed that PTS and PTSO do not cause mortality by contact or oral exposure at the maximum field application dose (1000 μg/mL, i.e. 1 μg/bee) in either winter or spring honey bees. It should be noted that, despite 1000 μg/mL being established as the maximum dose for field application, the tests carried out by our team on olive seedlings infected with *V. dahliae* already showed effects on all the parameters evaluated (incidence and severity of the disease, and population of the fungus in soil and vascular tissue) with concentrations of 250 and 500 μg/mL significant differences were observed with respect to the control. However, the response of spring honey bees to the dose established by OECD guidelines to assess the acute oral and contact toxicity

by limit test, 100 μg/bee (100,000 μg/mL), is different from that of winter bees. While none of the compounds resulted in significant toxic effect when they were tested at 100 μg/bee in winter honey bees, contact or ingestion of one of them at that concentration caused 100% spring honey bee mortality after 24 h of exposure. This dose, established to consider whether a product is safe, represents a 100-fold increase over the actual use dose under real field conditions. In both LD_{50} determinations it was observed that PTS is slightly more toxic than PTSO. These results were not as expected, since in previous contact toxicity tests carried out on adult individuals of the cotton aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae) PTSO showed greater biocidal activity than PTS (Falcón-Piñeiro et al. 2023).

The difference in susceptibility between winter and spring honey bees to PTS and PTSO may be related to their physiological differences. Winter honey bees have a considerably longer life span, since they emerge in late August, survive the winter and initiate brood rearing in spring (Dostálková et al. 2021). They have bigger fat body size, therefore they accumulate larger reserves of nutrients (Jabal-Uriel et al. 2022), as well as a higher protein concentration in the haemolymph (Kunc et al. 2019). The protein content of winter and spring honey bees mainly differs in the concentration of vitellogenin, that is synthetised in the fat body (Dostálková et al. 2021). Vitellogenin is highly expressed during fall and winter and is related to the greater longevity of winter honey bees (Ricigliano et al. 2018), it improves tolerance to starvation and protects cells from oxidative damage (Rand et al. 2015; Christen et al. 2019). Another factor that could increase the resistance of winter honey bees to PPPs compared to spring bees is the differential microbiota (Kešnerová et al. 2020). In recent years, researchers have demonstrated a close relationship between honey bees gut microbiota and insecticide resistance (Raymann and Moran 2018; Kwong and Moran 2016). Honey bee gut microbiota has been reported to promote the expression of cytochrome P450 enzymes, that play a key role in the detoxification of pesticides and plant secondary metabolites (Wu et al. 2020). Furthermore, we consider age to be a critical factor when comparing susceptibility of winter and spring honey bees populations to PPPs (Dostálková et al. 2021). Adult winter honey bees, old bees that ensure the survival of the colony until spring, were collected from no-brood hive frames, since after summer queens stop laying eggs (Aamidor et al. 2022). In contrast, in spring it was not possible to collect uniformly aged adult honey bees directly from the hive due to the high rate of egg laying. For spring honey bees, it was necessary to keep brood frames in incubators and collect emerged individuals, that were kept for 2 days in incubator until the start of the trials. Honey bee microbiota is stable after 8 days. Furthermore, it has been demonstrated that the microbiota acquired in laboratory conditions is different from that acquired in the hive (Aguado López et al. 2024). Therefore, the design of research that aim to compare honey bees from different seasons is limited because it was not possible to use adult bees of the same age for both populations.

Pesticide formulations often include more than one active ingredient. There is evidence that the simultaneous use of different active ingredients may have a toxic effect

that is more than additive, i.e., could induce synergistic effects (H. M. Thompson et al. 2014). In this research, the risks associated with the joint use of PTS and PTSO in the formulation of PPPs have been assessed. Our data show that co-exposure to PTS and PTSO did not result in synergistic effects. While exposure to 50 μg PTS + 50 μg PTSO/bee did not cause an increase in toxicity compared to 100 μg/bee of one of the active ingredients, exposure to 100 μg PTS + 100 μg PTSO had an additive effect and doubled the mortality rate. This demonstrates the dose dependency of the mortality outcomes.

Feeder experiments play an important role in revealing the behaviour of honey bees to different substances. Understanding these responses is important to determine the potential risk that such substances pose to honey bees in agricultural systems (Ohlinger et al. 2022). In this study, we observed that both winter and spring honey bees do not voluntarily ingest feed supplemented with PTS or PTSO at 10,000 μg/mL. This suggests that both compounds exert a strong repellent effect at high concentrations, preventing honey bees from ingesting them voluntarily or accidentally. This is an advantage, since at that concentration both PTS and PTSO caused around 50% mortality. The intake of honey bees, both winter and spring, whose diet was supplemented with 1,000 μg/mL or less was nearly 100% and did not significantly differ from the intake of the negative control group, fed with unsupplemented diet. At these concentrations, no mortality or sublethal damage were observed. These data corroborate the results obtained in the acute oral toxicity evaluation using limit test and suggest that, at the maximum field application dose, neither PTS nor PTSO alter the feeding or foraging and recruitment activity of honey bees. The effect of both compounds was quite homogeneous in terms of repellent activity, since no significant differences were observed between the intake of PTS and PTSO by individuals from the same season. In agreement with these results, in a previous study we observed that, at the same concentrations, PTS and PTSO exerted a similar repellent effect on the cotton aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae) (Falcón-Piñeiro et al. 2023). Furthermore, it has been reported that onion and garlic volatile compounds alter field behaviour of *A. gossypii* but do not affect its main predator, ladybirds, from the family of beetles *Coccinellidae* (Yang et al. 2023). The repellent properties of onion-derived compounds, as well as from other species of the genus *Allium*, such as garlic (*Allium sativum*) or leek *(Allium porrum*), have been widely described against other pests including the green aphid *Myzus persicae* Sulzer (Hemiptera: Aphididae), the wheat aphid *Sitobion avenae* Fabricius (Hemiptera: Aphididae), the mealworm beetle *Tenebrio molitor* Linnaeus (Coleoptera: Tenebrionidae) and the black fungus gnat *Lycoriella ingénue* Dufour (Diptera: Sciaridae) (Baudry et al. 2021; Plata-Rueda et al. 2017). Nevertheless, there is a scarcity of studies reporting this repellent effect in pollinators. In our research, we provide specific insights into two active compounds from *Allium*, underscoring the need for further research on their long-term impacts.

Previous studies have suggested that plant derived compounds may be effective in controlling pathogens/parasites threatening honey bees (Begna et al. 2023). Growth inhibition of bee pathogens *Paenibacillus larvae* and *Ascosphaera apis* by allicin derived

from garlic has been assessed *in vitro* (Aronstein and Hayes 2015). The antiparasitic activity of garlic and onion extracts has been demonstrated in bee hives with honey bee colonies infested with the ectoparasitic mite *Varroa destructor* (Mazeed and El-Solimany 2020). These assays did not report the effect of the compound on honey bees. In our research, we establish the doses at which PTS and PTSO derived from onion did not result in toxic effects or alteration of the feeding of winter and spring honey bees. Taking this into account, in future trials the effect of PTS and PTSO on bee pathogens and parasites could be evaluated at safe concentrations according to what was established in this study.

Finally, numerous studies have confirmed PTS and PTSO as effective flavour enhancers for food and feed, leading to comprehensive toxicological safety assessments. These evaluations consistently show that neither compound poses significant toxic risks in cellular or animal models, including no evidence of oral, subchronic or mutagenic toxicity (P. Mellado-García et al. 2015; Cascajosa-Lira et al. 2020; Cascajosa-Lira, Pichardo, et al. 2022; Cascajosa-Lira et al. 2023). This extensive research supports the safe inclusion of PTS and PTSO in the agri-food industry for human and animal consumption, including honey bees, enhancing their status as botanical additives.

In conclusion, our study on the safety of two organosulfur compounds derived from onion in the Western honey bee (*A. mellifera*) provides promising evidence regarding their potential use in agriculture with minimal risk to these pollinators. While our findings suggest that spring honey bees exhibit greater sensitivity to these compounds, winter honey bees demonstrated notable resilience, indicating a degree of safety across different seasonal populations. However, it is imperative to recognize that this research is specific to *A. mellifera* and further studies are needed to assess the toxicity and impact of these compounds on other pollinators, such as bumblebees. The differential sensitivity observed between seasonal populations underscores the importance of considering environmental and biological variables in the evaluation of agrochemical safety. Despite the need for further research to deepen our understanding of the impacts on other pollinator species, we can conclude that the organosulfur compounds derived from onion are potentially safe for pollinators. Therefore, their use in agriculture could represent a sustainable solution that is compatible with the preservation of pollinator population. This study not only contributes to the ongoing efforts to ensure the safety of natural agrochemicals for pollinators but also highlights the potential for developing environmentally friendly agricultural practices that support pollinator health and biodiversity.

4. Materials and Methods

4.1. Compounds and Reagents

PTS and PTSO from *Allium cepa* at 20% purity, supported in polysorbate 80, were provided by DOMCA S.A.U. Polysorbate 80 (CAS: 9005-65-6), acetone ≥ 97% (CAS: 67-64-1) and dimethoate (CAS: 60-51-5) were purchased from Sigma-Aldrich Química S.L. (Madrid, Spain).

4.2. Honey bees

The experiments presented here were carried out in February and June 2023. Adult honey bees were used in all experiments. They were obtained from an experimental apiary of the Centro de Investigación Apícola y Agroambiental (CIAPA) in Marchamalo (Castilla-La Mancha, Spain). Considering the annual life cycle of the honey bee colony, the method by which the adult honey bees were picked depended on the season. Since egg laying slows down considerably during winter, winter honey bees, born in the fall, were picked at 9 a.m. during the month of February from no-brood frames by brushing them into cardboard boxes and taken immediately to the laboratory (R. Martín-Hernández et al. 2007) where they were caged (see below). Spring honey bees were collected during the month of June from frames of capped brood, that were kept in an incubator at 35 ± 1°C. After 24 h, emergent adult honey bees were removed from the brood combs and placed in cylindrical steel mesh cages (175 mm long, 45 mm in diameter), that were kept in an incubator for 2 days at 25 ± 1°C (Urbieta-Magro et al. 2019). These cages had a 9.5 mm diameter hole in one of the bases that allowed the introduction of a conical tube that contained the food. During this time, bees were fed *ad libitum* with 50% (w/v) sucrose solution in water (Raquel Martín-Hernández et al. 2011). In order to make easier the honey bee handling, to perform the different experiments, winter and spring honey bees were anaesthetised with carbon dioxide. They were randomly distributed into groups of 15 and confined to steel mesh cages previously described. Only healthy bees not exerting any clinical sign of disease were used for the test.

4.3. Acute Contact Toxicity Test

Acute contact toxicity of PTS and PTSO was assessed by limit test according to OECD Guideline for the Testing of Chemicals in Honeybees (OECD 1998b). Limit test was performed using dilutions of each active ingredient (a.i.) at 100 μg/μL (as established by the guideline) and 1 μg/μL (maximum concentration at which these compounds are applied in the field) in water. Additionally, the synergy between PTS and PTSO was evaluated. Blend solutions were prepared with PTS and PTSO at 50 μg/μL and 0.5 μg/μL each, so that 2 solutions with a final concentration of 100 μg a.i./μl and 1 μg a.i./μL were obtained. The toxicity of 2 blends of PTS and PTSO at 100 and 1 μg a.i./μL each was also studied. Dimethoate at 0.3 μg/μL in acetone was used as toxic standard. The toxicity of acetone and polysorbate, which were used as diluents for dimethoate and PTS and PTSO, respectively, was also evaluated to verify that these did not have a negative effect on honey bees by themselves. Doubled-distilled water was used as control. Dilutions used in the limit test are shown in Table 2. For each test dose and control, 3 replicates cages containing 15 bees each were included. A volume of 1 μL of the corresponding dilution was applied to the thorax of anaesthetized bees using a micropipette. After application, bees were placed in the corresponding cage and held in an incubator (Memmert® Mod. IPP500, Schwabach, Germany) in the dark at 25 ± 1°C. They were fed with sucrose solution *ad libitum* during test time. Mortality was recorded

after 24 h and 48 h. In cases where mortality was observed, a full study was carried out to calculate the median lethal dose (LD50).

Table 2. Concentrations of test substances evaluated, and controls used in the limit test for the determination of acute contact toxicity and acute oral toxicity.

Test substance	Concentration (μg/μL)	Active ingredient/bee (μg)
PTS	100	100
PTS	1	1
PTSO	100	100
PTSO	1	1
PTS + PTSO	50 + 50	100 (50 each)
PTS + PTSO	0.5 + 0.5	1 (0.5 each)
PTS + PTSO	100 + 100	200 (100 each)
PTS + PTSO	1 + 1	2 (1 each)
Dimethoate	0.3	0.3
Acetone	–	–
Polysorbate	–	–
Water [1]/ Sucrose solution [2]	–	–

[1] Control for acute contact toxicity; [2] Control for acute oral toxicity

For the determination of LD50, bees were exposed to a range of doses of the test substances dissolved in water. To ensure that the LD50 range was covered, in a first trial the toxicity of PTS and PTSO was evaluated at the following concentrations: 100; 90; 80; 70; 60; 50; 40; 30; 20; 10 and 1 μg/μL. Based on the results obtained, test doses were prepared from 80 μg/μl with a factor of 1.5: 53.3; 35.5; 23.7; 15.8 and 10.5 μg/μL. Bees were treated by topical application and mortality was recorded following the same procedure. Same controls and number of replicates were included.

Live honey bees were observed for sublethal damage. Specifically, difficulty in flying, atypical muscle movements, and drowsiness were assessed.

Tests were considered invalid if the average mortality for the total number of bees in the different control groups exceeded 10% as recommended in the OECD 1998 guideline.

4.4. Acute Oral Toxicity Test

Acute oral toxicity of PTS and PTSO was assessed by limit test according to OECD Guideline for the Testing of Chemicals in Honeybees (OECD 1998a), with some modifications. Just as for the determination of acute contact toxicity, a limit test was carried out in which the same concentrations of active ingredients, previously described in Table 2, were evaluated. In this case, dilutions were prepared in sucrose solution. Same toxic standard was used, and acetone and polysorbate, used as solvents for dimethoate and PTS and PTSO, were also included in the test. Sucrose solution was used as control. 3 cages containing 15 bees each were used per test dose/control. Bees were deprived of food for 2 h before treatment so that their intestinal contents at the beginning of the test

were similar. They were disoriented by applying carbon dioxide so that they were not anesthetized. Next, a volume of 1 µL of the corresponding dilution was provided to each bee directly in the mouth using a micropipette. It was verified that the bees appropriately ingested the provided volume. After given test doses, bees were given sucrose solution *ad libitum* as food during test time. Mortality was recorded after 24 h and 48 h. In cases where mortality was observed, a full study was carried out to calculate the LD_{50}.

LD50 was assessed by oral exposure of bees to a wide range of doses of PTS and PTSO dissolved in sucrose solution: 100; 80; 60; 40; 20; 10 and 1 µg/µL. This range-finding test allowed us to establish appropriate concentrations for the assay. In a second trial, the mortality of bees exposed to PTS and PTSO at 30; 25; 20; 15; 10; 5 and 1 µg/µL was determined. Same procedure, replicates and controls were used.

Live honey bees were observed for sublethal damage. Difficulty in flying, atypical muscle movements, and drowsiness were assessed.

Tests were considered invalid if the average mortality for the total number of bees in the different control groups exceeded 10%.

4.5. Determination of voluntary intake

To determine if the honey bees feed willingly on the products tested, the voluntary intake of different concentrations of PTS and PTSO by bees was studied according to OECD Guideline for the Testing of Chemicals in Honeybees (OECD 1998a). To that end, 2 h starved bees were exposed to the following concentration of each compound prepared in sucrose solution: 10; 4; 2; 1; 0.1; 0.05 and 0.01 µg/µL. Dimethoate at 0.3 µg/µL in acetone was used as toxic standard, and sucrose solution, acetone and polysorbate were used as controls. Each group was made up of 3 cages with 15 bees each. The weight of the feeders was recorded before and after depositing 200 µL of the corresponding dilution. The feeders were then attached to the bases of the cages. After 6 hours in the case of winter bees and 24 hours in the case of spring bees, the feeders were removed from the cages and weighed in order to assess the amount of treated diet consumed (mg food/bee). Afterwards, the tested products were removed and fresh sucrose solution was provided *ad libitum* and mortality was recorded 24 h and 48 h after the feeders containing the treatments were removed.

The exposure time to the treated diet was adapted to the needs of the winter and spring honey bees. In a preliminary test it was found that winter bees, collected directly from the hive, quickly ingested the sucrose solution. However, spring honey bees, which emerged from frames kept in incubators, needed 24 h to ingest the same amount of food.

4.6. Statistical treatment

GraphPad prism 8.0 software (GraphPad Software Inc., San Diego, California) was used for statistical analysis. A one-way ANOVA test supplemented with Tukey's post hoc was used for the evaluation of statistically significant differences between mortality percentages observed in Limits Tests. The LD50 values were obtained by probit

analysis, that calculates natural mortality using the Abbott formula with a confidence level of 95% (Finney 1952). A one-way ANOVA test supplemented with Dunnett's post hoc was used to compare the amount of treated diet voluntarily ingested, as well as the recorded mortality, with the data of the control group. Differences were considered statistically significant when $p < 0.05$.

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CAPÍTULO 3. CARACTERIZACIÓN DE CEPAS DE CONTROL BIOLÓGICO CON POTENCIAL APLICACIÓN EN OLIVO

3.1. *Bacillus altitudinis* GG-22: A Novel Plant Growth-Promoting Bacterium with Beneficial Agronomic Properties

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Abstract: *Bacillus altitudinis* GG-22, isolated from the phyllosphere of agricultural crops, has been identified as a promising biocontrol agent and plant growth-promoting bacterium with substantial potential in sustainable agriculture. In this study, whole-genome sequencing using Illumina technology, combined with ANI analysis, confirmed the strain's classification as *B. altitudinis*. The genome revealed a rich set of genes involved in biocontrol mechanisms, including siderophore biosyn-thesis (schizokinen, bacillibactin) and lipopeptides (fengycins, lichenysin). In vitro antagonism assays demonstrated significant inhibitory effects against phytopathogenic fungi and oomycetes, such as *Verticillium dahliae* and *Pythium* sp., though GG-22 showed limited efficacy against bacterial pathogens, including *Xylella fastidiosa*. Transcriptomic profiling of olive trees treated with GG-22 indicated early activation of auxin transport and systemic acquired resistance (SAR) pathways, alongside substantial downregulation of cell wall remodelling genes. These findings suggest that GG-22 not only primes plant defence responses but also modulates hormonal pathways critical for growth and stress resilience. Future research should prioritize optimizing application strategies and exploring synergies with other microbial agents to fully harness the biocontrol and growth-promoting potential of GG-22. This strain holds promise for sustainable agricultural practices, particularly in managing fungal diseases and improving plant performance under stress conditions.

Keywords: *Bacillus altitudinis*, plant-growth-promoting bacteria, biocontrol, olive tree, sustainable agriculture.

In progress.

1. Introduction

The olive tree dominates the landscape of the Mediterranean basin, where it has a great economic, environmental and social impact (Villalobos et al. 2023; Bizos et al. 2020). It is a fundamental pillar of the agri-food system of the southern Mediterranean countries, which encompass 95% of the global olive grove production (Manetsberger et al. 2023; Caselli and Petacchi 2021). The impact of climate change in the Mediterranean basin, highly susceptible to heat waves and drought, poses substantial threats to olive trees (Cramer et al. 2018; Caselli and Petacchi 2021). Moreover, the climate emergency scenario has been related to a higher incidence of endemic and emerging microbial diseases (Shaw and Osborne 2011; Eastburn, McElrone, and Bilgin 2011), since it alters the biology of the pathogen and may expand its geographic area and host range. Furthermore, climate change influences the development and metabolism of the host plant, thereby affecting disease development (Fraga et al. 2021; Sbeiti et al. 2023). Among the diseases that currently pose a greater threat to olive crops are those caused by the bacterium *Xylella fastidiosa* and the filamentous fungus *Verticillium dahliae*. *X. fastidiosa*, a xylem limited bacterium, is a re-emergent pathogen that has been associated to olive quick decline syndrome (OQDS) since it was first detected in Italy in 2013 (Sicard et al. 2018). Infections by this phytopathogen in olive trees have caused large economic losses and severe damage to olive-growing regions in the Mediterranean, particularly in the outbreak area (Sicard et al. 2018), mainly due to the efficient transmission by the vector *Philaenus spumarius* L. (Hemiptera: Aphrophoridae), a sap-sucking hopper insect (Bosso et al. 2016; Trkulja et al. 2022). Similarly, *V. dahliae* is the most devastating soil-borne fungal pathogen threatening olive trees (Falcón-Piñeiro et al. 2021). This endemic fungus causes Verticillium wilt and thrives notably in southern Spain, the world's leading grower of this crop, where it is responsible for yield losses and tree mortality (Requena-Mullor et al. 2020). Other soil-borne pathogenic filamentous microorganisms that cause wilt disease in olive trees are *Fusarium oxysporum* and *Pythium* sp. (Bizos et al. 2020). Finally, another endemic pathogen that causes severe economic damage is *Pseudomonas savastanoi* pv. *savastanoi*, responsible for olive knot disease (Turco et al. 2022). This disease, although it does not cause a high mortality rate, is associated with a progressive weakening and significant reduction in olive yield (Košcak et al. 2023).

Beyond the direct threats posed by these pathogens, the management of olive diseases is further complicated by growing restrictions on the use of agrochemicals. Increasing concerns about the environmental and health impacts of chemically-based fertilizers and fungicides have significantly limited the available options for farmers. Therefore, it is crucial to develop new strategies that increase the resilience of the olive trees against the threat posed by climate change and phytopathogenic microorganisms (Slama et al. 2019). In this context, research on beneficial plant-associated microorganisms is receiving increasing attention as a natural defence against biotic and abiotic stress (Anguita-Maeso et al. 2022), as they have a beneficial effect on plant physiology and contribute to their adaptation to the environment (Bonatelli et al. 2021).

The genus *Bacillus* comprises a large group of highly ubiquitous, endospore-forming bacteria that can colonize diverse ecological niches, such as plants phyllosphere, rhizosphere and soil (Fira et al. 2018; H. B. Guo et al. 2020). Numerous *Bacillus* species have been isolated and characterized for their beneficial effects on plants (Balderas-Ruíz et al. 2020). They have been reported to stimulate plant growth and exert biological control over phytopathogenic microorganisms through different mechanisms (Ahmed et al. 2021). *Bacillus* spp. possess several plant growth-promoting attributes, including nitrogen fixation, phosphorus solubilization and siderophore production, which facilitate iron mobility by its solubilization from minerals and organic compounds (Sawant, Song, and Seo 2022; Tsotetsi et al. 2022). Additionally, *Bacillus* spp. produce phytohormones, such as indole acetic acid, abscisic acid, gibberellins and a vast range of cytokines, that are involved in different plant developmental processes (Poveda and González-Andrés 2021). In addition to phytohormones, *Bacillus* strains are reported to produce volatile organic compounds (VOCs) and enzymes that also enhance plant growth. Some VOCs, such as albuterol and 1,3-propanediol, induce the expression of phytohormone biosynthetic genes (Tsotetsi et al. 2022; Tyagi et al. 2018). *Bacillus* strains that contain the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase significatively benefit plants by cleaving plant-produced ACC into ammonia (Glick 2005). This not only provides nitrogen available for absorption, but also prevent ACC from being converted to ethylene, which inhibits cell division, DNA synthesis and, therefore, plant growth (Gamalero, Lingua, and Glick 2023). On the other hand, *Bacillus* spp. exert biocontrol mechanisms that include competition for nutrients and space, induced systemic resistance and, most importantly, the secretion of secondary metabolites such as antibiotics, lytic enzymes and VOCs (Lu et al. 2021). Antibiotics produced by *Bacillus* spp. are diverse and include both ribosomally synthetized peptides (bacteriocins) and non-ribosomally molecules such as peptides, lipopeptides and polyketides (Li et al. 2020; Miljaković, Marinković, and Balešević-Tubić 2020). The antagonistic activity of *Bacillus* spp. may also be due to the secretion of lytic enzymes, which can hydrolyse the major components of fungal and bacterial cell walls (Kumar et al. 2018; Gautam et al. 2019). Lastly, besides those that enhance plant growth, it has been reported that *Bacillus* spp. produce VOCs that inhibit mycelial growth and spore germination of phytopathogenic fungi (Fessia et al. 2022).

In this view, the aim of this study was to characterize the phyllosphere-associated bacterial strain *Bacillus* GG-22 and evaluate its potential as a biological control agent and plant growth-promoting bacterium. The research included a comprehensive genomic analysis of strain GG-22, as well as an investigation of its antagonistic activity against phytopathogens affecting olive trees. Furthermore, the study examined the transcriptomic response of olive trees treated with GG-22 to assess the potential molecular mechanisms underlying its beneficial effects.

2. Materials and Methods

2.1. Microorganism and growth conditions

The *Bacillus* spp. strain GG-22 was isolated from the phyllosphere of a tomato plant in orchards located on an organic farm in Almería (Spain), as part of a biodiversity study conducted through a research project by the University of Granada and DOMCA (Granada, Spain). The isolated strains underwent a screening process to assess their antagonistic activity against *Clavibacter michiganensis* and *Pseudomonas syringae*, two phytopathogenic bacteria affecting tomato crops. A preliminary genetic identification using 16S rRNA sequencing initially classified the strain as *Bacillus pumilus*, with 97.8% similarity. Strain GG-22 was selected for further investigation due to its broad-spectrum and potent antagonistic activity. GG-22 was grown on tryptone soy agar (TSA) (Bioser, Barcelona, Spain) at 25°C for 48 h.

2.2. Genome sequencing

Genomic DNA was extracted from a pure liquid culture following a previously described procedure (Martín-Platero et al. 2007). The genome was sequenced with the Illumina HiSeq4000 platform by STAB VIDA (Caparica, Portugal). Assembly and annotation were carried out using Bacflux pipeline developed at the Austrian Institute of Technology (AIT), a workflow for bacterial short reads assembly, QC, annotation, and more (Antonielli et al. 2024). The workflow included preprocessing of reads, assembly, quality control, contamination, completeness assessment, taxonomic analysis, annotation, antimicrobial resistance genes search, plasmids and prophages. The Average Nucleotide Identity (ANI) analysis was done by including all available genomes of *Bacillus altitudinis* and *Bacillus pumilus* available in NCBI GenBank until January 2024. Bacflux integrates several platforms for annotation and functional analysis. The gene prediction and coding sequence identification was conducted using Prokka and Bakta. Genes were assigned to COG functional categories with eggNOG. Gene clusters related to the production of secondary metabolites was predicted using the antiSMASH online tool. For antimicrobial resistance (AMR) prediction, filtered reads were mapped to the CARD database using BBMap, and contigs were screened for antimicrobial resistance and virulence genes using ABRicate. The presence of plasmids was investigated using Platon. Finally, contigs were screened for viral sequences using VirSorter2. The protein sequence FASTA file generated was uploaded to PGPT-Pre in PlaBAse, a prediction tool for bacterial plant growth-promoting traits (Patz et al. 2021).

2.3. Evaluation of antagonistic activity

2.3.1. In vitro antagonistic activity against phytopathogenic bacteria

The antagonistic activity of *Bacillus* sp. GG-22 was evaluated against three phytopathogenic bacteria infecting olive trees, *P. savastanoi* pv. *savastanoi*, *Agrobacterium tumefaciens* and *X. fastidiosa* subsp. *pauca* strain De Donno ST53 (Table 1). The antagonistic activity was assessed on solid nutritive media by dual-culture method, previously described (Zicca et al. 2020). Dual-culture tests with *P. savastanoi* and *A. tumefaciens* were

performed in TSA medium. Antagonistic activity against *X. fastidiosa* was tested on PD3 agar (Davis, Purcell, and Thomson 1980). Pathogenic bacteria and *Bacillus* GG-22 suspensions were prepared in saline solution (NaCl 0.9%). Three parallels rows of the target strain were generated by placing 3 drops of 20 µL of bacterial suspension at 10^8 CFU/mL at the top of the petri dish and letting them to flow down to the opposite site. After 24 h incubation at 25°C, an 8 mm well was made in the agar and filled with 200 µL of *Bacillus* spp. GG-22 suspension at 10^8 CFU/mL. Plates were incubated at 25°C for 4 days in the case of *P. savastanoi* and *A. tumefaciens* and for 10 days in the case of *X. fastidiosa*. Growth inhibition area was measured as the inhibition halo between *Bacillus* sp. GG-22 growth and the target bacterium species. Each target bacterial species tested in triplicate.

Table 1. Bacterial strains used along with their references and source of isolation.

Phytopathogenic bacteria	Reference	Isolation source
Pseudomonas savastanoi	CECT 5023	Olive tree (*Olea*
Agrobacterium tumefaciens	DMC 47	Olive tree (*Olea*
Xylella fastidiosa subsp *pauca* strain De Donno ST53	CFBP 8402	Olive tree (*Olea*

2.3.2. In vitro antagonistic activity against phytopathogenic fungi and oomycetes

Growth inhibition of phytopathogenic fungi described in Table 2 by *Bacillus* spp. GG-22 was assessed through dual-culture method in potato dextrose agar ((PDA); Bi-okar Diagnostics, Allonne, France)) (Cheffi et al. 2019; Bruez et al. 2015). A loop of bacterial cells of 48 h old culture on TSA was streaked linearly on one side of the petri dish. A 6 mm of diameter mycelial plug from an actively growing margin of fungal culture was placed on the opposite side of the petri dish. As controls, agar plates were inoculated similarly with the pathogen only. Plates were incubated at 25°C for 14 days. The radial mycelial growth in dual-culture and control plates was measured 5, 7, 10 and 14 days after inoculation (Passera et al. 2019). Growth inhibition percentage was calculated using the following formula:

$$\% \text{ inhibition} = [(C - T)/C] \times 100$$

where C is the fungal colony radius in control plates, and T is the fungal colony radius in dual-culture plates. Each test was carried out in triplicate.

Table 2. Fungal and oomycetal strains used along with their references, and source of isolation.

Species	Reference	Isolation source
Verticillium dahliae	V136I	Olive tree (*Olea europaea*)
Fusarium oxysporum	DMC 03	Olive tree (*Olea europaea*)
Pythium sp.	DMC 11	Olive tree (*Olea europaea*)

2.4. Olive tree transcriptomic analysis

The gene expression response of the olive (*O. europaea* L.) cultivars Ogliarola salentina to *Bacillus* sp. GG-22 was studied on three one-year potted plants maintained in greenhouse in controlled conditions (25°C and 65% relative humidity). Tissues (of

twigs) were sampled at time 0 (T0, samples GG1, GG2 and GG3) and plants were successively treated, in the same day, with GG-22 by spray application (15g/L). Additional tissues from the same plants were sampled at 12 hours (T2, samples GG7, GG8 and GG9) and 15 days after GG2 application (T5, samples GG13, GG14 and GG15). Immediately after T5, plants were needle inoculated with *X. fastidiosa* subsp. *pauca* strain De Donno ST53. A final tissue sampling was made 10 days after *X. fastidiosa* inoculation (T6, samples GG19, GG20 and GG21). Table 3 shows the experimental design.

Table 3. Experimental design of GG-22 treatment and *X. fastidiosa*-inoculation assay on olive plants for transcriptomic analysis.

Time point	**T0**		**T2 (12hpt)**	**T5 (15dpt)**		**T6 (10dpi)**
Condition	**Untreated (UN_TR)**	**GG-22 Treatment (TR)**	**(TR)**	**(TR)**	**Inoculation by *X. fastidiosa* (DD_Xf)**	**(DD_Xf)**
Rep1	**GG1**		**GG7**	**GG13**		**GG19**
Rep2	**GG2**		**GG8**	**GG14**		**GG20**
Rep3	**GG3**		**GG9**	**GG15**		**GG21**

Notes: hours post treatment (hpt); days post treatment(dpt); days post inoculation (dpi)

Xylella inoculation was performed by the pinprick inoculation method, described in the EPPO PM7/24 (EPPO 2018). Drops (10-50 μL) of *X. fastidiosa* suspension adjusted to 10^9 CFU/mL in PBS (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM, pH 7.4) buffer were placed at three consecutive leaf nodes and punctured through five times using a sterile entomological needle. Five twigs were inoculated per plant. To favour absorption, plants were maintained in horizontal position for a few minutes. Plants were regularly checked for the occurrence of symptoms of desiccation until the end of the trial, 36 months after *Xylella* inoculation.

Transcriptomes of *X. fastidiosa* infected plants were obtained by mRNA sequencing. For this, 1 g of xylem tissue powdered in liquid nitrogen was used to extract total RNA from each sample. RNA concentrations were quantified by Nanodrop measurements (ThermoFisher Scientific, Waltham, USA) and its integrity was assessed by 1% agarose gel visualization. cDNA libraries were constructed using the NEB's polyA procedure (method i7) and sequenced in paired-end mode (2 x 100 bp) using Illumina technology at the Next Generation Sequencing Facility of the Vienna BioCenter Core Facilities (VBCF, https://www.viennabiocenter.org/vbcf/). RNAseq data was obtained as fastq files, including fastqc and multiQC data.

Raw reads were pre-processed using BBduk, part of BBTools v38.90 (Joint Genome Institute 2022) and high-quality reads were mapped on the olive reference genome assembly of *O. europaea* cv. Farga (Cruz et al. 2016) using the splice-aware aligner HISAT2 v2.2.1 (Kim, Langmead, and Salzberg 2015) with default parameters. The mapping was further quality checked with Qualimap v2.2.2a (Okonechnikov, Conesa, and García-Alcalde 2016). A read count matrix using the function featureCounts of the R package Rsubread v2.10.5 (Liao, Smyth, and Shi 2019) was calculated with default

parameters, which was further normalized using variance stabilizing transformation (vst) with DESeq2 v1.34.0 (Love, Huber, and Anders 2014). The obtained count dataset was first analysed by principal component analysis (PCA) using the software PCA explorer (Marini and Binder 2019). A further exploratory data analysis (EDA) by K-means clustering and subsequent study of genes enriched in the clusters were conducted to gain insights into the functions of co-expressed genes by iDEP explorer package (de Pascali et al. 2019).

Differential expression analysis was conducted with DESeq2 v1.34.0 (Love, Huber, and Anders 2014) to identify genes that were significantly differentially expressed (DEGs) at the time T2, T5 and T6 versus T0. Gene ontology enrichment analysis was performed on the obtained lists of DEGs by ShinyGo platform (Ge, Jung, and Yao 2020).

2.5. Statistical analysis

GraphPad prism 8.0 software (GraphPad Software Inc., San Diego, California) was used for statistical analysis. Shapiro–Wilk normality tests were used to determine normal distribution of data subjected to ANOVA. Repeated measures two-way ANOVA test supplemented with Sidak's post-hoc test was used for the evaluation of statistically significant differences in mycelial growth. Differences were considered statistically significant when $p > 0.05$.

3. Results

3.1. Genome analysis

3.1.1. Genomic features, taxonomic affiliation and COG functional categories

The genome of strain GG-22 was sequenced, assembled, and annotated. Assembly evaluation shows that the genome had a total size of 3.84 Mb as well as an average G + C content of 41.23 % (Table 4). The coverage was 527.6X.

The assessment of genome quality based on 468 markers showed a completeness of 99.59% with a contamination of 0.21%. While initial 16S rRNA sequencing classified the GG-22 strain as *B. pumilus*, the taxonomic placement based on ANI analysis provided a match with *Bacillus altitudinis* (Figure 1). The phylogenetic tree clearly illustrates the genomic divergence between *B. altitudinis* and *B. pumilus* strains.

Table 4. Genome properties of *Bacillus* sp. GG-22 from QUAST (quality assembly tool for genome assemblies by CAB) report. All statistics are based on contigs of size >= 500 bp, unless otherwise noted (e.g., "# contigs (>= 0 bp)" and "Total length (>= 0 bp)" include all contigs).

Contigs_sel	Values
# contigs (>= 0 bp)	29.0
# contigs (>= 1000 bp)	26.0
# contigs (>= 5000 bp)	23.0
# contigs (>= 10000 bp)	23.0
# contigs (>= 25000 bp)	20.0
# contigs (>= 50000 bp)	19.0
Total length (>= 0 bp)	3840524.0
Total length (>= 1000 bp)	3837968.0
Total length (>= 5000 bp)	3832516.0
Total length (>= 10000 bp)	3832516.0
Total length (>= 25000 bp)	3786266.0
Total length (>= 50000 bp)	3755921.0
# contigs	29.0
Largest contig	551385.0
Total length	3840524.0
GC (%)	41.23
N50	306798.0
N90	92549.0
auN	284225.9
L50	5.0
L90	15.0
# N's per 100 kbp	0.0

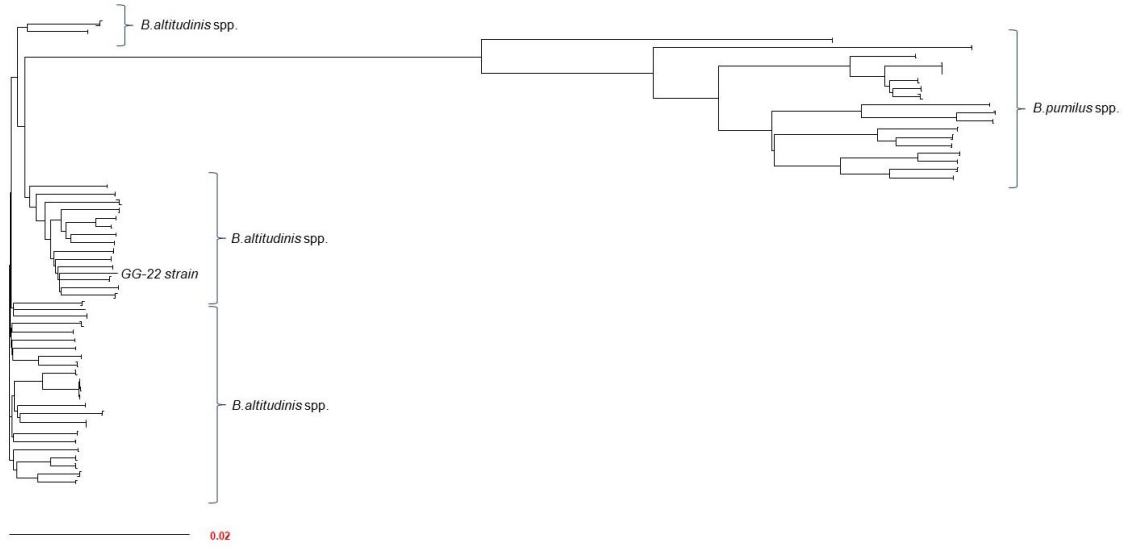


Figure 1. Phylogenetic tree showing the relationships between strains of *Bacillus altitudinis* and *Bacillus pumilus* based on whole-genome comparisons and Average Nucleotide Identity (ANI) distance. The GG-22 strain is highlighted within the *B. altitudinis* clade. The scale bar indicates 0.02 expected changes per site.

Genome annotation predicted 3,922 coding DNA sequences, 4,074 genes, 79 misc_RNA, 10 rRNA, 62 tRNA and 1 tmRNA. We analysed Clusters of Orthologous Groups (COG) categories and gene numbers with 1,225 genes with unknown functions (Figure 2).

The most abundant categories include transcription (347 genes) and translation, ribosomal structure, and biogenesis (199), which reflects the organism's capacity to efficiently regulate and produce proteins, essential for its growth, adaptation, and survival in various environments (de Souza et al. 2019). The predominance of genes related to amino acid, carbohydrate, and inorganic ion transport and metabolism (331, 252 and 25, respectively) indicates a high metabolic versatility. These categories play a pivotal role in nutrient exchange, and are key in plant-microbe and microbe-microbe interactions where competition for resources is critical (Lidbury et al. 2021). 211 genes are involved in cell wall/membrane/envelope biogenesis, which play a key role in adhesion and interaction with other organisms. Other categories of genes related to plant-microbe and microbe-microbe interactions include genes associated with signal transduction mechanisms (166 genes), cell motility (68 genes), defence mechanisms (67 genes), and intracellular trafficking and secretion (43 genes) (Barret, Morrissey, and O'Gara 2011; de Souza et al. 2019).

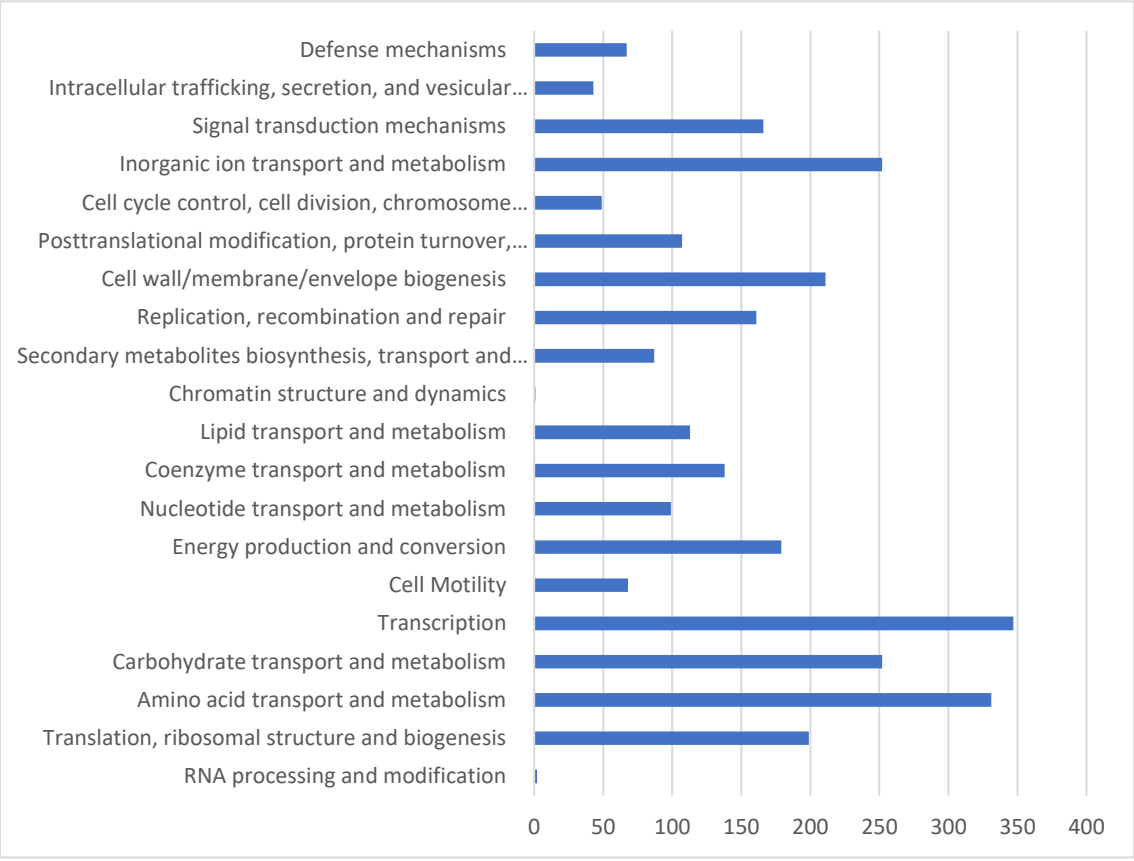


Figure 2. Functional classification of annotated genes in *Bacillus altitudinis* GG-22 based on COG (Clusters of Orthologous Groups) categories.

The AMR prediction analysis of the *B. altitudinis* GG-22 genome revealed the presence of two key genes associated with antibiotic resistance. First, the gene encoding chloramphenicol acetyltransferase (CAT) was identified, an enzyme that confers resistance to chloramphenicol by acetylating the antibiotic, thereby inactivating it and preventing its action on bacterial protein synthesis. Second, a gene related to BPU beta-lactamase was detected, an enzyme that degrades beta-lactam antibiotics such as penicillin and cephalosporins, providing the bacterium with resistance to these compounds (data not shown). Regarding plasmid presence, the analysis performed with Platon software found no evidence of plasmids in the GG-22 sample. This suggests that the detected resistance genes are chromosomally encoded, rather than located on extrachromosomal elements, potentially limiting their horizontal transfer to other bacteria. Additionally, four double-stranded DNA (dsDNA) phage sequences were identified in the GG-22 genome. These integrated prophages are remnants of previous bacteriophage infections and may play a role in genome evolution by facilitating the acquisition of new genes through horizontal transfer (Brüssow, Canchaya, and Hardt 2004). The presence of these prophages suggests that the GG-22 genome has been exposed to viral infections in the past, which could influence its genetic plasticity and adaptation to different environments.

3.1.2. Genes predicted to be involved in plant–microbe and fungi-bacteria interaction

Using the PLaBAse database, we found several plant growth-promoting traits in *B. altitudinis* GG-22. The effects of this strain can be divided into direct and indirect mechanisms. Direct effects include mechanisms involved in bio-fertilization, bioremediation and plant growth regulation. On the other hand, indirect effects involve mechanisms responsible for biotic and abiotic stress control, such as biocontrol, induction of plant systemic resistance (ISR) and systemic acquired resistance (SAR), microbial host colonization and competitive exclusion.

The direct effects of the strain are shown in Figure 3. The most prominent traits were heavy metal detoxification, xenobiotics degradation, phosphate and potassium solubilization, and iron acquisition, suggesting its capability to contribute to both nutrient cycling and availability and bioremediation. *B. altitudinis* GG-22 also demonstrated a strong potential for plant vitamin production, particularly in the metabolism of key vitamins such as folate (vitamin B9), thiamine (vitamin B1), pantothenic acid (vitamin B5), and riboflavin (vitamin B2). Furthermore, it was found to produce signaling volatiles and to stimulate seed germination and terpenoid derivatives production.

The indirect effects exerted by *B. altitudinis* GG-22 are presented in Figure 4. The most prominent traits include colonization and plant-derived substrate usage, highlighting the strain's ability to effectively colonize plant tissues and utilize available nutrients to support growth. The strain also demonstrated significant potential for neutralizing abiotic stress, suggesting its role in helping plants withstand adverse environmental conditions such as drought, salinity and extreme temperatures. Additionally, several traits related to cellular envelope (CE) functions were prominent, including bacterial fitness, which encompasses resistance to antimicrobial and toxic compounds, as well as multidrug resistance. Other CE functions include cell envelope remodelling, exopolysaccharide production, and quorum sensing response and biofilm formation, that further emphasize the ability of GG-22 to coordinate microbial activity and create robust microbial communities. Moreover, the strain exhibited traits related to

spore production, which, together with CE functions, enhance microbial survival, and neutralizing biotic stress, suggesting a role in suppressing pathogens.

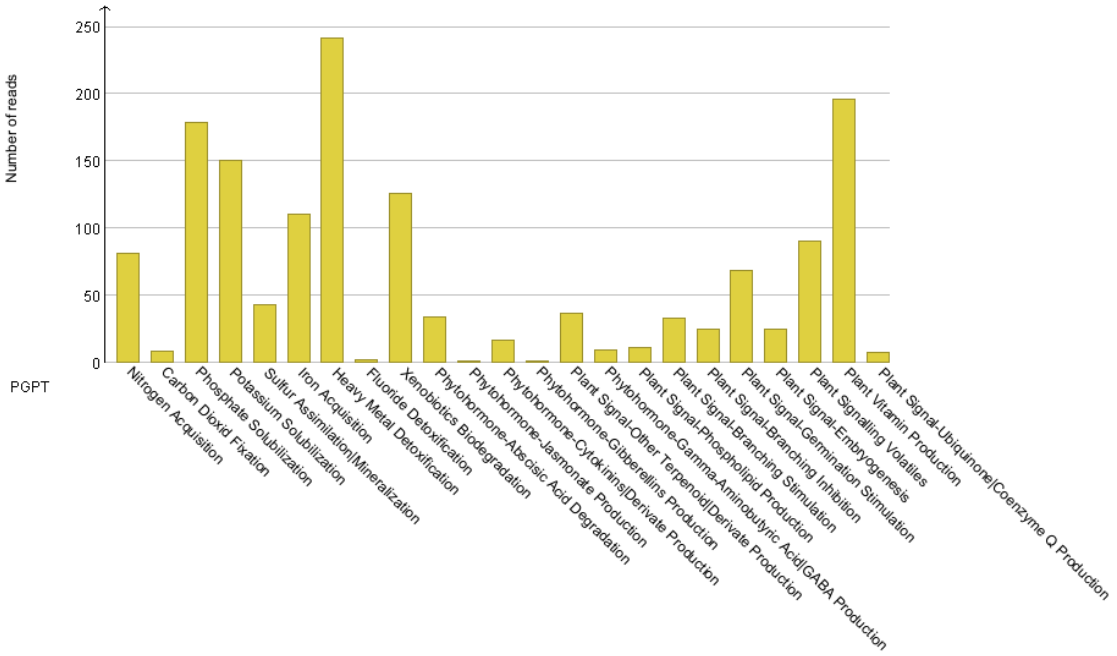


Figure 3. Direct plant growth-promoting traits of *Bacillus altitudinis* GG-22 identified using PLaBAse.

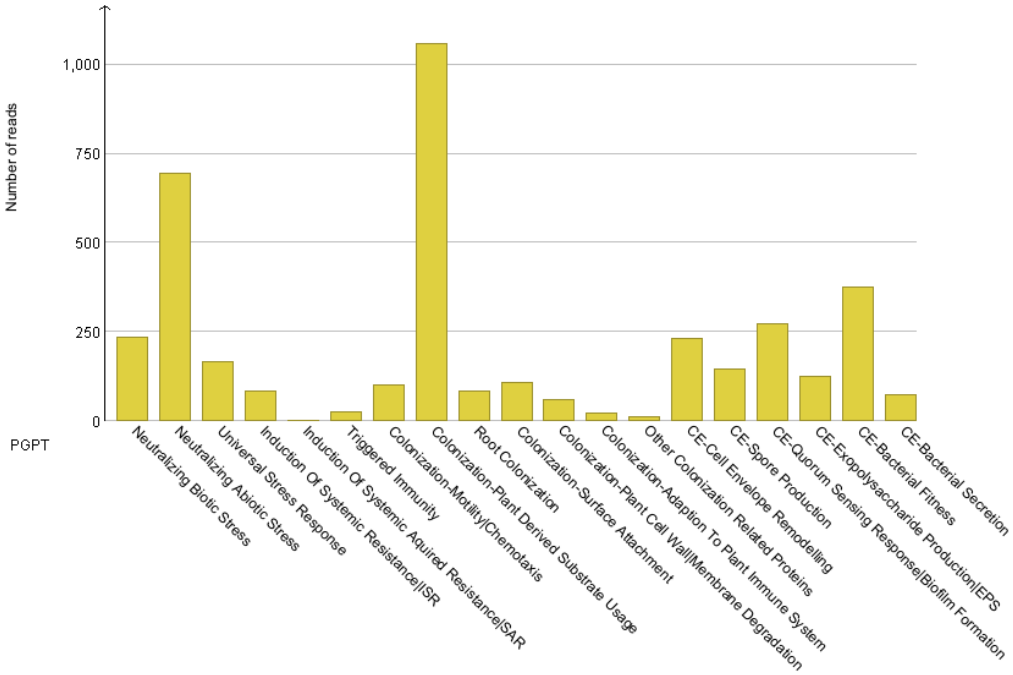


Figure 4. Indirect plant growth-promoting traits of *Bacillus altitudinis* GG-22 identified using PLaBAse.

3.1.3. Biosynthetic gene clusters involved in secondary metabolite production

A total of nine gene clusters likely involved in secondary metabolite production were identified in strain GG-22, using AntiSmash 7.1 (Table 5). Of these, one non-

ribosomal peptide synthetase (NRPS) cluster encodes a lichenysin type, as indicated by 85% identity at the protein level of the biosynthesis genes, which is a lipopeptide biosurfactant which structure is similar to surfactin. Other secondary metabolite gene cluster are related to schizokinen (60%), a citrate-containing dihydroxamate that is a siderophore; fengycin (53%), a cyclic lipopeptide; and bacillibactin (80%) a catechol-based siderophore. The others are unknown. Clusters for betalactone and RRE-containing regions indicate possible novel biosynthetic pathways. These findings highlight the potential of GG-22 to produce a wide range of secondary metabolites, including siderophores and surfactants, that enhance its competitiveness and adaptability in diverse environments and could be valuable for applications in biocontrol and iron acquisition.

Raw sequence data for the bacterial genome has not been yet deposited in the National Center for Biotechnology Information (NCBI).

Table 5. Identification of secondary metabolite regions in *Bacillus altitudinis* GG-22 using AntiSmash analysis.

Region	Type	From	To	Most similar known cluster		Similarity
1.1	Terpene	63.094	84.971			
1.2	T3PKS	124.522	165.619			
1.3	RiPP-like	471.024	481.350			
2.1	Betalactone	289.748	322.164			
4.1	NRPS	179.804	263.528	Lichenysin	NRP	85%
9.1	NI-siderophore, terpene	92.389	120.982	Schizokinen	Other	60%
11.1	RRE-containing	9.226	30.131			
12.1	Betalactone	7.736	36.144	Fengycin	NRP	53%
13.1	NRP-metallophore, NRPS	26.306	78.035	Bacillibactin/bacillibactin E/bacillibactin F	NRP	80%

3.2. Inhibition of plant pathogens by *B. altitudinis* GG-22

The antagonistic potential of *B. altitudinis* GG-22 was assessed against plant pathogenic bacteria and fungi in dual-culture assays. GG-22 was barely able to inhibit the growth of the pathogenic bacteria evaluated (Figure 5). In particular, *B. altitudinis* did not cause any inhibitory effect on *A. tumefaciens* and *X. fastidiosa* growth, whereas small inhibition halos were observed against *P. savastanoi* (Table 6).

In contrast, *B. altitudinis* GG-22 significantly reduced the growth of all the phytopathogenic fungi tested in dual-culture assay, based on the radial mycelial growth after 5, 7, 10 and 14 days of incubation in comparison with the control plates. with varying efficacy. GG-22 strain exhibited varying degrees of inhibition against each fungal strain. Mycelial growth inhibition of *V. dahliae*, showed in Figure 6, was 50.45% after 5 days of incubation and reached a maximum of 55.97% on day 14. Similarly, *B. altitudinis* GG-22 significantly influenced the growth of *Pythium* sp., as presented in Figure 7. *Bacillus* GG-22 reduced the mycelial development of *Pythium* sp. by 62.66% on the 5th day of incubation. The inhibitory effect remained stable over time, since after 14

days of incubation, 63.43% inhibition was observed. While the growth inhibition rate of *V. dahliae* and *Pythium* sp. displayed by *Bacillus* GG-22 exceeded 50% only after five days of incubation, it exhibited a lower and more gradual effect against *F. oxysporum* (Figure 8). *Bacillus* GG-22 significatively inhibited mycelial growth of *F. oxysporum* by 12.75%, 15.94% and 19.78% on the 5th, 7th and 10th days of incubation. The highest inhibition (41.88%) was displayed after 14 days of incubation.

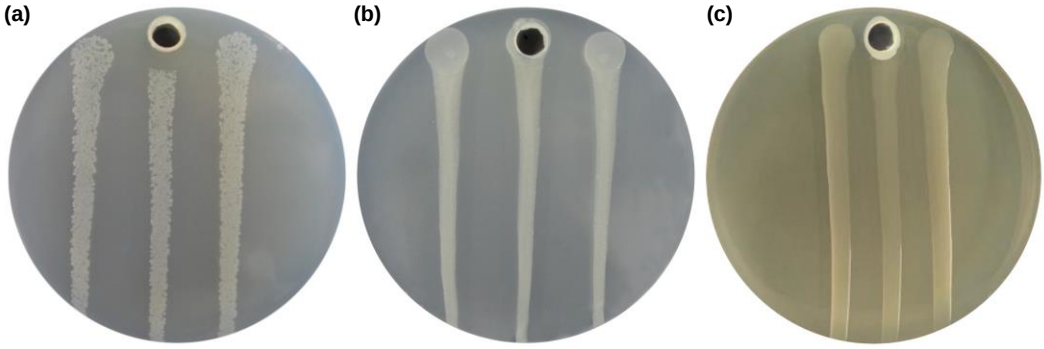


Figure 5. Dual-culture assay of antagonistic activity of *B. altitudinis* GG-22 against **(a)** *P. savastanoi* **(b)** *A. tumefaciens* **(c)** *X. fastidiosa*.

Table 6. Antagonistic activity of *B. altitudinis* GG-22 against phytopathogenic bacteria affecting olive trees.

Species	Growth inhibition halo (mm ± SD)
Pseudomonas savastanoi	8.7 ± 0.76
Agrobacterium tumefaciens	0
Xylella fastidiosa ST53 subsp *pauca*	0

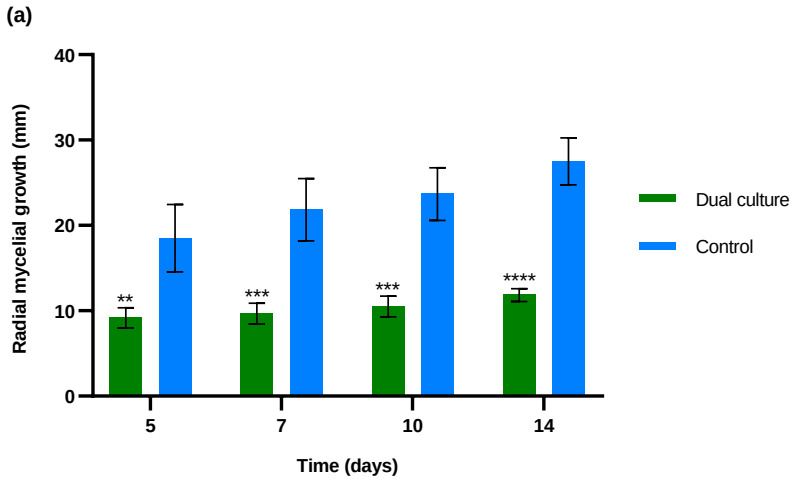


Figure 6. Cont.

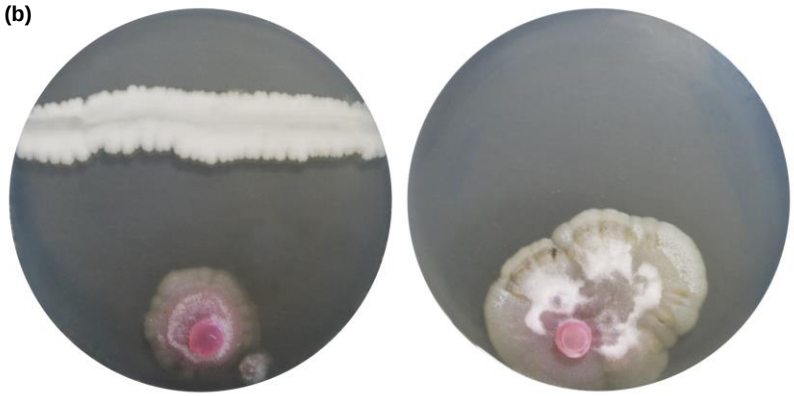


Figure 6. Antagonistic activity of *B. altitudinis* GG-22 against *V. dahliae* in vitro. **(a)** Mycelial growth of *V. dahliae* after 5, 7, 10 and 14 days in dual-culture and control plates. Significant mycelial growth inhibition in comparison with the control according to Sidak's multiple comparison test are reported with asterisks (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001). Values are means with SD in bars **(b)** Dual-culture (left) and control (right) plates 14 days after incubation.

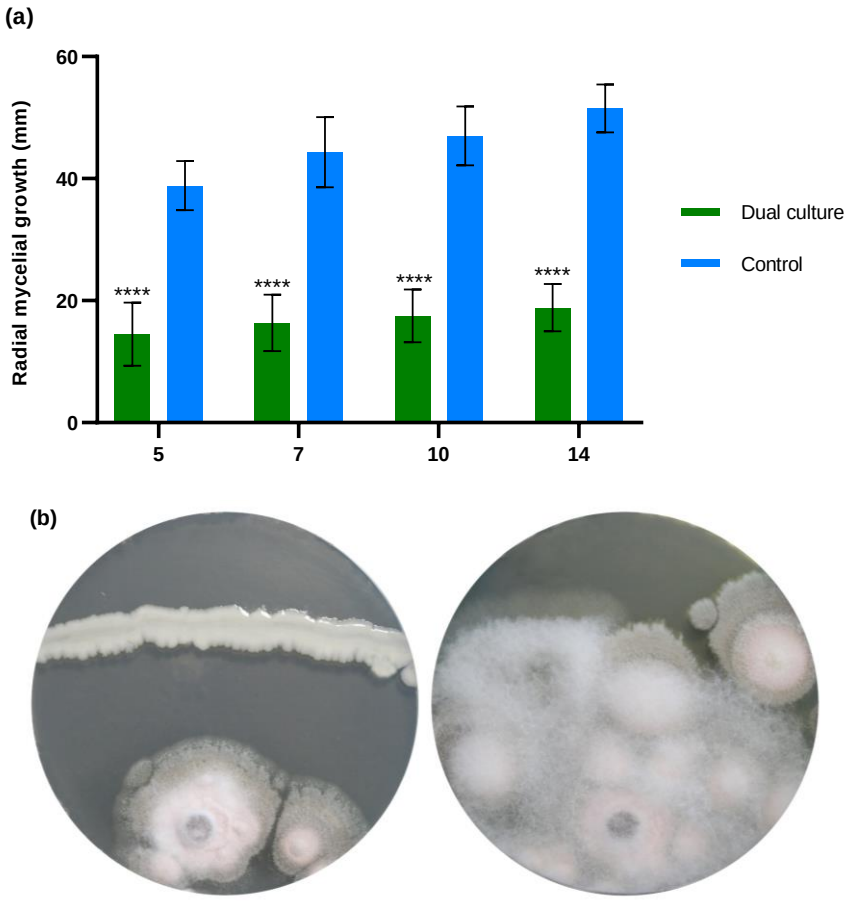


Figure 7. Antagonistic activity of *B. altitudinis* GG-22 against *Pythium* sp. in vitro **(a)** Mycelial growth of *Pythium* sp. after 5, 7, 10 and 14 days in dual-culture and control plates. Significant mycelial growth inhibition in comparison with the control according to Sidak's multiple comparison test are reported with asterisks (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001).

Values are means with SD in bars **(b)** Dual-culture (left) and control (right) plates 14 days after incubation.

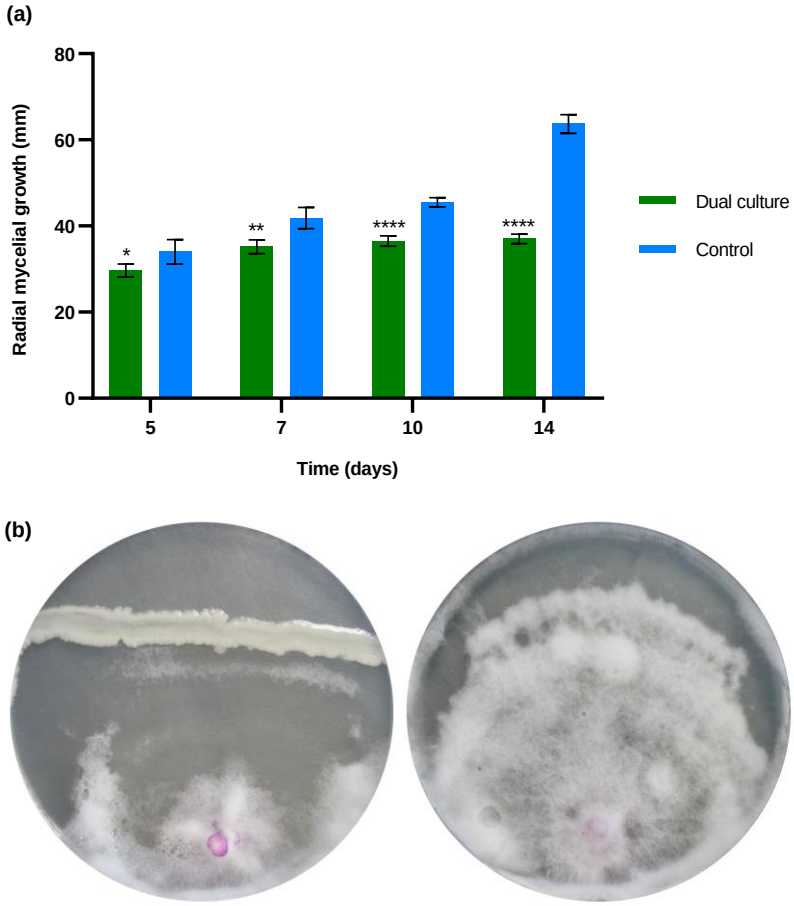


Figure 8. Antagonistic activity of *B. altitudinis* GG-22 against *F. oxysporum* in vitro. **(a)** Mycelial growth of *F. oxysporum* after 5, 7, 10 and 14 days in dual-culture and control plates. Significant mycelial growth inhibition in comparison with the control according to Sidak's multiple comparison test are reported with asterisks (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001). Values are means with SD in bars **(b)** Dual-culture (left) and control (right) plates 14 days after incubation.

3.3. Transcriptome profile of *X. fastidiosa* infected olives treated with *B. altitudinis* GG-22

3.3.1. Mapping & read count data

The obtained count dataset was analysed by principal component analysis (PCA) using the software PCA explorer (Figure 9). This analysis correctly clustered all the samples based on their time of sampling except for the sample GG2 which was an outlier at T0 and among the untreated (UN_TR) samples (Figure 9a). We therefore decided to eliminate this sample from the successive analysis. The final PCA graph clearly distinguished samples from T2 and T6 while grouping those from T0 and T5 (Figure 9b). The obtained clustering show that the gene expression of olive sprayed with GG-22 is altered 12 hours after the application (T2), while the effect of *B. altitudinis* GG-22 almost terminates 15 days after the application (T5). Successively, *X. fastidiosa* induces a further modification of gene expression (T6) 10 days after the inoculation.

(a) **(b)**

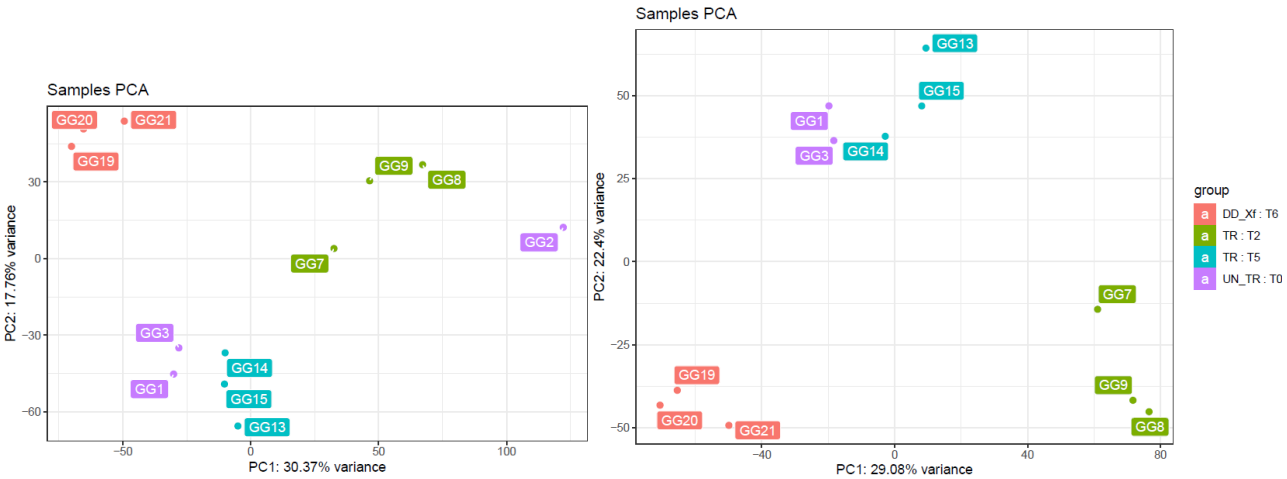
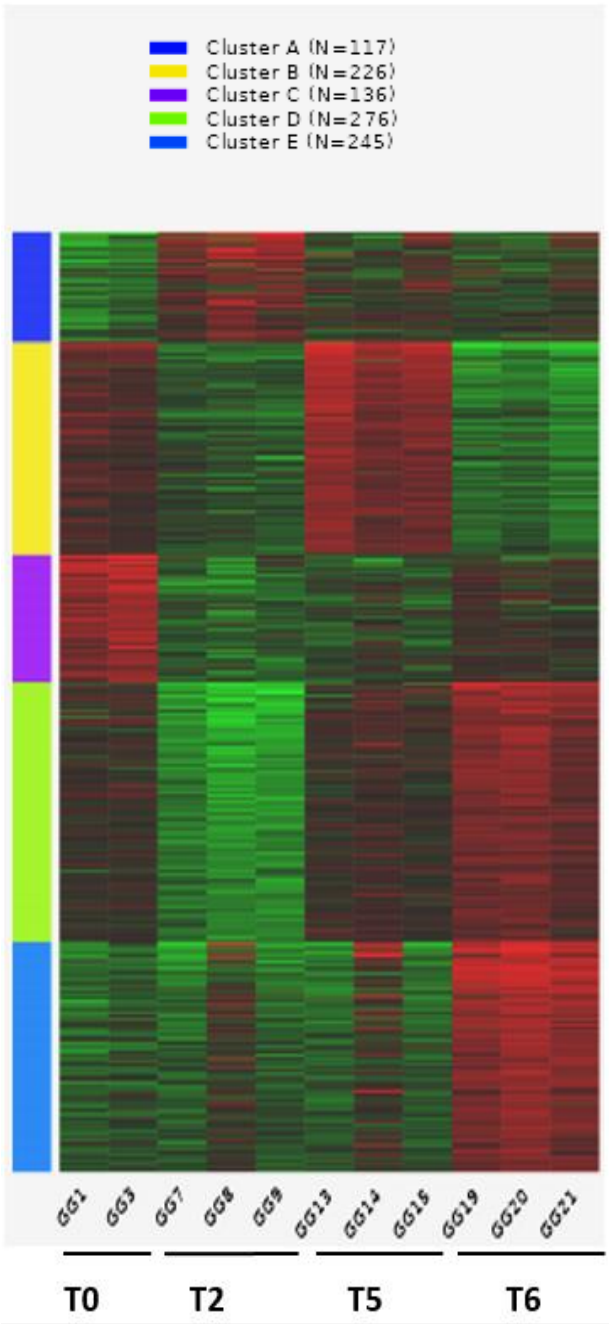


Figure 9. Principal component analysis of VST-transformed data from RNASeq analysis of the *B. altitudinis* GG-22-treated olives. **(a)** Group of samples from the different time points (T0, T2, T5 and T6) and different condition (DD_Xf, TR and UN_TR) are identified by different colours **(b)** The same analysis after elimination of the GG2 outlier sample.

K-means clustering is performed on a dataset of 1000 most variable genes and identifies five clusters of genes (A, B, C, D, E) with different expression patterns (Figure 10). Cluster D clearly groups genes showing a down regulated expression pattern (compare GG7, GG8 and GG9 to GG1 and GG3 characteristic of the GG-22 early treatment (T2) (Figure 10). A main term in this cluster indicates a downregulation of the "aspartic-type endopeptidase activity" an enzyme class involved in the response to multiple stresses (Figueiredo, Santos, and Figueiredo 2021). The simultaneous down regulation of the "cell wall" gene pathway pleads for a general remodelling of the cell wall. Of note is the downregulation of gene components of the "auxin polar transport" term, indicating a possible GG-22 activity on the auxin regulated plant development. Specific associated genes are "probable auxin efflux carrier component 1c" (OE9A004471, OE9A100299) and "auxin-responsive SAUR68-like" (OE9A028896, OE9A037445). This early GG-22 effect is no more observed at 15 days post application (T5), as can be inferred by comparing cluster B for GG1 and GG3 with GG13, GG14 and GG15. *Xylella* infection has a clear effect on the inoculated olives at T6, which is visible from the downregulation of gene cluster C and the upregulation of clusters B, D and E (Figure 10). Among the strongly statistically supported pathways (i.e. highest P value), are the upregulation of "polygalacturonase activity" and "extracellular region", two terms which fit with the presence of *Xylella* in the apoplast, in close contact with the cell wall.

The analysis of differentially expressed genes (DEGs) after *B. altitudinis* GG-22 treatment was carried out by comparing T2, T5 and T6 with T0. Confirming the PCA clustering GG-22 induced a higher alteration of gene expression at 12 hours after its application, when 1,772 genes with a fold change >+2 or <-2, were differentially regulated. This number decreased to 654 genes 15 days after treatment. An increase of the plant response was observed 10 days after *Xylella* inoculation, since 1,174 genes had

an altered expression with a fold change >+2 or <-2 (Figure 11). Moreover, a similar number of DEGs (1314) was altered at T6 when compared with T5.



Cluster	adj.Pval	nGenes	Pathways
A	0.000188	20	Oxidation-reduction process
A	0.000829	3	Chitin catabolic process
A	0.000829	4	2-alkenal reductase [NAD(P)] activity
A	0.000829	2	High-affinity hydrogen:glucose symporter activity
A	0.003761	2	Alcohol dehydrogenase (NAD) activity
A	0.008208	2	Chitin binding
A	0.008396	2	Embryo development
B	2.52E-19	15	Xyloglucan:xyloglucosyl transferase activity
B	9.10E-17	20	Hydrolase activity, hydrolyzing O-glycosyl compounds
B	1.15E-16	13	Xyloglucan metabolic process
B	7.24E-15	16	Apoplast
B	7.48E-09	18	Calcium ion binding
B	1.15E-07	23	Transcription factor complex
B	2.08E-07	22	Transcription factor activity, sequence-specific DNA binding
B	3.15E-07	9	Calmodulin binding
B	4.24E-07	29	Regulation of transcription, DNA-templated
B	1.05E-05	3	Response to bacterium
B	1.54E-05	8	Cell wall organization
B	0.000607	25	DNA binding
B	0.000796	2	Cellular glucan metabolic process
B	0.000975	6	Response to stress
B	0.001396	2	Sulfate reduction
C	2.29E-05	3	L-phenylalanine catabolic process
C	2.29E-05	3	Alkane 1-monooxygenase activity
C	2.29E-05	9	Extracellular region
C	5.45E-05	4	Alkaloid biosynthetic process
C	0.000408	5	Vacuole
C	0.000962	4	Pectin catabolic process
C	0.001555	3	Lyase activity
C	0.001602	3	Signal transducer activity
C	0.001765	4	Tyrosine metabolic process
C	0.002066	8	Iron ion binding
C	0.006029	3	Protein catabolic process
C	0.008222	6	Starch metabolic process
C	0.008515	3	Peptidase activity
C	0.008515	16	Oxidation-reduction process
C	0.009277	7	Heme binding
D	1.81E-07	14	Cell wall
D	6.70E-05	7	Aspartic-type endopeptidase activity
D	0.000264	7	Plant-type cell wall
D	0.000756	4	Auxin polar transport
D	0.000981	3	Cell periphery
D	0.001563	2	Regulation of organ growth
D	0.001997	4	Plant-type cell wall organization
D	0.001997	2	Leaf shaping
D	0.001997	5	Cell wall biogenesis
D	0.001997	5	Pectin catabolic process
D	0.001997	2	Desacetoxyvindoline 4-hydroxylase activity
D	0.001997	3	Polysaccharide biosynthetic process
D	0.003086	2	Nicotianamine synthase activity
D	0.003086	4	Aspartyl esterase activity
D	0.003498	3	Indole biosynthetic process
E	5.19E-23	18	Polygalacturonase activity
E	1.75E-22	27	Extracellular region
E	1.13E-12	21	Starch metabolic process
E	1.13E-12	15	Cell wall organization
E	3.83E-09	8	Plant-type cell wall organization
E	4.84E-09	40	Oxidation-reduction process
E	3.72E-08	9	Response to biotic stimulus
E	1.13E-05	7	Oxidoreductase activity, acting on CH-OH group of donors
E	1.58E-05	3	Pinoresinol reductase activity
E	1.69E-05	10	Cell wall
E	2.26E-05	11	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of
E	2.84E-05	14	Iron ion binding
E	5.84E-05	3	Serine-type endopeptidase inhibitor activity
E	8.10E-05	4	Guiding stereospecific synthesis activity
E	8.10E-05	8	Flavin adenine dinucleotide binding

Figure 10. Heat map of K-means clustering by iDEP tool of 1,000 most variably expressed genes at each time point. The colours in the map display the relative values of all tiles within four time points; green indicates the lowest expression, black indicates intermediate expression, and red indicates the highest expression. Genes were grouped into five clusters (A, B, C, D, E) on the basis of the expression similarity. The number of the expressed genes in each enriched pathway are reported in the Table with the adjusted P value significance.

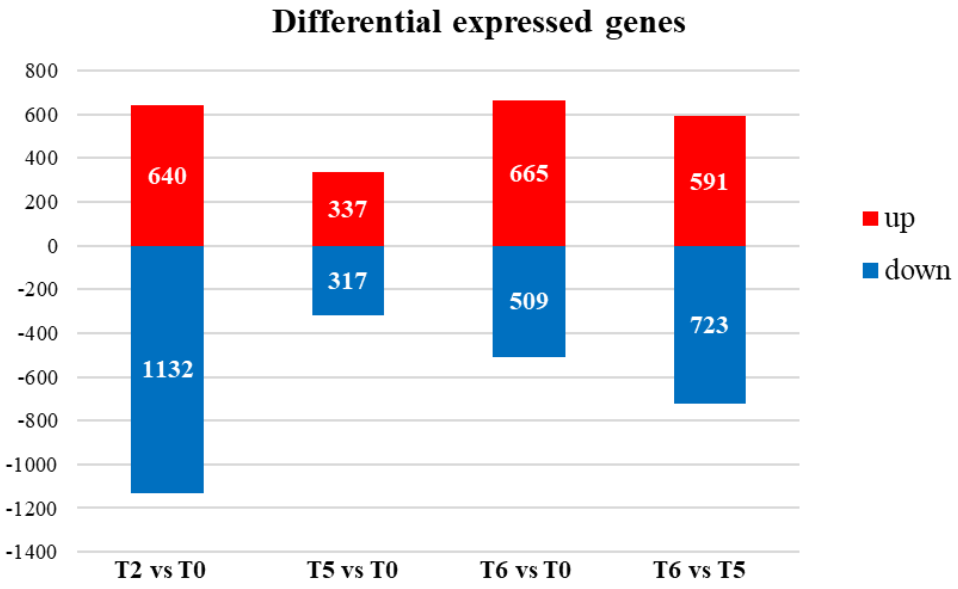


Figure 11. Differentially Expressed Genes (DEGs) among the different time points comparisons. Up and down regulated genes are indicated respectively in red and blue colours.

3.3.2. Gene Ontology of the early and late response of olive to *B. altitudinis* GG-22 application

Twelve hours after the GG-22 application, 640 and 1132 genes were respectively up- and down-regulated in the T2 vs T0 comparison (Figure 11). The Gene Ontology analysis of the 640 upregulated genes clearly shows the enrichment of "Nucleosome" (GO:0000786), "Nucleosome assembly" (GO:0006334) and "Regulation of systemic acquired resistance" (GO:0010112) Biological Processes (Figure 12). This last Biological Process is univocally enriched of NIM1-INTERACTING 1-like and 2-like genes (OE9A054971, OE9A105151, OE9A022404, OE9A009045), whose orthologues in *Arabidopsis thaliana* regulates the Systemic Acquired Resistance (SAR) response (Hermann et al. 2013). In parallel, *B. altitudinis* GG-22 likely induces a chromatin structure formation and remodelling as evidenced by up regulation of all members of the core histones complex, H2A, H2B, H3, H4 and H1-like (not shown) (Ding and Wang 2015).

The 1,132 downregulated genes (Figure 12) contribute to the enrichment of "Apoplast" (GO:0048046), Xyloglucan:xyloglucosyl transferase activity" (GO:0016762), "Cell wall biogenesis" (GO:0042546) and "Hydrolase activity, hydrolyzing O-glycosyl compounds" (GO:0004553), which are all processes having the involvement of cell-wall related proteins. Laccases and xyloglucan endotransglucosylase hydrolase 2-like enzymes are inhibited in their expression, which determines respectively a possible alteration of the lignin polymerization (Bai et al. 2023) and cell wall organization and biogenesis (Sharples, Nguyen-Phan, and Fry 2017).

Fifteen days after the application, the effects of GG-22 seem to be reduced as showed by the lower number of DEGs (654, Figure 11). The 337 upregulated DEGs

contribute to the enrichment (Figure 12, T5 vs T0) of several processes (FDR enrichment 10-2.8) having "serine-type carboxypeptidase activity" (GO:0004185) and "DNA-binding transcription factor activity" among the other. Enriched genes in these categories are several serine carboxypeptidase-like proteins whose role in protecting plants from biotic and abiotic stresses is known (Xiaomin Xu et al. 2021) and WRKY transcription factors, the more expressed, WRKY40, is induced by pathogens (Xinping Xu et al. 2006).

The 317 downregulated genes (Figure 12) enrich several processes related to the biosynthesis of secondary metabolites ("terpene synthase activity" (Singh and Sharma 2015), GO:0010333; Cinnamic acid biosynthetic process, GO:0009800) and the aromatic compounds ("chorismate biosynthetic process", GO: 0009423), involving enzymes like germacrene D synthase and chorismate synthase 1).

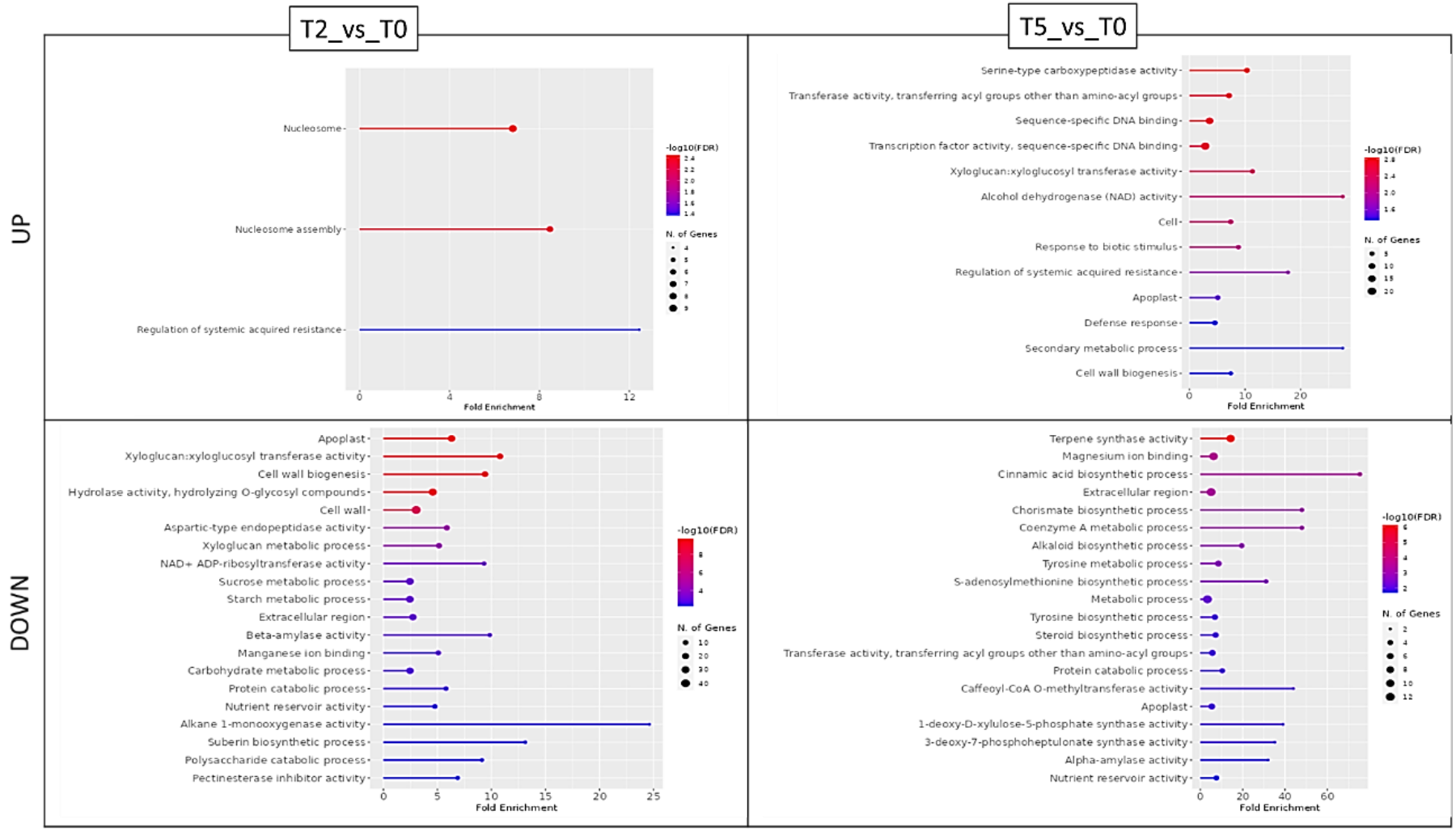


Figure 12. Gene Ontology analysis of T2 and T5 time points compared to T0. GO terms enriched in up and downregulated genes are showed together with their Fold enrichment values. Significance and number of gene in the term are respectively expressed by the log10FDR (False discovery rate) and the size of circles.

3.3.3. Gene Ontology of the response to *Xylella infection*

The transcriptome response of olives inoculated with *Xylella*, 10 days after the inoculation and 25 days after the start of the trial (T6), clearly differs from uninoculated plants at T0 (Figure 13). The 665 upregulated genes (Figure 11) point to the enrichment of terms "Response to biotic stimulus" (GO:0009607) and several others related to the olive response to *X. fastidiosa* infections, such as "response to auxin" (GO:9733), "defence response" (GO:0006952) and "extracellular region" (GO:0005576). Indeed, all the elements of a xylem-limited bacterium, the immune and hormone response and the involvement of the extracellular region are represented by the Gene Ontology. In detail, genes enriched in these processes are several pathogenesis-related proteins (major allergen Pru ar 1, pathogenesis-related STH-2-like, pathogenesis-related 1-like, thaumatin), hormone response (auxin-induced 15A-like, Auxin responsive SAU) and cell wall organization (polygalacturonase At1g48100-like, polygalacturonase-like, expansin A10 and B2), some of them already reported in previous studies on *Xylella* infections (Rodrigues et al., 2013; Giampetruzzi et al., 2016). Downregulated genes enrich several molecular functions and cellular components related to the signaling and cell-wall remodelling, such as "calcium ion binding" (GO:0005509), calmodulin binding" (GO:0005516), "xyloglucan:xyloglucosyl transferase activity" (GO:0016762), and "apoplast" (GO:0048046), which were also observed in the above reported transcriptome studies

We also analysed the response (T6 vs T5, Figure 13) of plants treated with GG-22 and successively inoculated with *Xylella* (10 days after inoculation). "Iron ion binding" (GO:0005506) and "Oxidoreductase activity, acting on paired donors" (GO:0016705) are the two top enriched molecular functions. A "gibberellin 2-beta-dioxygenase 1-like" gene, an enzyme that regulates endogenous gibberellin levels thereby affecting plant defence and growth (Schomburg et al. 2003), contributes to enrich the GO:0016705 term. Conversely, Gene Ontology of downregulated genes showed that "Xyloglucan:xyloglucosyl transferase activity" (GO:0016762) and "Transcription factor activity, sequence-specific DNA binding" (GO:0003700) are the two enriched molecular functions. Major downregulated genes in these two terms are a xyloglucan endotransglucosylase hydrolase which is an enzyme involved in cell wall remodelling, and several ethylene-responsive transcription factors ERF114-like, ERF113-like and ERF003-like.

In line with the lack of direct antagonism of *B. altitudinis* GG-22 toward *Xylella* and its decreased stimulation of gene expression observed at T5, a single treatment with the bacterium did not protect the olives from this strain of *X. fastidiosa*, as plants were severely desiccated at the end of the trial, 36 months after the inoculation (Figure 14).

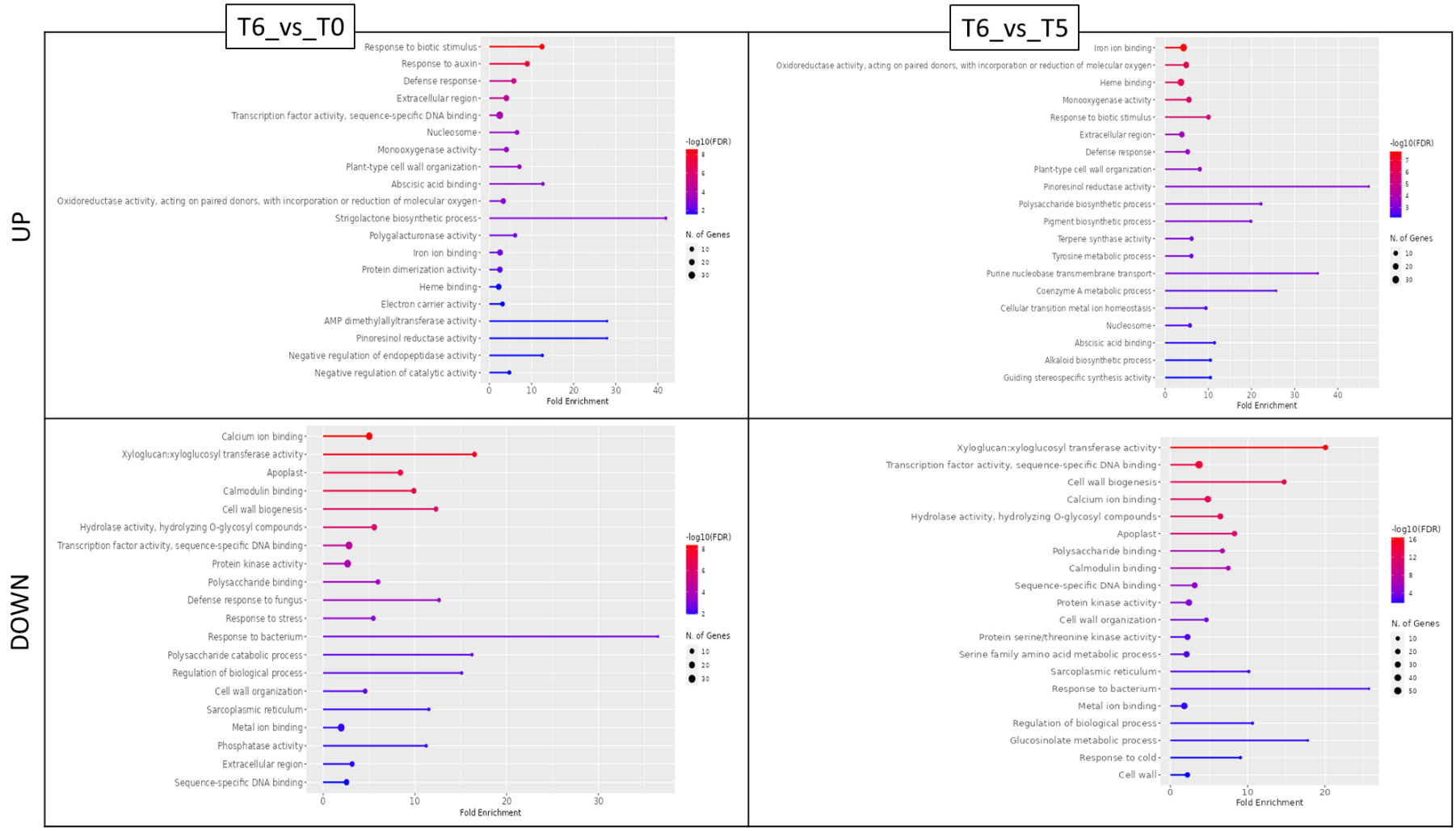


Figure 13. Gene Ontology analysis of T6 vs T0 and T5 respectively. GO terms enriched in up and downregulated genes are showed together with their Fold enrichment values. Significance and number of gene in the term are respectively expressed by the log10FDR (False discovery rate) and the size of circles.

Figure 14. Symptoms of desiccation on olives preventively treated with B. altitudinis GG-22 and successively inoculated with X. fastidiosa. Photos are taken 36 months after Xylella inoculation.

4. Discussion

Genome sequencing has been a helpful strategy to identify potential growth promoting bacteria and biological control agents. We sequenced, assembled and annotated the genome of strain GG-22. Although initial 16S rRNA sequencing identified the GG-22 strain as *B. pumilus*, complete genome analysis confirmed that GG-22 belongs to *Bacillus altitudinis*, which has several genes involved in plant growth stimulation or in the biocontrol of plant diseases. This highlights the limitations of 16S rRNA in distinguishing between closely related species, particularly within the *Bacillus* genus, where high sequence conservation in the 16S gene can mask species-level differences. The application of whole-genome sequencing, including ANI analysis, provides a more comprehensive understanding of the genetic relationships, showing a clear separation between *B. altitudinis* and *B. pumilus*. The GG-22 strain clusters tightly with other *B. altitudinis* strains, reinforcing its reclassification and underscoring the necessity of genome-wide approaches to resolve taxonomic ambiguities that cannot be addressed by single-gene markers like 16S rRNA.

The PLaBAse analysis revealed a significant number of genes in *B. altitudinis* GG-22 associated with biofertilization, bioremediation, and plant growth regulation mechanisms, particularly those involved in heavy metal detoxification, xenobiotics degradation, phosphate and potassium solubilization, and iron acquisition. These genetic traits, that are commonly associated with plant growth-promoting bacteria, suggest that this strain has the potential to contribute to both nutrient cycling and

availability, while its bioremediation potential also supports plant growth in contaminated environments (Dias et al. 2019; Ortúzar et al. 2020). The results of the AntiSmash assay are consistent with the potential for iron acquisition reflected by the PLaBAse assay. *B. altitudinis* GG-22 synthesizes two siderophores, schizokinen and bacillibactin. Bacillibactin and hydroxamate schizokinen siderophores have already been described in plant growth-promoting *B. altitudinis* strains from leaf surface and sediment (Narayanasamy et al. 2023; Jankoski et al. 2023), as well as in other *Bacillus* species with growth-promoting properties, such as *B. subtilis*, *B. thuringiensis* and *B. megaterium* (Timofeeva, Galyamova, and Sedykh 2023; Khan, Doshi, and Thakur 2016).

Furthermore, the strain possesses genes linked to the production of essential plant vitamins, especially those involved in the metabolism of folate (vitamin B9), thiamine (vitamin B1), pantothenic acid (vitamin B5), and riboflavin (vitamin B2). These vitamins are crucial for plant growth and development, playing an important role in root colonization and acting as elicitors or priming agents that stimulate plant defence mechanisms against pathogens and abiotic stress. Specifically, thiamine has been demonstrated to activate systemic acquired resistance (SAR) in plants, enhancing their ability to defend against pathogens. B-complex vitamins function as coenzymes in several metabolic processes such as glycolysis, the Krebs cycle, and nucleic acid synthesis. The strain's ability to supply essential vitamins can significantly enhance plant health and resilience (Riesco et al. 2022).

In this work, *B. altitudinis* GG-22 has demonstrated significant antagonistic potential against phytopathogenic fungi and oomycete but has showed limited efficacy against plant pathogenic bacteria. The antagonistic activity of *B. altitudinis* against *V. dahliae* and *F. oxysporum* has already been reported in a study where *B. altitudinis* also exhibited broad-spectrum inhibition activity against a wide variety of phytopathogenic fungi, including *Colletotrichum gloeosporioides*, *Fusarium graminearum*, *Botrytis cinerea* and *Magnaporthe oryzae*, through in vitro co-culture assays and VOCs release experiments (Shan et al. 2024). Other authors have demonstrated its strong antifungal activity against *Sclerotinia sclerotiorum*, *Corynespora cassiicola* and *Fusarium verticilloides in vitro*, further supporting its broad-spectrum efficacy (Goswami and Deka 2020). Beyond *in vitro* assays, the biocontrol effect of *B. altitudinis* has also been evaluated *in vivo*. In a potted planting experiment, *B. altitudinis* effectively controlled cotton Verticillium wilt. Treated plants showed stronger growth and significantly higher resistance to the disease compared to the control group, highlighting the protective effect of the strain against the infection (Shan et al. 2024). Similarly, *B. altitudinis* was tested on tea plants (*Camellia sinensis*) affected by *C. gloeosporioides*, the causal agent of anthracnose, and mustard plants (*Brassica juncea*) infected with *S. sclerotiorum*, responsible for white mold. In both cases, *B. altitudinis* significantly reduced disease incidence (Goswami and Deka 2020; Wu et al. 2024). The efficacy observed in our study against *Pythium*, an oomycete, aligns with previous research where *B. altitudinis* exhibited strong antagonistic activity against *Phytophthora sojae*, responsible for soybean root rot, and four other species of oomycetes in *Phytophthora* (Cao et al. 2024). This broad antimicrobial efficacy further emphasizes

the potential of *B. altitudinis* as a biological control agent with activity across a diverse range of fungal and oomycetal pathogens.

The limited antibacterial activity observed is consistent with findings from previous studies. In an antimicrobial screening of olive sporobiota isolates, *B. altitudinis* showed no inhibitory effect against various bacterial strains, including *Listeria innocua*, *Salmonella enterica*, *Escherichia coli*, *Enterococcus faecalis*, and *Bacillus cereus*, displaying activity only against *Staphylococcus aureus*. Furthermore, no antimicrobial effect was observed against *X. fastidiosa* (Manetsberger et al. 2024). Similarly, in another investigation, *B. altitudinis* exhibited activity against fewer than 50% of the bacterial strains tested. However, it demonstrated strong antifungal activity against *Botrytis cinerea* and *Lecanicillium fungicola* (Pino-Hurtado et al. 2024). These results further underscore the selective antimicrobial spectrum of *B. altitudinis*, showing greater efficacy against fungal pathogens compared to bacterial species. The synthesis of the lipopeptides lichenysin and fengycin by *B. altitudinis* GG-22, revealed by AntiSmash analysis, could explain the efficacy against phytopathogenic fungi. Fengycin, that was first described in *B. subtilis* strains as a novel antifungal lipopeptide, specifically inhibits filamentous fungi while being significatively less effective against yeast and bacteria (Vanittanakom et al. 1986; Dong et al. 2022). Aggregation of fengycin on the membrane surface disorders the lipid hydrophobic region and causes cell disruption. Sur, Romo and Grossfield (2018) reported that, although aggregates form in both bacterial and eukaryotic membranes, larger aggregates are only stable in the eukaryotic-like membranes. Therefore, fungal membranes are damaged while bacterial membranes are not (Sreyoshi Sur, Romo, and Grossfield 2018). Moreover, lichenysin, which has already been described in *B. altitudinis* strains, exert antifungal activity by altering the permeability of the fungal membrane, leading to the outflow of molecules from the cytoplasm (P. Guo et al. 2023).

A specific greenhouse trial was carried out in the frame of the EU project BIO-VEXO (887281) to study the olive gene expression response to *B. altitudinis* GG-22 and evaluate its possible protection against *X. fastidiosa*. A single treatment of olives with GG-22 by spray did not protect trees of *Xylella*, which underwent severe desiccations.

GG-22 was sensed by olives as observed by the early effects on gene expression, an alteration that decreased 15 days after the treatment as showed by the lower number of DEGs. A possible GG-22 plant growth promoting activity can be envisaged at T2, with the involvement of genes involved in the "auxin polar transport". Indeed, the downregulation of "probable auxin efflux carrier component 1c" (also known as PIN proteins) and "auxin-responsive SAUR68-like" may be due to a prolonged GG-22 auxin production, as observed in rice (Manna et al. 2022). Indeed, the observed stimulation of the systemic acquired resistance was not maintained up to T5 therefore ceasing to protect the plants from the *Xylella* inoculation. Conversely, a clear response to *Xylella* prevailed in gene expression analysis at 10 days (T6) after the bacterial inoculation. Strongly upregulated genes point to a plant defence reaction involving pathogenesis related proteins and a downregulation of genes related to the cell wall metabolism, indicating a

plant cell wall remodelling in response to an extracellular pathogen such as *Xylella*. Future attempts should consider repeated application of GG-22, in close proximity or even after *Xylella* inoculation, to control this slow replicating pathogen. Additionally, exploring the use of GG-22 in combination with other biocontrol agents or under different environmental conditions could enhance plant resilience against *Xylella* and other bacterial pathogens, while also investigating its potential in integrated pest management strategies. Research on optimizing dosage, application timing, and delivery methods will be crucial to fully harness its plant growth-promoting and biocontrol capabilities in diverse agricultural settings.

5. Conclusions

The comprehensive genomic and experimental analysis of *B. altitudinis* GG-22 reveals its dual potential as a biocontrol agent and plant growth-promoting bacterium. Genomic data, supported by functional analyses, demonstrate significant antagonistic activity against a broad spectrum of phytopathogenic fungi and oomycetes, mediated by the production of antifungal lipopeptides, which enhance its competitive fitness in both rhizosphere and phyllosphere environments. However, its limited efficacy against bacterial pathogens, such as *X. fastidiosa*, underscores a selective antimicrobial spectrum that requires further investigation. Gene expression analysis in treated olive plants revealed that GG-22 stimulates early transcriptional changes related to the auxin transport system and systemic acquired resistance (SAR), suggesting that it plays a role in modulating plant hormonal balance and priming defence responses. This was not sustained long-term, which may explain the lack of protection against *X. fastidiosa* in inoculation trials. Moreover, genes involved in cell wall remodelling and plant immune responses were differentially regulated post-treatment, suggesting that the interaction of GG-22 with host plants extends beyond nutrient facilitation to actively shaping plant defence strategies. GG-22 has demonstrated a notable ability to enhance plant growth and resilience through different mechanisms, such as the production of siderophores, that promote iron acquisition. This strain also possesses genetic traits for heavy metal detoxification, phosphate and potassium solubilization, and the biosynthesis of essential vitamins. These attributes underscore its utility in promoting plant growth under stress conditions, such as in contaminated or nutrient-deficient soils, while stimulating systemic resistance pathways.

Future research should focus on refining GG-22's application strategies, including dosage optimization and timing, to maximize its biocontrol efficacy and plant growth-promoting potential. Integration into multi-agent biocontrol systems, particularly in conjunction with other microbial antagonists, could broaden its spectrum of activity, especially against bacterial pathogens. Moreover, understanding the precise molecular mechanisms behind its short-term gene expression effects will be key to enhancing its long-term efficacy, particularly in the context of pathogen defence. In conclusion, *B. altitudinis* GG-22 represents a promising agent for sustainable agricultural practices, especially in mitigating fungal and oomycetal pathogens and promoting plant growth under various environmental stress conditions. The development of advanced

formulations and further field validation under diverse agricultural settings will be crucial in realizing its full potential as a biocontrol agent and biofertilizer.

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COMUNICACIONES CIENTÍFICAS

Congreso en red de Olivicultura, Citricultura y Fruticultura de la Sociedad Española de Ciencias Hortícolas
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Evaluación *in vitro* de la actividad antimicrobiana de extractos del género *Allium* frente a *Verticillium dahliae*

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La verticilosis del olivo, causada por el hongo *Verticillium dahliae*, es una de las enfermedades más amenazadoras para este cultivo en todo el mundo. Durante los últimos años se ha extendido a nuevas zonas olivareras españolas, afectando de forma severa principalmente a plantaciones de regadío. La incidencia creciente de verticilosis supone un gran problema para el sector olivarero, que debe atajarse con la mayor brevedad posible para evitar las pérdidas que esta enfermedad pueda ocasionar. La ineficacia de los tratamientos químicos unida a la actual corriente de sostenibilidad en el campo ha fomentado la búsqueda de nuevas estrategias de control de plagas, entre las que se incluye el uso de extractos vegetales con actividad antagonista y/o bioestimulante. El objetivo del presente trabajo ha sido evaluar la actividad antifúngica de un extracto de cebolla (EC) y un extracto de ajo (EA) ricos en compuestos organosulfurados (OSCs) frente a distintos patotipos de *V. dahliae* procedentes de aislados salvajes y diferentes colecciones de cultivo. Para ello se han seguido diferentes metodologías de estudio *in vitro* como el test difusión en agar, determinación de las concentraciones mínimas fungicidas, influencia en el desarrollo micelial y evaluación de la actividad antimicrobiana ligada a la fase gaseosa.
Los resultados obtenidos han demostrado que tanto el EC como el EA poseen una significativa actividad antifúngica, exhibiendo un doble efecto biocida y fungiestático. EC resultó más eficaz frente a la mayoría de cepas ensayadas, resultando efectivo en un intervalo de concentraciones de 30-50 mg/mL. Además, los extractos demostraron una importante actividad antifúngica ligada a la volatilidad, lo que revela la capacidad de la fase gaseosa de los compuestos predominantes en ambos extractos para inhibir el crecimiento de las diferentes cepas de *V. dahliae*.
A falta de posteriores ensayos de eficacia *in planta* se puede concluir que los extractos de aliáceas ricos en compuestos organosulfurados (OSCs) son herramientas prometedoras en el control integrado de *la verticilosis*, posicionándose como una alternativa eficaz y respetuosa con el medio ambiente que permitirá reducir el uso de productos fungicidas y fitosanitarios de origen químico.

Palabras Clave: *Verticillium dahliae*, aliáceas, compuestos organosulfurados, antifúngicos, olivo

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Grupo Operativo Salud Olivar: Desarrollo de estrategias innovadoras para el control de enfermedades endémicas y emergentes en olivo en España.

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Ciertas enfermedades que afectan al olivar, como la Verticilosis u otras emergentes como la causada por *Xylella fastidiosa*, se caracterizan por su virulencia y causan gran preocupación entre los agricultores. Estas enfermedades suponen un problema que el sector olivarero debe afrontar cuanto antes para evitar importantes pérdidas económicas.
El Grupo Operativo Salud Olivar está abordando esta problemática desde tres vertientes distintas: la prevención, la detección temprana y el tratamiento de árboles ya infectados. Los avances en el ámbito de la prevención han estado relacionados con el diagnóstico inicial de los campos de Dcoop afectados por Verticilosis y el aislamiento de cepas específicas. Atendiendo a las estrategias de biocontrol y aumento de la productividad, se ha avanzado en el diseño de un producto bioestimulante basado en microorganismos, así como en un producto a base de extractos vegetales para incrementar el crecimiento de los olivos y reducir el estrés de agentes externos. Respecto al desarrollo de soluciones enfocadas al tratamiento de *Xyllella fastidiosa*, los objetivos planteados en el proyecto recogen, por un lado, la obtención de una endolisina recombinante a partir de la secuencia obtenida del genoma de virus bacteriófagos, y por otro, el desarrollo de productos basados en extractos vegetales.

Palabras claves: Verticilosis, *Xylella fastidiosa*, olivar, biocontrol, extracto vegetal, microorganismos, bioestimulantes, endolisinas.

II Congreso Investigación PTS Granada
Spain, 2022

Evaluación de la actividad antimicrobiana de compuestos de Allium frente a fitopatógenos

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El incremento en la última década de enfermedades y plagas que afectan a los cultivos, y las consiguientes pérdidas económicas ocasionadas en el sector han propiciado la búsqueda de nuevas estrategias de manejo de plagas. Por otro lado, el impacto medioambiental derivado del incremento del uso de productos fungicidas y fitosanitarios de origen químico ha derivado en la búsqueda de alternativas naturales y sostenibles. Entre las soluciones que destacan por su efectividad se encuentran el uso de microorganismos de control biológico y de extractos botánicos. Entre los últimos, los extractos de aliáceas y sus principios activos de tipo azufrado ocupan un lugar destacado al resultar potencialmente inocuos y ambientalmente sostenibles.

El presente trabajo tiene como objetivo evaluar la capacidad antimicrobiana de dos extractos ricos en compuestos organosulfurados del tipo tiosulfinato y tiosulfonato, derivados de ajo (*Allium sativum*) y cebolla (*Allium cepa*). Para ello, se llevaron a cabo distintas metodologías de estudio in vitro, tales como el test de difusión en agar según Kirby-Bauer y la determinación de las Concentraciones Mínimas Bactericidas/Fungicidas (CMB/F). Además, se determinó la actividad antimicrobiana ligada a la fase gaseosa de los compuestos predominantes en ambos extractos mediante un test de difusión aérea y, en el caso de hongos fitopatógenos, el efecto sobre el desarrollo micelial. Ambos extractos se evaluaron frente a una amplia colección de microorganismos diana que incluye bacterias fitopatógenas de los géneros *Pseudomonas*, *Erwinia, Xanthomonas* y *Clavibacter*, y hongos como *Penicillium, Fusarium, Botrytis* y *Alternaria*, entre otros.

Tanto el extracto de cebolla como el de ajo han mostrado una actividad antimicrobiana significativa frente a la totalidad de fitopatógenos ensayados, destacando la actividad antimicrobiana ligada a la fase gaseosa de sus metabolitos. Estos resultados ponen de manifiesto el elevado potencial de estos productos como biopesticidas para agricultura, si bien es necesario llevar a cabo nuevos estudios en condiciones reales de campo, así como profundizar en los aspectos relativos a su seguridad e inocuidad ambiental.

Palabras clave: *Allium*, Fitopatógenos, Antimicrobiano, Biopesticidas, Plagas

II Congreso Investigación PTS Granada
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Evaluación de la actividad antimicrobiana de dos compuestos organosulfurados derivados de Allium cepa frente a patógenos de interés acuícola

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La intensificación de la acuicultura en los últimos años está propiciando la aparición de brotes infecciosos que causan importantes pérdidas económicas a nivel mundial. Aunque tradicionalmente se han utilizado antibióticos para controlar la aparición de distintas enfermedades bacterianas y micóticas, en la actualidad las restricciones legales y el incremento de las resistencias a antibióticos a limitado el uso de estos fármacos. Por otro lado, el desarrollo y aplicación de vacunas es costoso e inaccesible para muchos medianos y pequeños productores. Por ello, desde hace varias décadas se buscan alternativas naturales para combatir las enfermedades que afectan a la acuicultura y mejorar el sistema inmune de los peces. Estas alternativas han sido lideradas por prebióticos, probióticos y fitogénicos de plantas. Entre los últimos, los extractos de aliáceas ocupan un lugar destacado, habiéndose descrito por su capacidad para potenciar la inmunidad de los peces.

El presente trabajo tiene como objetivo evaluar in vitro la actividad antimicrobiana de dos compuestos organosulfurados presentes en la cebolla (*Allium cepa*), el propil propano tiosulfinato (PTS) y propil propano tiosulfonato (PTSO). Para ello, se han utilizado diferentes metodologías de estudio como el test de difusión en agar, la determinación de las concentraciones mínimas bactericidas (CMB) o la influencia en el desarrollo micelial, en el caso de microorganismos responsables de micosis como Saprolegnia spp. Ambos compuestos se han ensayado frente a una amplia selección de bacterias patógenas de peces, incluyendo *Flavobacterium columnare, Tenacibaculum maritimum, Pseudomonas anguiliseptica, Photobacterium damselae* subsp. *piscicida* o *Lactococcus garvieae*, entre otros.

Los resultados han demostrado una significativa actividad antimicrobiana de amplio espectro frente a todas las cepas bacterianas ensayadas, tanto Gram-positivas, como Gram-negativas y hongos micóticos, con valores de CMB comprendidos entre 15-125 μg/mL. Además, PTS y PTSO ejercieron un efecto fungiestático frente a *Saprolegnia parasítica* a la menor dosis ensayada.

Dado que estos fitogénicos de aliáceas se han usado con éxito en otros sectores ganaderos como el avícola o porcino como ingredientes funcionales para mejorar la salud y el bienestar animal, estos resultados suponen un interesante antecedente para desarrollar su aplicación en acuicultura. No obstante, se requieren ensayos de eficacia in vivo que aporten mayor evidencia científica sobre los beneficios de usar estos fitogénicos en la nutrición de peces.

Palabras clave: *Allium cepa*, acuicultura, fitogénicos, antimicrobiano

I Reunión del grupo de trabajo de Interacciones Bióticas en Plantas de la Sociedad Española de Biología de Plantas
Spain, 2024

Evaluación de la capacidad bioestimulante y antifúngica de un extracto de cebolla estandarizado en compuestos organosulfurados en cultivo de olivo

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El hongo *Verticillium dahliae* es responsable de la verticilosis del olivo, una de las enfermedades más amenazadoras para este cultivo y que se encuentra ampliamente distribuida en los países de la cuenca mediterránea. Durante los últimos años, *V. dahliae* ha causado importantes pérdidas económicas, afectando de forma severa principalmente a plantaciones de regadío, que presentan una tendencia al alza debido al impacto del cambio climático en las plantaciones de secano. Además, el escenario actual de emergencia climática, caracterizado por cambios en la intensidad y estacionalidad de las precipitaciones, se ha relacionado con una mayor incidencia de verticilosis. La búsqueda de soluciones más eficaces y sostenibles es una prioridad para el sector oleícola. El objetivo del presente trabajo ha sido evaluar la actividad bioestimulante y antifúngica de un extracto de cebolla estandarizado en compuestos organosulfurados en dos fincas experimentales de olivo de regadío en las que la presencia de *V. dahliae* estaba confirmada. Para ello se procesaron las muestras foliares procedentes de las parcelas control y tratamiento, se determinó la acumulación de malondialdehído (MDA) y reducción de Fe3+ a Fe2+ como marcadores de estrés, y se llevó a cabo la detección y cuantificación de *V. dahliae* mediante qPCR. Finalmente se llevó a cabo el análisis de los frutos. Los resultados obtenidos sugieren que el extracto de cebolla reduce la peroxidación lipídica, al observarse un descenso significativo de MDA en los olivos tratados, así como la severidad de la infección a través de la disminución de la población del hongo. Además, se ha establecido una correlación directa entre la aplicación del extracto y el porcentaje de grasa del fruto. A falta de más ensayos in planta, se puede concluir que el extracto de cebolla estandarizado en compuestos organosulfurados es una herramienta prometedora en el control integrado de la verticilosis.

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Onion Extract: biostimulant effect in olive plantlets under controlled conditions and in vitro antibacterial activity against *Xylella fastidiosa*

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Olive trees dominate the landscape of the Mediterranean basin since it is one of the species best adapted to the Mediterranean climate and soil. Nevertheless, the effects of climate change in the Mediterranean basin poses substantial impact to olive trees, since it has been related to a higher incidence of endemic and emerging diseases. Among these, *Xylella fastidiosa* stands out as the main threat for this crop. It is therefore crucial to develop new strategies that increase the resilience of the olive trees against it. The present work aims to evaluate the biostimulant activity of an onion extract standardized in organosulfur compounds (OE), applied both by foliar spray and by irrigation, in 6-month-old olive plantlets in growth chambers. To this end, root and leaf samples from control and treated plantlets were processed. Four different stress markers were determined: accumulation of malondialdehyde (MDA), reduction of Fe3+ to Fe2+, amino acid content and expression of stress response genes. The length of the roots and the number of leaves were also determined. Additionally, in vitro tests were carried out to evaluate the antimicrobial activity of OE against *X. fastidiosa*. Results suggest that OE increases the reducing capacity while it reduces lipid peroxidation, as a significant decrease in MDA was observed in the treated olive trees. Greater root development was observed in olives treated by irrigation. Lastly, OE inhibited the growth of *X. fastidiosa* in agar diffusion test. Even though further in planta trials are needed, it can be concluded that onion extract standardized in organosulfur compounds is a promising tool to increase resilience of olive trees in an integrated pest management policy, and specifically against *Xylella fastidiosa*.

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Reduction of *Verticillium dahliae* by onion extract: a biostimulant and antifungal effect

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Verticillium wilt caused by fungus *Verticillium dahliae* is consider one of the most threatening diseases of olive trees, and is widely distributed in all countries of the Mediterranean basin. In recent years, *V. dahliae* has caused significant economic losses, severely affecting mainly irrigated plantation. Furthermore, the current climate emergency scenario has been related to a higher incidence of verticillium wilt. Therefore, the search for more effective and sustainable solutions to maintain productivity is a priority for the olive sector. The present work evaluates the biostimulant and antifungal activity of an onion extract standardized in organosulfur compounds (OE) in two experimental irrigated olive orchards in which the presence of *V. dahliae* was confirmed. To this end, leaf samples from control and OE-treated smallholdings were processed. The accumulation of malondialdehyde (MDA) and the reduction of Fe+3 to Fe+2 were determined as stress markers. Detection and quantification of *V. dahliae* was carried out using qPCR. Additionally, weight, volume and moisture and fat content of the fruits were also analysed. Results suggest that the OE reduces lipid peroxidation, as a significant decrease in MDA was observed in the treated olive trees. Application of OE also reduced the severity of the infection, with a significant decrease in the fungal population of the leaf samples. Lastly, a direct correlation between the application of the extract and the fat percentage of the fruit was established. Even though further in planta trials are needed, it can be concluded that onion extract standardized in organosulfur compounds might be promising tool in the integrated control of Verticillium wilt.

DISCUSIÓN INTEGRADORA

El olivo, un cultivo de gran relevancia económica y social en la región mediterránea, enfrenta actualmente retos crecientes debido a las consecuencias del cambio climático. Las alteraciones en las condiciones ambientales ejercen un estrés abiótico significativo, afectando a su crecimiento y productividad (Orlandi et al. 2020). Este debilitamiento fisiológico compromete la capacidad del árbol para adaptarse a condiciones adversas, lo que a su vez lo hace más susceptible a infecciones de patógenos como *V. dahliae* o *X. fastidiosa* (Sbeiti et al. 2023). Esta combinación de factores pone en riesgo la sostenibilidad de la industria del aceite de oliva, vital para muchas economías rurales (Benítez-Cabello, Delgado, and Quintas 2023). Ante estos desafíos, es fundamental implementar estrategias que no solo controlen las enfermedades, sino que también fortalezcan la resiliencia del olivo frente a los factores de estrés ambiental.

La creciente necesidad de implementar prácticas agrícolas más sostenibles y respetuosas con el medio ambiente ha llevado a la búsqueda de alternativas naturales a los pesticidas y productos químicos tradicionales (Khursheed et al. 2022). En este contexto, los compuestos organosulfurados (OSCs) derivados de la cebolla, como el Propil-Propano-Tiosulfato (PTSO) y el Propil-Propano-Tiosulfinato (PTS), han demostrado ser candidatos prometedores tanto por su capacidad para controlar patógenos, como por su acción bioestimulante. Los estudios realizados en este trabajo ofrecen una visión integrada del impacto de estos compuestos en la salud del suelo, la resiliencia de los cultivos y la seguridad para polinizadores, lo que resalta su potencial para formar parte de programas de manejo integrado de plagas y estrategias de economía circular. A continuación, se discuten los hallazgos más relevantes de estas investigaciones, destacando las implicaciones agronómicas y ecológicas de su uso, así como las perspectivas futuras para su aplicación en la agricultura moderna.

1. Actividad antimicrobiana de los OSCs

Los estudios presentados coinciden en que PTS y PTSO presentan una destacada actividad antimicrobiana dependiente de la dosis, tanto frente a bacterias como a hongos fitopatógenos. En el primer estudio, se evaluó la actividad antibacteriana frente a seis cepas de bacterias y ocho hongos y ascomicetos, mientras que el segundo trabajo se centró en la actividad antifúngica frente a tres aislados de *V. dahliae*. En todos los casos, se observó una superioridad en la eficacia del PTSO frente al PTS, lo que ya había sido reportado en estudios anteriores (Sorlozano-Puerto et al. 2018). PTSO mostró valores de Concentración Mínima Bactericida (MBC) más bajos en comparación con PTS, especialmente frente a *Xanthomonas campestris* y *Agrobacterium tumefaciens*. Esta tendencia también fue observada en el caso de *V. dahliae*, donde la Concentración Mínima Fungicida (MFC) fue consistentemente más baja para PTSO en los tres aislados probados, siendo especialmente notable en el aislado V136I 1A. Otros microorganismos en los que se observó esta diferencia incluyeron *Fusarium graminearum* y *Phyllosticta* spp. Estos datos corroboran que PTSO es más efectivo en la inhibición de microorganismos,

tanto bacterianos como fúngicos, lo cual podría estar relacionado con su mayor estabilidad química y volatilidad (Cabello-Gómez et al. 2022).

En el caso de *X. fastidiosa*, a diferencia de lo observado en otros patógenos, no se encontraron diferencias significativas en la eficacia entre PTS y PTSO, aunque ambos compuestos mantuvieron su actividad bactericida de manera dependiente de la dosis. Esta falta de variación en la eficacia contrasta con los resultados obtenidos en otros microorganismos, lo que podría deberse a las técnicas *in vitro* específicas empleadas para *X. fastidiosa*. Esta bacteria presenta importantes desafíos para su cultivo en laboratorio debido a que requiere formar *biofilms* para crecer adecuadamente en medios de cultivo, lo que complica su manejo experimental (de la Fuente, Navas-Cortés, and Landa 2024). Las técnicas empleadas para los otros microorganismos no son aplicables en este caso, ya que la bacteria no crece adecuadamente bajo las condiciones estándar utilizadas en medios sólidos. Estos factores podrían haber influido en los resultados obtenidos, limitando la observación de diferencias entre PTS y PTSO que sí se evidenciaron frente a otros patógenos. Según los conocimientos actuales, este es el primer estudio que evalúa la eficacia de los OSCs derivados de cebolla frente a *X. fastidiosa*, lo que sugiere nuevas posibilidades para su aplicación en el control de esta bacteria en el marco de la agricultura sostenible. Los resultados obtenidos son coherentes con los observados en otros miembros de la familia Xanthomonadaceae, como *Xanthomonas campestris*, frente a los cuales los extractos de cebolla han demostrado ser efectivos en inhibir el crecimiento bacteriano (Chung et al. 2022; Hussain et al. 2021).

Por otro lado, al evaluar la actividad antimicrobiana de ambos compuestos, se observó que el efecto antifúngico de PTS y PTSO fue superior a su efecto antibacteriano. Estos resultados coinciden con investigaciones *in vitro* previas, en las que se analizó la eficacia antimicrobiana de estos compuestos frente a levaduras y bacterias, destacándose su potencial para aplicaciones terapéuticas en salud humana (Sorlozano-Puerto et al. 2021). Una posible explicación para la mayor susceptibilidad de los hongos a estos compuestos radica en la mayor permeabilidad de la pared celular fúngica de quitina, en comparación con la pared de peptidoglicano presente en las bacterias (Lemar, Turner, and Lloyd 2002). Además, la posible interacción de PTS y PTSO con los grupos tiol de las SSCPs, proteínas pequeñas secretadas ricas en cisteína, específicas de hongos, podría contribuir a la capacidad antifúngica de ambos compuestos, ya que estas proteínas desempeñan un papel clave en la reproducción, dispersión y colonización de sustratos por parte de hongos fitopatógenos (Feldman, Yarden, and Hadar 2020; Zhao et al. 2021).

Uno de los resultados más relevantes de esta tesis es la capacidad de estos OSCs para reducir la severidad de los síntomas causados por *V. dahliae* en olivo (*Olea europaea* cv. Picual). Este aspecto es de gran importancia debido a la elevada incidencia de este patógeno, que se establece en el sistema vascular del olivo y representa una seria amenaza para la producción (Valverde et al. 2023). En este trabajo se ha demostrado que los plantones que crecieron en cámara climática en sustratos infestados con el hongo y tratados con 250 y 500 µg/mL de una mezcla de PTS y PTSO (1:1) experimentaron una reducción significativa en la incidencia y severidad de la enfermedad, con tasas de

incidencia del 60% y 20%, respectivamente, en comparación con el 100% en el control positivo no tratado. Aún más destacable es la ausencia total de síntomas en los plantones tratados con la mezcla de ambos compuestos seguida de una aplicación adicional vía riego (100 mL) una semana después del trasplante.

Este enfoque de tratamiento combinado en higienización de sustratos antes del trasplante y riego destaca el potencial de estos compuestos para reducir la concentración de *V. dahliae* en el suelo y para proteger el sistema vascular de las plantas tras el trasplante, cuando son especialmente vulnerables debido a la necesidad de adaptarse al nuevo entorno. En este trabajo, se observó una disminución significativa en la densidad de *V. dahliae* en los suelos tratados con OSCs, alcanzando reducciones de hasta 4-5 unidades logarítmicas a una dosis de 1.000 μg/mL. Aunque el patógeno no fue erradicado por completo, la reducción en su población fue suficiente para disminuir notablemente los síntomas de la enfermedad en los plantones. Asimismo, los ensayos de higienización de sustratos infectados con *F. oxysporum* y *A. tumefaciens* realizados en el marco de esta tesis corroboran la efectividad de los compuestos PTS y PTSO para reducir la concentración de fitopatógenos de suelo. Estos resultados son consistentes con investigaciones previas que evaluaron la efectividad de extractos de *Allium* mediante la biofumigación de suelos infestados por *Ralstonia solanacearum*. En dicho estudio, aunque el patógeno sobrevivió en suelo y pudo infectar las raíces de tomate, no logró colonizar a la planta huésped (Deberdt et al. 2012). De manera similar, otro estudio demostró que la incorporación de residuos de ajo en suelo redujo la incidencia de marchitez por *Fusarium* en pepino, y aumentó la altura de las plantas, el desarrollo foliar y el rendimiento del cultivo (A. Ali et al. 2020). Por otro lado, los resultados del ensayo de higienización de sustrato indican que la actividad antifúngica de PTS y PTSO es superior a su actividad antibacteriana, lo cual ya había sido sugerido por las pruebas de actividad antimicrobiana *in vitro*.

En el ensayo en cámara climática también se demostró una reducción significativa de la colonización vascular por *V. dahliae*, que se redujo del 60% al 43%, reforzando la hipótesis de que los compuestos PTS y PTSO alteran la capacidad del hongo pata colonizar los tejidos vegetales. Esta estrategia se alinea con estudios previos que sugieren que reducir la presión del inóculo y la severidad de la infección contribuye a mitigar el impacto de la enfermedad en la planta, incluso en casos de infecciones persistentes (Keykhasaber, Thomma, and Hiemstra 2018). Los resultados obtenidos en los olivares de Linares y Santaella, naturalmente infectados con *V. dahliae* corroboran esta hipótesis. Aunque el tratamiento no redujo sustancialmente el porcentaje de árboles infectados, la cuantificación de *V. dahliae* por RT-qPCR demostró una disminución significativa en la densidad del patógeno en las hojas tratadas, evidenciada por el menor número de copias del gen diana en ambas localizaciones. Esto refuerza el potencial del extracto de cebolla para limitar la severidad de la enfermedad en condiciones de campo. A pesar de la capacidad antifúngica que han demostrado los extractos de *Allium* frente a hongos fitopatógenos in vivo en otros modelos vegetales (Carreón-Delgado et al. 2023; Elsherbiny et al. 2023; Oladejo and Imani 2022), hasta donde sabemos su aplicación para

el control de la verticilosis el olivo en condiciones de campo no ha sido investigada. En contraste, extractos de otras plantas como la granada (*Punica granatum*) y el algarrobo (*Ceratonia siliqua*) han mostrado eficacia para reducir la severidad de la verticilosis en olivo cuando se aplican mediante riego en condiciones de campo (Antón-Dominguez et al. 2024).

2. Volatilidad y persistencia en el suelo de los OSCs

La eficacia de los compuestos PTS y PTSO en el control de fitopatógenos está estrechamente relacionada con su volatilidad y persistencia en el ambiente. En los ensayos de actividad de la fase gaseosa de PTS y PTSO *in vitro*, ambos compuestos demostraron una acción antimicrobiana efectiva en su fase gaseosa, inhibiendo el crecimiento de todos los patógenos evaluados sin estar en contacto directo.

La cuantificación de PTS y PTSO en el suelo mediante HPLC-UV evidenció que la actividad de estos compuestos se ve afectada por su rápida volatilización, lo que provoca una disminución acusada de su concentración tras la aplicación. Esto permite que los microorganismos se recuperen, lo que resalta la necesidad de establecer protocolos de aplicación óptimos basados en aplicaciones repetidas. Aplicaciones sucesivas de concentraciones más bajas han resultado ser más efectivas para mantener el control de los patógenos de suelo a lo largo del tiempo que una única aplicación a una concentración elevada. Además, este ensayo confirmó la menor volatilidad de PTSO frente a PTS, que se traduce en una mayor estabilidad en el suelo y una acción más prolongada, lo cual puede explicar su mayor capacidad biocida.

La volatilidad de estos compuestos y la actividad antimicrobiana de su fase gaseosa los convierten en candidatos ideales para su uso en sistemas de manejo integrado de plagas que involucren procesos de higienización de sustrato, un método que utiliza compuestos volátiles para eliminar plagas y enfermedades del suelo. Estos compuestos se difunden en el suelo tras su aplicación, permitiendo una interacción efectiva con los fitopatógenos presentes. El potencial de las especies del género *Allium*, como las cebollas y los puerros, para su uso en biofumigación ha sido respaldado por diversos autores (Arnault et al. 2013; Wang et al. 2022). Estos estudios han evidenciado que los OSCs no solo presentan capacidad biofumigante, sino que también promueven el crecimiento vegetativo, incrementando la productividad de los cultivos. Este enfoque es particularmente relevante para el manejo de fitopatógenos persistentes, como *V. dahliae*, que presenta una gran capacidad de supervivencia en el suelo debido a sus estructuras de resistencia.

La volatilidad de los OSCs derivados de cebolla, ajo y otras aliáceas, así como de plantas aromáticas como tomillo (*Thymus vulgaris*) y clavo (*Caryophyllus aromaticus*), minimiza los riesgos de acumulación en el suelo y reduce el impacto prolongado sobre organismos beneficiosos, posicionándolos como candidatos ideales para su integración en esquemas de control biológico, especialmente en sistemas donde el uso de pesticidas sintéticos es limitado o no deseado (Isman 2016; Steglinska et al. 2022). En contraste con productos químicos tradicionales como el bromuro de metilo, cuyos efectos colaterales

negativos sobre el ambiente y la salud han restringido su uso (Rogers et al. 2024), los extractos y aceites esenciales presentan una mayor biodegradabilidad y, por consiguiente, menor toxicidad para el medio ambiente (Jiménez-Reyes et al. 2019). Los compuestos de *Allium* ofrecen una alternativa eficaz, sostenible y segura. De hecho, algunos estudios han informado que los tiosulfinatos y disulfuros derivados de especies de *Allium* poseen un espectro de actividad pesticida comparable al del bromuro de metilo, lo que las convierte en herramientas efectivas para la gestión integrada de enfermedades (Panth, Hassler, and Baysal-Gurel 2020).

3. Otras aplicaciones en el manejo de plagas

Además de la actividad antimicrobiana, en este trabajo se ha demostrado el potencial insecticida y repelente de PTS y PTSO contra *Aphis gossypii*, un áfido de importancia agrícola que causa importantes daños en cultivos como el algodón, melón y pepino, al alimentarse de la savia de las plantas y transmitir virus fitopatógenos (Cocuzza 2024). Aunque ambos compuestos mostraron actividad frente al áfido, PTSO presentó una mayor actividad biocida, mientras que el PTS mostró una mayor capacidad repelente. La mayor volatilidad del PTS, demostrada mediante el análisis de suelo mediante HPLC-UV, explica su superior capacidad repelente en comparación con el PTSO. Las tasas de mortalidad alcanzadas por ambos compuestos fueron comparables a las obtenidas con insecticidas químicos comerciales, mientras que la capacidad repelente de ambos compuestos a concentraciones del 0,1% superó significativamente la eficacia del control DEET. Esta propiedad biocida, combinada con su alta volatilidad, sugiere que PTS y PTSO podrían ser utilizados no solo para el manejo de patógenos, sino también en el control de plagas de insectos. La actividad insecticida y repelente de compuestos volátiles derivados de cebolla y ajo ya había sido documentada. Una mezcla no estandarizada de extractos acuosos de cebolla y ajo redujo significativamente la población del pulgón *Cerataphis orchidearum* en palmeras datileras (Ali Al-Shuraym 2022) y *Myzus persicae* en plantas de tomate (Badar et al. 2022). Asimismo, un estudio reciente ha demostrado que ellos compuestos volátiles del puerro influyen en el comportamiento alimentario de *M. persicae*, afectando negativamente la ingesta de savia en plantas de pimiento (Baudry et al. 2021).

4. Actividad bioestimulante de los OSCs

El uso del extracto de cebolla rico en OSCs en condiciones controladas y en campo mostró una clara actividad bioestimulante en los olivos, evidenciada por la reducción del daño oxidativo y el estímulo del crecimiento de raíces y brotes. En los ensayos en cámara climática con plantones no estresados, los niveles de malondialdehído (MDA) se redujeron significativamente en las hojas de olivos tratados con el extracto mediante irrigación. El MDA es un producto de la peroxidación lipídica y por tanto un indicador de estrés oxidativo. Estos resultados sugieren que el extracto de cebolla podría reducir el daño de las membranas celulares bajo condiciones de estrés (Del Buono et al. 2021). La capacidad antioxidante de los OSCs de cebolla ya había sido descrita anteriormente en otros ámbitos, como en la medicina y la industria alimentaria

(Mellado-García et al. 2016). Por otro lado, el aumento significativo en la longitud de las raíces en las plantas tratadas, tanto en aplicaciones foliares como por riego, refuerza la idea de que estos compuestos no solo protegen contra el estrés oxidativo, sino que también estimulan el desarrollo radicular, mejorando la capacidad de la planta para absorber agua y nutrientes. Este efecto es especialmente relevante en el contexto de olivos sometidos a condiciones de sequía, una situación cada vez más frecuente debido al cambio climático. La promoción del desarrollo radicular también es crucial en el contexto de enfermedades vasculares, como la verticilosis, ya que una mayor longitud y densidad de raíces puede mejorar la conductividad hidráulica y, por consiguiente, la resiliencia de la planta frente a la obstrucción de los vasos del xilema causada por *V. dahliae* (Pedranzani et al. 2016; Fradin and Thomma 2006). Estos resultados son coherentes con estudios en los que compuestos derivados de *Allium* han mostrado propiedades antioxidantes y de bioestimulación del crecimiento. Otros autores has observado que extractos de ajo reducen los niveles de MDA y promueven el crecimiento de la raíz en plantas de berenjena infectadas artificialmente con *V. dahliae* (M. Ali et al. 2021).

La capacidad bioestimulante también se observó en los ensayos realizados en finca experimental con olivos de cuatro años sometidos a estrés abiótico, la aplicación del extracto de cebolla a través del riego resultó en un aumento significativo en la brotación y longitud de los brotes. Este efecto se incrementó después de la tercera aplicación, lo que indica que un régimen de tratamiento continuo es más efectivo. Cabe destacar que la idoneidad de realizar aplicaciones repetidas ya había sido observada en los ensayos de actividad antimicrobiana en suelo. Estos hallazgos sugieren que la frecuencia de aplicación del extracto desempeña un papel crucial en la maximización del efecto beneficioso del extracto de cebolla, independientemente de si el objetivo es mejorar el crecimiento vegetal o potenciar la resistencia frente a agentes patógenos. La mayor eficiencia observada en la aplicación por irrigación en comparación con la pulverización foliar, especialmente después de tres aplicaciones sucesivas, destaca la relevancia de optimizar las estrategias de aplicación de bioestimulantes para maximizar su eficacia en condiciones de campo y garantizar un efecto consistente en escenarios de estrés múltiple. La influencia de extractos del género *Allium* sobre la brotación ya ha sido documentada en frutales como el manzano, donde la aplicación de un extracto de ajo mediante irrigación se relacionó con un incremento en la floración, brotación y rendimiento (Seif El-Yazal and Rady 2018). De manera similar, se ha documentado que el extracto de ajo (*Allium sativum* L.) también promueve la brotación en vid (*Vitis vinifera* L.) y melocotonero (*Prunus persica* L.) (Fradin and Thomma 2006; Maia et al. 2013). Este resultado es particularmente relevante en el contexto del cambio climático, ya que las temperaturas más altas están reduciendo el número de horas de frío requeridas para una adecuada brotación, afectando negativamente a la producción de olivos (Benlloch-González et al. 2019).

Por otro lado, la capacidad antioxidante del extracto de cebolla también se observó en los ensayos de campo realizados en olivares comerciales afectados de

verticilosis. La aplicación de este extracto mediante irrigación redujo el contenido de MDA en hoja, corroborando los resultados obtenidos en cámara climática. Estos resultados respaldan la hipótesis de que el extracto de cebolla mitiga el estrés oxidativo en árboles sometidos a estrés biótico. Los mecanismos antioxidantes de los OSCs como PTS y PTSO presentes en la cebolla han sido bien definidos en estudios previos, destacándose su capacidad para eliminar radicales libres, inducir la actividad de enzimas antioxidantes celulares y proporcionar protección antioxidante no enzimática (Llana-Ruiz-Cabello et al. 2015; Cascajosa-Lira et al. 2024).

Un hallazgo adicional y de gran relevancia fue el aumento del contenido graso en los frutos de los olivos tratados con el extracto de cebolla, particularmente en el olivar ubicado en Santaella. Este incremento podría estar relacionado con la mejora en el desarrollo radicular, que facilitaría la absorción de agua y nutrientes con la reducción del estrés oxidativo en los tejidos y con la reducción de la severidad de la colonización de *V. dahliae*. El contenido graso de los frutos es un parámetro clave de calidad en la producción de aceite de oliva (Volakakis et al. 2022). El déficit hídrico, impuesto por el cambio climático, así como por la incidencia de *V. dahliae*, que causa oclusión vascular, reduce el tamaño de los frutos y su contenido de aceite, afectando negativamente la calidad del producto final (Greven et al. 2009). Estos resultados subrayan el potencial del extracto de cebolla como una herramienta efectiva para mejorar la calidad del aceite de oliva en condiciones de estrés biótico y abiótico, lo que es especialmente relevante ante los desafíos actuales del cambio climático.

La reducción significativa de la densidad del patógeno en las hojas de los olivos tratados en las fincas de Linares y Santaella, previamente descrita, respalda la hipótesis de que el extracto de cebolla ejerce un efecto antifúngico directo *in planta*. Además, su capacidad bioestimulante mejora los mecanismos de defensa de la planta y reduce el daño oxidativo, incrementando así la resiliencia del olivo frente al estrés biótico y abiótico. Esta acción dual, que combina la protección directa contra *V. dahliae* con el refuerzo general de la salud del árbol, optimiza el manejo de la verticilosis y mejora la capacidad del olivo para enfrentar los desafíos del cambio climático, maximizando el rendimiento y la calidad del cultivo.

5. Evaluación del perfil de seguridad de OSCs en polinizadores

Los resultados de toxicidad aguda oral y por contacto en abejas melíferas de invierno y primavera proporcionan información crucial para validar el uso de compuestos derivados de la cebolla en sistemas agrícolas sin poner en peligro a las abejas. La ausencia significativa de mortalidad en abejas de invierno y primavera al ser expuestas a la dosis máxima de aplicación en campo (1 μg/abeja) destaca la seguridad potencial de estos compuestos en las prácticas agrícolas a escalas reales. Sin embargo, las abejas de primavera mostraron mayor sensibilidad, con un aumento considerable de la mortalidad al ser expuestas a concentraciones mucho más altas (100 μg/abeja), lo que destaca la importancia de considerar variaciones estacionales y fisiológicas en las evaluaciones de toxicidad de agroquímicos. La diferencia en la susceptibilidad entre las

abejas melíferas de invierno y primavera a PTS y PTSO puede deberse a sus diferencias fisiológicas (Jabal-Uriel et al. 2022). Las abejas de invierno tienen un cuerpo graso de mayor tamaño, lo que les permite acumular más reservas de nutrientes y proteínas como la vitelogenina, asociada con su longevidad, resistencia a la inanición y protección contra el daño oxidativo (Kunc et al. 2019). Otro factor importante estaría relacionado con la microbiota intestinal, que varía entre las abejas de invierno y primavera. En las abejas de invierno, la microbiota promueve la expresión de enzimas del citocromo P450, cruciales para la desintoxicación, lo que podría explicar su mayor resistencia a los metabolitos secundarios de plantas (Wu et al. 2020; Kešnerová et al. 2020).

Además de la evaluación de la toxicidad en abejas, es importante tener en cuenta el posible efecto repelente que los OSCs pueden ejercer sobre otros polinizadores como abejorros (*Bombus* sp.) y depredadores naturales de plagas. En investigaciones previas, se observó que los compuestos volátiles de cebolla y ajo ejercen un efecto repelente sobre plagas como el áfido del algodón (*Aphis gossypii*) sin afectar a su depredador natural, la mariquita (Yang et al. 2023). Este resultado es alentador, ya que indica que los compuestos derivados de *Allium* pueden ser eficaces contra plagas sin afectar a los enemigos naturales. Sin embargo, los estudios sobre polinizadores son más escasos. Los resultados presentados en este estudio sugieren que las concentraciones utilizadas en campo no alterarían el comportamiento de alimentación o forrajeo de las abejas, lo que respaldaría su uso seguro en la agricultura.

Aunque los estudios presentados demuestran una baja toxicidad de PTS y PTSO y un perfil seguro para las abejas melíferas, es fundamental llevar a cabo investigaciones a largo plazo, así como evaluar los efectos de estos compuestos en otros organismos del ecosistema agrícola, incluidos otros polinizadores, y sus posibles interacciones con los enemigos naturales de las plagas.

6. El papel de *B. altitudinis* en el control biológico y promoción del crecimiento vegetal

El uso de microorganismos beneficiosos como *Bacillus altitudinis* GG-22 en la agricultura moderna plantea un enfoque biológico prometedor tanto para el control de enfermedades como para la promoción del crecimiento vegetal (El-Saadony et al. 2022). Esta cepa, aislada de la filosfera de cultivos agrícolas, ha mostrado un notable potencial en la inhibición de patógenos fúngicos del suelo y en la mejora de la resiliencia de los cultivos, como el olivo, a través de la activación de mecanismos de defensa natural de las plantas.

Los ensayos *in vitro* han demostrado la capacidad antagonista de GG-22 frente a *V. dahliae* y de otros hongos y oomicetos del suelo, subrayando su capacidad para el control de patógenos fúngicos que penetran a través de la raíz. Sin embargo, la cepa mostró escasa o nula actividad frente a las bacterias evaluadas, incluida *X. fastidiosa*, lo que revela una limitación en su rango de acción y limita su aplicación en el manejo de patógenos bacterianos. Esta actividad antimicrobiana selectiva ha sido observada en otras cepas de *B. altitudinis* empleadas en control biológico, que han mostrado una fuerte inhibición de hongos fitopatógenos como *Fusarium oxysporum* y *Botrytis cinerea*, pero con

actividad antibacteriana limitada frente a varias especies, incluida *X. fastidiosa*. Estos resultados destacan el espectro antifúngico predominante de *B. altitudinis* y su limitada eficacia frente a bacterias (Manetsberger et al. 2024; Shan et al. 2024). *B. altitudinis* GG-22 ejerce su acción de biocontrol principalmente mediante la producción de los lipopéptidos fengicina y lichenisina, ambos caracterizados por su capacidad para alterar las membranas fúngicas y comprometer la integridad celular. La fengicina altera la región hidrofóbica lipídica, lo que provoca disrupción celular, mientras que la lichenisina aumenta su permeabilidad, lo que resulta en la pérdida de componentes citoplasmáticos esenciales (Guo et al. 2023; Sreyoshi Sur, Romo, and Grossfield 2018).

Además, GG-22 produce los sideróforos bacilibactina y esquizocina, que contribuyen al control biológico al restringir el acceso de los patógenos al hierro, un elemento clave para su desarrollo. Al monopolizar este recurso, GG-22 limita el crecimiento de organismos competidores, reforzando su eficacia como agente de biocontrol (Deb and Tatung 2024). Asimismo, la capacidad de producir sideróforos favorece la adquisición de hierro por la planta, mejorando su nutrición y promoviendo el crecimiento vegetal, lo que sitúa a GG-22 como una bacteria promotora del crecimiento además de su función antagonista (Garg, Kumar, and Bhati 2021).

La capacidad de esta cepa para suministrar vitaminas esenciales, entre ellas la tiamina, también contribuye significativamente a la salud y resiliencia de las plantas (Riesco et al. 2022). La tiamina, en particular, está implicada en la activación de la resistencia sistémica adquirida (SAR) (Mora et al. 2024). Además, el análisis transcriptómico de olivos tratados con GG-22 reveló una activación temprana de genes relacionados con la regulación de auxinas, lo que sugiere una modulación de rutas hormonales clave para el desarrollo de la planta y el aumento de la resistencia a condiciones de estrés (Sosnowski, Truba, and Vasileva 2023; Marzi et al. 2024). Estos hallazgos refuerzan el potencial de GG-22 como un agente integral en la agricultura sostenible, capaz de equilibrar la protección y el crecimiento bajo condiciones de estrés múltiple. Sin embargo, la aplicación de GG-22 no logró proteger a los olivos de los síntomas de *X. fastidiosa*, ya que la estimulación de la resistencia sistémica adquirida no se mantuvo en el tiempo. Tras la infección, se observó la regulación al alza de genes relacionados con proteínas patogénicas y una regulación a la baja de genes del metabolismo de la pared celular, sugiriendo una remodelación estructural frente al patógeno (Sosnowski et al. 2023). Esto subraya la necesidad de considerar aplicaciones repetidas de GG-22, además de investigar su uso en combinación con otros agentes de biocontrol y bajo distintas condiciones ambientales podría optimizar su eficacia. Además, es crucial estudiar como interactúa *B. altitudinis* GG-22 con el microbioma del suelo y las dinámicas que pueden influir en su actividad, para garantizar que el uso de esta cepa en la agricultura no tenga efectos adversos. Entender mejor la dosis, el momento de aplicación y los métodos de administración es clave para maximizar su potencial tanto en la promoción del crecimiento vegetal y la respuesta a estrés como en el control biológico de microorganismos fitopatógenos en diversos sistemas agrícolas.

Finalmente, la estabilidad genética también representa un punto crítico. Aunque la cepa GG-22 no presenta plásmidos que puedan facilitar la transferencia horizontal de genes de resistencia a otros microorganismos, la presencia de profagos integrados en su genoma sugiere que ha estado expuesto a infecciones virales en el pasado, lo que podría afectar su plasticidad genética y adaptación a diferentes ambientes (Liu et al. 2021; Gonçalves et al. 2021). Este aspecto plantea interrogantes sobre su estabilidad y eficacia a largo plazo en ambientes agrícolas donde las condiciones ambientales y las comunidades microbianas pueden variar considerablemente.

7. Implicaciones para la agricultura sostenible

En el contexto de la agricultura moderna, tanto *B. altitudinis* GG-22 como los OSCs derivados de *Allium* representan avances cruciales hacia la sostenibilidad. La cepa *B. altitudinis* GG-22 emerge como una herramienta biotecnológica, capaz de enfrentar los desafíos de patógenos fúngicos del suelo, como *V. dahliae*, mientras simultáneamente promueve el crecimiento vegetal a través de la activación de rutas hormonales y defensivas en las plantas. Sin embargo, el potencial de este microorganismo no está exento de desafíos. Su especificidad de acción plantea preguntas sobre su eficacia en diversos tipos de suelo y cultivos, y su interacción con el microbioma circundante aún requiere un estudio más profundo. A medida que continuemos explorando su capacidad para integrarse en sistemas agrícolas complejos, será esencial garantizar que *Bacillus* no solo controle patógenos, sino que también fortalezca la estabilidad del ecosistema del suelo, favoreciendo interacciones positivas con otros microorganismos beneficiosos y evitando desplazamientos microbianos no deseados.

Por su parte, los OSCs como el PTSO y el PTS, ofrecen una alternativa biocida igualmente innovadora, con una alta volatilidad que les permite difundir en suelo, lo cual resulta especialmente efectivo para la reducción de inóculos de patógenos en esta matriz. Además, estos compuestos destacan por su actividad bioestimulante y promotora del crecimiento vegetal, lo que es crucial en escenarios de estrés biótico y abiótico, como la sequía o la salinidad. Al ser compuestos naturales y biodegradables, su uso minimiza los riesgos de contaminación ambiental, una ventaja clave frente a los productos químicos convencionales. No obstante, su volatilidad también plantea una limitación potencial; su persistencia tras la aplicación puede ser insuficiente para un efecto a largo plazo sin aplicaciones repetidas. Esto subraya la necesidad de optimizar protocolos de aplicación, ajustando las dosis y frecuencias para garantizar una protección continua sin generar efectos adversos en organismos no objetivo, como polinizadores y enemigos naturales de plagas.

Lo que resulta particularmente intrigante de estos dos enfoques es su capacidad de adaptación a diferentes contextos de manejo agrícola. Por un lado, GG-22, al poder colonizar la rizosfera y tejidos vegetales, ofrece una solución a largo plazo que no solo controla enfermedades, sino que también mejora la salud general de la planta a través de la promoción del crecimiento. Mientras tanto, los OSCs, aunque más volátiles y con efectos inmediatos sobre los patógenos, podrían ser especialmente útiles en fases críticas

del ciclo de cultivo. Esto abre la posibilidad de que ambas soluciones no sean únicamente herramientas efectivas de control biológico, sino bioestimulantes que podrían ser clave en cultivos afectados por distintos tipos de estreses.

A pesar de este potencial, ambos enfoques requieren de nuevos estudios para maximizar su eficacia en el campo. En definitiva, tanto *B. altitudinis* GG-22 como los compuestos derivados de *Allium* representan alternativas viables y sostenibles en la protección de cultivos. No obstante, su verdadera fortaleza residirá en la capacidad de integrarse en sistemas de manejo agrícola más amplios, donde su acción conjunta o complementaria ofrezca una protección continua contra patógenos, sin comprometer la salud del suelo ni la biodiversidad. La agricultura del futuro, ante el cambio climático y la presión por reducir el uso de agroquímicos, probablemente percibirá en estos enfoques no solo recursos temporales, sino soluciones fundamentales para una producción agrícola más resiliente, saludable y respetuosa con el medio ambiente.

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CONCLUSIONES

1. Los análisis químicos del extracto de cebolla (*Allium cepa*) mediante GC/MS y HPLC confirmaron que contiene una alta concentración de compuestos organosulfurados (OSCs), siendo PTS y PTSO los más abundantes. Estos compuestos demostraron propiedades bioactivas relevantes.
2. Los estudios *in vitro* demostraron que tanto PTS y PTSO como el extracto de cebolla inhiben eficazmente el crecimiento de diversos fitopatógenos, incluidos *Xylella fastidiosa* y *Verticillium dahliae*, destacando su utilidad en estrategias integradas de manejo de enfermedades fúngicas y bacterianas en el olivo. La actividad antimicrobiana de estos compuestos mostró un amplio espectro de acción y una clara dependencia de la dosis. Además, su eficacia en fase gaseosa, y volatilidad sugiere un gran potencial para su uso como higienizantes de sustrato, evitando la acumulación de residuos en el suelo.
3. En condiciones controladas, los OSCs mostraron un efecto positivo sobre la fisiología de los plantones de olivo de la variedad Picual, estimulando el crecimiento radicular y la resistencia al estrés oxidativo en plantas no estresadas. En olivos con verticilosis, los compuestos organosulfurados contribuyeron a disminuir la presencia del patógeno y los síntomas de la enfermedad. Este efecto bioestimulante, combinado con las propiedades antimicrobianas observadas, sugiere que estos compuestos tienen un doble rol en la protección y bioestimulación de los cultivos.
4. Los ensayos toxicológicos realizados en abejas melíferas (*Apis mellifera*) indicaron que PTS y PTSO no presentan toxicidad oral ni por contacto a la dosis de aplicación, lo que sugiere que estos compuestos pueden ser empleados de manera segura en sistemas agrícolas sin comprometer la salud de los polinizadores. Además, las abejas melíferas de invierno presentaron una mayor resistencia frente a estos compuestos que las abejas de primavera, lo que resalta la importancia de considerar factores estacionales en la evaluación de la seguridad de agroquímicos naturales. Si bien los resultados son alentadores en cuanto a la seguridad de los OSCs, se requieren estudios adicionales para evaluar su impacto en otros polinizadores.
5. Las formulaciones prototipo basadas en PTS y PTSO fueron optimizadas para su aplicación en el cultivo del olivo, tanto foliar como mediante fertiirrigación. Los ensayos demostraron que la eficacia del tratamiento se incrementa con aplicaciones repetidas a dosis bajas, debido a la rápida degradación y baja persistencia de los compuestos en el suelo. Esto indica que la correcta frecuencia y la dosificación de las aplicaciones son factores determinantes para maximizar su efectividad en el manejo de enfermedades, manteniendo al mismo tiempo un perfil ambiental seguro.
6. El extracto de cebolla rico en OSCs ha mostrado no solo propiedades bioestimulantes, sino también un efecto protector frente a la verticilosis en cultivos de olivos. Asimismo, los tratamientos promovieron el crecimiento de los brotes en condiciones de estrés abiótico. Además, se observó una reducción en la carga genética de *V. dahliae* y un aumento en el contenido de grasa en los frutos en árboles infectados, lo que sugiere beneficios adicionales en la calidad del producto final.

7. El análisis de *Bacillus altitudinis* GG-22 revela su doble función como agente de biocontrol y promotor del crecimiento vegetal, destacando por su capacidad para inhibir hongos y oomicetos mediante la producción de lipopéptidos antifúngicos. A nivel del transcriptoma, el tratamiento con GG-22 activa de manera temprana genes relacionados con el transporte de auxinas y la resistencia sistémica adquirida (SAR), lo que sugiere que modula el equilibrio hormonal y las defensas de la planta. Sin embargo, estos efectos no se mantienen a largo plazo, lo que podría explicar su limitada eficacia contra patógenos bacterianos como *Xylella fastidiosa*. Además, GG-22 mejora la adquisición de nutrientes esenciales como el hierro y posee genes relacionados con la solubilización de fosfatos y la detoxificación de metales pesados, lo que lo hace valioso en suelos pobres o contaminados.